Hypovirulence-associated Mycovirus Epidemics Cause Pathogenicity Degeneration of Beauveria bassiana in the Field

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Research Article

Keywords: mycovirus, epidemic, entomogenous fungus, Beauveria bassiana, BbCV2, biological control, hypovirulence mechanism

DOI: https://doi.org/

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Additional Declarations: No competing interests reported.
Hypovirulence-associated Mycovirus Epidemics Cause Pathogenicity Degeneration of *Beauveria bassiana* in the Field

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Abstract

Background

The entomogenous fungus *Beauveria bassiana* is used as a biological insecticide worldwide, but its application is affected by pathogenicity degeneration in the field. Previous studies showed that multiple factors contribute to this phenomenon. Mycovirus
infection causes hypovirulence of phytopathogenic fungi and mycoviruses have been used for plant disease biocontrol. However, it remains unknown whether mycovirus epidemics are key factors causing hypovirulence of *B. bassiana* naturally in the field.

Methods

A mycovirus Beauveria bassiana chrysovirus 2 (BbCV2) we have previously identified was employed to clarify its impact on the pathogenicity of host fungi *Beauveria bassiana* against the larvae of insect pest *Ostrinia furnacalis*. Wild strains of *B. bassiana* were collected from different geographic locations in Jilin Province, China, to clarify the epidemic and genetic diversity of the mycovirus BbCV2. The serological analysis was conducted by preparing polyclonal antibody against a BbCV2 coat protein, to determine whether the it can dissociate outside the host fungi cells and subsequently infect new hosts. Transcriptome analysis was used to reveal the interactions between viruses and hosts.

Results

We found that the mycovirus BbCV2 possessed efficient and stable horizontal and vertical transmission capabilities and caused *B. bassiana* hypovirulence. Surprisingly, this virus was prevalent in the field as a core virus in wild *B. bassiana* strains, without obvious
genetic differentiation. The serological results showed that the virus could not only replicate within the host cell, but also dissociate outside to infect new hosts. Transcriptome analysis revealed decreased expression of genes related to insect epidermis penetration and toxin metabolism in *B. bassiana* caused by mycovirus infection.

**Conclusion**

*B. bassiana* infected by hypovirulence-associated mycovirus can spread the virus to new host strains after infecting insects, and cause the virus epidemics in the field. The findings confirmed that mycovirus infection may be an important factor affecting the pathogenicity degradation of *B. bassiana* in the field.

**Keywords**: mycovirus, epidemic, entomogenous fungus, *Beauveria bassiana*, BbCV2, biological control, hypovirulence mechanism

**Background**

Mycoviruses can infect phytopathogenic fungi, reducing their pathogenicity toward plants, and providing a means to achieve biological control of plant disease [1]. For example, hypovirulent strains of *Cryphonectria parasitica* infected with mycovirus Cryphonectria hypovirus 1 (CHV1) were released in a forest, and
the expansion and spread of chestnut blight were effectively controlled by the epidemic [1]. Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) can directly infect hyphae of the host phytopathogenic fungus *Sclerotinia sclerotiorum* *in vitro* with high efficiency, and direct spraying of the virion on plant leaves can control Sclerotinia disease [2]. Similarly, Sclerotinia sclerotiorum partitivirus 1 (SsPV1) can inhibit the growth of *S. sclerotiorum* and significantly decrease host pathogenicity [4], and the mycovirus *Heterobasidion* partitivirus 13 strain an1 (HetPV13-an1) can diminish the pathogenicity and growth rate of host fungi [5].

*Beauveria bassiana* is an entomogenous fungus that has been used worldwide and developed into a variety of commercial agents for biological control of pest insects in forestry and agriculture [Error! Reference source not found.-8]. However, virulence toward insect pests decreases during the preservation and application of *B. bassiana* strains, limiting the efficient application of waste resources [9]. The mechanism of its hypovirulence has been extensively studied, including host specialisation and genetic diversity [10, 10], the impact of environmental factors [12, 13], nutritional factors [14, 15], morphology and physicochemical characteristics of plant surfaces where fungi are applied [16, 17].
Moreover, insects have pathogen recognition systems and immune defence capabilities that can also counteract entomogenous fungi [18-Error! Reference source not found.].

In recent years, more and more mycoviruses have been isolated and characterised from *B. bassiana* [20-25], most of which do not impact the virulence of *B. bassiana*. However, a few mycoviruses possess the ability to alter the biological characteristics and virulence of *B. bassiana*. Kotta and Coutts (2017) found that 16 of 75 strains of *B. bassiana* from different locations around the world contained mycoviruses, among which mycoviruses Beauveria bassiana polymycovirus 1 (BbPmV-1) and Beauveria bassiana polymycovirus 3 (BbPmV-3) were found to affect pigment deposition, spore production and colony growth of their host fungus by interfering with basic metabolic pathways [26, 27]. However, it is unclear whether mycoviruses have the ability to cause epidemics, and thereby induce hypovirulence of *B. bassiana* populations in the field, even though mycoviruses are able to undergo interspecific, intraspecific and vertical transmission in *Beauveria* spp. [29]. Therefore, whether mycoviruses are transmitted through direct hyphal contact or migrate outside to infect other host cells following their replication in host fungal cells remains to be elucidated.
To explore these questions, we selected the mycovirus Beauveria bassiana chrysoviruses 2 (BbCV2) that have been identified in previous work [30], which infects various Beauveria species [29], and investigated whether the virus (1) can decrease the pathogenicity of the host B. bassiana strain; (2) is prevalent and genetically stable in wild B. bassiana populations in the field; and (3) can dissociate outside host fungus cells to infect other strains; we also (4) probed the mechanism through which mycoviruses affect host fungi. The results help to reveal whether mycoviruses are key factors causing B. bassiana hypovirulence in preservation and field applications.

**Materials and methods**

**Fungal material and horizontal transmission of viruses**

B. bassiana strain BbOFZK152 is naturally infected by mycovirus BbCV-2 alone (30), while BbOFDH1-5-GFP is a virus-free isogenic strain isolated from Ostrinia furnacalis and labelled with the phosphinothricin resistance gene bar and green fluorescence protein (GFP), preserved in the China General Microbiological Culture Collection Center (CGMCC No. 15673). The two strains were cultured on potato dextrose agar (PDA) at 26 °C, and stored on PDA slants at 4 °C in the Jilin Key Laboratory of Agricultural
Microbiology. Isogenic strains with and without BbCV2 infection were obtained via the insect co-infection method as described previously [29]. Coinfected insect bodies harbouring the virus-infected donor strain BbOFZK152 and virus-free recipient strain BbOFDH1-5-GFP were fed at 25 °C for 7 to 10 days as described previously [Error! Reference source not found.] to obtain muscardine cadavers. Conidia from fungus-infected muscardine cadavers were inoculated on Czapek-Dox Agar medium containing 200 μg/mL phosphinothricin for screening of BbOFDH1-5-GFP strains, and monoclones of 50 of these were incubated on PDA medium for 10 days to obtain mycelia for virus detection. The presence of the virus in BbOFDH1-5-GFP strains was confirmed by double strand RNA (dsRNA) extraction[32] and Reverse Transcription-polymerase Chain Reaction (RT-PCR) with primers CVR (5’-TCCGTAGGTGAACCTGCGG-3’) and CVF (5’-TCCTCCGCTTATTGATATGC-3’) specific to BbCV2. Three virus-infected BbOFDH1-5-GFP strains, designated BbOFDHC1, BbOFDHC2 and BbOFDHC3, as well as three virus-free strains BbOFDH1, BbOFDH2 and BbOFDH3 derived from monospores of BbOFDH1-5-GFP, were randomly selected for virulence evaluation, virus detection and transcriptomic analysis. Evaluation of vertical spread efficiency of BbCV2 was performed as described previously
[29], in which the three virus-infected strains above were subcultured on PDA medium for three generations and verified by dsRNA extraction and RT-PCR. Tests for each strain were repeated three times.

**Virulence assays**

Fungal virulence toward *O. furnacalis* was determined using the dip method. Third-instar *O. furnacalis* larvae were dipped into 2 mL suspensions (10⁷ spores/mL) for 20 s and reared normally on artificial feed. *O. furnacalis* larvae were divided into seven groups (BbOFDH1, BbOFDH2, BbOFDH3, BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3, and sterile 0.05% Tween-80 as a blank control). Each group included three replicates with 20 larvae in each replicate. The number of dead insects was recorded every 24 h, beginning on day 2 and ending on day 8, and survival curves was plotted using GraphPad Prism 8 (Dotmatics Ltd, Windhill, UK).

A 1 mL volume of conidia (1×10⁷ conidia/mL) of *B. bassiana* strains with and without virus infection were added in the same volume (50 mL) of Sabouraud Dextrose Medium with Yeast Extract (SDY) for shaking cultivation at 26 °C for 72 h, then centrifuged at 12,000 rpm for 10 min. The supernatant was removed, dried, and
fungal bodies were placed in a drying oven at 40 °C for 2 days. The dry weight of all samples was measured using a 1/10000 balance (OLABO, Jinan, China). Each fungal strain included six replicates.

**Detection of mycoviruses in wild strains of *B. bassiana***

In the autumn of 2020, we collected and identified 106 strains of *B. bassiana* isolated from *O. furnacalis* muscardine cadavers in corn fields in seven different locations in Jilin Province, China (Table S1). All strains were stored at -80°C. The tested strains were activated and cultured on PDA medium. Ten strains from each collection site were randomly selected, and the forementioned methods were used for dsRNA extraction and RT-PCR detection to determine the BbCV2 infection status.

**Table S1 Tested strains**

<table>
<thead>
<tr>
<th>Collection locations</th>
<th>Geographical coordinates</th>
<th>Codes</th>
<th>Numbers of stains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baishan</td>
<td>E 128°11'21&quot;, N41°24'32&quot;</td>
<td>BS</td>
<td>21</td>
</tr>
<tr>
<td>Fusong</td>
<td>E 127°48'41&quot;, N42°17'55&quot;</td>
<td>FS</td>
<td>12</td>
</tr>
<tr>
<td>Yongji</td>
<td>E 126°50'44&quot;, N43°67'87&quot;</td>
<td>YJ</td>
<td>15</td>
</tr>
<tr>
<td>Antu</td>
<td>E 129°48'11&quot;, N42°21'18&quot;</td>
<td>AT</td>
<td>15</td>
</tr>
<tr>
<td>Location</td>
<td>Coordinates</td>
<td>Code</td>
<td>Number</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------</td>
<td>------</td>
<td>--------</td>
</tr>
<tr>
<td>Changling</td>
<td>E 123°96'75&quot;, N44°27'59&quot;</td>
<td>CL</td>
<td>16</td>
</tr>
<tr>
<td>Lishu City</td>
<td>E 123°46'28&quot;, N43°22'30&quot;</td>
<td>LS</td>
<td>13</td>
</tr>
<tr>
<td>Dongfeng</td>
<td>E 125°53'10&quot;, N42°67'69&quot;</td>
<td>DF</td>
<td>14</td>
</tr>
</tbody>
</table>

**Genetic diversity analysis**

Three strains of *B. bassiana* were randomly selected from each different collection location confirmed to be infected by virus BbCV2 by dsRNA extraction and RT-PCR. Three pairs of primers (Table S2) were designed for full-length amplification of nucleotide sequences of the RNA-dependent RNA polymerase (RdRp) gene of virus BbCV2 (GenBank no. MW314841.1). Products obtained by RT-PCR amplification were extracted from 1% agarose electrophoresis gels, cloned using a TA/Blank Zero Cloning Kit (Vazyme, Nanjing, China), transformed into competent *Escherichia coli* DH5-α cells (Vazyme) by heat shock, and sequenced by Sangong Bioengineering Co. Ltd. (Shanghai, China). The complete sequence of the BbCV2 RdRp gene was obtained by splicing using DNAstar7.1 (DNASTAR, Inc, Madison, USA) and DNAMAN version 9 (Lynnon Biosoft, Vaudreuil, Canada). Modification and splicing of the measured cDNA gene sequence were assessed using Snap
Gene 6.0.2 (Dotmatics Ltd, Windhill, UK) and DNAMAN (LynnonBiosoft, Vaudreuil, Canada) 9. MEGA11 (Mega Limited, Auckland, New Zealand) and DNAsp5 (University of Barcelona, Barcelona, Spain) were used to construct a phylogenetic tree of virus BbCV2 in the selected *B. bassiana* strains collected from different locations in Jilin Province, and the genetic distance was calculated.

**Table S2 Primers for RdRp gene amplification**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5´-&gt;3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCV1-2F</td>
<td>CTACCGCAAAAGAGAAAAAGC</td>
</tr>
<tr>
<td>RCV1-2R</td>
<td>GGTCAGCGATCTCCTTAGCC</td>
</tr>
<tr>
<td>RCV2-2F</td>
<td>GACGTGGGCCGGATAATAC</td>
</tr>
<tr>
<td>RCV2-2R</td>
<td>AGCCGGGTCTACAACCTTCG</td>
</tr>
<tr>
<td>RCV3-2F</td>
<td>ATTCGGGCATGGGATGTGT</td>
</tr>
<tr>
<td>RCV3-2R</td>
<td>CGGTAGATTGTTGTGCAATCTG</td>
</tr>
</tbody>
</table>

**Antibody preparation and virus-specific detection of BbCV2 virus**

The dsRNA of *B. bassiana* strain BbOFZK152 was extracted for reverse transcription using an Oligo dT primer kit (TakaRa, Dalian, China), and the cDNA of gene BbCV2 coat protein gene
(BbCV2-CP) (GenBank No. MW314842.1) was amplified by PCR using specific primers, which were (CV2-F, 5´-CCGCTCGAGATGGCCGCGTCCTCAGCACCAGT-3´) and (CV2-R, 5´-CGCGGATCTCACCCACGGTCCTCCTGTCCAGC-3´) possessing XhoI and BamHI restriction enzyme sites. Construction of recombinant vector, genetic transformation and protein expression were performed as previously described [33]. Recombinant BbCV2-CP protein was purified from inclusion bodies using Ni NTA affinity chromatography (SOLARBIO, Beijing, China), and protein concentration was determined using a BCA protein quantification kit (Vazyme). Purified BbCV2-CP protein was desalinated and concentrated using a protein ultrafiltration concentration tube, and collected protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Animal immunisation for polyclonal antibody preparation was performed as described previously [33] using six Japanese large-eared rabbits (Liaoning Changsheng Biotechnology Co. Ltd. Shenyang, China). Three rabbits were immunised with purified recombinant protein, and three (controls) were immunised with a mixture of normal saline and incomplete Freund’s adjuvant. All animal experiments complied with relevant experimental animal
welfare regulations. The titre of polyclonal antibody detection was determined using indirect enzyme linked immunosorbent assay (ELISA) as previously described [34, 34]. OD450 values of polyclonal antibodies (P) and of negative serum wells (N) were determined. Antibody titre tests were conducted three times and average values of P and N were calculated at each dilution. An average P/N ration >2 was considered positive.

Virus-infected and virus-free strains of *B. bassiana* were cultured on PDA for 10 days, fungal hyphae was scraped for total protein extraction using a Fungal Protein Extraction Kit (SOLARBIO) according to the manufacturer’s instructions, and protein lysis solution was assessed by western blotting as described previously [36].

**Virus detection within and outside *B. bassiana* cells**

Five randomly selected strains of *B. bassiana* strains harbouring virus BbCV2 verified by RT-PCR were used for virus detection *in vitro* and *in vivo* by indirect ELISA. Five randomly selected virus-free *B. bassiana* strains verified by RT-PCR served as negative controls, purified BbCV2-CP protein served as a positive control, and double-distilled served as a blank control.
Fungal strains were cultured in SDY medium until hyphae formation, the supernatant and pellet were separated by centrifugation, the sediment was washed three times with sterile water to remove culture medium residues, frozen in liquid nitrogen and thawed three times, and lysed by Scientz JY98-IIIDN ultrasonic instrument (Ningbo, Zhejiang, China). Samples were centrifuged, the supernatant was removed, and the pellet was resuspended in phosphate-buffered saline (PBS). These samples and the liquid culture medium used for fungi cultivation were subjected to virus detection by indirect ELISA.

**Detection of virus in insect bodies**

Second instar larvae of Asian corn borer (ACB) were infected with conidia of virus-infected strain BbOFDHCV1 and virus-free strain BbOFDH1. Larvae without *B. bassiana* infection served as blank controls. Ten insects were used in each treatment, and mycelia of strain BbOFDHCV1 cultured in liquid medium served as positive controls. Four days after inoculation, insect bodies were dissected with a sterilised scalpel to obtain epidermis, which was ground in PBS for indirect ELISA detection. Additionally, longitudinally cut insect epidermis from each
treatment was mixed with 500 μL 4% paraformaldehyde, fixed for 30 min, centrifuged at 4000 rpm for 15 min to remove paraformaldehyde, rinsed three times in PBS, and blocked with 5% skimmed milk at 37°C for 1 h. Primary antibody against BbCV2-CP was diluted 1:5000, incubated with samples at 37°C for 1 h, and secondary antibody, FITC-labelled goat anti-rabbit IgG (Thermo Fisher Scientific, Shanghai, China) at a 1:6000 dilution was incubated at 37°C in the dark for 1 h. Samples were observed using a Leica DM RBE laser scanning confocal microscope (Zeiss, Oberkochen, Germany) at a wavelength of 488 nm for hyphae of *B. bassiana* and 495 nm for hyphae of *B. bassiana* and virions of BbCV2.

**RNA-Seq data processing and analysis**

Based on the results of virulence against pests, virus-infected strains BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3, as well as virus-free strains BbOFDH1, BbOFDH2 and BbOFDH3 were subjected to transcriptome sequencing. Following incubation at 26°C for 10 days, total RNA was extracted, and RNA samples were purified and used to construct six libraries. Libraries were sequenced on an Illumina HiSeq 6000 platform at Novogene
Bioinformatics Technology Co. Ltd. (Beijing, China). Raw data (raw reads) were filtered and checked for sequencing error rate and GC content distribution to obtain clean reads for subsequent analysis. The genome of *B. bassiana ARSEF 2860* (NCBI accession number: ADAH00000000) was employed as the reference genome. Clean reads from each library were searched against the *B. bassiana* genome database using the HISAT2 program [36]. Gene expression levels were quantified and corrected for sequencing depth and gene length using fragments per kilobase of exon per million mapped fragments (FPKM) [38]. Differential expression analysis between groups was performed using the DESeq2 R package (1.20.0) [38] and *p*-values were adjusted to control the false discovery rate (FDR) [40]. Differentially expressed genes (DEGs) were assigned when an absolute value of log$_2$Ratio (fold change) >1 at a threshold q-value <0.05 was obtained (5% FDR). Gene function enrichment analysis is used to categorise the functions of genes using different databases. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analyses of DEGs were performed using the clusterProfiler R package (3.8.1). Significantly enriched functional categories were assigned when the corrected *p*-value was less than the threshold of 0.05 [40].
**Statistical analysis**

One-way analysis of variance (ANOVA) was performed using SPSS software version 26.0 (SPSS Inc., Chicago, IL, USA). Significant differences were determined with Duncan’s multiple range test.

**Results**

**Horizontal transmission of BbCV2**

BbCV2 viruses were successfully transmitted from virus-infected strain BbOFZK152 to virus-free strain BbOFDH1-5-GFP by co-infection assay. After 10 days of coinfection, conidia from muscardine cadavers were isolated and cultured on Czapek-Dox Agar medium containing phosphinothricin, and monocolonies were cultured on PDA medium for virus infection detection. Viral dsRNA extraction and RT-PCR amplification results showed that 39 of 50 BbOFDH1-5-GFP isolates were positive. Three virus-infected strains (BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3) and three randomly selected virus-free strains (BbOFDH1, BbOFDH2 and BbOFDH3) were used for subsequent experiments (Fig. 1). The results revealed stable and efficient vertical transmission of BbCV2.
in *B. bassiana*. After two generations of subculture, in each generation of strains BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3, the virus-harbouring rate was >73.33 ± 5.77%, revealing no significant differences between different generations of each strain or the same generation among the three strains (Table S3).

**Fig. 1** Horizontal transmission of BbCV2 via insect coinfection of *B. bassiana* strains. (A) Muscardine cadavers of insects resulting from coinfection with virus-free and virus-infected strains; (B) Detection of viral dsRNA in recipient strains via treatment with DNase I and S1 nuclease; (C) RT-PCR amplification of BbCV2 in recipient strains. Reverse transcription products from strain BbOFZK152 served as positive controls (+). Reverse transcription products were replaced by distilled deionised water in negative controls (-).
**Table S3 Efficiency of virus vertical transmission of *B. bassiana* via subculture**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Virus-harbouring rate of the 2nd generation</th>
<th>Virus-harbouring rate of the 3rd generation</th>
<th>Significance of the differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbOFDHCV1</td>
<td>73.33 ± 5.77%</td>
<td>76.67 ± 5.77%</td>
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</tr>
<tr>
<td>BbOFDHCV2</td>
<td>80.00 ± 10.00%</td>
<td>80.00 ± 0.00%</td>
<td>ns</td>
</tr>
<tr>
<td>BbOFDHCV3</td>
<td>76.67 ± 11.55%</td>
<td>76.67 ± 5.77%</td>
<td>ns</td>
</tr>
<tr>
<td>Significance of differences</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Data were analysed using SPSS software ver. 26.0 (one-way ANOVA) and Duncan’s multiple range test (*p* <0.05). ns, no significant difference.

**BbCV2 infection causes fungal host hypovirulence**

Infection with BbCV2 virus caused the hypovirulence and biomass decline of *B. bassiana*. The survival rate of *O. furnacalis* larvae infected with three virus-free strains and three virus-infected strains was significantly higher than that of virus-infected strains post-inoculation from 3 days to 8 days. Thus, compared with virus-free strains BbOFDH1, BbOFDH2 and BbOFDH3, the virulence of virus-infected strains BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3 was decreased significantly (Fig. 2A), and the biomass of the virus-free strains was significantly higher than that of the virus-infected strains, there were no
differences within the group (Fig. 2 B).

**Fig. 2** Virulence and biomass evaluation of *B. bassiana* strains with and without BbCV2 infection. (A) Evaluation of virus-infected and virus-free *B. bassiana* strains; (B) Biomass of virus-infected and virus-free *B. bassiana* strains. Error bars indicate standard deviation; ns = no significant difference; ** = significant difference. Duncan’s multiple range test (*p* <0.01).

**Epidemics and genetic diversity of BbCV2**

BbCV2 virus showed an extremely high virus-harbouring rate in wild *B. bassiana* strains. The results of dsRNA extraction morphology showed that *B. bassiana* strains from all different geographical sites were infected by different dsRNA viruses, and RT-PCR detection showed that BbCV2 was the major virus (Fig. 3A).
The statistical results showed that the lowest infection proportion of virus BbCV2 in *B. bassiana* strains was 80% from Yongji, compared with up to 100% from Baishan, Fusong, Antu and Changling (Fig. 3B), and the average virus-harbouring rate of all tested strains was 90%. BbCV2 virus was genetically stable independent of geographical location.

Fig. 3 Detection and infection proportion of BbCV2 virus in *B. bassiana* strains, and analysis of genetic diversity from different collection sites. (A) dsRNA mycovirus detection of *B. bassiana* strains. Left: dsRNA extraction of all tested fungal strains; (+) = dsRNA from strains without DNase I and S1 nuclease treatment; (-) = dsRNA from strains with...
DNase I and S1 nuclease treatment. Right: detection of BbCV2 infection in B. bassiana strains by RT-PCR; (+) = original virus-infected B. bassiana strain of BbOFZK152 as positive control; (-)=original virus-free B. bassiana strain of BbOFDH-bar as negative control. (B) Proportion of BbCV2 infection in B. bassiana strains from different collection sites. (C) Nucleotide phylogenetic tree of virus BbCV2 collected from different regions and 32 mycoviruses.

Nucleotide sequences of the BbCV2 RdRp gene from 21 strains from different sites were concatenated and corrected, resulting in a total length of 3345 bp. The (+G) model of Tamura Nei (1993) was used to evaluate the base composition of the full-length RdRp gene sequences of all BbCV2 viruses from different collection sites (Table S4). The average A, U, C, and T content of RdRp gene fragments of BbCV2 viruses was 21.60%, 20.08%, 25.37% and 32.93%, respectively. The G+C content (58.31%) was higher than the A+U content (41.69%). There was no significant difference in the base content of RdRp sequences of BbCV2 viruses among different collection sites. Multiple comparison results showed that the nucleotide sequences of the RdRp gene of BbCV2 viruses in Jilin Province shared very high nucleotide sequence similarity of 99.78% and amino acid sequence similarity of up to 99.98%. Phylogenetic tree construction showed that the nucleotide
sequence of the RdRp gene of 21 selected BbCV2 viruses formed an evolutionary cluster with 32 RdRp mycovirus sequences (Fig. 3C and Table S5), and there was no significant differentiation phenomenon, hence the BbCV2 virus was genetically stable during epidemics in the field.

Table S4 Base content of RdRp gene sequences of BbCV2 viruses

<table>
<thead>
<tr>
<th>Regions</th>
<th>A</th>
<th>U</th>
<th>C</th>
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<td>25.38%</td>
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<td>BS</td>
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<td>20.11%</td>
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<td>CL</td>
<td>21.61%</td>
<td>20.10%</td>
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<td>DF</td>
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<tr>
<td>FS</td>
<td>21.61%</td>
<td>20.10%</td>
<td>25.36%</td>
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Table S5 Sequence information

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Efficient and specific polyclonal antibody production

A highly efficient and specific polyclonal antibody was successfully prepared. The results showed that a large amount of protein was expressed with a relative molecular weight of ~85 kDa, similar to the expected size of BbCV2-CP (Fig. S1A). The protein was purified using denaturing Ni\textsuperscript{2+} affinity chromatography, and high-purity recombinant protein was obtained by elution with 250 mM imidazole (Fig. S1B). Before immunising animals with proteins as antigens, purified proteins were concentrated and desalted using ultrafiltration tubes (Fig. S1C).

Fig. S1 SDS-PAGE analysis of purified recombinant BbCV2-CP protein.

(A) SDS-PAGE analysis of recombinant BbCV2-CP protein. Lane 1, supernatant without IPTG induction; Lane 2, supernatant with IPTG induction; Lane 3, pellet with IPTG induction; Lane 4, pellet without IPTG induction; (B) SDS-PAGE analysis of recombinant protein purification. Lane 1, crude lysate; Lane 2, eluted protein. Lane 3-5, fractions eluted with 10, 20 and 250 mM imidazole, respectively; (C) Ultrafiltration of recombinant
BbCV2-CP. Protein. Lane 1, recombinant BbCV2-CP protein following ultrafiltration.

The titre of BbCV2-CP polyclonal antibody was measured from 1:500 to 1:8,129,000 at multiple dilution ratios, and the highest dilution of the polyclonal antibody was considered its titre (Fig. S2). The results showed that the purified polyclonal antibody had a titre of 1:8,192,000, indicating that the polyclonal antibody obtained by immunising rabbits with purified BbCV2-CP protein as antigen had a good antigenic immune response.

![Fig. S2 Determination of the polyclonal antibody titre of BbCV2-CP protein](image-url)
Western blotting was performed on virus-infected and virus-free *B. bassiana* strains (Fig. 4). Clear specific bands of 85 kDa were observed for virus-infected strains but not virus-free fungal strains, indicating that the prepared antibody had good immunological activity and good specificity, and could be used for virus detection in host fungi.

![Western blot image](image)

**Fig. 4 Detection of BbCV2 virus in *B. bassiana* by Western blot**

Lane M, protein markers; Lane 1-2, blank controls; Lane 3-5, virus-free BbOFDH1-3 strains; Lane 6-8, virus-infected BbOFDHCV1-3 strains.

**BbCV2 is secreted outside host fungal cells**

BbCV2 virus displayed the ability to disperse outside host fungi not only in liquid culture media but also in insect bodies. The prepared BbCV2-CP polyclonal antibody was used to detect the presence of virus both in and outside fungal strains infected by BbCV2 by indirect ELISA. The results showed that both fungal
hyphae and liquid medium samples were positive (P/N value >2; Fig. S3A). Furthermore, identical results were observed for all insect epidermis samples infected with the virus-infected fungal strain, for which the $p$-value was more than twice that of the negative control and the group infected with the virus-free fungal strain, confirming the presence of the virus in insect bodies (Fig. S3B).

**Fig. S3** Detection of BbCV2 virus in and outside host cells by **indirect-ELISA.** (A) Liquid culture medium. 1, blank control; 2, positive control (BbCV2-CP); 3, negative control (supernatant of virus-free strains); 4, supernatant of virus-harbouring strains; 5, negative control (pellet of virus-free strains); 6, pellet of virus-harbouring strains; (B) Insect bodies. 1-2, negative control (larvae without *B. bassiana* infection); 3-4, larvae infected
by BbOFDH; 5-6, larvae infected by BbOFDHCV; 7, positive control (BbCV2-CP).

The virus distribution in insect bodies following host fungi infection was also determined by immunofluorescence assay. The results showed that after infection with the virus-infected *B. bassiana* strain, at 488nm and 495 nm, hyphae of *B. bassiana* and virions were clearly observed. Furthermore, for merged 488nm and 495 nm wavelengths, either hyphae of *B. bassiana* or virions could be observed, with virions distributed around mycelia of *B. bassiana*, showing that the virus was secreted from host fungi cells following infection (Fig. 5A). For samples infected with the virus-free *B. bassiana* strain, only hyphae of *B. bassiana* were visible and virions could not be observed (Fig. 5B). For samples without *B. bassiana* infection, neither hyphae of *B. bassiana* nor virions were visible (Fig. 5C).
Fig. 5 Fluorescence observation of virus distribution in insect bodies.

(A) Insect bodies infected with virus-harbouring *B. bassiana* strains; (B) Insect bodies infected with virus-free *B. bassiana* strains; (C) Insect bodies without *B. bassiana* infection.

**Transcriptome analysis of virus-infected *B. bassiana***

*Overview of RNA-Seq data*

Raw data (raw reads) were filtered and checked for sequencing error rates and GC content distribution. The procedure yielded 40.32 Gb of clean data (clean reads) for subsequent analysis. All libraries had a Q20 value >97% and Q30 >93%. The percentage of
total sequencing reads successfully matching the genome of Bb2860 exceeded 94%, confirming the quality and accuracy of the data (Table S6). Correlation coefficients were calculated from the FPKM values of all genes in each sample and plotted as heatmaps. The intra-group R2 for strains BbOFDH1, BbOFDH2, BbOFDH3, BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3 were all >0.72 and close to 1 (Fig. S4). Values for BbOFDH1, BbOFDH2 and BbOFDH3 virus-free strains were all similar in terms of principal component 1 (PC1) and PC2. The BbOFDHCV1, BbOFDHCV2 and BbOFDHCV3 strains in the BbCV2 group were also similar for PC1 but different for PC2. However, they were also well separated from the virus-free group, indicating good biological reproducibility within groups (Fig. 6A). Following BbCV2 infection, B. bassiana yielded 1563 statistically significant DEGs, of which 835 were upregulated and 728 were downregulated (green dots in Fig. 6B).
**Figure S4 Correlation heatmap of strain samples**

**Figure 6 Transcriptome profile of RNA-Seq data.** (A) Principal component analysis of virus-free and BbPmV-4 groups; (B) Volcano plot of RNA-Seq data using log$_2$ fold change and log$_{10}$ $p$-value. Red and green dots denote up- and downregulated genes, respectively, and blue dots indicate
Table S6: Summary of sequencing data

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GO enrichment analysis of DEGs

To gain insight into the underlying gene functions regulated by BbCV-2 infection, we performed GO analysis. GO analysis of 835 upregulated genes showed that, in the biological process category, the GO terms “response to oxidative stress”, “nucleoside metabolic process”, “glycosyl compound metabolic process”,

genes with no significant expression.
“phosphorylation”, “carbohydrate biosynthetic process”, “tetrapyrrole metabolic process”, “organic acid biosynthetic process” and “carboxylic acid biosynthetic process” were the most highly enriched (Fig. 7A). The main biological processes related to down-regulated genes were “monocarboxylic acid metabolic process”, “lipid metabolic process”, “cellular lipid metabolic process”, “monocarboxylic acid biosynthetic process”, “cellular carbohydrate metabolic process” and other metabolic processes (Fig. 7B).

KEGG enrichment analysis of DEGs

To assess the pathways that play important roles in fungus-mycovirus interactions, DEGs were further categorised using KEGG pathway analysis. The 20 most significant KEGG pathways were selected for analysis. Upregulated genes were mainly enriched in “biosynthesis of secondary metabolites”, “galactose metabolism”, “starch and sucrose metabolism”, “amino sugar and nucleotide sugar metabolism” and “fructose and mannose metabolism” (Fig. 7C). Downregulated genes were mainly enriched in “carbon metabolism”, “biosynthesis of antibiotics”, “glyoxylate and dicarboxylate metabolism”, “pyruvate metabolism”
and “pentose phosphate pathway” (Fig. 7D).

**Fig. 7 GO and KEGG enrichment analyses of DEGs.** (A) GO enrichment analysis of upregulated genes; (B) GO enrichment analysis of downregulated genes; (C) KEGG enrichment analysis of upregulated genes; (D) KEGG enrichment analysis of downregulated genes.

*Effects of BbCV2 on virulence-related genes of B. bassiana*

Pathogenicity of *B. bassiana* is a complex biological process involving the adsorption of conidia onto the pest epidermis, the
formation of appressoria, and the metabolism of hydrolytic enzymes for epidermis penetration. After entering the epidermis, mycelia formed by conidia germination exploit insect nutrition and evade the host immune system [42]. During this process, toxins are metabolised and blastospores are produced, which germinate into hyphae and penetrate the pest epidermis to form new conidia [43]. Transcriptome analysis allows us to identify numerous *B. bassiana* genes potentially linked to BbCV2 virus infection.

Expression of genes encoding functional enzymes that degrade the insect cuticle was significantly downregulated following BbCV2 infection of *B. bassiana*, similar to fasciclin domain-containing proteins [43] and genes encoding BBA_00614 and BBA_05349, and fatty acid hydroxylase superfamily protein-encoding genes BBA_08427, BBA_06832, BBA_05271, BBA_03495, BBA_03308 and BBA_02581 [44]. The epicuticle of insects is rich in proteins, chitin and lipids. Subtilisin-like protease (Pr1), chitinases and cytochrome P450 (CYP) enzymes all contribute to penetration of the insect cuticle by *B. bassiana* [44-46]. Expression of Pr1 and chitinase-related gene BBA_04617 was significantly downregulated following BbCV2 infection. Genes encoding CYP52 and other CYPs, including *BBA_03806, BBA_06473, BBA_08111* and *BBA_09022*, were significantly downregulated.
B. bassiana can overcome host insect defences by expressing genes associated with stress management. Several genes encoding antioxidant enzymes, including glutathione S-transferase (GST), superoxide dismutase (SOD), catalase and oxidoreductase, are upregulated during infection [47, 48]. Polyketide synthases (PKSs) synthesize secondary metabolites such as oosporein that inhibit polyphenol oxidase (PPO) activity, which leads to inhibition of the expression of insect antimicrobial peptides [49, 50]. Infection with mycovirus Stemphylium lycopersiciernavirus 1 (SlAV1) caused downregulation of polyketone synthase 1 (PKS1), leading to specific interference and colony pigmentation of Stemphylium lycopersici, reducing its pathogenicity toward plants [51]. In the present study, PKS-related genes BBA_06613, BBA_03616 and BBA_09856 were significantly downregulated. Mitogen-activated protein kinase (MAPK) participates in the process by enhancing the ability to penetrate the insect cuticle from inside to outside [53]. Expression of MAPK-related gene BBA_09043 was significantly downregulated in the BbCV2 infected strain, although its function needs to be clarified.

Nonribosomal peptide synthases (NRPSs) are closely related to the metabolism of microbial toxins and antibiotics. B. bassiana can metabolise various toxins through NRPS pathways, including
beauveritin 14, 2-pyridone tenellin, bassianin 25, beauveritin and bassianolide [53-54]. Three virulence-related NRPS genes (BBA_04028, BBA_08222 and BBA_03671) were identified in B. bassiana isolated from Plutella xylostella [56]. Here, we identified four NRPS-related genes (BBA_09856, BBA_03616, BBA_01841 and BBA_04028) that were significantly downregulated. BBA_04028 encodes BbLaeA, and knockout of BBA_04028 diminished beauvericin and bassiatin metabolism, while overexpression of BbLaeA increased the production of toxins [56].

*Gene expression level determination by qRT-PCR*

To validate the results obtained in RNA-Seq experiments, four upregulated and six downregulated genes were randomly selected and specific primers were designed for qRT-PCR. Relative expression measured by qRT-PCR was consistent with expression levels measured by RNA-seq for all tested genes (Fig. S5), confirming that the transcriptome data were reliable.
Fig. S5 qRT-PCR verification of RNA-Seq gene expression levels

Discussion

Mycoviruses can not only reduce the pathogenicity of phytopathogenic fungi, but also convert host pathogenic fungi into a biocontrol agent [51, 57]. Unfortunately, mycoviruses can also cause hypovirulence of entomopathogenic fungi. Wang et al. (2023) found that infection with partitivirus Metarhizium majus partitivirus 1 (MmPV1) decreased conidiation and tolerance to heat shock and UV-B irradiation, and especially hypovirulence toward the insect pest of the host entomogenous fungus *Metarhizium majus* [59]. There have been few reports on mycoviruses causing *B.*
bassiana hypovirulence. In the present study, we found that mycovirus BbCV2 caused hypovirulence of B. bassiana toward the insect pest ACB, and decreased biomass.

Diminished virulence of B. bassiana during field usage and strain preservation is important for its biocontrol applications. Epidemics of mycoviruses in phytopathogenic fungi provide a biological control opportunity for plant diseases. However, whether mycoviruses that infect entomopathogenic fungi are prevalent in the field and can thereby cause hypovirulence of wild B. bassiana remains unknown. Here, we collected and characterised B. bassiana strains from infected ACB in different geographical locations in fields in Jilin Province, China, a discovered that BbCV2 virus infection was widely present in wild B. bassiana strains, with a virus-harbouring rate up to 90%. Furthermore, the virus-harbouring proportion was not correlated with collection site, indicating that the virus is likely to spread naturally within the region. Jia et al. monitored the interannual dynamics and abundance of mycovirus infections in S. sclerotiorum; 24 kinds of mycoviruses classified as members of the local core virus group exhibited persistence and relatively high transmissibility in a single crop field [59]. In the present study, the BbCV2 virus was the core virus infecting B. bassiana isolated from ACB from different sites,
and epidemics of this virus may be a key factor responsible for hypovirulence of *B. bassiana* in the field.

During interactions with host fungi, mycoviruses can mutate, leading to genetic diversity. Viral genetic diversity has important implications for virus persistence, pathogenesis and transmission [60], and is the result of long-term interactions between heritability including mutation, gene recombination, gene flow (gene migration), random drift and natural selection [62]. Previous studies demonstrated that the RdRp gene and its protein product are the only universal gene and protein among RNA viruses, making them primary targets for genetic diversity analysis of RNA viruses [63, 63]. In the present study, there were no significant differences in the RdRp base content of the tested BbCV2 viruses from different collection sites, and the G+C content was higher than that the A+T content. A higher G+C base content can endow the nucleic acid structure with greater stability and reduce genetic variations such as mismatches. No minimalist information sites were found, and phylogenetic tree analysis revealed high amino acid sequence identity, further indicating that viruses were genetically stable in their natural environments, and that the *B. bassiana* host fungus places no significant selection pressure on the virus, suggesting that the virus takes the initiative in interactions
Mycoviruses can transmit interspecifically and intraspecifically between the two.

Mycoviruses can transmit interspecifically and intraspecifically between host fungi [65, 65]. Liu et al. found that SsHADV-1 could not only infect its host fungus extracellularly, but also the mycophagous insect *Lycoriella ingenua*, which was used as a transmission vector for virus spread, and more importantly, this virus could multiply and transmit vertically within the life cycle of insects [67]. Our previous research found that BbCV2 could spread from *B. bassiana* to another two *Beauveria* species via not only hyphal anastomosis but also insect coinfection [29]. In the present study, we observed efficient transmission from virus-infected strains to virus-free strains of *B. bassiana*, as well as vertical transmission in the same strain. However, it is not clear whether the virus transmitted through direct fusion of mycelia by coculture on medium and coinfection in insect bodies, or whether virions could be released outside host fungi cells and then infect other strains. Here, we detected the virus both inside host fungal cells and in the culture medium by indirect-ELISA. Furthermore, the virus could be secreted into the insect body after infection of the host fungal strain, as determined by both indirect-ELISA and immunofluorescence assay, indicating that BbCV2 is able to escape from the original host and subsequently infect new fungal
hosts and spread, leading to virus epidemics within the *B. bassiana* population, thereby diminishing the virulence of wild *B. bassiana* strains in the field. Further research is needed to explore whether the BbCV2 mycovirus can infect insects and multiply in the insect body.

In studies on the interaction mechanisms between mycoviruses and host fungi, transcriptome data are important for clarifying the influence of viruses on the main functional genes and pathways of hosts, and transcriptomics is widely used to screen interacting genes. In *Botrytis cinerea* infected by Botryosphaeria dothidea chrysovirus 1 (BdCV1) and Botryosphaeria dothidea partitivirus 1 (BdPV1) viruses, differential genes between virus-infected and virus-free strains were identified, and DEGs enrichment analysis identified genes related to metabolic processes, cellular processes, catalytic activity, transporter activity, signal transduction and other biological pathways. Subsequent KEGG analysis identified numerous DEGs associated with metabolism, transcription and signal transduction (68). In *S. sclerotiorum*, 958 mRNAs were affected by the hypovirulent hypovirus2-L, with over 100 genes involved in basic metabolism and sugar and lipid transport [68]. In our previous research, transcriptome analysis of *B. bassiana* infected with hypervirulent
mycovirus BbPmV-4 showed that virus infection significantly increased the expression levels of some genes related to MAPK (BBA_09022) and cytochrome P450 (BBA_03806, BBA_06473 and BBA_08111) in *B. bassiana* [69]. By contrast, these genes were downregulated under BbCV2 infection. Furthermore, some genes related PKS and NRPS pathways were identified in this study. In addition, biomass was significantly decreased following virus infection, which likely causes a decrease in toxin metabolism. The hypovirulence mechanism of partitivirus MmPV1 toward host fungi is known to involve a decrease in toxin metabolism caused by infection, including effects on triterpenoids and metarhizins A and B [59]. Hence, interactions between mycoviruses and genes/pathways related to insect epidermis penetration and toxin metabolism may be involved in the mechanisms by which BbCV2 causes hypovirulence of *B. bassiana*, but this needs to be verified at the protein level though co-immunoprecipitation assays [70, 71] and pull-down assays [72], both of which are efficient methods for probing virus-host interactions. Here, a polyclonal antibody with high titre and specificity against mycovirus BbCV2 coat protein was prepared, providing a useful tool for investigating interactions between BbCV2 and its *B. bassiana* fungal host in future.
In the past, acquisition of high-virulence strains of *B. bassiana* has mainly involved isolation and screening of wild strains collected from the field using biological testing, which is time-consuming and labour-intensive [74, 75]. Here, we found that mycoviruses may be a key factor causing the decline of *B. bassiana* virulence, and virus detection could be used to exclude hypovirulent virus-infected strains, and to screen strains of wild *B. bassiana* with high virulence, thereby improving the efficiency of high virulence strain screening. To the best of our knowledge, detection of mycoviruses has mainly been performed using dsRNA and RT-PCR methods, which require complex software and can be used for qualitative but not quantitative testing. Efficient and specific serological techniques have been widely used for quantitative and qualitative detection of animal and plant viruses [76, 77], but there are few reports that they have been applied for detection of mycoviruses. Here, we prepared a highly efficient and specific polyclonal antibody against mycovirus BbCV2 coat protein, and established a serological system that expands the detection methods available for mycoviruses, especially for quantitative detection.

**Declarations**

**Ethics approval and consent to participate**
This study was approved by the ethics committee of Jilin Academy of Agricultural Sciences. We certify that the study was performed in accordance with the 1964 declaration of HELSINKI and later amendments. Written informed consent was obtained from all the participants prior to the publication of this study.

Consent for publication

A copy of the written consent is available for review by the Editor-in-Chief of the journal.

Availability of data and materials

All raw data of RNA-seq are available at Sequence Read Archive (PRJNA822034 and PRJNA993422). All materials can be obtained from corresponding author.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by the Jilin Project for Outstanding Talents (Teams) for Innovation and Entrepreneurship in Science and Technology [grant number 20230508011RC] and the Jilin Agricultural Science and Technology Innovation Project [grant number CXGC 202109GH].
Authorship contributions

Zhengkun Zhang: methodology, formal analysis, data curation, writing-original manuscript, visualization. Wenbo Guo: immunologic tests. Yang Lu: biological tests and analysis of genetic diversity. Qin Kang: Insects collection and feeding, transcriptome data analysis. Li Sui: software, validation, Hongyu Liu: virus transmission and identification. Yu Zhao: data analysis. Xiaowei Zou: transcriptome data analysis. Qiyun Li: conceptualization, resources, writing-original manuscript, project administration, funding acquisition. All authors read and approved the final version of the manuscript.

Acknowledgements

We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

References


2. Milgroom MG, Cortesi P. Biological control of chestnut blight


13. Jaronski ST, Mascarin GM. The production and uses of *Beauveria bassiana* as a microbial insecticide. World J Microbiol


41. Young, M.D, Wakefield, M.J, Smyth, G.K and Oshlack, A. Gen


expression patterns of *Botryosphaeria dothidea* infection with mycoviruses chrysoviruses 1 (BdCV1) and partitiviruses 1 (BdPV1). Virol J. 2018; 15: 126.


73. Louche A, Salcedo SP, Bigot S. Protein-Protein Interactions:


Figure 1
Figure 2

A

B

Figure 3
Figure 4

Figure 5
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Figure 7
Figure 8
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Figure 10
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Figure 11
Figure 12
Figure 13

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