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A Mycovirus in Aspergillus fumigatus Shapes Fungal Fitness and Modulates Aflatoxin Biosynthesis in Competing Aspergillus Species

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Abstract

Mycoviruses are emerging as important regulators of fungal biology, yet their roles in shaping interspecific interactions and mycotoxin production remain largely unresolved. Here, we investigated the ecological and metabolic effects of a naturally occurring mycovirus in *Aspergillus fumigatus* AAA12 on fungal fitness and its interactions with aflatoxin-producing species, including *Aspergillus flavus* and *Aspergillus nomiae*. The virus, designated Aspergillus fumigatus partitivirus AAA12 (AfuPV-AAA12), possesses a bipartite double-stranded RNA (dsRNA) genome (1.6–1.8 kb per segment), forms isometric particles of approximately 30–40 nm in diameter, and is phylogenetically assigned to the family Partitiviridae. Virus-infected (VI) and virus-free (VF) isogenic lines were generated to directly assess virus-associated phenotypes. Mycovirus infection significantly enhanced radial growth and increased conidiation by approximately 10-fold, without affecting biomass production, indicating a shift toward a hyper-sporulating phenotype. In dual-culture assays, both VI and VF lines inhibited the growth of competing fungi, however, their effects on aflatoxin production were highly context-dependent. In *A. flavus*, total aflatoxin levels were significantly reduced by up to 37.37% (AAA19) and 34.27% (AAA20), whereas in *A. nomiae*, responses varied, with minimal inhibition (< 5.86% in AAA21) or significant stimulation (− 18.02% in AAA22). Toxin profiling revealed pronounced modulation of aflatoxin biosynthesis, including a marked reduction in aflatoxin G1 (from 175.47 to 44.59 μg/kg) accompanied by increased aflatoxin B1 production in *A. nomiae.* In summary, these findings demonstrate that mycovirus infection enhances fungal fitness while indirectly modulating aflatoxin biosynthesis in competing species, highlighting a context-dependent role of mycoviruses in shaping fungal ecological interactions and mycotoxin risk.

Introduction

The genus *Aspergillus* comprises diverse filamentous fungi that play critical ecological, medical, and agricultural roles. Several species are opportunistic human pathogens, most notably *Aspergillus fumigatus*, whereas others, including *Aspergillus flavus* and *Aspergillus parasiticus*, are major producers of aflatoxins, highly carcinogenic secondary metabolites, that contaminate staple crops and pose significant risks to food safety and public health[1,2]. Aflatoxin biosynthesis is governed by a complex regulatory network integrating genetic and environmental signals, and increasing evidence indicates that inter-fungal interactions can significantly influence toxin production in co-occurring species[3,4].

Mycoviruses are widespread across the fungal kingdom and are increasingly recognized as important modulators of fungal biology. These viruses typically possess RNA genomes, lack an extracellular phase, and are transmitted vertically via spores or horizontally through hyphal anastomosis. Although often asymptomatic, mycoviral infections can alter host phenotypes, including growth, sporulation, stress tolerance, pathogenicity, and secondary metabolism[5,6]. While early studies focused primarily on hypovirulence in plant pathogens, recent work has revealed diverse and sometimes fitness-enhancing effects of mycoviruses across a broad range of fungal hosts. *Aspergillus fumigatus*, the leading cause of invasive aspergillosis, is globally distributed and highly adapted to environmental and host-associated

stress conditions[1]. The discovery of persistent mycoviruses in this species has provided new insights into virus–host interactions. Notably, a dsRNA polymycovirus (AfuPmV-1M) has been shown to enhance stress tolerance, sporulation, and overall fitness in vitro and in vivo, suggesting that mycoviruses can act as modulators of fungal adaptation rather than solely as deleterious agents[7,8]. These findings raise important questions about how mycoviral infection influences fungal ecological performance beyond the host itself.

In natural and agricultural environments, *Aspergillus* species frequently coexist and compete for resources, and such interactions play a key role in shaping community structure and mycotoxin contamination. Competitive dynamics can influence both fungal fitness and secondary metabolite production, with co-culture studies demonstrating that interspecific interactions can either suppress or enhance aflatoxin biosynthesis depending on environmental conditions and strain combinations[3]. However, the potential role of mycoviruses in modulating these interspecific interactions remains poorly understood. Mechanistically, mycoviruses may influence regulatory pathways linked to secondary metabolism, including oxidative stress responses and transcriptional control of biosynthetic gene clusters. These pathways are known to govern aflatoxin production and fungal development, suggesting that virus-induced changes in host physiology could indirectly alter toxin biosynthesis in competing species[5,6,9]. Despite this potential, the impact of mycovirus infection on competitive interactions involving aflatoxin-producing *Aspergillus* species has not been systematically investigated.

In this study, we examined the effects of a naturally occurring mycovirus in *A. fumigatus* AAA12 on fungal fitness and interspecific interactions with aflatoxin-producing isolates. Virus-infected and virus-free isogenic lines were generated to enable direct comparison of virus-associated phenotypes. Using dual-culture assays, we evaluated how mycovirus infection influences antagonistic activity and whether it modulates aflatoxin production in competing *Aspergillus* species. By integrating phenotypic analyses with confrontation assays, this work aims to elucidate how mycoviral infection in one fungal species can reshape ecological interactions and alter toxin biosynthesis in multispecies fungal communities.

Results

Identification of aflatoxigenic Aspergillus isolates from environmental samples

A total of 74 *Aspergillus* isolates were obtained from soil and air samples collected from diverse environmental locations. Initial screening using a rapid aflatoxin detection assay revealed that only five isolates (6.75%) were capable of producing detectable levels of aflatoxins. These isolates were designated AAA19, AAA20, AAA21, AAA22, and ASA58. Macroscopic and microscopic morphological characteristics were examined after 5 days of incubation on PDA (Fig. 1A). Isolates AAA19 and AAA20 exhibited similar colony morphology, characterized by white mycelia with abundant dark-green conidia, a white peripheral margin, and a wrinkled colony surface toward the center. The reverse side of the colonies was pale with corresponding radial wrinkling. In contrast, isolates AAA21, AAA22 and ASA58 formed green colonies with a distinct white margin and produced dark-green sclerotia; the reverse side

of the colony was also pale. Microscopic examination revealed that isolates AAA19 and AAA20 possessed sub-globose vesicles with uniseriate conidial heads and globose conidia. Isolates AAA21, AAA22 and ASA58 exhibited globose vesicles with uniseriate conidiation and globose conidia. These morphological features were consistent with descriptions of *Aspergillus flavus* and *Aspergillus nomiae* reported in standard taxonomic keys.

To confirm species identity, molecular identification based on ITS rDNA sequencing was performed, and phylogenetic analysis using the maximum-likelihood method clustered isolates AAA19 and AAA20 with reference strains of *A. flavus*, whereas isolates AAA21, AAA22 and ASA58 grouped with *A. nomiae* (Fig. 1C). The combined morphological and molecular evidence therefore confirmed that two isolates belonged to *A. flavus* and three isolates to *A. nomiae*, all of which are known aflatoxin-producing species. The ITS rDNA sequences of aflatoxigenic *Aspergillus* isolates have been deposited in the GenBank database under accession number PZ295726 – PZ295730.

Screening, purification, and characterization of mycoviruses from Aspergillus isolates

A total of 74 *Aspergillus* isolates were screened for mycovirus infection using dsRNA extraction by the phenol–chloroform method. Nine isolates (12.16%) were found to contain dsRNA elements, including B2.1.M5, AAA12, ASA24, ASA67, ASW08, ASW39, KW1-3, AI18, and NJ01. Following viral particle purification by PEG precipitation and CsCl density gradient ultracentrifugation, stable viral particles were successfully recovered from only five isolates: B2.1.M5, AAA12, ASW08, ASW39, and KW1-3. Agarose gel electrophoresis of purified viral nucleic acids revealed distinct dsRNA profiles among isolates (Fig. S1). Isolate B2.1.M5 exhibited two bands of approximately 1.0–2.0 kb, while AAA12 showed bands ranging from approximately 1.6–1.8 kb. Isolates ASW08 and ASW39 displayed three dsRNA segments with sizes of approximately 1.0–1.8 kb and 2.0–3.8 kb, whereas KW1-3 produced two bands of approximately 2 and 3 kb. These genome sizes fall within the reported range of mycoviruses associated with *Aspergillus* species. Morphological examination of the mycovirus-infected isolates grown on PDA revealed colony characteristics consistent with *Aspergillus* species, including velvety to floccose colonies with various conidial pigmentation and typical conidiophore structures (Fig. 1B). Microscopic observation showed globose to sub-globose vesicles, uniseriate conidiation, and globose conidia typical of species within the genus *Aspergillus*. To confirm species identity, molecular identification was performed based on ITS rDNA sequencing. Phylogenetic analysis using the maximum-likelihood method clustered the isolates with reference sequences of known *Aspergillus* species (Fig. 1C), confirming that the mycovirus-infected isolates belonged to distinct *Aspergillus* taxa. The ITS rDNA sequences of mycovirus-infected *Aspergillus* isolates have been deposited in the GenBank database under accession numbers PZ295731–PZ295737.

Molecular and structural characterization of mycovirus associated with Aspergillus fumigatus AAA12

The *Aspergillus fumigatus* AAA12 isolate was selected for further analysis because of its stable viral particle recovery and availability of sequence data. When cultured on PDA, the isolate initially produced white mycelia that developed into blue-green, velvety colonies within 5–7 days, with dense conidiophore

production characteristic of *A. fumigatus*. The conidial heads were bluish-green, with spherical conidia measuring approximately 2–3.5 μm in diameter (Fig. 2A).

Partial viral sequences were obtained from dsRNA segments using a random-priming amplification approach based on the Froussard method[10], with minor modifications. cDNA clones were generated from heat-denatured dsRNA, followed by PCR amplification, cloning, and sequencing. To obtain additional cDNAs covering the complete sequences, purified dsRNAs were subjected to a single-primer, genome-walking RT-PCR protocol as previously described[11]. Sequence analysis indicated that the virus associated with isolate AAA12 belongs to the family *Partitiviridae* and was designated Aspergillus fumigatus partitivirus AAA12 (AfuPV-AAA12). The sequences obtained were partial and lacked complete 5' and 3' untranslated regions (UTRs).

The partial genome of AfuPV-AAA12 consists of two dsRNA segments approximately 1.6–1.8 kb in length (Fig. 2B). The larger segment encodes the putative RNA-dependent RNA polymerase (RdRp), whereas the smaller segment encodes the putative capsid protein (CP), a genomic organization typical of members of the family *Partitiviridae* (Fig. 2C). Analysis of purified viral proteins by SDS-PAGE revealed a predominant protein band of approximately 50 kDa (Fig. 2D), consistent with the expected size of the viral capsid protein. Purified viral particles were further examined by transmission electron microscopy (TEM). The virions displayed an isometric, icosahedral morphology with diameters of approximately 30–40 nm (Fig. 2E), consistent with structural features reported for *Partitiviridae*. The buoyant density of viral particles in CsCl gradients was approximately 1.2 g cm^{-3}, which is typical of dsRNA mycoviruses (Fig. 2F). The genomic nature of the virus was confirmed through nuclease digestion assays. Viral nucleic acids were resistant to DNase and S1 nuclease but susceptible to RNase digestion, confirming the presence of a double-stranded RNA genome (Fig. 2G). Phylogenetic analysis based on the partial viral RdRp sequences and those of partitiviruses selected from all genera (Table S1) further supported the classification of AfuPV-AAA12 within the family *Partitiviridae* (Fig. 2H), showing close evolutionary relationships with previously reported *A. fumigatus* partitiviruses.

Curing of mycovirus and assessment of horizontal and vertical transmission

The AAA12 isolate was successfully cured of mycovirus infection using cycloheximide treatment at a concentration of 150 μM. Virus-free isogenic lines were confirmed by the absence of dsRNA bands following phenol–chloroform extraction and agarose gel electrophoresis, as well as by negative RT-PCR amplification of virus-specific sequences, whereas dsRNA was consistently detected in the wild-type virus-infected isolate. After 5 days of growth on PDA, virus-free colonies exhibited reduced radial expansion and altered pigmentation compared with virus-infected controls. In addition, the enlarged white peripheral zone observed in virus-free colonies suggested decreased sporulation relative to virus-infected isolates (Fig. 3A).

Horizontal transmission of the mycovirus was evaluated using hyphal anastomosis assays, with the virus-infected AAA12 strain serving as the donor and the cured isogenic line as the recipient (Fig. 3B). Following mycelial contact, recipient colonies sampled from the interaction zone tested positive for dsRNA by extraction and gel electrophoresis, confirming successful virus transfer via hyphal fusion. These results indicate that the AAA12 isolate is vegetatively compatible with its cured derivative, allowing efficient virus transmission through anastomosis. Vertical transmission was assessed through single-spore isolation from the virus-infected AAA12 strain. dsRNA was detected in 9 out of 12 progeny isolates, corresponding to a vertical transmission rate of approximately 75% (Fig. 3C). These findings indicate that the AAA12 mycovirus is efficiently transmitted through asexual spores, although transmission via hyphal fusion appears to be more efficient under the tested conditions. Together, these results demonstrate that the AAA12 mycovirus can be effectively eliminated by cycloheximide treatment and is capable of both horizontal and vertical transmission, supporting its stable maintenance and ecological dissemination within compatible *A. fumigatus* populations

Effects of mycovirus infection on fungal fitness

The biological effects of mycovirus infection on *A. fumigatus* AAA12 were evaluated by comparing virus-infected (VI) and virus-free (VF) isogenic lines in terms of radial growth, biomass production, sporulation, and spore size (Fig. 4). On potato dextrose agar (PDA), radial colony expansion was monitored over 7 days. VI isolates exhibited significantly faster radial growth than VF isolates, with significant differences observed on days 2 and 4–7 ($p < 0.05$), while no differences were detected on days 1 and 3 (Fig. 4A). This pattern suggests that mycovirus infection promotes sustained hyphal expansion following initial colony establishment. In contrast, no significant differences in biomass production were observed between VI and VF isolates in potato dextrose broth (PDB). Wet biomass averaged 833 mg for VI isolates and 903 mg for VF isolates, while dry biomass averaged 96.6 mg and 103 mg, respectively (Fig. 4B), indicating that the growth advantage is primarily associated with surface expansion rather than total biomass accumulation.

Mycovirus infection had a pronounced effect on asexual reproduction. VI isolates produced approximately ten-fold more conidia than VF isolates ($p < 0.05$; Fig. 4C), accompanied by darker pigmentation and dense conidial layers. In contrast, VF colonies displayed broader, poorly sporulating white peripheral zones lacking conidiation. Despite these marked differences in sporulation, no significant difference in spore size was observed, with mean diameters of 2.75 μm for VI and 2.96 μm for VF isolates (Fig. 4D), consistent with previously reported values for *A. fumigatus*[12]. In addition, mycovirus infection significantly reduced aflatoxin production in *A. fumigatus* AAA12 (Table S2). Virus-infected isolates produced markedly lower levels of total aflatoxin (1.57 μg/kg) compared to virus-free isolates (11.43 μg/kg), representing an approximately seven-fold decrease. Specifically, levels of aflatoxin B1 and G1 were substantially reduced in VI isolates (0.85 and 0.72 μg/kg, respectively) relative to VF isolates (3.15 and 8.28 μg/kg), while aflatoxin B2 and G2 were not detected in either condition. Overall, these findings demonstrate that mycovirus infection confers an increased fitness-related traits in

A. fumigatus AAA12, characterized by enhanced radial growth and markedly increased sporulation, coupled with a substantial reduction in aflatoxin production, without affecting biomass production or spore morphology.

Effects of mycovirus infection on antagonistic activity and aflatoxin modulation

The ecological effects of mycovirus infection were evaluated using dual-culture assays between *A. fumigatus* AAA12 (virus-infected [VI] and virus-free [VF] isogenic lines) and aflatoxin-producing isolates, including *A. flavus* (AAA19, AAA20) and *A. nomiae* (AAA21, AAA22) (Fig. 5). Both VI and VF lines inhibited the growth of all tested isolates, with inhibition levels generally exceeding 20%, although the magnitude of inhibition varied among isolates. No consistent trend was observed between VI and VF lines across all species, indicating that mycovirus infection does not uniformly enhance antagonistic activity but rather modulates interspecific interactions in an isolate-dependent manner. To further assess whether AfuPV-AAA12 could be transmitted via hyphal fusion, agar plugs from the interzone regions of aflatoxin-producing *Aspergillus* isolates grown in dual culture with *A. fumigatus* AAA12 were analyzed. No evidence of viral transmission was detected, as dsRNA extraction followed by agarose gel electrophoresis and RT-PCR failed to detect the presence of AfuPV-AAA12 in the fungal hyphae of the aflatoxin-producing isolates.

The impact of these interactions on secondary metabolism was assessed by quantifying aflatoxin production (Table 1). In *A. flavus* isolates, both VI and VF treatments resulted in significant reductions in total aflatoxin production compared with monoculture controls. In AAA19, aflatoxin levels decreased by 35.85% (VI) and 37.37% (VF), with no significant difference between treatments. In AAA20, inhibition was also significant but differed between treatments, with VF showing greater suppression (34.27%) than VI (19.12%). These results demonstrate that *A. fumigatus* AAA12 effectively suppresses aflatoxin production in *A. flavus*, but mycovirus infection does not consistently enhance this inhibitory effect. In contrast, the response of *A. nomiae* isolates differed markedly. In AAA21, total aflatoxin production was not significantly affected by either VI or VF treatments, with only minor reductions observed (3.57% and 5.86%, respectively). In AAA22, however, both VI and VF treatments resulted in a significant increase in total aflatoxin production, as reflected by negative inhibition values (− 18.02% and − 17.71%, respectively), indicating stimulation rather than suppression. These findings highlight a species- and isolate-dependent response to dual-culture interactions.

Analysis of individual aflatoxin components (Table S3) revealed that these overall trends were associated with toxin-specific and pathway-level changes. In *A. flavus*, reductions in total aflatoxin were primarily driven by significant decreases in aflatoxins B1 and B2. Notably, in AAA20, aflatoxin G1 was detected exclusively in the VI treatment, suggesting that mycovirus infection may induce shifts in secondary metabolism under competitive conditions. In *A. nomiae*, dual-culture interactions resulted in a marked redistribution of aflatoxin profiles rather than uniform suppression. In both AAA21 and AAA22,

aflatoxin B1 levels increased significantly in VI and VF treatments, whereas aflatoxin G1 levels were strongly reduced. Despite these opposing trends, total aflatoxin levels remained unchanged in AAA21 and increased in AAA22, indicating that antagonistic interactions alter toxin biosynthesis pathways rather than simply inhibiting them. These results demonstrate that while *A. fumigatus* AAA12 exhibits antagonistic activity against aflatoxin-producing fungi, its effect on aflatoxin biosynthesis is complex and context-dependent. Mycovirus infection does not consistently enhance aflatoxin suppression but instead appears to influence the regulation and redistribution of aflatoxin biosynthetic pathways, particularly in *A. nomiae*.

Table 1
Inhibition of total aflatoxin production in dual-culture assays by virus-infected (VI) and virus-free (VF) *A. fumigatus* AAA12

Isolate	Species	Total Aflatoxin (μg/kg) Control	VI (μg/kg)	VF (μg/kg)	VI Inhibition (%)	VF Inhibition (%)
AAA19	*Aspergillus flavus*	153.45 ± 2.10^{a}	98.44 ± 1.35^{b}	96.10 ± 0.80^{b}	35.85	37.37
AAA20	*Aspergillus flavus*	119.37 ± 0.63^{a}	96.55 ± 1.53^{b}	78.47 ± 0.46^{c*}	19.12	34.27
AAA21	*Aspergillus nomiae*	592.27 ± 6.98^{a}	571.12 ± 2.50^{a}	557.55 ± 8.12^{a}	3.57	5.86
AAA22	*Aspergillus nomiae*	719.19 ± 5.42^{b}	848.81 ± 6.69^{a}	846.55 ± 9.65^{a}	−18.02	−17.71

Values are presented as mean ± SD. Different superscript letters within the same row indicate significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's HSD test). Negative values indicate stimulation of aflatoxin production. *Confirm VI vs VF significance for AAA20.

Discussion

This study demonstrates that mycovirus infection in *Aspergillus fumigatus* AAA12 significantly influences fungal fitness and modulates interspecific interactions with aflatoxin-producing *Aspergillus* species. The characterized virus, AfuPV-AAA12, belongs to the family *Partitiviridae*, a group of persistent dsRNA mycoviruses typically defined by bipartite genomes encoding an RNA-dependent RNA polymerase (RdRp) and a capsid protein (CP), isometric virions of approximately 30–40 nm in diameter, and efficient vertical transmission[13]. These features were confirmed through molecular, structural, and biochemical analyses, including genome organization, particle morphology, and buoyant density, establishing AfuPV-AAA12 as a typical member of this family. Partitiviruses are commonly associated with asymptomatic or beneficial host phenotypes, often maintaining long-term, stable infections without causing disease symptoms[14]. Consistent with growing evidence that mycoviruses can enhance host

performance, AfuPV-AAA12 infection increased radial growth and resulted in approximately a ten-fold rise in conidiation, indicating improved fitness-related traits. Similar growth-promoting and sporulation-enhancing effects have been reported in other mycovirus–fungus systems, supporting the view that mycoviruses can function as mutualistic partners that enhance host adaptability rather than solely inducing hypovirulence[5,8].

Despite this enhanced fitness, the ecological consequences of mycovirus infection were complex. Both virus-infected (VI) and virus-free (VF) lines inhibited the growth of competing fungi; however, their effects on aflatoxin production were strongly species- and isolate-dependent. In *A. flavus*, significant reductions in total aflatoxin production were observed, consistent with previous studies showing that interspecific competition can suppress mycotoxin biosynthesis through nutrient limitation or stress-mediated regulation[3,15]. In contrast, other systems have reported increased mycotoxin production following mycovirus infection, such as Aspergillus ochraceus virus (AoV)-induced ochratoxin A (OTA) overproduction in *A. ochraceus*[16]. This inconsistency suggests that mycovirus effects on secondary metabolism are highly context-dependent and influenced by host species, virus identity, and environmental conditions. Aflatoxin production is also highly sensitive to environmental cues and is regulated by interconnected signaling pathways, including oxidative stress responses and transcriptional control of biosynthetic gene clusters[9]. In *A. nomiae*, responses were more variable, ranging from minimal inhibition to stimulation of aflatoxin production. Notably, increased toxin levels in AAA22 suggest that competitive stress may activate secondary metabolism under certain conditions. This observation aligns with genomic and transcriptomic studies indicating that aflatoxin biosynthesis is governed by complex regulatory networks that respond to ecological context and strain-specific factors[17,18]. Thus, antagonistic interactions do not necessarily correlate with toxin suppression and may instead trigger compensatory metabolic responses.

A key finding of this study is the shift in aflatoxin composition in *A. nomiae*, characterized by increased aflatoxin B1 and reduced aflatoxin G1 levels. This shift suggests that interspecific interactions, potentially influenced by mycovirus-mediated changes in host physiology, can alter metabolic flux within the aflatoxin biosynthetic pathway. Supporting this, the AoV study reported that increased toxin production was not correlated with viral load or transcriptional activation of known biosynthetic genes[16], implying regulation at post-transcriptional or metabolic levels. This supports the hypothesis that mycoviruses may influence metabolic flux rather than simply gene expression. Variability in B- and G-type aflatoxin production has been linked to differences in gene cluster regulation and enzymatic activity, indicating that environmental and biological factors can redirect pathway outputs[19,20]. The detection of aflatoxin G1 exclusively in the VI treatment of AAA20 further suggests that mycovirus infection may indirectly influence secondary metabolism by altering competitive dynamics or stress responses. Importantly, mycovirus infection did not consistently enhance antagonistic activity or aflatoxin suppression, indicating a context-dependent ecological role. Rather than acting as a universal inhibitor of toxin production, AfuPV-AAA12 appears to modulate the balance between growth, competition, and secondary metabolism. These findings are consistent with plant-based studies showing that

suppression of *A. flavus* and aflatoxin production is associated with enhanced antioxidant defense systems in the host. For example, *A. fumigatus* treatment in maize has been shown to reduce oxidative stress by lowering lipid peroxidation and H_2O_2 accumulation while increasing phenolic compounds, proline levels, and antioxidant enzyme activities[21]. Such mechanisms may indirectly affect fungal competition and toxin biosynthesis by modifying the oxidative environment, which regulates aflatoxin gene expression.

No evidence of AfuPV-AAA12 transmission to aflatoxin-producing *Aspergillus* isolates was observed following dual-culture interactions, indicating that the virus was not transferred via hyphal fusion under the tested conditions. This lack of interspecific transmission suggests the presence of biological barriers, such as vegetative incompatibility or host specificity, which are known to restrict mycovirus movement between genetically distinct fungi. This observation is consistent with previous findings that mycovirus transmission is often limited by vegetative incompatibility and primarily occurs within compatible hosts[14,22]. Such constraints are commonly reported for partitiviruses and other dsRNA mycoviruses, which rely mainly on vertical transmission or intraspecific hyphal anastomosis for dissemination[23]. The inability of AfuPV-AAA12 to infect competing *Aspergillus* species indicates that its ecological impact is mediated indirectly through changes in host physiology and competitive interactions rather than direct cross-species transmission. Consequently, the persistence and spread of AfuPV-AAA12 are likely restricted to compatible *A. fumigatus* populations.

From an applied perspective, these findings have important implications for biocontrol strategies targeting aflatoxin contamination. Although *A. fumigatus* AAA12 exhibited antagonistic activity against aflatoxin-producing fungi, the potential for toxin stimulation in certain species highlights the need for caution when considering mycovirus-associated fungi in agricultural applications. Supporting this concern, previous studies have shown that mycovirus-mediated effects on mycotoxin production can vary and, in some cases, increase toxin levels[16], emphasizing the need for case-by-case evaluation. Moreover, the apparent restriction of AfuPV-AAA12 transmission reduces the likelihood of unintended spread to non-target fungal species, which may be advantageous for biosafety. However, a deeper understanding of species-specific responses and environmental conditions is essential for predicting outcomes in complex microbial communities. In conclusion, this study provides new insights into the ecological roles of mycoviruses in fungal systems. By demonstrating that AfuPV-AAA12 exhibits typical characteristics of Partitiviridae, enhances host fitness, and exerts context-dependent effects on aflatoxin biosynthesis without interspecific transmission, these findings highlight mycoviruses as important yet underappreciated modulators of fungal interactions and mycotoxin dynamics in natural and agricultural environments.

Methods

Aspergillus isolates and aflatoxin screening

All *Aspergillus* isolates used in this study were obtained from the Mycology Laboratory, Division of Biological Science, Prince of Songkla University, Thailand, and maintained on potato dextrose agar (PDA) slants at 4°C. The isolates were originally recovered from soil and air samples collected from various locations and buildings in Songkhla Province, southern Thailand[24]. Prior to experimentation, strains were subcultured on fresh PDA plates and incubated at 25°C in the dark for 5–7 days to obtain actively growing mycelia and conidia. Preliminary screening for total aflatoxin production (AFB1, AFB2, AFG1, and AFG2) was performed using a commercial Total Aflatoxins Rapid Test Kit (Meizheng, PerkinElmer Company) based on a competitive lateral-flow immunoassay, with a detection range of 5–50 μg kg^{-1}.

Extraction and detection of viral double-stranded RNA (dsRNA)

Viral dsRNA was extracted from fungal mycelia using a modified phenol–chloroform method[25]. Briefly, approximately 0.1 g of fresh mycelium was ground in 350 μL extraction buffer and incubated at 70°C for 60 min. The homogenate was then extracted sequentially with Tris-HCl–saturated phenol and chloroform, followed by centrifugation. The aqueous phase was collected and nucleic acids were precipitated using ethanol and sodium acetate. After centrifugation, the pellet was washed with 70% ethanol, air-dried, and resuspended in nuclease-free water. dsRNA profiles were analyzed by agarose gel electrophoresis.

Purification and characterization of viral particles

Viral particles were further purified by polyethylene glycol (PEG) precipitation and cesium chloride (CsCl) density gradient ultracentrifugation[26]. Mycelia were homogenized in TE buffer (1:2, w/v), filtered, and centrifuged at 7,800 rpm for 25 min at 4°C. The supernatant was mixed with 10% (w/v) PEG 6000 and 0.6 M NaCl, stirred overnight at 4°C, and centrifuged at 7,800 rpm for 30 min at 4°C. The resulting pellet was resuspended in TE buffer and ultracentrifuged at 25,000 rpm for 3.5 h at 4°C. The suspension was layered onto a CsCl gradient and centrifuged at 105,000 rpm for 90 min at 4°C. Fractions were collected and screened for dsRNA by phenol–chloroform extraction and agarose gel electrophoresis. Purified viral particles were dialyzed against TE buffer at 4°C for 24 h, examined by transmission electron microscopy (TEM), and characterized by nuclease digestion using RNase, DNase, and S1 nuclease to confirm genome type. Purified viral proteins were separated in 12% SDS-PAGE. Samples of 5 μL were mixed with an equal volume of 2X Laemmli buffer containing β-mercaptoethanol (Sigma-Aldrich, USA) and then denatured at 99°C for 5 min. The denatured samples were immediately cooled on ice and then loaded into each well. Precision Plus Protein™ Dual Xtra standards (Bio-Rad, USA) were used as protein markers.

Identification of Aspergillus spp.

Morphological identification of *Aspergillus* isolates was based on macroscopic and microscopic characteristics, including colony growth rate, color, texture, pigment and exudate production, hyphal

septation, conidiophore structure, vesicle seriation, conidial head arrangement, and conidial morphology. Identification was performed using standard taxonomic keys described by Wingfield et al[27]. For molecular confirmation, genomic DNA was extracted from fresh mycelium using the DNeasy® Plant Mini Kit (QIAGEN, The Netherlands) with minor modifications. The internal transcribed spacer (ITS) region was amplified using primer pairs ITS5/ITS4N or ITS1/ITS4N. PCR conditions followed those described by Wingfield et al[27]. Amplicons were resolved on 1% agarose gel in 1× TAE buffer, stained with SYBR Safe (Invitrogen, USA), purified using the MinElute® Gel Extraction Kit (QIAGEN), and subjected to Sanger sequencing. Sequences were compared to the NCBI GenBank database using BLAST. Multiple sequence alignments were generated using MUSCLE, and phylogenetic analysis was conducted in MEGA version 12.1 using the maximum-likelihood method with 1,000 bootstrap replicates to assess branch support[28].

Construction of cDNA library from dsRNA by random-PCR

cDNA libraries were constructed from purified viral dsRNA using the sequence-independent amplification method of Froussard[10] with minor modifications. Viral dsRNA (0.5 µg) was denatured with Froussard-FOR primer (5'-GCCGGAGCTGTGCAGAATTCNNNNNN-3') at 95°C for 10 min and immediately chilled on ice. First-strand cDNA synthesis was performed using SuperScript™ III reverse transcriptase (Invitrogen, USA) following Kanhayuwa et al[26], with incubation at 50°C for 1 h and enzyme inactivation at 70°C for 15 min. cDNA was size-fractionated using a Nanosep 30K centrifugal filter (Millipore, USA) and subsequently amplified by PCR using the Froussard-REV primer (5'-GCCGGAGCTGTGCAGAATTC-3'). To obtain additional cDNAs covering the complete sequences, purified dsRNAs were subjected to a single-primer, genome-walking RT-PCR protocol as previously described[11]. PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN, The Netherlands), analyzed by agarose gel electrophoresis, and cloned into the pGEM®-T Easy vector (Promega, USA). Recombinant plasmids were transformed into *Escherichia coli* JM109 competent cells, and positive clones were selected by blue–white screening. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN), and insert sizes were verified by *Eco*RI digestion and agarose gel electrophoresis prior to sequencing.

Curing of mycovirus

Elimination of mycovirus from fungal isolates was carried out using cycloheximide treatment combined with hyphal tip isolation, with minor modifications from previously described protocols[29]. Briefly, hyphal tips from virus-infected isolates were transferred onto potato dextrose agar (PDA) supplemented with cycloheximide (150 µM) and incubated at 25°C in the dark for 10 days. This procedure was repeated for three consecutive passages. Subsequently, treated hyphae were transferred to fresh PDA without cycloheximide and incubated at 25°C for an additional 5 days to allow recovery of fungal growth. Regenerated isolates were then screened for the presence or absence of mycovirus by dsRNA extraction, followed by confirmation using RT-PCR amplification.

Horizontal and vertical transmission of mycovirus

Horizontal transmission

Horizontal transmission of mycovirus was assessed using a hyphal anastomosis assay[30]. Virus-infected donor isolates and virus-free recipient isolates were inoculated at opposite sides of the same PDA plate and incubated at 25°C until mycelial contact occurred. Mycelial plugs were then collected from three positions on the recipient colony (near, middle, and far from the donor side). These plugs were transferred to fresh PDA plates and incubated at 25°C for 5 days. Mycovirus infection in recipient colonies was evaluated by dsRNA extraction followed by agarose gel electrophoresis and confirmed using RT-PCR amplification.

Vertical transmission

Vertical transmission was examined via asexual spores (conidia)[31]. Virus-infected isolates were cultured on PDA at 25°C, and conidia were harvested using sterile water containing 0.05% (v/v) Tween 80. Serial dilutions were prepared and plated onto PDA, followed by incubation at 25°C for 48 h. Individual germinated colonies were transferred to fresh PDA plates and incubated for 3–5 days. dsRNA extraction was then performed to determine the presence of mycovirus in progeny isolates, followed by confirmation using RT-PCR amplification.

Phenotypic analysis of mycovirus infection

The effects of mycovirus infection on fungal biology were evaluated by measuring growth rate, sporulation, spore size, and biomass production. For growth rate assessment, equal numbers of conidia from virus-infected and virus-free isogenic lines were inoculated at the center of PDA plates and incubated at 25°C. Colony diameters were measured in millimeters (mm) every 24 h for 7 days. To quantify sporulation, equal numbers of conidia (1×10^6 conidia) were inoculated onto PDA plates and incubated at 25°C for 7 days. Conidia were harvested with sterile water containing 0.05% Tween 80, and mycelia were removed by filtration. Spore concentrations were determined using a hemocytometer and expressed as conidia per square centimeter of colony area. Spore diameters were measured using an ocular micrometer under a light microscope. For biomass determination, equal numbers of conidia were inoculated into 10 mL of potato dextrose broth (PDB) in 50 mL conical tubes and incubated at 25°C for 7 days under static conditions. Mycelia were harvested by filtration through Whatman No. 1 filter paper, and wet weights were recorded. Samples were then dried at 70°C for 48 h, and dry biomass weights were measured. Agar plugs from virus-infected and virus-free isolates were collected and processed for total aflatoxin quantification using high-performance liquid chromatography (HPLC), according to established protocols[32].

Dual-culture antagonism assays and total aflatoxin analysis

Dual-culture assays were conducted to evaluate antagonistic interactions between *A. fumigatus* AAA12 (virus-infected and virus-free isogenic lines) and aflatoxin-producing *Aspergillus* isolates. Equal numbers

of conidia from each strain were inoculated at a distance of 2 cm from the edge of PDA plates and incubated at 25°C for 7 days. Radial growth was measured, and percentage inhibition was calculated as follows: Inhibition (%) = [(A − B)/A] × 100, where A represents the radial growth of the test fungus in monoculture and B represents the radial growth in dual culture[3]. Agar plugs from the interaction zones of dual-culture plates involving aflatoxin-producing *Aspergillus* isolates were collected and processed for total aflatoxin quantification using HPLC, according to established protocols[32]. Mycovirus infection in aflatoxin-producing *Aspergillus* colonies was assessed by dsRNA extraction followed by agarose gel electrophoresis and subsequently confirmed using RT-PCR amplification.

Statistical analysis

All experiments were performed with three independent biological replicates, each measured in duplicate. Data are presented as mean ± standard deviation (SD). Statistical significance between two groups was assessed using Student's t-test, while comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test to identify differences among group means. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA).

Data availability

Sequence data for the *Aspergillus* isolates generated in this study have been deposited in the NCBI GenBank database under accession numbers PZ295726 – PZ295737. All other data generated or analyzed during this study are included in this article and its Supplementary Information.

Declarations

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Author contributions statement

H.I.N. and L.K.W. conceived and designed the experiments, conducted the experiments, analyzed the results. L.K.W. drafted the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests

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Figures

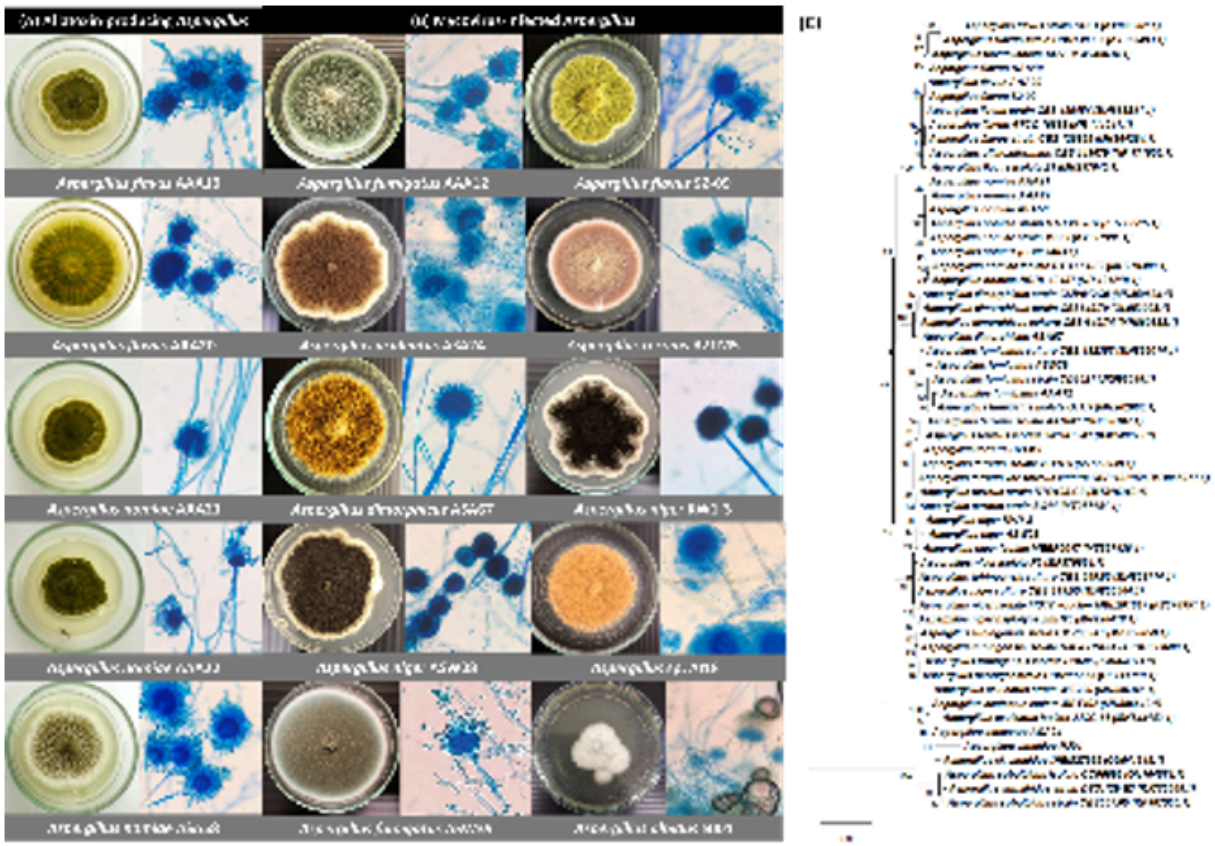


Figure 1

Morphological and molecular identification of aflatoxin-producing and mycovirus-infected *Aspergillus* isolates. (A–B) Colony morphology on PDA after 5 days of incubation at 25 °C and corresponding microscopic structures observed at 40× magnification. (C) The phylogenetic tree based on ITS rDNA sequences constructed using the maximum-likelihood method with 1,000 bootstrap replicates, showing the relationships between the studied isolates and reference *Aspergillus* species.

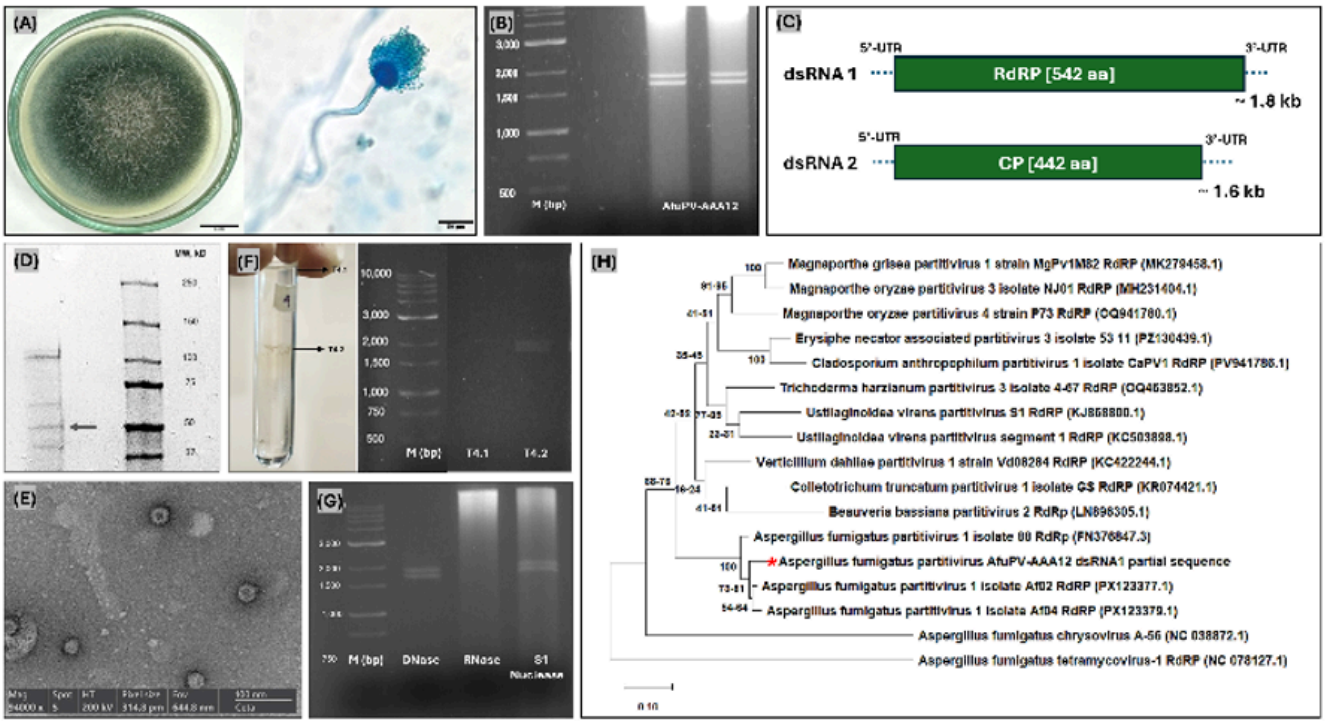


Figure 2

Molecular and structural characterization of the mycovirus associated with *Aspergillus fumigatus* AAA12. (A) Morphology of *A. fumigatus* AAA12 grown on PDA after 5 days of incubation. (B) Agarose gel electrophoresis showing dsRNA segments (1.6–1.8 kb) extracted from the AAA12 isolate. (C) Genomic organization of partial sequences of AfuPV-AAA12 showing two dsRNA segments encoding the putative RNA-dependent RNA polymerase (RdRp) and capsid protein (CP). (D) SDS-PAGE analysis of purified viral particles showing a major protein band of approximately 50 kDa corresponding to the capsid protein. (E) Transmission electron microscopy (TEM) showing isometric viral particles with diameters of approximately 30–40 nm. (F) CsCl density gradient analysis indicating the buoyant density of viral particles (1.2 g cm^{-3}). (G) Nuclease digestion assay demonstrating resistance to DNase and S1 nuclease but sensitivity to RNase, confirming a dsRNA genome. (H) Phylogenetic analysis based on partial viral RdRP sequences showing the placement of AfuPV-AAA12 within the family *Partitiviridae*.

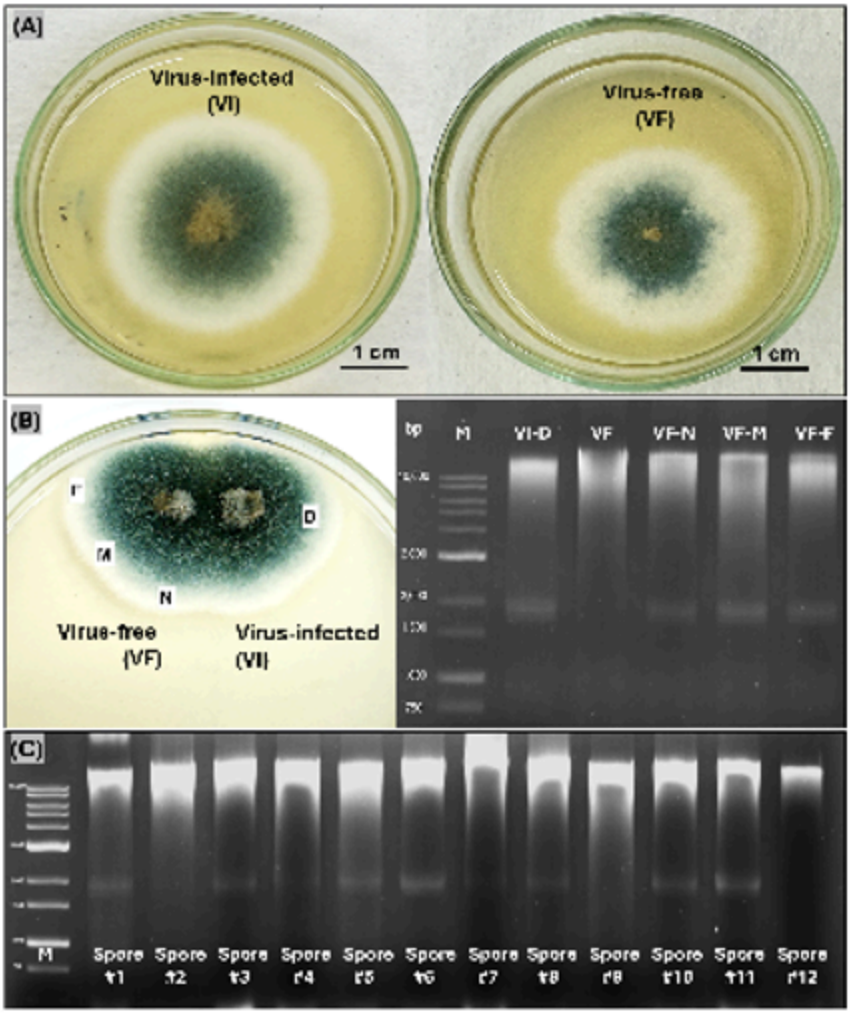


Figure 3

Confirmation of mycovirus curing and transmission in *A. fumigatus* AAA12. (A) Colony morphology of virus-infected (VI) and virus-free (VF) isogenic lines following cycloheximide treatment after 5 days of incubation on potato dextrose agar (PDA). (B) Hyphal fusion assay showing horizontal transmission of the AfuPV-AAA12 mycovirus from donor (virus-infected; VI-D) to recipient (virus-free; VF) isolates (left panel), with subsequent confirmation of dsRNA elements in recipient colonies by electrophoresis (right panel). (C) Vertical transmission analysis via single-spore isolation, followed by detection of dsRNA in progeny isolates, indicating inheritance of the mycovirus.

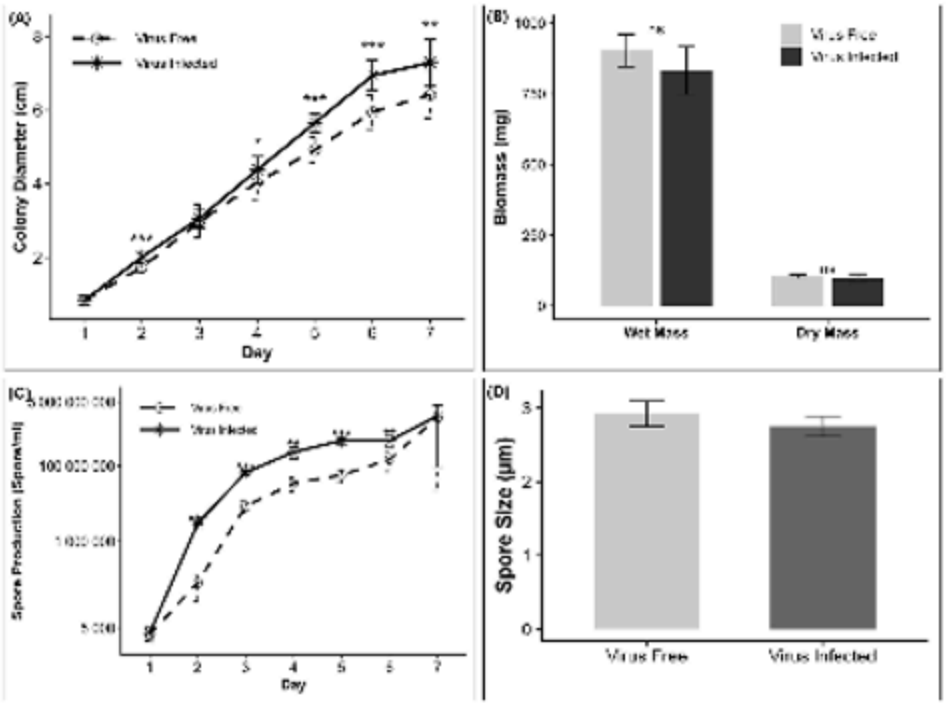


Figure 4

Effects of mycovirus infection on growth, biomass, and sporulation of *A. fumigatus* AAA12. (A) Radial growth of virus-infected (VI) and virus-free (VF) isogenic lines on potato dextrose agar (PDA) measured over 7 days. (B) Biomass production in potato dextrose broth (PDB). (C) Conidial production. (D) Spore size distribution. Data are presented as mean ± standard deviation (SD). Asterisks indicate statistical significance as assessed by two-way ANOVA: *, P-value<0.05; **, P-value<0.01; ***, P-value<0.001.

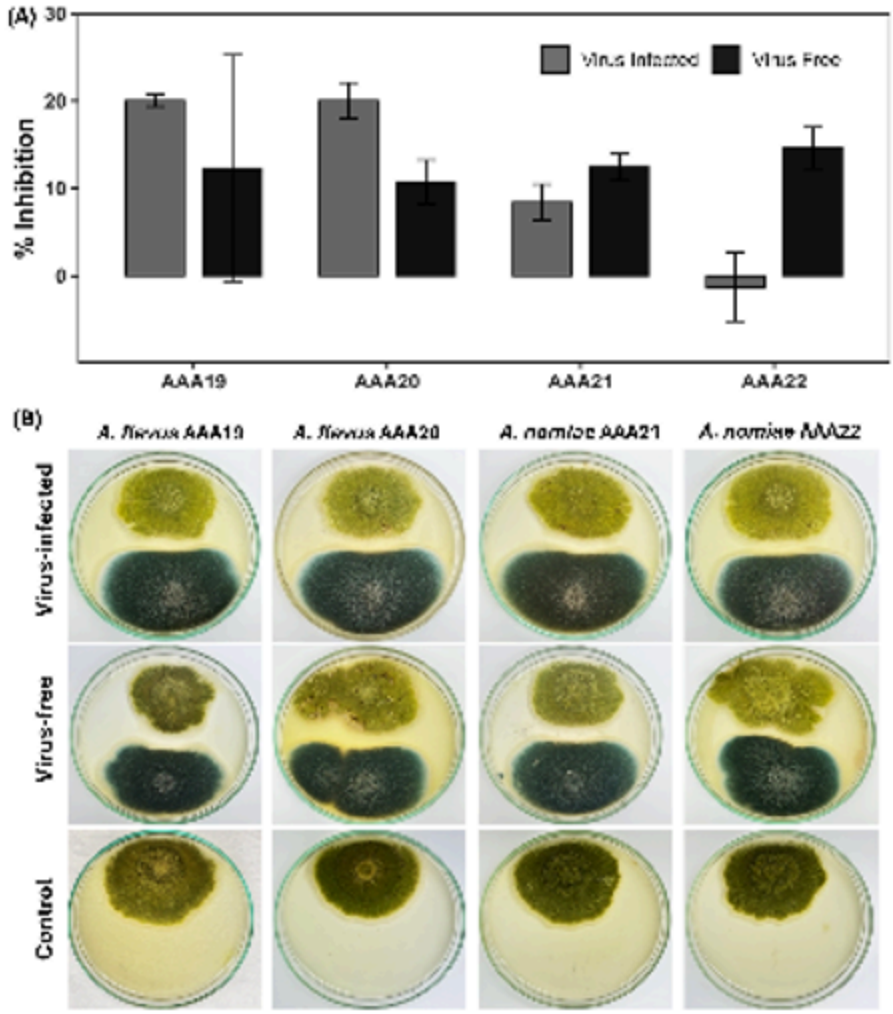


Figure 5

Effects of mycovirus infection on the antagonistic activity of *A. fumigatus*AAA12. (A) Growth inhibition (%) of aflatoxin-producing isolates AAA19 and AAA20 (*A. flavus*), and AAA21 and AAA22 (*A. nomiae*), in dual-culture assays with virus-infected (VI) and virus-free (VF) AAA12 isogenic lines. Bars represent mean ± SD (n = 3). (B) Representative images of dual-culture assays illustrating colony interactions between AAA12 (VI or VF) and each isolate, alongside monoculture controls.

Supplementary Files

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