Hypovirulence and Double-Stranded RNA in *Botrytis cinerea*

M. D. Wu, L. Zhang, G. Q. Li, D. H. Jiang, M. S. Hou, and H.-C. Huang

First, second, third, fourth, and fifth authors: The Key Laboratory of Plant Pathology of Hubei Province, Huazhong Agricultural University, Wuhan 430070, Hubei, China, and the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, Hubei, China; and sixth author: Agriculture and Agri-Food Canada, Research Centre, Lethbridge, Alberta, T1J 4B1, Canada.
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ABSTRACT

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Twenty-one strains of *Botrytis cinerea* isolated from 13 species of plants grown in China were compared for pathogenicity on *Brassica napus*, mycelial growth on potato dextrose agar, and presence of double-stranded (ds)RNA. The results showed that the strain CanBc-1 was severely debilitated in pathogenicity and mycelial growth, compared with the 20 virulent strains. A dsRNA of approximately 3.0 kb in length was detected in CanBc-1 and 4 hypovirulent single-conidium (SC) isolates of CanBc-1, but was not detected in the 20 virulent strains of *B. cinerea* and 4 virulent SC isolates of CanBc-1. Results of the horizontal transmission

experiment showed that the hypovirulent trait of CanBc-1 was transmissible and the 3.0-kb dsRNA was involved in the transmission of hypovirulence. Analysis of a 920-bp cDNA sequence generated from the 3.0-kb dsRNA of CanBc-1 indicated that the dsRNA element was a mycovirus, designated as *B. cinerea* debilitation-related virus (BcDRV). Further analyses showed that BcDRV is closely related to *Ophiostoma mitovirus* 3b infecting *O. novo-ulmi*, the causal agent of Dutch elm disease. Mitochondria and cytoplasm in hyphal cells of CanBc-1 became degenerated, compared with the virulent isolate CanBc-1c-66 of *B. cinerea*. This is the first report on the occurrence of *Mitovirus*-associated hypovirulence in *B. cinerea*.

Additional keywords: gray mold.

Botrytis cinerea Pers.: Fr. (teleomorph *Botryotinia fuckeliana* [de Bary] Whetzel) is a ubiquitous phytopathogenic fungus, which attacks more than 200 plant species and causes diseases of gray mold, leaf blight, blossom blight, or post-harvest fruit rots (16). In temperate climates, it has become a severe problem on numerous field and greenhouse crops, such as cucumber, tomato, grapes, and strawberry (16,30). Source of cultivars resistant to *B. cinerea* remains unavailable in most cultivated crops because of the necrotrophic infection nature of this pathogen (47). Therefore, control of *B. cinerea* mainly relies upon the use of fungicides. However, the chemical control measure is unsustainable due to problems such as fungicide resistance of *B. cinerea*, fungicide residues in plant produce, and environmental pollution. Exploitation of alternative measures to control *B. cinerea*, including biological control, has attracted the interest of many researchers (17).

Successful control of chestnut blight caused by *Cryphonectria* (*Endothia*) *parasitica* with hypovirulent (HV) strains of this pathogen in Europe (19), has inspired further studies on fungal hypovirulence and its potential for control of other plant pathogenic fungi (38). Studies on hypovirulence of *C. parasitica* showed that the mechanism involved in this biocontrol strategy is through the transmission of hypovirulent elements or factors from HV strains to virulent strains via hyphal anastomosis, thereby resulting in debilitation of pathogenicity of virulent strains (1).

The presence of single-stranded (ss)RNA- or dsRNA-mycoviruses in mycelia of *B. cinerea* has been reported in previous

studies (7,21,22,23,48). However, pathogenicity tests showed that hypovirulence was not always associated with infection of *B. cinerea* by mycoviruses. For example, Howitt et al. (21) reported that there was no significant difference in infection of beans between dsRNA-containing and dsRNA-free strains of *B. cinerea*. On the other hand, Castro et al. (8) found that the strain CCg425 of *B. cinerea* infected by a mycovirus (6.8-kb dsRNA) was less aggressive in infecting leaves of bean than the mycovirus-free strain CKg54 of this pathogen.

B. cinerea is a pathogen of numerous economically important crops in China (30). However, there are no reports on the presence of dsRNA or mycoviruses in *B. cinerea* in this country. The objectives of this study were (i) to report the occurrence of a hypovirulent strain of *B. cinerea* in China, (ii) to establish the association of the presence of a 3.0-kb dsRNA element with hypovirulence of *B. cinerea*, and (iii) to partially characterize the 3.0-kb dsRNA element in the hypovirulent strain CanBc-1 of *B. cinerea*.

MATERIALS AND METHODS

Source of *B. cinerea* strains. Twenty-one wild type strains of *B. cinerea* used in this study were collected in 2003 to 2005 from 13 species of host plants grown in China (Table 1). Twenty of these strains were collected by our laboratory staff and the other strain, designated as T₈, was courtesy of H. M. Zeng, the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. Derivative isolates were obtained from the hypovirulent strain CanBc-1 of *B. cinerea* in the single-conidium isolation experiment, or from the virulent strains CanBc-1c-66 or CanBc-2 in the horizontal transmission experiment as described. Stock cultures of each isolate or strain of *B. cinerea* were maintained on potato dextrose agar (PDA) made of fresh potato and stored in a refrigerator at 4°C. Working cultures were established

Corresponding author: G. Q. Li; E-mail address: guoqingli@mail.hzau.edu.cn

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains supplemental material not included in the print edition. Figure 1 appears in color online.

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by transferring a stock agar plug containing mycelial mats of each isolate or strain of *B. cinerea* onto PDA in a petri dish and incubating at 20°C for 1 to 2 weeks.

Determination of mycelial growth and colony morphology. Mycelial agar plugs (MAP) (6-mm-diameter) were removed from colony margins of 3-day-old PDA cultures of each isolate or strain of *B. cinerea*, and transferred to the center of petri dishes (9 cm diameter) containing 20 ml of PDA, one plug per dish. There were five dishes (replicates) for each isolate or strain. The dishes were incubated at 20°C in the dark. Colony diameter of each dish was measured after incubation for 2 to 15 days to determine the colony morphology and the radial growth rate (RGR), and for 30 days to determine formation of sclerotia for some isolates or strains of *B. cinerea*. $RGR\ (cm/day) = (D_3 - D_2)/2$, where D_2 and D_3 represent the diameter of 2- and 3-day-old colony in each dish, respectively. The test was repeated once.

Pathogenicity assay. Detached leaves of oilseed rape (*Brassica napus*, cultivar Zhongyou Za No. 4) were used for testing the pathogenicity of isolates or strains of *B. cinerea*. Seeds were sown in a field plot of the Huazhong Agricultural University, Wuhan, China, in early October 2004, and plants were watered as required. Leaves were excised from the central part of these seedlings of oilseed rape in early December 2004. Eight leaves of approximate size (10 × 8 cm) were selected and placed in two lines on moist towels in an enamel tray (45 × 30 × 2.5 cm, L × W × H). MAPs were removed from the colony margin of PDA cultures of each isolate or strain of *B. cinerea* using a sterilized cork borer and placed on the leaves with the mycelial side of each plug facing the leaf surface, two plugs per leaf and four leaves for each isolate or strain (Fig. 1). The trays were individually sealed with a transparent plastic film to maintain high humidity and incubated in a growth chamber at 20°C under fluorescent light (12 h light/12 h dark). Lesion diameter around each MAP on the leaves of oilseed rape was measured after incubation for 72 h. The test was repeated once.

Hypovirulence of single-conidium isolates. In order to understand the asexual inheritance of hypovirulence in the hypovirulent strain CanBc-1 of *B. cinerea*, single conidial isolates were estab-

lished. MAPs of CanBc-1 were placed on PDA in petri dishes (9-cm-diameter) and incubated at 20°C in the dark for 30 days. Dishes showing conidial production on colonies were selected. Conidia in each dish were washed off by flooding each colony with 5 ml of sterile distilled water and scraping the surface of colonies using a sterile triangle-shaped glass rod. The mixtures were filtered through a four-layered sterilized cheesecloth to remove mycelial fragments. The resulting conidial suspensions of CanBc-1 containing 3 to 5×10^4 conidia/ml were diluted 10-fold with sterile distilled water. Aliquots of 0.1 ml of the diluted conidial suspensions of CanBc-1 were pipetted onto PDA plates (9.0 cm diameter) and evenly spread with a sterilized glass rod. The plates were incubated in the dark at 20°C for 12 h. Germ tubes were individually cut from germinated conidia of CanBc-1 using a fine needle under a Zeiss dissecting microscope (29) and placed on PDA slants in glass tubes, one germ tube per slant. The slants were incubated at 20°C for 10 days and then stored at 4°C. Each slant culture was regarded as a single-conidium (SC) isolate of CanBc-1. They were tested for pathogenicity on detached leaves of oilseed rape and for mycelial growth on PDA using the parental hypovirulent strain CanBc-1 and the virulent strain CanBc-2 of *B. cinerea* as controls. The SC isolates of CanBc-1 showing average lesion diameters of less than 0.5 cm (20°C, 72 h) and the symptoms of abnormal growth on PDA were considered hypovirulent, whereas the SC isolates of CanBc-1 showing average lesion diameters of larger than 0.5 cm and normal mycelial growth on PDA were considered virulent.

Extraction, purification, and identification of dsRNA. An agar plug containing mycelial mats of each isolate or strain of *B. cinerea* was placed in a petri dish (9-cm-diameter) containing 20 ml of potato dextrose broth made of fresh potato. There were 20 dishes for each isolate or strain. The dishes were incubated at 20°C in the dark for 3 to 6 days. Mycelial mats of each isolate or strain of *B. cinerea* were harvested from dishes using a sterilized glass hook, rinsed in sterilized distilled water three times at 3 min each, blotted on sterilized paper towels to remove excessive moisture, transferred to centrifuge tubes (1.5 ml) and stored at -20°C until use. Extraction and purification of dsRNA in mycelia

TABLE 1. Origin, pathogenicity, radial growth rate, and the presence/absence of double-stranded (ds)RNA in strains of *Botrytis cinerea* collected from host plants in China

Strain	Origin (host, location, and collection time)	LD (cm) ^a	RGR (cm/day) ^b	dsRNA ^c
CanBc-1	<i>Brassica napus</i> , Wuhan, Hubei, 2003	0.0	0.5	+
CanBc-1a	<i>Lycopersicon esculentum</i> , Wuhan, Hubei, 2004	1.1	1.4	-
CanBc-1b	<i>Lycopersicon esculentum</i> , Wuhan, Hubei, 2004	1.1	1.2	-
CanBc-2	<i>Brassica napus</i> , Wuhan, Hubei, 2004	1.9	1.7	-
CanBc-4	<i>Brassica napus</i> , Xianning, Hubei, 2004	1.7	1.5	-
CarBc-1	<i>Dianthus caryophyllus</i> , Shaoshan, Hunan, 2004	2.0	1.5	-
ElaBc-1	<i>Ficus elastica</i> , Xinxiang, Henan, 2005	1.5	1.4	-
GBc-1	<i>Vitis vinifera</i> , Wuhan, Hubei, 2004	2.1	1.7	-
LetBc-1	<i>Lactuca sativa</i> , Wuhan, Hubei, 2004	1.6	1.6	-
OniBc-1	<i>Allium schoenoprasum</i> , Shengnongjia, Hubei, 2004	2.2	1.6	-
OniBc-2	<i>A. schoenoprasum</i> , Shengnongjia, Hubei, 2004	2.1	1.4	-
PeaBc-1	<i>Lathyrus odoratus</i> , Wuhan, Hubei, 2004	1.0	1.7	-
PoinBc-1	<i>Euphorbia pulcherrima</i> , Xinxiang, Henan, 2005	1.7	1.6	-
RedBc-1	<i>Codiaeum variegatum</i> , Xinxiang, Henan, 2005	1.6	1.8	-
RoseBc-1	<i>Rosa chinenses</i> , Wuhan, Hubei, 2004	2.3	1.6	-
RoseBc-2	<i>Rosa chinenses</i> , Wuhan, Hubei, 2004	1.4	1.3	-
RoseBc-3	<i>Rosa chinenses</i> , Wuhan, Hubei, 2004	1.5	1.7	-
SwpBc-1	<i>Ipomoea batatas</i> , Wuhan, Hubei, 2004	1.5	1.7	-
T8	<i>Lycopersicon esculentum</i> , Beijing, 2003	1.6	1.2	-
TagBc-1	<i>Tagetes patula</i> , Changyang, Hubei, 2004	2.1	1.7	-
TagBc-2	<i>Tagetes patula</i> , Wuhan, Hubei, 2004	1.9	1.6	-
LSD ($P = 0.05$)		0.4	0.2	

^a The pathogenicity test was conducted on detached leaves of oilseed rape at 20°C under humid conditions for 72 h. LD = lesion diameter. Each value within the column is the mean of 16 replicates.

^b The growth rate test conducted on potato dextrose agar at 20°C. Radial growth rate (RGR) was calculated on the basis of two measurements of the diameter of each colony after incubation for 48 and 72 h. Each value within the column represents the mean of 10 replicates.

^c dsRNA was extracted and purified using the cellulose CF-11, separated by electrophoresis on agarose gel, which was viewed on an UV trans-illuminator after being stained with ethidium bromide. The symbols + and - within the column represent the presence and the absence of dsRNA, respectively.

of each isolate or strain of *B. cinerea* were conducted using the procedures described by Morris and Dodds (35). dsRNA in extracts of each isolate or strain of *B. cinerea* was separated by electrophoresis on agarose gel (1%, wt/vol). The gels were stained with ethidium bromide (1.5 µg/liter) in water and viewed on an UV trans-illuminator. Confirmation of dsRNA was conducted using RNase-free DNase I, S1 nuclease, and RNase A as recommended by the manufacture (TAKARA Biotechnology Co., Ltd., Dalian, China). The molecules that can be digested by RNase A under the low salt condition (0.03 M NaCl), but cannot be digested by RNase A under the high salt condition (0.3 M NaCl) by DNase or by S1 nuclease were considered dsRNA (35).

Transmission of hypovirulence and dsRNA. The pairing culture technique (1,4,6,9,12,25,29) was used to test the transmissibility of the hypovirulent trait and the specific dsRNA element (3.0 kb) from the hypovirulent strain CanBc-1 to the virulent isolate CanBc-1c-66 or the virulent strain CanBc-2 of *B. cinerea*. CanBc-1 was used as donor, whereas CanBc-1c-66 and CanBc-2 as recipients. There were five kinds of cultures in this experiment, two kinds of double-strains-pairing cultures: CanBc-1 + CanBc-1c-66 and CanBc-1 + CanBc-2, and three kinds of single-strain cultures: CanBc-1, CanBc-1c-66, and CanBc-2. For the pair-culture treatments, an MAP inoculum of CanBc-1 (6-mm-diameter) was placed on PDA in a petri dish (9-cm-diameter) at 1-cm distance from the rim of the dish. An MAP inoculum of CanBc-1c-66 or CanBc-2 was placed beside the inoculation plug of CanBc-1 at 0.5 cm apart. For the single-strain cultures, dishes were inoculated with CanBc-1, CanBc-1c-66, or CanBc-2 alone at one MAP per dish. There were 3 dishes for each kind of culture. The dishes were incubated at 20°C in the dark and mycelial growth of CanBc-1, CanBc-1c-66, or CanBc-2 on both single-strain cultures and double-strains-pairing cultures was determined after 6 days. An agar plug (6-mm-diameter) containing mycelial mats

was removed from each colony of CanBc-1c-66 at the point far from the inoculation plug of CanBc-1 on double-strains-pairing cultures to establish three recipient derivative isolates for CanBc-1c-66, designated as CanBc-1c-66a, CanBc-1c-66b, and CanBc-1c-66c. Meanwhile, a mycelial agar plug was taken from each colony of CanBc-2 on double-strains-pairing cultures to establish three recipient derivative isolates for CanBc-2, designated as CanBc-2a, CanBc-2b, and CanBc-2c. These derivative isolates of *B. cinerea* were tested for pathogenicity on leaves of oilseed rape, radial growth rate, and production of conidia and sclerotia on PDA (20°C, 30 days), and the presence of dsRNA in mycelia. The experiment was repeated two more times.

cDNA synthesis, cloning, and sequence analyses. The strategy used to partially characterize the 3.0-kb dsRNA element in CanBc-1 of *B. cinerea* was to analyze the cDNA sequence originated from that element. CanBc-1 and CanBc-1c-78, a hypovirulent SC isolate of CanBc-1, were used in this experiment. CanBc-1 contained the 3.0-kb dsRNA element, whereas CanBc-1c-78 had 2 dsRNA elements, one is 2.3 kb and the other is 3.0 kb (Fig. 2). The 2.3-kb dsRNA might be derived from the 3.0-kb dsRNA through the internal deletion mechanism according to the previous study by Nuss and Koltin (37) with mycovirus-infected hypovirulent strain EP713 of *C. parasitica*. Firstly, the gel-purified 2.3-kb dsRNA extracted from mycelia of CanBc-1c-78 was used as a template to generate cDNA fragments following the procedure described by Xie et al. (49) with the random hexadeoxyribonucleotide primer mixture (TaKaRa Biotechnology) as primers. After cloning, sequencing and analyzing the resultant cDNA fragments, a 2152-bp cDNA sequence for the 2.3-kb dsRNA element was obtained (GenBank accession no. EF583556). It is highly homologous to the *Ophiostoma mitovirus 3b* (20) based on the result of the alignment analyses using the program of the Basic Local Alignment Search Tool (BLAST) through the

Fig. 1. A, Pathogenicity, B, colony morphology, and C, the presence of double-stranded (ds)RNA in the virulent strain CanBc-2 and the hypovirulent strain CanBc-1 of *Botrytis cinerea*. A, Two leaves of oilseed rape inoculated with CanBc-2 (left) and CanBc-1 (right) and incubated at 20°C for 72 h. Note severe infection by CanBc-2, whereas no infection by CanBc-1. B, Two 15-day-old cultures of *B. cinerea* on potato dextrose agar (20°C) showing a normal colony with numerous dark brown sclerotia for CanBc-2 (left) and an abnormal colony with mycelial sectors and without formation of sclerotia by CanBc-1 (right). C, Agarose gel electrophoregram of dsRNA extracted from CanBc-1 and CanBc-2. Digestion of each extract with S1 nuclease and DNase I was conducted before electrophoresis. Marker = wide range DNA marker (TaKaRa).

National Center for Biotechnology Information (NCBI) website (M. D. Wu and G. Q. Li, *unpublished data*). Then, a pair of specific oligonucleotide DNA primers, BCV5, 5'-TCCTAATGGTGGTGTATCA-3' and BCV6, 5'-CTGGAGAAGCTGGA-GAAA-3', was designed on the basis of the 2,152-bp cDNA sequence using the program PrimerSelect 5.00 in the DNASTar software (DNASTAR Inc., Madison, WI). Finally, reverse transcription-polymerase chain reaction (RT-PCR) was performed with the gel-purified 3.0-kb dsRNA extracted from mycelia of CanBc-1 as template and BCV5/BCV6 as primers following the procedure described by Xie et al. (49). The RT-PCR product (a cDNA fragment) was separated by 1% agarose gel electrophoresis, purified using the Fermentas DNA Extraction Kit (Jingmei Biotech. Co., Ltd., Shengzhen, China), ligated into the pMD18-T vector (TaKaRa Biotechnology), and transformed into competent cells of *Escherichia coli* JM109. Positive *E. coli* clones grown on Luria-Bertani agar medium containing ampicillin (50 µg/ml) were selected and individually tested for the size of the cDNA insert by the enzymatic method (44). One of the positive clones containing about 900-bp cDNA insert was designated as p1R2a. The cDNA insert in that clone was sequenced by the dideoxynucleotide termination method using a Big Dye Terminator Sequencing Kit (BigDye terminator version 2.0, ABI) on an ABI PRISM 377-96 automatic sequencer (Beijing AuGCT Biotechnol. Co., Ltd., Beijing, China). The cDNA sequence was used to deduce for amino acid residues using the ORF (open reading frame) Finder program in the NCBI website with the mold mitochondrial codon usage, as the deduction or translation was disrupted if the standard codon usage was applied. Multiple sequence alignment and bootstrapping analysis of the putative protein encoded by the cDNA sequence in p1R2a with those for related viruses were accomplished with the CLUSTAL W programs (46). A phylogenetic tree was displayed using the TREEVIEW program (39).

Northern hybridization. Northern hybridization analysis was performed by the method of Jiang and Ghabrial (26) to verify the authenticity of the cDNA sequence (p1R2a) generated from the purified 3.0-kb dsRNA in CanBc-1. p1R2a was labeled with [³²P] dCTP using the radiolabeling kit (TaKaRa Biotechnology Co.) and used to probe the 3.0-kb dsRNA of CanBc-1 blotted on a piece of Immobilon-Ny⁺ transfer membrane (Millipore, Bedford, MA). Autoradiographic method was used for detection of hybrid-

ization signals on the Fuji Fluorescent Image Analyzer (Model Fla-5000, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Transmission electron microscopy. A mycelial agar plug of the hypovirulent strain CanBc-1 or the virulent isolate CanBc-1c-66 of *B. cinerea* was inoculated on the center of a piece of sterilized cellophane film (8 cm diameter) placed on PDA in a petri dish (9-cm-diameter). The dishes were incubated at 20°C for 3 days for CanBc-1c-66 and 6 days for CanBc-1. Small pieces (3 × 3 mm) of the films containing mycelia of CanBc-1 or CanBc-1c-66 were sampled from the colony margin areas by cutting the films with a pair of sterilized scissors and fixed in 2% of glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.0) at 4°C overnight. The specimen film pieces were washed in 0.2 M sodium phosphate buffer (pH 7.0) three times at room temperature (20 to 25°C), 10 min each time, and post-fixed in 2% osmium tetroxide in 0.2 M sodium phosphate buffer for 2 h, and stained in 5% of uranyl acetate in 50% of ethanol for 1 h. Then, they were dehydrated in graded series of ethanol, infiltrated with Spurr's low viscosity embedding medium, and polymerized at 70°C for 8 h. Ultra-thin sections (50 to 60 nm in thickness) for the specimens of each strain of *B. cinerea* were cut with a diamond knife, mounted on slotted and formvar-coated grids, stained with 5% of aqueous lead citrate and 5% of uranyl acetate, and examined with a Hitachi transmission electron microscope (H-7000FA) at 75 kv.

Statistical analysis. Data on radial growth rates, lesion diameters, production of conidia, and sclerotia by isolates or strains of *B. cinerea* were analyzed using analysis of variance (ANOVA) in SAS (SAS Institute, Cary, NC, version 8.0, 1999). The numerous data on conidial yield of *B. cinerea* were log₁₀-transformed prior to the ANOVA. Treatment means for tested isolates or strains of *B. cinerea* in each experiment were separated using the least significant difference test at *P* = 0.05 level.

RESULTS

Hypovirulence and abnormal colony morphology of *B. cinerea* strain CanBc-1. When inoculated on detached leaves of oilseed rape, 20 strains of *B. cinerea* collected from 13 species of host plants in China, including strain CanBc-2, were virulent (Fig. 1A, Table 1), whereas the strain CanBc-1 of *B. cinerea* collected

Fig. 2. A, Mycelial growth rate, **B,** pathogenicity, and **C,** the presence of double-stranded RNA of the *Botrytis cinerea* strains CanBc-1, CanBc-2, and eight single-conidium isolates of CanBc-1. Results were expressed as means ± standard error of the mean in both pathogenicity test (*n* = 16) and the growth rate test (*n* = 10). Bars in each figure headed with the same letters are not significantly different (*P* > 0.05) according to least significant difference test. Marker = wide range DNA marker (TaKaRa). HV = hypovirulent, V = virulent.

from *Brassica napus* grown in Wuhan, China, was hypovirulent (Fig. 1A). After incubation at 20°C for 72 h, the lesion diameter varied among the virulent strains of *B. cinerea*, ranging from 1.0 to 2.3 cm (Table 1), whereas the hypovirulent strain CanBc-1 caused no infection on leaves of oilseed rape (Fig. 1A, Table 1). Mycelial growth by this hypovirulent strain around each inoculated mycelial agar plug was evident (Fig. 1A).

The 20 virulent strains of *B. cinerea* grew rapidly on PDA at 20°C with average radial growth rate ranging from 1.2 to 1.9 cm/day (Table 1). Colonies of these strains developed normally and covered the entire dish (9 cm diameter) within 3 days. Aerial mycelia, conidia, and/or sclerotia were formed in 15-day-old cultures of these strains such as CanBc-2 (Fig. 1B). In contrast, the hypovirulent strain CanBc-1 of *B. cinerea* grew slowly with the average radial growth rate of 0.5 cm/day and covered the entire dish after 10 days. It often produced sparse conidia and did not produce any sclerotia on the colonies after incubation for 15 days (Fig. 1B).

Presence of dsRNA in virulent and hypovirulent strains of *B. cinerea*. A dsRNA element was detected in mycelia of the hypovirulent strain CanBc-1 of *B. cinerea* (Fig. 1C, Table 1). The size of dsRNA element was approximately 3.0 kb as indicated by the DNA marker (Fig. 1C). In contrast, dsRNA was not detected in mycelia of the 20 virulent strains of *B. cinerea* investigated, including CanBc-2 (Table 1, Fig. 1C).

Pathogenicity and dsRNA of single-conidium isolates derived from CanBc-1. Differentiation in pathogenicity and mycelial growth was observed among single-conidium (SC) isolates derived from the hypovirulent strain CanBc-1 of *B. cinerea*. Of the total of 94 SC isolates tested, 75 isolates (79.8%) were hypovirulent (HV), whereas the other 19 isolates (20.2%) were virulent (V). For example, CanBc-1 and the four HV-type SC isolates of CanBc-1, including CanBc-1c-50, CanBc-1c-73, CanBc-1c-78, and CanBc-1c-82, grew slowly on PDA with the average radial growth rate ranging from 0.3 to 1.1 cm/day (Fig. 2A), and they failed to cause infection on leaves of oilseed rape (Fig. 2B).

The specific 3.0-kb dsRNA was detected in CanBc-1 and the four HV-type SC isolates of CanBc-1 (Fig. 2C). Moreover, a small dsRNA molecule of approximately 2.3 kb in length was detected in CanBc-1c-78 (Fig. 2C). In contrast, the four V-type SC isolates of CanBc-1, including CanBc-1c-55, CanBc-1c-64, CanBc-1c-66, and CanBc-1c-93, grew rapidly with the average radial growth rate ranging from 1.4 to 1.6 cm/day (Fig. 2A) and they caused infection on leaf tissues of oilseed rape with average lesion diameter ranging from 1.2 to 1.4 cm after inoculation for 72 h (Fig. 2B). There was no significant difference ($P > 0.05$) in lesion diameter (Fig. 2B) between these four V-type SC isolates of CanBc-1 and the virulent strain CanBc-2 of *B. cinerea*. dsRNA was not detected in mycelia of these four V-type SC isolates of CanBc-1 and CanBc-2 (Fig. 2C).

Transmission of hypovirulence and dsRNA in CanBc-1. On single-strain cultures, the virulent isolate CanBc-1c-66 and the virulent strain CanBc-2 grew rapidly and covered the entire dish after incubation for 6 days, whereas the hypovirulent strain CanBc-1 grew slowly and covered approximately one third of the dish (Fig. 3). On double-strains-pairing cultures of CanBc-1 + CanBc-2, CanBc-2 still grew rapidly and covered the entire dish after 6 days, whereas on double-strains-pairing cultures of CanBc-1 + CanBc-1c-66, CanBc-1c-66 grew rapidly during the first 3 days, followed by slow growth with the formation of profuse sporulations around the inoculation plug of CanBc-1 and failed to colonize the entire dish after 6 days (Fig. 3).

Three derivative isolates of CanBc-1c-66 (CanBc-1c-66a, CanBc-1c-66b, and CanBc-1c-66c) were obtained from the three recipient colonies of CanBc-1c-66 on double-strains-pairing cultures. These three isolates were similar to CanBc-1 in slow mycelial growth with the average radial growth rate of less than 0.7 cm/day (Fig. 4A), presence of mycelial sectors on their colonies, and reduced production of conidia (3 to 5×10^6 conidia/dish) on PDA (Table 2), and attenuated pathogenicity on leaves of oilseed rape with the average lesion diameter of less than 0.1 cm (Fig. 4A). However, these three isolates were different from their

Fig. 3. Transmission of the hypovirulence trait and double-stranded RNA from the hypovirulent strain CanBc-1 to the virulent strains CanBc-1c-66 or CanBc-2 of *Botrytis cinerea* on potato dextrose agar. All dishes were incubated at 20°C for 6 days. Note colonization of the entire dish by *B. cinerea* in single cultures of CanBc-1c-66 and CanBc-2, and in the pair culture of CanBc-2 + CanBc-1, but not in the single culture of CanBc-1 and in the pair culture of CanBc-1c-66 + CanBc-1. Also note the profuse sporulation by CanBc-1c-66 behind the colony front in the pair culture of CanBc-1c-66 + CanBc-1 (dark gray area). At the location on the colony of each pair culture marked with an asterisk "*", a mycelial agar plug was removed from the colony of CanBc-1c-66 to establish derivative isolates CanBc-1c-66a, CanBc-1c-66b, or CanBc-1c-66c, or from the colony of CanBc-2 to establish derivative isolates CanBc-2a, CanBc-2b, or CanBc-2c.

parental isolate CanBc-1c-66 which grew rapidly with the average growth rate of 1.3 cm/day, mass production of conidia (2.5×10^8 conidia/dish) on PDA (Table 2) and caused severe infection on leaves of oilseed rape with the average lesion diameter of 2.3 cm (Fig. 4A). The specific 3.0-kb dsRNA was detected in these three hypovirulent derivative isolates of CanBc-1c-66, as well as in CanBc-1, but not in CanBc-1c-66 (Fig. 4C).

CanBc-2a, CanBc-2b, and CanBc-2c were obtained from the three colonies of CanBc-2 on double-strains-pairing cultures. These three isolates were similar to CanBc-2 in rapid mycelial growth with the average radial growth rate ranging from 1.5 to 1.6 cm/day (Fig. 4B), formation of normal colony morphology, and production of conidia (5 to 7×10^5 conidia/dish) and sclerotia on PDA (Table 2), and high virulence on leaves of oilseed rape

with the average lesion diameter ranging from 2.0 to 2.6 cm after 72 h (Fig. 4B), but were significantly different ($P < 0.05$) from CanBc-1 which caused no infection and no lesions (Fig. 4A). The specific 3.0-kb dsRNA was not detected in these three derivative isolates of CanBc-2 and the parental strain CanBc-2 (Fig. 4C).

Identification of the 3.0-kb dsRNA element in CanBc-1. A cDNA fragment of 920 bp in length (p1R2a, GenBank accession no. EF580100) was obtained from the RT-PCR using BCV5 and BCV6 as primers and the 3.0-kb dsRNA extracted from mycelia of the hypovirulent strain CanBc-1 of *B. cinerea* as template. Northern hybridization confirmed the authenticity of that cDNA fragment (Fig. 5). The cDNA fragment had an open reading frame (ORF) putatively encoding a protein containing 295 amino acid residues. The putative protein was homologous to the RNA-

Fig. 4. A and B, Pathogenicity, radial growth rates and **C**, the presence of dsRNA in strains or isolates of *Botrytis cinerea*. The three derivative isolates of CanBc-1c-66 (CanBc-1c-66a, CanBc-1c-66b, and CanBc-1c-66c) and the three derivative isolates of CanBc-2 (CanBc-2a, CanBc-2b, and CanBc-2c) were obtained from the pair cultures showing in Figure 3. The results were expressed as means $\pm$ standard error of the mean for each isolate or strain in both pathogenicity test ($n = 16$) and the growth rate test ($n = 10$). Bars in each figure for each parameter headed with the same letters are not significantly different ($P > 0.05$) according to least significant difference test. HV = hypovirulent, V = virulent.

TABLE 2. Comparison of number and size of conidia and sclerotia produced by virulent (V) and hypovirulent (HV) strains or isolates of *Botrytis cinerea*

Strain	Pathotype	Conidia ^a		Sclerotia ^a	
		Log ₁₀ conidia/dish ($n = 5$)	Size (μ m) ($n = 30$)	Sclerotia/dish ($n = 5$)	Size (mm) ($n = 30$)
CanBc-1	HV	4.7	10.8 $\times$ 8.6	14	1.1 $\times$ 1.0
CanBc-1c-66	V	8.4	10.7 $\times$ 7.5	0	NS
CanBc-1c-66a	HV	6.6	11.0 $\times$ 7.9	0	NS
CanBc-1c-66b	HV	6.7	11.0 $\times$ 7.8	0	NS
CanBc-1c-66c	HV	6.5	10.9 $\times$ 8.2	0	NS
CanBc-2	V	5.7	10.9 $\times$ 8.8	52	4.7 $\times$ 3.3
CanBc-2a	V	5.6	10.5 $\times$ 7.7	68	4.9 $\times$ 3.0
CanBc-2b	V	5.8	10.5 $\times$ 8.1	68	4.0 $\times$ 2.8
CanBc-2c	V	5.9	11.6 $\times$ 8.8	87	3.9 $\times$ 2.8
LSD ($P = 0.05$)		0.30		35.8	

^a Conidia and sclerotia were collected from 30-day-old cultures grown on potato dextrose agar (20°C). NS = no sclerotial formation.

dependent RNA polymerase (RdRp) of seven members of the mycoviruses belonging to the genus *Mitovirus* (Fig. 6A), including *Cryphonectria cubensis mitovirus* 2-a (CeMV2a), *Cryphonectria parasitica mitovirus* 1-NB631 (CpMV1-NB631), *Ophiostoma novo-ulmi mitovirus* 1a-Ld (OnuMV1a), *Ophiostoma novo-ulmi mitovirus* 1b-Ld (OnuMV1b), *Ophiostoma novo-ulmi mitovirus* 3a-Ld (OnuMV3a), *Ophiostoma novo-ulmi mitovirus* 3b-Ld (OnuMV3b), and *Sclerotinia homoeocarpa mitovirus* (ShMV). Therefore, the 3.0-kb dsRNA element in the hypovirulent strain CanBc-1 of *B. cinerea* is a mycovirus. We designate it as *Botrytis cinerea* debilitation-related virus (BcDRV). Further analyses showed that the putative RdRp of BcDRV and those for the seven members of the genus *Mitovirus* contained six conserved amino acid sequence motifs (I to VI) (Fig. 6A). The motif I was considered to be a characteristic of mitoviruses according to Hong et al. (20). It contains 13 amino acids which are identical and 17 amino acids which are chemically similar in all these eight RdRp sequences, including that for BcDRV (Fig. 6A). Phylogenetic analysis on the basis of the RdRp sequences indicated that BcDRV was clustered to a group containing seven members of the genus *Mitovirus*, especially closely related to OnuMV3b (Fig. 6B), but far from two members of the genus *Narnavirus* (Fig. 6B), including *Saccharomyces cerevisiae Narnavirus* 20S (ScNV20S) and *Saccharomyces cerevisiae Narnavirus* 23S (ScNV23S), and two *Enterobacteria* phages (Fig. 6B), including M12 and MX1.

Degeneration of cytoplasm and mitochondria in CanBc-1. Observations of ultra-thin sections of hyphal cells showed that the cytoplasm and mitochondria differed greatly between the hypovirulent strain CanBc-1 and the virulent isolate CanBc-1c-66 of *B. cinerea*. CanBc-1c-66 had the electronically dense and evenly distributed cytoplasm in hyphal cells and formed numerous mitochondria with normal cristae inside each mitochondrion (Fig. 7). In contrast, CanBc-1 showed the electronically-sparse and unevenly distributed cytoplasm in hyphal cells and produced numerous malformed mitochondria without formation of cristae inside each mitochondrion (Fig. 7).

DISCUSSION

This study provides the first evidence that hypovirulence exists in populations of *B. cinerea* in China. The morphological and cultural characteristics of the hypovirulent strain CanBc-1 of

B. cinerea collected from oilseed rape in Wuhan in 2003 are similar to that of hypovirulent strains reported in other phytopathogenic fungi, including *C. parasitica* (1), *Fusarium graminearum* (10), *H. sativae* (31), *R. solani* (6,25), *O. novo-ulmi* (5), *S. minor* (34), *S. sclerotiorum* (4,29), and *S. homoeocarpa* (50). Reduced production of conidia by CanBc-1 is similar to that by the hypovirulent strain CCg425 of *B. cinerea* reported by Castro et al. (8). However, CanBc-1 appears more severely debilitated than CCg425 in conidial production on PDA medium, as the conidial production by CanBc-1 was reduced by 93.7 and 99.8%, compared to the virulent strains CanBc-2 and CanBc-1c-66 of *B. cinerea*, respectively (Table 2), whereas the conidial production by CCg425 was reduced by about 50%, compared to the virulent strain CKg54 of *B. cinerea* (8). The abnormal traits expressed in hypovirulent fungal strains, including CanBc-1 of *B. cinerea*, might reduce the ecological fitness of fungi under natural conditions, compared to the strains expressing normal traits, and consequently they might reduce the survival and dissemination of the "hypovirulent element." Prospero et al. (41) reported that the saprophytic colonization of chestnut tissues by *C. parasitica* plays an important role in the epidemiology of hypovirulence-causing mycoviruses. This mechanism may occur in *B. cinerea*, as the saprophytic phase is important for this fungus to survive and to infect plant tissues (47).

This study reveals that the 3.0-kb dsRNA element in CanBc-1 may be responsible for hypovirulence of *B. cinerea*, as it is closely associated with hypovirulence of this fungus. Further analysis of the partial cDNA sequence generated from that dsRNA element demonstrates that it is a mycovirus (BcDRV) closely related to seven members of the genus *Mitovirus* reported previously (14,15,20,40), especially to *Ophiostoma mitovirus* 3b. This is the first report on the occurrence of member of the genus *Mitovirus* in *B. cinerea*. Meanwhile, this study showed that the mitochondria in hyphal cells of CanBc-1 of *B. cinerea* became malformed without the formation of cristae inside each mitochondrion (Fig. 7). The 920-bp cDNA generated from the 3.0-kb dsRNA in CanBc-1 was translatable (to RdRp) only with the mold mitochondrial codon usage, but was not translatable with the standard codon usage. Therefore, mitochondria may be the target for this *Botrytis cinerea* debilitation-related virus (BcDRV). Additional studies by locating the 3.0-kb dsRNA element and/or BcDRV in hyphal cells of CanBc-1 are warranted. Rogers et al. (43) reported that mitoviruses targeting at mitochondria of *Ophiostoma novo-ulmi* caused deficiency in cytochrome aa3, thereby resulting in the debilitation disease for this plant pathogenic fungus. Whether or not this mechanism occurs in CanBc-1 of *B. cinerea* remains unknown and needs further studies.

Previous studies indicated that mycoviruses or dsRNA elements can be transmitted vertically to asexual spores, such as conidia (37,41), and horizontally to other virulent fungal strains of the same or different taxons through hyphal fusions (anastomosis) or heterokaryon formation (1,11,13,32,33,36,41). Horizontal transmissions of mycoviruses or dsRNA elements are usually restricted by vegetative incompatibility (1,13,32,33,36). The incompatible hyphal interaction between strains of most ascomycetous fungal species containing different vegetative incompatibility genes (*vic*) may inhibit or restrict the movement of dsRNA elements or mycoviruses (45). Death of hyphal cells was observed in intermingled areas of two incompatible colonies of fungi, including *C. parasitica* (3) and *S. sclerotiorum* (27). On the other hand, Ihrmark et al. (24) reported that dsRNA elements could be transmitted among different vegetative incompatible isolates of the basidiomycetous fungus *Heterobasidion annosum*, the causal agent of pine root rot. Death of hyphal cells was not obviously observed in hyphal interactions between different isolates of *H. annosum* (18). This study demonstrates that hypovirulence and the specific dsRNA (3.0 kb) or BcDRV were detected in four single-conidium isolates of CanBc-1 of *B. cinerea*. This result indicates that

Fig. 5. Northern blotting detection of the 3.0-kb double-stranded (ds)RNA extracted from mycelia of *Botrytis cinerea* strain CanBc-1. The plasmid p1R2a containing a cDNA sequence generated from the 3.0-kb dsRNA of CanBc-1 was used as probe with λ -HindIII digest DNA (TaKaRa) as marker.

hypovirulence and BcDRV in CanBc-1 can be vertically transmitted to its conidia. The horizontal transmission of hypovirulence and the 3.0-kb dsRNA or BcDRV was successful from CanBc-1 to the virulent strain CanBc-1c-66 of *B. cinerea*, but unsuccessful from CanBc-1 to another virulent strain CanBc-2 of *B. cinerea*. This strain-specific hypovirulence-transmission behavior might be regulated by the vegetative incompatibility existing in *B. cinerea* (2). CanBc-1c-66, a single-conidium isolate of CanBc-1, is closely related to CanBc-1 and may have the same or similar alleles at *vic* loci as CanBc-1, which may confer a compatible interaction between hyphal cells of CanBc-1 and CanBc-1c-66. In contrast, CanBc-1 and CanBc-2 collected in different years in Wuhan, China (Table 1) might be different in alleles at *vic* loci, which may confer an incompatible interaction between hyphal cells of these two strains of *B. cinerea*. Additional studies to elucidate the mechanisms restricting the transmission of the

hypovirulence trait and the 3.0-kb dsRNA or BcDRV from CanBc-1 to CanBc-2 would be helpful in developing this hypovirulent strain as a biocontrol agent for grey mould diseases.

Spontaneous appearance of genetically distinct dsRNA elements has been reported in *R. solani* (6,25,28). Previous studies showed that these dsRNAs were closely related to hypovirulence of this pathogenic fungus (6,25,28). In the present study, a small dsRNA of approximately 2.3 kb in length was detected in the hypovirulent strain CanBc-1c-78 of *B. cinerea*, a single-conidium isolate of CanBc-1 (Fig. 2). The effect of the presence of this small dsRNA on pathogenicity of *B. cinerea* remains unknown, as CanBc-1c-78 was as hypovirulent as its parental strain CanBc-1 on leaves of oilseed rape. Nuss and Koltin (37) indicated that small dsRNA elements (about 1.0 kb) in the hypovirulent strain Ep713 of *C. parasitica* were derived from the large dsRNA elements (12 to 13 kb) through the internal deletion mechanism.

Fig. 6. A, Conserved amino acid sequence motifs of the putative RNA-dependent RNA polymerases of *Botrytis cinerea* debilitation-related virus (BcDRV) and seven members of the genus *Mitovirus*. Abbreviations (database accession numbers in parentheses): CcMV2a, *Cryphonectria cubensis mitovirus* 2-a (AAR01973); CpMV1-NB631, *Cryphonectria parasitica mitovirus* 1-NB631 (AAA61703); OnuMV1a, *Ophiostoma novo-ulmi mitovirus* 1a-Ld (CAJ32466); OnuMV1b, *Ophiostoma novo-ulmi mitovirus* 1b-Ld (CAJ32467); OnuMV3a 3a-Ld (CAA06228); OnuMV3b, *Ophiostoma novo-ulmi mitovirus* 3b-Ld (CAJ32468), and ShMV, *Sclerotinia homoeocarpa mitovirus* (AAO21337). Sequences were aligned with the CLUSTAL W program. “*” indicates identical amino acid residues; “:” and “.” indicate higher and lower chemically similar residues, respectively, as defined in CLUSTAL W; **B**, Evolutionary relationships between the putative RdRps of BcDRV, seven members of *Mitovirus* mentioned above, two members of *Narnavirus* and two bacteriophages. Abbreviations (Database accession numbers in parentheses): ScNV20S, *Saccharomyces cerevisiae Narnavirus* 20S (AAC98925); ScNV23S, *Saccharomyces cerevisiae Narnavirus* 23S (AAC98708); M12, *Enterobacteria* phage M12 (AAF19635); MX1, *Enterobacteria* phage MX1 (AAC14701). The bootstrap analysis was done with the CLUSTAL W program and the tree was displayed using the TREEVIEW program. The numbers on the branches of the tree are the results of the bootstrap analyses (1,000 replicates).

Fig. 7. Transmission electron micrographs showing the difference in cytoplasm and mitochondria between CanBc-1c-66 (virulent, left) and CanBc-1 (hypovirulent, right) of *Botrytis cinerea*. Note numerous normal mitochondria containing cristae in CanBc-1c-66, whereas malformed mitochondria without formation of cristae in CanBc-1. M = mitochondria, N = nucleus.

This mechanism may occur in the hypovirulent strain CanBc-1 of *B. cinerea* in the process of asexual reproduction. This study showed that 3.0-kb dsRNA in CanBc-1 and the 2.3-kb dsRNA in CanBc-1c-78 are closely related, as the DNA primers designed on the basis of the cDNA sequence of the 2.3-kb dsRNA element yielded a 920-bp cDNA in the RT-PCR reaction when the 3.0-kb dsRNA from CanBc-1 was used as template. Future studies to compare the full length cDNA sequences of these two dsRNA elements occurring in the isolate CanBc-1c-78 of *B. cinerea* are warranted.

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LITERATURE CITED

- Anagnostakis, S. L. 1982. Biological control of chestnut blight. *Science* 215:466-471.
- Beever, R. E., and Parkes, S. L. 2003. Use of nitrate non-utilising (Nit) mutants to determine vegetative compatibility in *Botryotinia fuckeliana* (*Botrytis cinerea*). *Eur. J. Plant Pathol.* 109:607-613.
- Biella, S., Smith, M. L., Aist, J. R., Cortesi, P., and Milgroom, M. G. 2002. Programmed cell death correlates with virus transmission in a filamentous fungus. *Proc. R. Soc. Lond. B* 269:2269-2276. (DOI: 10.1098/rspb.2002.2148)
- Boland, G. J. 1992. Hypovirulence and double-stranded RNA in *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* 14:10-17.
- Brasier, C. M. 1983. A cytoplasmically transmitted disease of *Ceratocystis ulmi*. *Nature* 305:220-223.
- Castanho, B., Butler, E. E., and Shepherd, R. J. 1978. The association of double-stranded RNA with *Rhizoctonia* decline. *Phytopathology* 68:1515-1519.
- Castro, M., Kramer, K., Valdivia, L., Ortiz, S., Benavente, J., and Castillo, A. 1999. A new double-stranded RNA mycovirus from *Botrytis cinerea*. *FEMS Microbiol. Lett.* 175:95-99.
- Castro, M., Kramer, K., Valdivia, L., Ortiz, S., and Castillo, A. 2003. A double-stranded RNA mycovirus confers hypovirulence-associated traits to *Botrytis cinerea*. *FEMS Microbiol. Lett.* 228:87-91.
- Chen, B., Chen, C.-H., Bowman, B. H., and Nuss, D. L. 1996. Phenotypic changes associated with the wild-type and mutant hypovirus RNA transinfection of plant pathogenic fungi phylogenetically related to *Cryphonectria parasitica*. *Phytopathology* 86:301-310.
- Chu, Y.-M., Jeon, J.-J., Yea, S.-J., Kim, Y.-H., Yun, S.-H., Lee, Y.-W., and Kim, K.-H. 2002. Double-stranded RNA mycovirus from *Fusarium graminearum*. *Appl. Environ. Microbiol.* 68:2529-2534.
- Coenen, A., Kevei, F., and Hoekstra, R. F. 1997. Factors affecting the spread of double-stranded RNA viruses in *Aspergillus nidulans*. *Genet. Res. Camb.* 69:1-10.
- Cortesi, P., McCulloch, C. E., Song, H.-Y., Lin, H.-Q., and Milgroom, M. G. 2001. Genetic control of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* 159:107-118.
- Dalzoto, P. R., Glienke-Blanco, C., Kava-Cordeiro, V., Ribeiro, J. Z., Kitajima, E. W., and Azevedo, J. L. 2006. Horizontal transfer and hypovirulence associated with double-stranded RNA in *Beauveria bassiana*. *Mycol. Res.* 110:1475-1481.
- Deng, F., and Boland, G. J. 2004. A satellite RNA of *Ophiostoma novo-ulmi* mitovirus 3a in hypovirulent isolates of *Sclerotinia homoeocarpa*. *Phytopathology* 94:917-923.
- Doherty, M., Coutts, R. H. A., Brasier, C. M., and Buck, K. W. 2006. Sequence of RNA-dependent RNA polymerase genes provides evidence for three more distinct mitoviruses in *Ophiostoma novo-ulmi* isolate Ld. *Virus Genes* 33:41-44.
- Elad, Y., Williamson, B., Tudzynski, P., and Delen, N. 2004. *Botrytis* spp. and diseases they cause in agricultural systems—an introduction. Pages 1-8 in: *Botrytis: Biology, Pathology and Control*. Y. Elad, B. Williamson, P. Tudzynski, and N. Delen, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
- Fravel, D. R. 2005. Commercialization and implementation of biocontrol. *Annu. Rev. Phytopathol.* 43:337-359.
- Hansen, E. M., Stenlid, J., and Johansson, M. 1993. Somatic incompatibility and nuclear reassortment in *Heterobasidion annosum*. *Mycol. Res.* 97:1223-1228.
- Heiniger, U., and Rigling, D. 1994. Biological control of chestnut blight in Europe. *Annu. Rev. Phytopathol.* 32:581-599.
- Hong, Y., Dover, S. L., Cole, T. E., Brasier, C. M., and Buck, K. W. 1999. Multiple mitochondrial viruses in an isolate of the Dutch elm disease fungus *Ophiostoma novo-ulmi*. *Virology* 258:118-127.
- Howitt, R. L. J., Beever, R. E., Pearson, M. N., and Forster, R. L. S. 1995. Presence of double-stranded RNA and virus-like particles in *Botrytis cinerea*. *Mycol. Res.* 99:1472-1478.
- Howitt, R. L. J., Beever, R. E., Pearson, M. N., and Forster, R. L. S. 2001. Genome characterization of *Botrytis* virus F, a flexuous rod-shaped mycovirus resembling plant 'potex-like' viruses. *J. Gen. Virol.* 82:67-78.
- Howitt, R. L. J., Beever, R. E., Pearson, M. N., and Forster, R. L. S. 2005. Genome characterization of a flexuous rod-shaped mycovirus, *Botrytis* virus X, reveals high amino acid identity to genes from plant 'potex-like' viruses. *Arch. Virol.* 151:563-579.
- Ihrmark, K., Johannesson, H., Stenström, E., and Stenlid, J. 2002. Transmission of double-stranded RNA in *Heterobasidion annosum*. *Fungal Genet. Biol.* 36:147-154.
- Jian, J. H., Lakshman, D. K., and Tavantzis, S. M. 1997. Association of distinct double-stranded RNAs with enhanced or diminished virulence in *Rhizoctonia solani* infecting potato. *Mol. Plant-Microbe Interact.* 10:1002-1009.
- Jiang, D., and Ghabrial, S. A. 2004. Molecular characterization of *Penicillium chrysogenum* virus: Reconsideration of the taxonomy of the genus *Chrysovirus*. *J. Gen. Virol.* 85:2111-2121.
- Kohn, L. M., Carbone, I., and Anderson, J. B. 1990. Mycelial interactions in *Sclerotinia sclerotiorum*. *Exp. Mycol.* 14:255-267.
- Lakshman, D. K., and Tavantzis, S. M. 1994. Spontaneous appearance of genetically distinct double-stranded RNA elements in *Rhizoctonia solani*. *Phytopathology* 84:633-639.
- Li, G. Q., Huang, H. C., Laroche, A., and Achaya, S. N. 2003. Occurrence and characterization of the hypovirulence in the tan-sclerotial isolate of *Sclerotinia sclerotiorum*. *Mycol. Res.* 107:1350-1360.
- Li, M.-Y., Li, X.-H., Yan, H., and Zhang, T.-T. 2002. Occurrence and control of grey mould diseases on vegetables in China. *Plant Protection Technology and Extension* 221:31-33. (in Chinese with an English abstract)
- Lindberg, G. D. 1960. Reduction in pathogenicity and toxin production in diseased *Helminthosporium victoriae*. *Phytopathology* 50:457-460.
- Liu, Y., and Milgroom, M. G. 1996. Correlation between hypovirus transmission and the number of vegetative incompatibility (*vic*) genes different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology* 86:79-86.
- McCabe, P. M., Pfeiffer, P., and van Alfen, N. K. 1999. The influence of dsRNA viruses on the biology of plant pathogenic fungi. *Trends Microbiol.* 7:377-381.
- Melzer, M. S., and Boland, G. J. 1996. Transmissible hypovirulence in *Sclerotinia minor*. *Can. J. Plant Pathol.* 18:19-28.
- Morris, T. J., and Dodds, J. A. 1979. Isolation and analysis of double-stranded RNA virus-infected plant and fungal tissue. *Phytopathology* 69:854-858.

36. Newhouse, J. R., and MacDonald, W. L. 1990. The ultrastructure of hyphal anastomoses between vegetative compatible and incompatible virulent and hypovirulent strains of *Cryphonectria parasitica*. *Can. J. Bot.* 69:602-614.
37. Nuss, D. L., and Koltin, Y. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. *Annu. Rev. Phytopathol.* 28:37-58.
38. Nuss, D. L. 2005. Hypovirulence: Mycoviruses at the fungal-plant interface. *Nature Rev. Microbiol.* 3:632-642.
39. Page, R. D. M. 1996. TreeView: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12:357-358.
40. Polashock, J. J., Bedker, P. J., and Hillman, B. I. 1997. Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: Ascospore inheritance and implications for mitochondrial recombination. *Mol. Gen. Genet.* 256:566-571.
41. Prospero, S., Conedera, M., Heiniger, U., and Rigling, D. 2006. Saprophytic activity and sporulation of *Cryphonectria parasitica* on dead chestnut wood in forests with naturally established hypovirulence. *Phytopathology* 96:1337-1344.
42. Rogers, H. J., Buck, K. W., and Brasier, C. M. 1986. Transmission of double-stranded RNA and a disease factor in *Ophiostoma ulmi*. *Plant Pathol.* 35:277-287.
43. Rogers, H. J., Buck, K. W., and Brasier, C. M. 1987. A mitochondrial target for double-stranded RNA in diseased isolates of the fungus that causes Dutch elm disease. *Nature* 329:558-560.
44. Sambrook, J., Frisch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
45. Saup, S. J. 2000. Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. *Microbiol. Mol. Biol. Rev.* 64:489-502.
46. Thompson, J. D., Higgins, D. G., Gibson, T. J., and Clustal, W. 1994. Improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4680-4697.
47. van Kan, J. A. L. 2006. Licensed to kill: The lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* 11:247-253.
48. Vilches, S., and Castillo, A. 1997. A double-stranded RNA mycovirus in *Botrytis cinerea*. *FEMS Microbiol. Lett.* 155:125-130.
49. Xie, J., Wei, D. M., Jiang, D. H., Fu, Y. P., Li, G. Q., Ghabrial, S. A., and Peng, Y. L. 2006. Characterization of debilitation-associated mycovirus infecting the plant-pathogenic fungus *Sclerotinia sclerotiorum*. *J. Gen. Virol.* 87:241-249.
50. Zhou, T., and Boland, G. J. 1997. Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa*. *Phytopathology* 87:147-153.