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Factors affecting the spread of double-stranded RNA viruses in *Aspergillus nidulans*

ALEX COENEN[1]*, FERENC KEVEI[2] AND ROLF F. HOEKSTRA[1]
[1] *Department of Genetics, Wageningen Agricultural University, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands*
[2] *Department of Microbiology, Attila József University, P. O. Box 533, Szeged H-6701, Hungary*

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Summary

Viruses are common in asexual Aspergilli but not in sexual Aspergilli. We found no viruses in 112 isolates of the sexual *Aspergillus nidulans*. We have investigated factors that could play a role in preventing the spread of mycoviruses through populations of *A. nidulans*. Experiments were performed with *A. nidulans* strains infected with viruses originating from *A. niger*. Horizontal virus transmission was restricted but not prevented by somatic incompatibility. Viruses were transmitted vertically via conidiospores but not via ascospores. Competition experiments revealed no effect of virus infection on host fitness. Outcrossing was found to limit the spread of viruses significantly more than selfing. It is concluded that the exclusion of viruses from sexual Aspergilli could be due to the formation of new somatic incompatibility groups by sexual recombination.

1. Introduction

Aspergillus nidulans is a homothallic filamentous ascomycete (Raper & Fennel, 1965). *A. nidulans* grows vegetatively by forming a mycelium of thread-like cells (hyphae) and reproduces by means of asexual conidiospores and sexual ascospores. One fruiting body (cleistothecium) contains 10^5–10^6 ascospores formed by repeated mitosis, fusion and meiosis of the same two nuclei (Alexopoulos & Mims, 1979; Burnett, 1976). Mycoviruses are common in filamentous ascomycetes (Buck, 1986). They have isometric protein capsules with a diameter of 25–50 nm (Bozarth, 1986) and a double-stranded (ds) RNA genome varying in size from 3·5 to 10 kb consisting of one or more segments (Buck, 1986). Viruses have been found in 10 asexual *Aspergillus* species (Buck, 1986; Varga *et al.*, 1994) but never in sexual Aspergilli. The same is true for the closely related fungal genus *Penicillium*. Viruses have been observed in 11 asexual Penicillia (Buck, 1986) but never in sexual Penicillia. However, viruses do occur in some sexual filamentous ascomycetes. Viruses have been observed in two sexual species of *Neurospora* (Myers *et al.*, 1988) and in many sexual plant pathogenic species (Lemke, 1977). The object of this study was to investigate factors affecting the spread of viruses in strains of the sexual *A. nidulans* infected with a dsRNA virus from the asexual *A. niger*. Factors investigated were: host resistance to virus infection, vertical and horizontal transmission, the effects of virus infection on host fitness and the consequences of genetic recombination on virus infection.

Resistance to virus infection is demonstrated by the absence of infection after cytoplasmic contact with a virus-infected strain. Resistance has been observed in some members of the *A. niger* aggregate (Varga *et al.*, 1994) and in *Cryphonectria parasitica* (Polashock *et al.*, 1994). Vertical virus transmission is between generations, from parent to progeny. The evidence to date is that mycoviruses are transmitted vertically through conidiospores (Lecoq *et al.*, 1979) but only rarely through ascospores (Rawlinson *et al.*, 1973; Day *et al.*, 1977; Rogers *et al.*, 1986). Horizontal transmission is between related or unrelated individuals of the same generation. Horizontal virus transmission is greatly restricted by somatic incompatibility in *Ceratocystis ulmi* (Brasier, 1984) and *Cryphonectria parasitica* (Anagnostakis & Day, 1979; Liu & Milgroom, 1996). Somatic incompatibility is the inability of two fungal mycelia to fuse and form a heterokaryon (for overviews on somatic incompatibility see Carlile, 1987; Glass & Kuldau, 1992; Leslie, 1993). In *A. nidulans* somatic incompatibility is regulated by heterokaryon incompatibility genes (*het*

* Corresponding author: Fax: +31-(0)317–483146.
e-mail: Alex.Coenen@popgen.el.wau.nl

genes: Jinks & Grindle, 1963). When two strains have different alleles for one or more *het* genes they are somatically incompatible (Jinks *et al*., 1966). When two strains have a different allele for a *partial-het* gene they are partially somatically incompatible (Coenen *et al*., 1994). Most pairs of *A. nidulans* isolates are somatically incompatible (Grindle, 1963*a*, *b*; Croft & Jinks, 1977; Anwar *et al*., 1993; Dales *et al*., 1993).

A negative effect of virus infection on host fitness will prevent the spread of a virus through a population if it is not compensated by an increased transmission rate. We have used competition experiments to determine fungal fitness. Determining relative fitness in competition experiments has two advantages over determining absolute fitness by counting progeny numbers. Firstly, in fungi, ratios are easier to measure experimentally than absolute numbers. Secondly, competition experiments determine fitness under conditions when there is actually selection on higher fitness.

Genetic recombination creates progeny that are genetically different from each other and from their parents. This can hinder virus transmission either because viruses are adapted to the parental genotype (the Red Queen theory: Van Valen, 1973) or, as we expected to be the case in *A. nidulans*, because recombination creates some progeny that are somatically incompatible with each other and with their parents. If this is the case progeny formed by outcrossing will be less susceptible to infection by their parents and by each other than progeny formed by selfing. In this study this hypothesis was tested by comparing the sib-infection rates of progeny formed by selfing and progeny formed by outcrossing.

2. Materials and methods

(i) *Strains and Media*

A. niger strain 341 is an adenine-requiring mutant of ATCC strain 22343 (Varga *et al*., 1994). *A. niger* strains 1.1.16, 1.7A.8, 1.8A.7 and 1.8A.16 are *nia* mutants from the Wageningen collection of A. van Diepeningen. All five *A. niger* strains contain dsRNA viruses. Strains 341 and 1.1.16 virus pattern 1, strain 1.7A. 8 virus pattern 2, strain 1.8A.7 virus pattern 7 and strain 1.8A.16 virus pattern 8 (Varga *et al*., 1994). All *A. nidulans* isolates are from the Birmingham collection of Dr J. H. Croft. The 112 isolates listed in Table 1 belong to at least 40 somatic compatibility groups. Isolates 700 to 713 are all somatically incompatible with each other. *A. nidulans* strains with AC numbers are recombinants. The genotypes for *het* genes *A* and *B* and *partial-het* gene *d* relevant for this paper were determined previously (Coenen *et al*., 1994) and are as follows: 701 (*A B d*), 713 (*A B′ d′*), AC-2 (*A B d′*), AC-4 (*A B′ d*), AC-7 (*A′ B′ d*) and AC-10 (*A′ B d*). Isolates 701 and 704 have different alleles for three *het* genes.

All media have been described previously (Coenen *et al*., 1994). MMU is minimal medium with 0·5 g/l urea as sole nitrogen source. $MMNO_3$ is minimal medium with 0·5 g/l nitrate as sole nitrogen source. *y* is a conidiospore colour mutation resulting in yellow conidiospores (Pontecorvo, 1953). *nia* and *cnx* are nitrate utilization mutants (Cove, 1976). Strains with a *nia* or the *cnx* mutation cannot grow on $MMNO_3$ but can grow on MMU. Strains with the same number are from the same isolate. Thus 701*y* and 701*y*;virus differ only in virus infection. Triton X-100 was added to some media (0·01 % final concentration) to reduce colony size.

(ii) *Determination of virus infection*

The extraction protocol was adapted from A. Van Diepeningen (unpublished data). Tubes containing 3·5 ml liquid medium were inoculated with conidiospores and incubated at 30 °C for 2 days. The resulting mycelial mats were transferred to Eppendorf tubes, frozen in liquid nitrogen and ground to a powder. After addition of 300 μl of extraction buffer (20 mM EDTA, 20 mM Tris/HCl pH7·5, 1 M NaCl, 1 % SDS) the Eppendorf tubes were incubated at 70 °C for 1 h. After addition of 300 μl phenol and 300 μl chloroform:isoamyl alcohol (24:1) the Eppendorf tubes were centrifuged at 10000 rpm for 15 min. The aqueous phase was transferred to a new Eppendorf tube and after addition of 500 μl chloroform:isoamyl alcohol (24:1) centrifuged for 10 min at 10000 rpm. The aqueous phase was again transferred to a new Eppendorf tube and after addition of 800 μl ethanol stored at −20 °C for 4 h. The DNA/RNA was pelleted by 10 min centrifugation at 10000 rpm. The supernatant was removed and the pellet vacuum-dried. The pellet was resuspended overnight in 15 μl water. Samples were run on 0·8 % agarose gels containing ethidium bromide. DNA and RNA bands were made visible with ultraviolet light.

(iii) *Virus transmission by protoplast fusion*

Protoplast fusion was performed as described previously (Coenen *et al*., 1996). For interspecies virus transmission protoplasts of a virus-containing *ade* or *nia A. niger* donor strain and a wild-type *A. nidulans* acceptor strain were fused. The total fusion mixture was plated on a single plate of a $MMNO_3$. This medium selected for the acceptor strain and against the *A. niger* donor strain. After 5 days all conidiospores were collected from the plates in 5 ml saline, pelleted in a centrifuge (10 min 3500 rpm) and the pellet inoculated in the centre of a new plate of $MMNO_3$. After 5 days the process was repeated. This was done to enable the virus to increase in frequency so that even a very low frequency of virus transmission would be detected. After another 5 days a conidiospore sample was taken and tested for presence of the virus.

(iv) *Somatic virus transmission*

Somatic virus transmission was attempted from virus-containing *A. niger* (*nia*) or *A. nidulans* (*nia*;*y*) donor strains to wild-type *A. nidulans* acceptor strains. Bottles containing 10 ml MMU were each inoculated with 10^5 conidiospores of each strain and incubated at 30 °C. After 7 days sterile glass beads (3 mm diameter) and 3 ml saline (0·8 % w/v NaCl) were added to the bottles and a spore suspension was made. A bottle containing 10 ml $MMNO_3$ was inoculated with 500 μl of the spore suspension. This medium selected for the acceptor strain and against the donor strain. After 5 days this process was repeated. In this way the virus was enabled to reach detectable levels in the acceptor. After another 5 days a new spore suspension was made and 100 μl inoculated in the centre of a plate of $MMNO_3$. After several days a conidiospore sample was taken from the colony growing on the $MMNO_3$ plate and tested for presence of the virus.

(v) *Quantitative analysis of somatic virus transmission*

Quantitative analysis of somatic virus transmission was performed with an *A. nidulans* (*nia*;*y*;*dsRNA virus from A. niger* 341) donor strain and a wild-type *A. nidulans* acceptor strain. Approximately 10^6 conidiospores of each strain were mixed, plated on MMU and incubated at 30 °C. After 4 days a conidiospore suspension was made from these plates. Dilutions of the spore suspension were plated on $MMNO_3 + 0{\cdot}01$ % Triton X-100 so that small isolated colonies grown from single conidiospores of the acceptor strain could be collected. Either single colonies were tested for presence of the virus or groups of 10 colonies were tested as follows. Eppendorf tubes containing 700 μl $MMNO_3$ were inoculated with conidiospores from 10 colonies of the acceptor strain. After 5 days 100 μl saline was added and the tubes were vortexed so that a conidiospore suspension was formed. Fifty microlitres of the spore suspension was inoculated into a new Eppendorf tube containing 700 μl $MMNO_3$. After 5 days the process was repeated. After another 5 days a sample of conidiospores was taken from the Eppendorf tube and tested for presence of the virus. By allowing the conidiospores to grow for three successive periods of 5 days even very low initial virus concentrations had time to reproduce to a detectable level. Absence of the virus meant that none of the 10 acceptor colonies had contained virus. Presence of the virus meant that 1 or more of the 10 acceptor colonies had contained the virus. This method was tested by inoculating Eppendorf tubes nine times with *A. nidulans* 701 (wild-type) and once with *A. nidulans* 701 (wild-type containing dsRNA virus from *A. niger* 341). In all of 10 Eppendorf tubes inoculated in this way and treated as described above the presence of the virus was detected. This shows that this method is sensitive enough to detect the presence of the virus in only 1 of the 10 colonies inoculated into the Eppendorf tube. Consequently this method can be used to observe low frequencies of virus transfer without performing excessive numbers of DNA/RNA extractions. From the results an approximate frequency of virus transfer can be determined with the following equation:

$$F = 1 - \sqrt[10]{\left(\frac{X}{N}\right)}.$$

where F is the frequency of transfer, X is the number of virus-free groups of 10 acceptor spores, N is the total number of groups of 10 acceptor spores tested and X/N is the probability that 10 out of 10 spores do not contain virus.

(vi) *Isolation of colonies grown from single conidiospores or ascospores*

To obtain ascospore suspensions cleistothecia were rolled on 3 % water agar to free them of conidiospores and then crushed in an Eppendorf tube containing saline (0·8 % w/v NaCl). A *y* mutation in one of the parental strains makes it possible to distinguish cleistothecia produced by selfing from cleistothecia produced by outcrossing. Conidiospore suspensions were obtained by suspending a sample of conidiospores in saline. Appropriate dilutions of spore suspensions were plated on media containing Triton X-100 so that colonies grown from single spores could be isolated.

(vii) *Competition experiments*

Competition experiments between strain 701*y* and both the virus-free and the virus infected-strains of the same *A. nidulans* isolate were performed simultaneously using the same spore suspension from strain 701*y* so that virus infection was the only variable. For each strain a plate of $MMNO_3$ was inoculated with approximately 10^6 conidiospores, incubated for 3 days at 30 °C and then stored for 1 day at 4 °C. In this way it was ensured that all strains had the same physiological history. For each competition experiment a mixture containing approximately equal amounts of strain 701*y* and the other strain and a total of approximately 10^7 spores/ml was made. Suitable dilutions of this mixture (± 100 colonies per plate) were plated on $MMNO_3$ + Triton X-100. After 3 days the exact ratio of the two strains before competition could be determined by counting the number of wild-type colour and the number of yellow colonies on these plates. Two hundred microlitres of the mixture was plated on each of two plates of $MMNO_3$ (duplicate competition plates). After incubation at 30 °C for 4 days a spore suspension was made from both of these competition plates. Suitable dilutions of these spore suspensions were plated on

$MMNO_3$ + Triton X-100 to determine the ratio of the two strains after competition.

(viii) *Sib-infection experiment*

Sib-infection experiments were carried out to determine to what extent colonies grown from virus-free ascospores would produce virus-infected conidiospores due to infection by conidiospores of the parental strains or by virus-infected ascospores. Sib-infection experiments were performed with strains 701*y*;virus and 704virus. The contents of a cleistothecium were suspended in 1 ml saline. Then 0·1 ml of this suspension was diluted and plated on $MMNO_3$ so that colonies grown from single ascospores could be tested for presence of the virus. Approximately 10^5 conidiospores of strain 701*nia*;*y*;virus and 10^5 conidiospores of strain 704*cnx*;virus (nitrate utilization mutants of the parental strains) were added to the remaining 0·9 ml. All the spores were pelleted by centrifugation (10 min 10000 rpm). The pellet was suspended in 100 μl saline and then plated on MMU (sib-infection plate). On this plate virus-free mycelium originating from the ascospores could be infected by virus-containing mycelium originating from the conidiospores. After 4 days at 30 °C a conidiospore suspension was made from the sib-infection plates and dilutions were plated on $MMNO_3$ + Triton X-100. On this medium only conidiospores originating from the ascospores could grow and not conidiospores originating from the nitrate utilization mutants of the parental strains. Colonies grown from single conidiospores were tested for presence of the virus. Vegetative compatibility reactions between parent and progeny could not be tested because the *nia* and *cnx* mutations had already been used to select against the parental conidiospores.

3. Results

(i) *Strains screened for virus*

The 112 *A. nidulans* isolates listed in Table 1 were screened for the presence of dsRNA viruses. Although most isolates were from England and Wales, isolates from India, Africa, USA, Trinidad, Hungary and Barbados were also tested. No virus was found in any of the isolates.

(ii) *Transmission experiments*

All attempts to transfer the virus from *A. niger* to *A. nidulans* by protoplast fusion were successful. The 16 *A. niger*/*A. nidulans* strain combinations in which the virus was transferred by protoplast fusion are listed in Table 2. All further experiments were performed with *A. nidulans* strains infected with the virus from *A. niger* strain 341. Interspecies virus transmission was also attempted without the aid of protoplast fusion. Interspecies somatic virus transmission was successful in the two combinations with *A. niger* strain 1.1.16 as donor but not in the two combinations with *A. niger* strain 1.8A.7 as donor (Table 2). Virus transmission by somatic fusion of *A. nidulans* strains was undertaken with strains 701*y*;*nia*;virus and 704*y*;*nia*;virus as donors and the wild-types of strains 701 to 710 as acceptors (Table 2). This is a total of 18 somatically incompatible combinations and two somatically compatible combinations (701*y*;*nia*;virus/701 and 704*y*;*nia*;virus/704). In a first experiment the virus was transferred in 19 of the 20 combinations; in a second experiment the virus was transferred in all 20 combinations.

The quantitative analysis of somatic virus transmission was performed to determine the effect of *het* genes and *partial-het* genes separately and collectively on virus transmission. The strain combinations tested and their allelic differences for *het* genes *A* and *B* and *partial-het* gene *d* are shown in Table 3. Fig. 1 shows the gel from the combination with strain AC-2 as donor and strain 701 as acceptor (Table 3, expt. 2). In some strain combinations single acceptor conidiospores were tested for presence of the virus. In combinations where low transmission frequencies were expected groups of 10 acceptor conidiospores were tested. In these cases the equation given in Sect. 2(v) was used to determine the transmission frequency. For each strain combination the transmission frequency was determined from the average of the two independent experiments. The results show that virus transmission between somatically compatible strains is highly efficient and that *het* genes *A* and *B* and *partial-het* gene *d* restrict but do not prevent somatic virus transmission. The frequencies in Table 3 suggests that *het* genes reinforce each other's action in limiting somatic virus transmission, since in all experiments transmission was limited more by two *het* genes or a *het* gene and a *partial-het* gene together than by either separately.

(iii) *Competition experiments*

Competition experiments were performed between strain 701*y* and virus-free and virus-infected strains of isolates 701, 702, 703 and 704. The total numbers counted and the relative frequencies before and after competition are shown in Table 4. The change in frequency for strain 701*y* before and after competition and chi-squared for the significance of this change are also shown in Table 4. A chi-squared test was used to determine the significance of the variation between the duplicate competition experiments. In 3 of the 12 mixtures tested there was a significant ($P < 0{\cdot}05$) variation between the duplicates (0·85/0·99, 1·23/1·38 and 1·34/153). In the other 9 mixtures there was no significant variation between the duplicates. The 3 competition experiments between strains 701*y* and

Table 1. A. nidulans *strains screened for double-stranded RNA virus*

Isolate number(s)	Place of isolation	Year
1–3	Birmingham, England	1954
5	Bombay, India	1960
6	Zimbabwe	1960
26	Birmingham, England	1964
27–29, 31, 33–37, 65–67, 69	Birmingham, England	1962
38, 40, 42–49, 51, 52	Durham, England	1962
63	London, England	1962
80–86, 103, 113–118	Pembrokeshire, Wales	1962
89	Cardiganshire, Wales	1962
92	Monmouthshire, Wales	1962
93, 94, 99	Hampshire, England	1962
106	Warwickshire, England	1962
109	Kent, England	1962
133–137, 144–146, 155, 157–159	Northumberland, England	1963
140	Lincolnshire, England	1963
138, 139, 156, 160–163	Cardiganshire, Wales	1963
191, 192	Trinidad	1963
228	Gold Coast	1982
229	Pretoria, South Africa	1982
230	Arizona, USA	1982
257	Hungary	1981
258, 261	California, USA	1984
277	India	1986
278	India	1987
290–293	Barbados	1987
700–704, 726, 727, 745	Birmingham, England	1992
705–709, 722	Pembrokeshire, Wales	1992
710–718, 723, 724	Cardiganshire, Wales	1992

Table 2. *Transmission of double-stranded RNA viruses with and without the aid of protoplast fusion*

Donor strain[a]	Acceptor strains[b]
Transmission via protoplast fusion	
A. niger 341	700, 701, 702, 703, 704
A. niger 1.7A.8	701, 702, 703, 704
A. niger 1.8A.7	701, 702, 704
A. niger 18A.16	701, 702, 703, 704
Transmission via somatic fusion	
A. niger 1.1.16	701
A. niger 1.1.16	704
A. niger 1.8.A7	701 No transmission
A. niger 1.8.A7	704 No transmission
A. nidulans 701	701, 702, 703, 704, 705, 706, 707, 708, 709, 710
A. nidulans 704	701, 702, 703, 704, 705, 706, 707, 708, 709, 710

Transmission was successful unless otherwise indicated.
[a] All donor strains were *nia*; *A. nidulans* donor strains were also *y*.
[b] All acceptor strains were *A. nidulans* wild types.

704 resulted in varying fitness advantages for strain 701*y*. This could be due to variation in uncontrollable factors between independent experiments. Strain 701*y* showed a significant competitive advantage over strains 702, 703 and 704. No difference in competitive performance was observed in the competition experiments between strain 701*y* and strain 701, demonstrating that the *y* mutation does not affect competitive performance.

The effect of the virus on host fitness was computed from the sum of the numbers of colonies counted on the duplicate competition plates. The expected results after competition between strain 701*y* and a virus-infected strain in the case when the virus has no effect on host fitness was determined by extrapolating the ratio between before and after competition with the uninfected strain to the results before competition with the infected strain. Expected and observed results

Table 3. *Quantitative analysis of somatic virus transmission*

Donor[a]	Acceptor[b]	*het* gene tested[c]	Expt 1	Expt 2	Frequency[d]
701	701	–	17/20[e]	18/18	0·92
AC-2	701	*d*	9/20	5/18	0·37
AC-4	701	*B*	4/10	5/10	0·06
713	701	*Bd*	0/10	3/10	0·02
713	713	–	19/20	17/18	0·95
AC-4	713	*d*	11/20	7/18	0·47
AC-2	713	*B*	0/10	0/10	< 0·01[f]
701	713	*Bd*	0/10	0/10	< 0·01[f]
701	701	–	10/10	10/10	< 0·99[g]
AC-10	701	*A*	10/10 3/20	10/10 6/20	0·23
AC-4	701	*B*	6/10	8/10	0·11
AC-7	701	*AB*	1/10	7/10	0·05

[a] All donor strains were *y*;*nia*;virus from *A. niger* strain 341.
[b] All acceptor strains were wild-type.
[c] These are the alleles for *het* genes in which the donor and acceptor differ.
[d] Determined from the average of expts 1 and 2.
[e] Number of virus-containing acceptor spores out of the total number tested except when the total number tested is 10, when the number of virus-containing groups of 10 acceptor spores out of 10 groups of 10 acceptor spores is given.
[f] Too low to determine with this method
[g] Too high to determine with this method

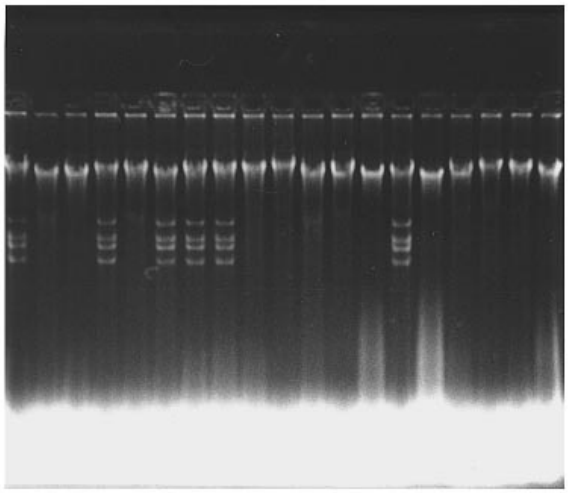

Fig. 1. DNA/RNA gel electrophoresis. Gel showing positive control (*A. niger* 341, first lane on the left) and 18 progeny from the cross of strains 701 and AC-2*y*;*nia*;virus from *A. niger* 341. The band near the top of all lanes is DNA. The smear at the bottom of all lanes is single stranded RNA. The four bands in lanes 1, 4, 6, 7, 8 and 14 are the segmented double stranded RNA virus genome. The positive control and five of the 18 progeny are virus infected.

and statistical analysis are shown in Table 5. For the competition experiments between strain 701*y* and the virus-infected strains of isolates 702, 703 and 704 there was no significant variation between the observed results and the results to be expected in the case that the virus has no effect on host fitness. This means that under our experimental conditions the virus has no significant effect on the fitness of its host. In the competition experiment between strain 701*y* and the virus-infected strain of isolate 701 the virus appears to increase the fitness of its host. As this is solely due to the result in only one of the two duplicate plates we assume that this was caused by an experimental error.

(iv) *Virus in conidiospores and ascospores*

The virus was included in all conidiospores but excluded from most ascospores (Table 6). Measured low frequencies of virus infection in ascospores could be due to infection with conidiospores. It is difficult to ensure that the surface of a cleistothecium is completely free of conidiospores before it is crushed. Virus infection was observed in only 1 of the 158 spores from cleistothecia formed by outcrossing but in 22 of 254 (9%) of the spores from cleistothecia formed by selfing.

(v) *Sib-infection in selfing/outcrossing*

Sib-infection of ascospores was determined for eight cleistothecia. Two cleistothecia were formed by selfing of strain 701*y*;virus, two by selfing of strain 704virus and four by the crossing of these two strains (Table 6). Of the 80 ascospores from the four selfed cleistothecia 11 contained virus (14%). After sib-infection 69 of 80 conidiospores were virus infected (86%). Of the 80 ascospores from the four outcrossed cleistothecia one contained virus (1%). After sib-infection 15 of 80 conidiospores were virus-infected (19%). Therefore 72% of the colonies grown from selfed ascospores were sib-infected as against 18% from outcrossed ascospores. This difference is highly significant (chi-squared = 84, d.f. = 1, $P < 0{\cdot}001$).

Table 4. *Competition experiments*

Strains	Ratio		Frequency[a]	chi-squared[b]
	Before competition	After competition		
701*y*:701	107:105[b](50:50)[c]	180:174 (51:49)	1·01	0·01
		185:189 (49:51)	0·98	0·17
701*y*:701virus	128:116 (52:48)	189:237 (44:56)	0·85	10·9
		174:161 (52:48)	0·99	0·05
701*y*:702	140:140 (50:50)	268:157 (63:37)	1·26	29
		203:114 (64:36)	1·28	25
701*y*:702virus	111:107 (51:49)	221:121 (65:35)	1·27	26
		197:105 (65:35)	1·28	25
701*y*:703	120:102 (54:46)	237:237:51 (82:18)	1.52	92
		297:60 (83:17)	1·54	122
701*y*:703virus	129:118 (52:48)	214:57 (79:21)	1·51	77
		359:76 (83:17)	1·58	161
701*y*:704	338:350 (49:51)	280:172 (62:38)	1·26	30
		180:104 (63:37)	1·29	23
701*y*:704virus	399:331 (55:45)	327:161 (67:33)	1·23	30
		305:131 (70:30)	1·28	42
701*y*:704	340:313 (52:48)	318:178 (64:36)	1·23	29
		345:135 (72:28)	1·38	75
701*y*:704virus	343:332 (51:49)	263:126 (68:32)	1·33	43
		328:142 (70:30)	1·37	67
701*y*:704	110:114 (49:51)	208:90 (70:30)	1·42	52
		242:87 (74:26)	1·50	78
701*y*:704virus	135:118 (53:47)	226:89 (72:28)	1·34	43
		269:60 (82:18)	1·53	106

[a] $\frac{\text{Frequency } 701\gamma \text{ after}}{\text{Frequency } 701\gamma \text{ before}}$.
[b] The difference between before and after is significant when chi-squared is larger than 3·9 (d.f. = 1).
[c] Absolute numbers of colonies counted.
[d] Relative ratio of the two strains.

Table 5. *Expected and observed results for competition experiments*

Strains	Ratio		Chi-squared[b]
	Expected[a]	Observed	
701*y*:701virus	397:364	363:398	6·1[c]
701*y*:702virus	414:230	418:226	0·11
701*y*:703virus	577:129	573:133	0·15
701*y*:704virus	624:300	632:292	0·32
701*y*:704virus	574:285	591:268	1·52
701*y*:704virus	484:160	495:149	1·0

[a] Expected ratio if the virus has no effect on host fitness.
[b] The difference between the expected ratio and the observedratio is significant when chi-squared is larger than 3·9 (d.f. = 1).
[c] $P < 0{\cdot}05$

4. Discussion

There are no reports of viruses being found in sexual Aspergilli despite their abundance in asexual Aspergilli (Buck, 1986; Varga *et al*., 1994). In this study no viruses were found in 112 isolates of the sexual *A. nidulans*. The results presented in this paper show that *A. nidulans* is not resistant to virus infection. All attempts to infect *A. nidulans* with dsRNA viruses from *A. niger* were successful. Viruses have previously been transferred by protoplast fusion between strains belonging to the *A. niger* aggregate (Varga *et al*., 1994) and by electroporation of protoplasts between different species from the genus *Cryphonectria* (Chen *et al*., 1996). We have found that in some cases the virus was transmitted from *A. niger* to *A. nidulans* without the aid of protoplast fusion. This suggests that there is somatic fusion between *A. niger* and *A. nidulans* and that such interspecies virus transmission could also occur under natural conditions.

Horizontal virus transmission was observed in all 18 heterokaryon incompatible *A. nidulans* strain combinations tested. The quantitative analysis showed that horizontal transmission was highly efficient between heterokaryon compatible strains but greatly restricted between heterokaryon-incompatible strains. That horizontal virus transmission is only restricted but not prevented by somatic incompatibility has also been observed in *Ceratocystis ulmi* (Brasier, 1984) and *Cryphonectria parasitica* (Anagnostakis & Day, 1979; Liu & Milgroom 1996). Somatic incompatibility mechanisms will slow down, but not prevent, the spread of viruses through *A. nidulans* populations.

Table 6. *Viruses in conidiospores and ascospores before and after sib-infection*

Strain(s)	Type of spores	No. infected/no. tested[a]	Infected conidiospores after sib-infection
700virus	Conidiospores	19/19	
701virus	Conidiospores	19/19	
702virus	Conidiospores	30/30	
704virus	Conidiospores	38/38	
700virus	Ascospores selfing	6/18, 0/19, 2/20, 2/20	NT
701*y*virus	Ascospores selfing	0/20, 0/20	15/20, 20/20
704virus	Ascospores selfing	1/19, 0/18, 0/20, 0/20	NT
704virus	Ascospores selfing	9/20, 2/20	20/20, 14/20
700(♀)[b] × 704virus(♂)	Ascospores outcrossing	0/20	NT
700(♂) × 704virus(♀)	Ascospores outcrossing	0/20	NT
700virus(♀) × 700(♂)	Ascospores outcrossing	0/19	NT
700virus(♂) × 700(♀)	Ascospores outcrossing	0/19	NT
701*y*;virus × 704virus	Ascospores outcrossing[c]	1/20, 0/20, 0/20, 0/20	5/20, 5/20, 0/20, 5/20

NT, not tested.
[a] Results for ascospores are shown per cleistothecium.
[b] Strain marked ♀ is the strain from which progeny inherited their mitochondria (Coenen *et al*., 1996).
[c] Strains 701 and 704 have different alleles for three *het* genes.

The virus was vertically transmitted via conidiospores but was excluded from most ascospores. The inclusion of viruses in conidiospores has been reported for many sexual and asexual filamentous ascomycetes (Lecoq, *et al*., 1979). It is probably the result of the extensive contact between conidiospores and mycelium before their detachment. The exclusion of viruses from ascospores has been observed in *Gaeumannomyces graminis* (Rawlinson *et al*., 1973), *Ceratocystis ulmi* (Rogers *et al*., 1986) and *Cryphonectria parasitica* (Day *et al*., 1977). Exclusion of viruses from ascospores indicates a stringent separation of fertilization structures from parental mycelium. That the virus was included in some ascospores formed by selfing in *A. nidulans* may be because a fertilization mechanism is not essential for selfing. The absence of a fertilization mechanism in selfing has been observed in several homothallic *Neurospora* species (Raju, 1978). The exclusion of viruses from ascospores is in itself hardly an obstacle to the spread of viruses through *A. nidulans* populations because *A. nidulans* produces at least as many conidiospores as ascospores. The exclusion of viruses from ascospores would explain the absence of viruses from *A. nidulans* populations if the *A. nidulans* life cycle contains a stage consisting solely of ascospores. However, there is no evidence that this is the case.

In our competition experiments we counted the relative numbers of conidiospores formed by two strains before and after competition. A general virus-induced fitness loss resulting in reduced growth would be observed with this method. A specific virus-induced fitness loss resulting only in reduced ascospore production or reduced growth under specific conditions other than those used in our experiments would not be observed with this method. The competition experiments revealed a significant variation in fitness between fungal isolates irrespective of virus infection. Virus infection had no detectable effect on host fitness. Apparently the amount of the host's energy the virus uses for its own reproduction is insignificant. We conclude that the exclusion of viruses from *A. nidulans* populations is not due to negative effects of virus infection on host fitness.

That mycoviruses evolve towards benevolence is in accordance with the theory that there is a trade-off between virus transmission and virulence (Bull *et al*., 1991; Ewald, 1994; Lenski & May, 1994). Mycoviruses cannot exist outside their hosts (Lecoq *et al*., 1979; Buck, 1986). Therefore the transmission rate of the virus is directly proportional to the fitness of the host. The less a mycovirus reduces the fitness of its host the higher its transmission rate. Although no fitness comparisons have previously been made, the absence of a virus-induced phenotype has also been reported for virus-infected strains of the *A. niger* aggregate (Varga *et al*., 1994) and *Neurospora* species (Myers *et al*., 1988). In the plant pathogenic fungi *Cryphonectria parasitica* (the cause of chestnut blight) and *Ceratocystis ulmi* (the cause of Dutch elm disease) virus infection induces a reduction in growth and spore production (Brasier, 1986; Chen *et al*., 1996). The hosts plants of virus-infected fungi live significantly longer than the host plants of virus-free fungi (Brasier, 1986; Anagnostakis, 1988; see also Buck, 1988). Michalakis *et al*. (1992) suggested that the virus-induced reduction in the rate of fungal growth and reproduction in *C. parasitica* may be compensated by an extension of the fungal life span.

Sib-infection experiments were carried out to test the relative effects of selfing and outcrossing on the infection of progeny with parental viruses. We found that even under conditions that were ideal for somatic virus transmission only 19% of progeny formed by

outcrossing were virus infected compared with 86% of progeny formed by selfing. Presumably this is due to the recombination of *het* genes, although other factors may also play a role. The production of virus-free somatic compatibility groups (by sexual reproduction) at a higher rate than that with which they are infected (by somatic virus transmission) will result in the exclusion of viruses from fungal populations. Since one cleistothecium formed by outcrossing will contain progeny with all possible combinations of *het* genes present in the parents (Pontecorvo, 1953), even a limited amount of outcrossing will create a large number of new virus-free somatic incompatibility groups. As there is no genetic recombination in asexual Aspergilli we conclude that sexual reproduction can explain the reported absence of viruses in populations of sexual Aspergilli despite their ubiquity in populations of asexual Aspergilli.

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