Hypovirulence and double-stranded RNA in *Botrytis cinerea*

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Abstract

Twenty-one strains of *Botrytis cinerea* isolated from 13 species of crops/plants grown in China were compared for pathogenicity on detached leaves of *Brassica napus*, for mycelial growth on potato dextrose agar (PDA), and for the presence of double-stranded RNA in mycelia. The results showed that the strain CanBc-1 was greatly debilitated in pathogenicity and mycelial growth, compared to other tested strains of *B. cinerea*. A species of double-stranded (ds) RNA of about 3.0 kb in length was detected in the hypovirulent strain CanBc-1, but not in any of the virulent strains of *B. cinerea* investigated. Seventy-five out of 94 single-conidium (SC) isolates (79.8%) of CanBc-1 were still hypovirulent to *B. napus* and the 3.0-kb dsRNA was consistently detected in four of these hypovirulent SC isolates. However, other 19 single-conidium isolates (20.2%) of CanBc-1 became virulent and no dsRNA was detected in four of these virulent SC isolates. The results of the horizontal transmission experiment showed that the transmission of the hypovirulence trait and the 3.0-kb dsRNA to the virulent isolate CanBc-1c-66 of *B. cinerea*, which was closely related to CanBc-1 (a single-conidium isolate of CanBc-1) was successful in paired cultures of CanBc-1 and CanBc-1c-66. The trans-infected derivatives of CanBc-1c-66 became severely debilitated in pathogenicity, reduced in mycelial growth and suppressed in conidial production, compared to CanBc-1c-66. But the transmission to another virulent strain CanBc-2 of *B. cinerea*, which was unrelated to CanBc-1, was unsuccessful in paired cultures of CanBc-1 and CanBc-2. This study suggests that hypovirulence exists in populations of *B. cinerea* in China and the 3.0-kb dsRNA molecule is closely associated with hypovirulence of this pathogen.

Key words: *Botrytis cinerea*, hypovirulence, dsRNA

Botrytis cinerea Pers.: Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a ubiquitous phytopathogenic fungus which can attack more than 200 dicotyledonous plant species, causing diseases of gray mold, leaf blight, blossom blight, or post-harvest fruit rots (25). It has become a severe problem for industries of production of vegetables (cucumber and tomato) and fruits (grape and strawberry) in temperate climates (25), especially in areas where plastics-protected greenhouses are used to grow these crops (33). Resistant cultivars against *B. cinerea* are not available in most of the cultivated crops because of the necrotrophic nature in the infection process of this pathogen (47). Therefore, control of *B. cinerea* has been largely dependent on the use of fungicides. However, the chemical control measure is not as sustainable as expected due to the development of the fungicide resistance in populations of *B. cinerea* (50). Exploitation of alternative measures to control *B. cinerea*, including biological control, has attracted interests of many researchers (15, 16).

Successful control of chestnut blight caused by *Cryphonectria* (*Endothia*) *parasitica* with hypovirulent (HV) strains of this pathogen in Europe (18) has inspired scientists or researchers to study the insights of fungal hypovirulence and the possibility of exploiting this strategy to control other fungal pathogens, including *B. cinerea* (9). Further studies showed that the mechanism involved in this biocontrol strategy is through the transmission of hypovirulent elements from HV strains to virulent strains through hyphal anastomosis (1). So far, except for *C. parasitica*, hypovirulence has been reported in numerous other plant pathogenic fungi, including *Botrytis cinerea* (9), *Diaporthe ambigua* (43), *Fusarium graminearum* (11), *Helminthosporium victoriae* (34), *Monosporascus cannonballus* (2), *Ophiostoma novo-ulmi* (=*Ceratocystis ulmi*) (6), *Rhizoctonia solani* (7, 26), *Rosellinia necatrix* (27), *Sclerotinia homoeocarpa* (51), *Sclerotinia minor* (36), and *Sclerotinia sclerotiorum* (5, 31), and insect pathogenic fungus *Beauveria bassiana* (14). Mycoviruses or double stranded (ds) RNA elements were detected to be associated with or to be responsible for hypovirulence in the above-mentioned fungi (2, 5, 6, 7, 9, 11, 14, 26, 27, 31, 34, 36, 43, 51).

Presence of single-stranded (ss) RNA- or dsRNA-mycoviruses in mycelia of *B. cinerea* has been reported in previous studies (8, 9, 20, 21, 22, 48). However, pathogenicity tests showed that the mycovirus infection of *B. cinerea* was not consistently associated with hypovirulence in this fungus. For example, Howitt et al. (20) reported that dsRNA-containing strains of *B. cinerea* were not significantly different from dsRNA-free strains of this fungus in infection of bean leaves. On the other hand, Castro et al. (9) found that the mycovirus (6.8 kb dsRNA) -containing strain CCg425 of *B. cinerea* was much less aggressive (hypovirulent) also in infection of bean leaves than the mycovirus-free strain CKg54 of this fungus. We hypothesized that not all, but only particular species of mycoviruses or dsRNA elements inhabiting in *B. cinerea* may cause or be related to hypovirulence of this host fungus.

B. cinerea is widely distributed on numerous economically-important crops in China (33). However, information about the presence of mycoviruses or dsRNA elements in *B. cinerea* populations in this country is not available so far. Here, we report our research results on the hypovirulent strain CanBc-1 of *B. cinerea* isolated from oilseed rape (*Brassica napus* L.),

including characterization of the dsRNA element in CanBc-1 and establishment of the associative relationship between the specific dsRNA and hypovirulence of *B. cinerea* using this particular strain.

1. Materials and methods

1.1 Origin of Botrytis cinerea strains

Nineteen wild-type strains of *B. cinerea* used in this study were isolated from 13 species of crops/plants and listed in Table 1. Strains CanBc-1a and CanBc-1b were isolated from two small lesion spots (<1 mm diam.) on leaves of tomato (*Lycopersicon esculentum* Mill.) inoculated with mycelia of CanBc-1 using plant tissue-isolation procedures described by Li et al. (31). The strain T_8 was kindly provided by Dr. H. M. Zeng of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. Derivative isolates of CanBc-1 or CanBc-2 used in this study were obtained in the single-conidium isolation experiment or the transmission experiment described below. Stock cultures of each *B. cinerea* strain or isolate were incubated on potato dextrose agar (PDA) made of fresh potato and stored in a refrigerator at 4 ºC. Working cultures were established by transferring a stock agar plug containing mycelia of each strain onto PDA medium in a Petri dish (9 cm diam) and incubating at 20ºC for 1-2 weeks.

1.2 Determination of radial growth rates and colony morphology

Mycelial agar plugs (6 mm diam.) were removed from colony margins of 3-day-old PDA cultures of each investigated *B. cinerea* strain or isolate, and transferred to the centre of Petri dishes (9 cm diam.) containing 20 ml of PDA, one plug per dish. There were five dishes (replicates) for each strain. The dishes were incubated at 20ºC in the dark. Diameter of each colony was measured after incubation for 2 and 3 days for determination of radial growth rate (GR) using the formula: RGR (cm/d) = $(D_3-D_2)/2$, where D_2 and D_3 represented the diameters of each 2- and 3-day-old colony, respectively.

1.3 Pathogenicity assay

Pathogenicity of the investigated strains or isolate of *B. cinerea* was tested on leaves of oilseed rape. Seeds of oilseed rape (*B. napus*) cultivar Zhongyou Za No. 4 were sown in a field plot located in the campus of our university in the early October of 2004 and watered as required. Leaves were excised from the central part of these seedlings of oilseed rape plants in early December of the same year. Eight similarly-sized leaves (10 × 8 cm, L × W) were selected and placed in two lines on moist towels in an enamelware tray (45 × 30 × 2.5 cm, L × W × H). Eight mycelial agar plugs (6 mm diam.) were removed from the colony margin of PDA cultures of each strain or isolate of *B. cinerea* using a sterilized cork borer and inoculated on a line of four leaves in a tray by placing two inoculum plugs on the tissue area beside the main vein with the mycelial side of each plug facing the leaf surface (Fig. 1). The trays were individually sealed with clear plastic films to maintain high humid conditions and incubated in a growth chamber (20ºC) under fluorescent light (12 h light: 12 h dark). Lesion diameter around each inoculated agar plug was measured after incubation for 72 h.

1.4 Single conidium isolation

Mycelial agar plugs of CanBc-1 were inoculated on PDA and incubated at 20ºC in the dark for 30 days. Dishes with the conidial production on the surface of the colonies were selected. Conidia in each dish were washed off by flooding each colony with 10 ml of sterile distilled water and scraping the surface of colonies using a sterile triangle-shaped glass rod. The mixure was filtered through a four-layered sterilized cheesecloth to remove mycelial fragments. The resulting conidial suspension was properly diluted with SDW. Aliquots of 0.1 ml of each diluted conidial suspension were pippeted onto PDA plates and evenly spread with a glass rod. The plates were incubated at 20ºC for 24 h. Mycelia or germ tubes growing from each conidia were removed with the aid of a Zeiss dissecting microscope and transferred to PDA slants in a glass tube. The slants were incubated at 20ºC for 10 days and then stored at 4ºC. Each slant of culture was regarded 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 with its parental strain CanBc-1 and the virulent strain CanBc-2 as controls. Those with the average lesion diameters <0.5 cm and attenuated mycelial growth were regarded as hypovirulent isolates, whereas those with the average lesion diameters >0.5 cm and normal mycelial growth were regarded as virulent isolates.

1.5 Transmission of hypovirulence and dsRNA

The pairing cultural technique (1, 6, 7, 10, 13, 26, 32) was used to test the transmissibility of the hypovirulentce trait and the hypovirulence-associated dsRNA from the hypovirulent (HV) strain CanBc-1 to virulent (V) strains or isolates of *B. cinerea*, including CanBc-1c-66 and CanBc-2. CanBc-1 was used as the donor, whereas CanBc-1c-66 or CanBc-2 as the recipients. There were three treatments for each combination, the pairing-culture treatment and two single-culture treatments, one for CanBc-1 and the other for CanBc-1c-66 or CanBc-2. For the pairing cultural treatment, a mycelial agar plug inoculum of CanBc-1 (6 mm diam) was inoculated in a Petri dish (9 cm diam) containing 20 ml of PDA at 1 cm away from the rim of the dish. Then, CanBc-1c-66 or CanBc-2 was inoculated about 0.5 cm away beside the inoculation point of CanBc-1 in the same dish. The dishes for the control treatments were inoculated with CanBc-1, CanBc-1c-66 or CanBc-2. There were three dishes for each treatment in each combination. The dishes were incubated at 20°C in dark and mycelial growth by CanBc-1, CanBc-1c-66 or CanBc-2 in both single and pair cultures was observed after 6 days. A mycelial plug (6 mm diam.) was taken from the margin of each colony of CanBc-ic-66 or CanBc-2 in paired cultures at the point far from the inoculation plug of CanBc-1 and transferred to PDA and establish three recipient derivatives for CanBc-1c-66 (a, b and c), and three recipient

derivatives for CanBc-2 (a, b, and c). These derivative isolates were tested for pathogenicity on leaves of oilseed rape, for determination of the mycelial growth rate and production of conidia and sclerotia on PDA, and for the presence of dsRNA.

1.6 Extraction, purification and identification of double-stranded RNA

A mycelial agar plug of each strain or isolate of *B. cinerea* was inoculated in a Petri dishe (9 cm diam.) containing 20 ml of potato dextrose broth made of fresh potato. There were 20 dishes for each strain or isolate. The dishes were incubated at 20ºC in dark for 3 to 6 days. Mycelial mats of each strain or isolate were harvested from dishes using a sterilized glass hook, rinsed in sterilized distilled water three times, 3 min each time, blotted half dry on sterilized paper towels and transferred to centrifuge tubes (1.5 ml) and stored at -20ºC. Extraction and purification of dsRNA in mycelia of *B. cinerea* strains or isolates were conducted using the procedures described by Li et al. (32) with minor modifications. A mycelial sample of about 4 g of each strain or isolate was frozen in the liquid nitrogen and ground to fine powder using a sterilized mortar and pestle. The powder was transferred to a 50-ml centrifuge tube and 10 ml of extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4 and 0.6 M NaCl, pH 9.5) containing 2% (v/v) β-mercaptoethanol and 1% (w/v) SDS (sodium dodecyl sulfate) was added. The tube was shake-incubated at 200 rpm and 20ºC for 60 min. Then, an equal volume of Tris-phenol solution (pH 8.0) was added and the tube was gently agitated for 2 min. The mixture was centrifuged at 17000×g for 30 min at 4ºC. The supernatant was pipetted out and transferred to a 50-ml tube and an equal volume of chloroform: isoamyl alcohol mixture (24: 1) was added. After being agitated for 2 min, the mixture was centrifuged for 30 min under the same conditions. The supernatant was again pipetted out and transferred to a 50-ml tube, adjusted to contain 15% ethanol (v/v) by addition of water-free ethanol and mixed with 1 g of CF-11 powder (Sigma-Aldrich Inc., St Louis, MO, USA). The tube was agitated for 1 min to get the powder totally immerged in the aqueous phase and the mixture was centrifuged at 6000×g for 10 min at 4ºC. The supernatant containing single stranded RNA or DNA was discarded and 10 ml of ethanol (15%, v/v)-amended sodium Tris-EDTA (STE) buffer (0.1 M NaCl, 50 mM Tris, 10 mM Na_2EDTA, pH 7.0) was added to suspend the precipitate and the mixture was centrifuged again to collect the cellulose precipitate. This washing procedure using ethanol-containing STE buffer was repeated three times. The precipitate of the last centrifuge was suspended with 10 ml of complete 100% STE buffer to elute dsRNA molecules from cellulose. The mixture was centrifuged at 6000×g for 10 min at 4ºC to collect the supernatant. Two volumes of cold ethanol (95%) and 1/10 volume of sodium acetate solution (3 M) were added to the supernatant, thoroughly mixed and stored at -20ºC overnight. The mixture was subject to a centrifuge at 17000×g for 30 min to collect the precipitate. After drying at room temperature (20-25ºC), the precipitate was dissolved in 60 µl of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA, pH 8.0). A 10 µl sample of the extract for each *B. cinerea* strain or isolate was mixed with 5 µl of loading dye (bromophenol blue), loaded on a agarose gel (1%, w/v) with Wide Range DNA Marker (500-12, 000 bp, TAKARA Biotechnology Co., Ltd, Dalian, China) as marker, and electrophoresed in Tris-boric acid-EDTA (TBE) buffer (pH 8.3). Gels were viewed on an uv Trans-illuminator in Kodak Imaging System (Gel Logic 100, Upland, CA, USA) after being stained with ethidium bromide (0.5 µg/ml) for 30 min. The identity of dsRNA was confirmed using RNase-free DNase I, SI nuclease and RNase A as recommended by the manufacture (TAKARA Biotechnology Co., Ltd, Dalian, China). The molecules that can be digested by RNase under the low salt condition (0.03 M MaCl), but can not be digested by RNase under the high salt condition (0.3 M MaCl), by DNase and S1 nuclease were considered as dsRNA (39),

1.7 Data analysis

Analysis of variance (ANOVA) (SAS Institute, Cary, NC, USA, Version 8.0, 1999) was used to determine the statistical significance of differences in radial growth rate, lesion diameter, conidial production and sclerotial production among strains/isolates of *B. cinerea* in each experiment. The data on conidial production by *B. cinerea* were Log_{10}-transformed prior to the ANOVA. After each analysis, means were individually back-transformed to numerical values. Treatment means for tested strains or isolates of *B. cinerea* in each experiment were separated using Duncan's Multiple Range Test at $P = 0.05$ level.

2. Results

2.1 Hypovirulence and abnormal colony morphology of the B. cinerea *strain CanBc-1*

On leaves of oilseed rape, 20 out of 21 strains *B. cinerea*, including CanBc-2, caused infection and lesions developed after incubation at 20ºC for 24 h. The average lesion diameters ranged from 1.0 to 2.3 cm among these virulent strains of *B. cinerea* after 72 h (Table 1). On the other hand, CanBc-1 did not cause any infection and no lesions were developed on leaves of oilseed rape under the same incubation conditions (Table 1). Mycelial growth by this hypovirulent strain around each inoculated mycelial agar plug was evident.

On PDA media, these 20 virulent strains of *B. cinerea*, including CanBc-2, grew rapidly at 20 ºC with the average radial growth rates ranging from 1.2 to 1.9 cm/d (Table 1). Colonies of these strains developed normally and covered the entire dishes (9 cm diam.) after 3 days. Then, formation of aerial mycelia, conidiation or/and sclerotia were observed. On the other hand, the hypovirulent strain CanBc-1 grew slowly with the average radial growth rate being 0.5 cm/d and covered the whole dishes after 10 days. Colonies of this strain developed abnormally with the formation of mycelial sectors. Sparse conidiation and no sclerotial formation by CanBc-1 were observed after 15 days.

2.2 Presence of dsRNA in virulent and hypovirulent strains of B. cinerea

A species of dsRNA molecule was detected in mycelia of the hypovirulent strain CanBc-1 of *B. cinerea* (Table 1). The

size of that dsRNA molecule was about 3.0 kb according to the DNA marker. On the other hand, dsRNA was not detected in mycelia of other 20 virulent strains of *B. cinerea* (Table 1), including CanBc-1a and CanBc-1b, which were derived from infective mycelia of CanBc-1, and CanBc-2 (Table 1).

Table 1. Origin, radial growth rate, pathogenicity and double-stranded (ds) RNA of *Botrytis cinerea* strains collected from various host plants in central China.

Strains	Origin (host, place and collection time)	DL (cm)	GR (cm/d)	dsRNA
CanBc-1	*Brassica napus*, Wuhan, Huibei, 2003.05	0.0 j	0.5 i	+
CanBc-1a	CanBc-1-infected *Lycopersicon esculentum*, 2003.08	1.1 ih	1.4 fgh	-
CanBc-1b	CanBc-1-infected *L. esculentum*, 2003, 08	1.1 ih	1.2 h	-
CanBc-2	*Brassica napus*, Wuhan, Huibei, 2004.05	1.9 abcde	1.7 abc	-
CanBc-4	*Brassica napus*, Xianning, Hubei, 2004.04	1.7 bcdefg	1.5 bcdefg	-
CarBc-1	*Dianthus caryophyllus*, Shaoshan, Hunan, 2004.11	2.0 abcd	1.5 bcdefg	-
ElaBc-1	*Ficus elastica*, Xinxiang, Henan, 2005.02	1.5 fg	1.4 efg	-
GBc-1	*Vitis vinifera*, Wuhan, Hubei, 2004.08	2.1 ab	1.7 ab	-
LetBc-1	*Lactuca sativa*, Wuhan, Hubei, 2005.03	1.6 efg	1.6 bcdef	-
OniBc-1	*Allium schoenoprasum*, Shengnongjia, Hubei, 2004.05	2.2 a	1.6 abcd	-
OniBc-2	*A. schoenoprasum*, Shengnongjia, Hubei, 2004.05	2.1 abc	1.4 defgh	-
PeaBc-1	*Lathyrus odoratus*, Wuhan, Hubei, 2004.10	1.0 i	1.7 ab	-
PoinBc-1	*Euphorbia pulcherrima*, Xinxiang, Henan, 2005.02	1.7 cefg	1.6 abc	-
RedBc-1	*Codiaeum variegatum*, Xinxiang, Henan, 2005.02	1.6 defg	1.8 a	-
RoseBc-1	*Rosa chinenses*, Wuhan, Hubei, 2004.07	2.3 a	1.6 bcdef	-
RoseBc-2	*Rosa chinenses*, Wuhan, Hubei, 2004.07	1.4 gh	1.3 hg	-
RoseBc-3	*Rosa chinenses*, Wuhan, Hubei, 2004.07	1.5 fgh	1.7 ab	-
SwpBc-1	*Ipomoea batatas*, Wuhan, Hubei, 2004.05	1.5 gfh	1.7 ab	-
T8	*Solanum lycopersicum*, Beijing, 2003.06	1.6 defg	1.2 h	-
TagBc-1	*Tagetes patula*, Changyang, Hubei, 2004.07	2.1 ab	1.7 ab	-
TagBc-2	*Tagetes patula*, Wuhan, Hubei, 2004.10	1.9 abcdef	1.6 bcde	-
$LSD_{0.05}$		0.4	0.2	

Note: DL=Diameter of lesions; GR=Growth rate. "+"=presence, "-"=absence

2.3 Pathogenicity differentiation and dsRNA among single-conidium isolates of CanBc-1

Ninety-four single-conidium (SC) isolates were obtained from the parental strain hypovirulent strain CanBc-1 of *B. cinerea*. Differentiation in pathogenicity and mycelial growth was observed among these isolates, 75 isolates (79.8%) were identified as the hypovirulent (HV) type, whereas other 19 isolates (20.2%) as the virulent (V) type (Fig. 2). 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 radial growth rates ranging from 0.3 to 1.1 cm/d and did not cause any infection of leaf tissues of oilseed rape (Table 2). The specific 3.0 kb-dsRNA was detected in CanBc-1 and these four HV-type SC isolates of CanBc-1 (Table 2). On the other hand, 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 radial growth rates ranging from 1.4 to 1.6 cm/d and caused infection on leaf tissues of oilseed rape with the average lesion diameter ranging from 1.2 to 1.4 cm after incubation for 72 h, not significantly (P>0.05) different from the virulent strain CanBc-2 of *B. cinerea* (Table 2). dsRNA was not detected in mycelia of these four V type SC isolates of CanBc-1 and CanBc-2.

Table 2. Comparison of pathogenicity, mycelial growth rate and the presence of dsRNA among CanBc-1, eight single-conidium isolates of CanBc-1 and CanBc-2 of *Botrytis cinerea*.

Strain	GR(cm/d)	DL(cm)	dsRNA
CanBc-1	0.8 e	0 c	+
CanBc-1c-50	0.9 d	0 c	+
CanBc-1c-73	0.3 f	0 c	+
CanBc-1c-78	1.1 d	0 c	+
CanBc-1c-82	0.7 e	0 c	+
CanBc-1c-55	1.4 c	1.2 b	-
CanBc-1c-64	1.5 bc	1.3 ab	-
CanBc-1c-66	1.6 ab	1.2 b	-
CanBc-1c-93	1.6 ab	1.4 a	-
CanBc-2	1.7 a	1.4 ab	-
$LSD_{0.05}$	0.1	0.2	

Note: DL=Diameter of lesions; GR=Growth rate. "+"=presence, "-"=absence

2.4 Transmission of hypovirulence and dsRNA in CanBc-1

In single cultures, CanBc-1c-66 and CanBc-2 grew rapidly and covered the whole dishes after 6 days, whereas CanBc-1

grew slowly and covered about one third of the dishes. In paired cultures, CanBc-2 still grew rapidly and covered the whole dishes also after 6 days, whereas CanBc-1c-66 grew rapidly during the first three days, followed by the slow growth in the front, profuse sporulations behind the front and failure in colonization of the whole dishes.

Three derivative isolates of CanBc-1c-66 were obtained from three recipient colonies of CanBc-1c-66 in paired cultures and designated as CanBc-1c-66a, CanBc-1c-66b and CanBc-1c-66c. These three isolates were similar to CanBc-1 in slow mycelial growth with the average radial growth rates being <0.7 cm/d (Table 3), in formation of mycelial sectors, and in reduced production of conidia (3-5 × 10^6 conidia/dish) (Table 4) on PDA, and in attenuated pathogenicity on leaves of oilseed rape with the average diameter of 72-hour-old lesions being <0.1 cm. But they were greatly different from their parental strain CanBc-1c-66, which grew rapidly at 1.3 cm/d and produced large quantity of conidia at 2.5 × 10^8 conidia/dish on PDA, and caused severe infection with an average diameter of 72-hour-old lesions being 2.3 cm on leaves of oilseed rape (Table 3). The specific 3.0-kb dsRNA molecule was detected in these three hypovirulent isolates, as well as in CanBc-1, but not in CanBc-1c-66.

Table 3. Comparison of pathogenicity, mycelial growth rate and the presence of dsRNA among strains of *Botrytis cinerea* strains derived from the horizontal transmission experiment of this study.

Strain	GR(cm/d)	DL(cm)	dsRNA
CanBc-1	0.7 c	0 b	+
CanBc-1c-66	1.3 b	2.4 a	+
CanBc-1c-66a	0.6 d	0 b	+
CanBc-1c-66b	0.5 d	0.1 b	+
CanBc-1c-66c	0.6 d	0.1 b	+
CanBc-2a	1.6 a	2.2 a	-
CanBc-2b	1.6 a	2.0 a	-
CanBc-2c	1.5 a	2.6 a	-
CanBc-2	1.6 a	2.4 a	-
$LSD_{0.05}$	0.1	0.2	

Note: DL=Diameter of lesions; GR=Growth rate. "+"=presence, "-"=absence

Meanwhile, three derivative isolates of CanBc-2 were also obtained from three recipient colonies of CanBc-2 in paired cultures and designated as CanBc-2a, CanBc-2b and CanBc-2c. These three isolates were similar to CanBc-2 in rapid mycelial growth with the average radial growth rates being 1.5 to 1.6 cm/d (Table 3), in formation of normal colony morphology, and in production of conidia (5-7 × 10^5 conidia/dish) and sclerotia (Table 2) on PDA, and in strong pathogenicity on leaves of oilseed rape with average lesion diameters being 2.0 to 2.6 cm after 72 h, but were greatly different from CanBc-1 (Table 4). The specific 3.0-kb dsRNA molecule was not detected these three virulent recipient isolates of CanBc-2 and CanBc-2 (Table 4).

3. Discussion

A hypovirulent strain CanBc-1 of *B. cinerea* was screened from 21 strains of this pathogenic fungus isolated from 13 species of plants grown in China in the present study. This indicates that hypovirulence exists in natural populations of *B. cinerea* in this country. Abnormal symptoms, including suppressed mycelial growth and altered colony morphology, reduced conidial production and sclerotial formation, were observed on PDA cultures of CanBc-1. These results confirms previous observations in hypovirulent strains of other phytopathogenic fungi, including *H. sativae* (34), *R. solani* (7, 26), *O. novo-ulmi* (6), *S. minor* (38), *S. sclerotiorum* (5, 31) and *S. homoeocarpa* (51) and *Fusarium graminearum* (11) for suppressed mycelial growth, *C. parasitica* (19, 37) and *B. cinerea* (9) for suppressed conidial production, and *S. sclerotiorum* (32) for elimination of sclerotial formation. These abnormal traits might reduce the ecological fitness of hypovirulent fungal strains. How can hypovirulent fungal strains, including the *B. cinerea* strain CanBc-1, survive and disseminate in nature? Prospero et al. (44) reported that saprophytic colonization of chestnut tissues by *C. parasitica* play an important role in the epidemiology of hypovirulence-causing mycoviruses. This may occur in *B. cinerea*, as it is similar to *C. parasitica* in requirement of the saprophytic phase for infection of plant tissues and for production of conidia (47), through which hypovirulence is disseminated.

This study reveals that the 3.0-kb dsRNA molecule in CanBc-1 is closely related to hypovirulence of *B. cinerea*. The correlative evidences include: (i) the specific dsRNA was detected in the hypovirulent strain CanBc-1, but not in the eighteen wild-type virulent strains of *B. cinerea* and the two virulent derivatives isolated from infective mycelia of CanBc-1 (CanBc-1a and CanBc-1b) (Table 1); (ii) it was consistently detected in hypovirulent, but not virulent, single-conidium isolates of CanBc-1 (Fig. 3), suggesting that hypovirulence and hypovirulence-associated dsRNA in CanBc-1 might be of cytoplasmic, rather than nuclear, inheritance, as previously reported in the hypovirulent strains of *C. parasitica* (Nuss, 1992); and (iii) a concomitance of the specific dsRNA was observed in the successful transmission of hypovirulence from CanBc-1 to CanBc-1c-66; but not in the unsuccessful transmission of this trait from CanBc-1 to CanBc-2 (Fig. 5). All these evidences suggest that the "hypovirulence element" in CanBc-1 can be infective and might be of the mycovirus nature, as the mycoviruses reported in the hypovirulent strains of *C. parasitica* (42) and *S. sclerotiorum* (49). Infection of *B. cinerea* by virus-like particles (20), dsRNA elements (20), ssRNA mycoviruses (21, 22) or dsRNA mycoviruses (8, 9, 48) has been reported. However, only CCg425 was documented to be hypoviulent (9). CanBc-1 seems different from CCg425 in the degree

of pathogenicity attenuation, completely attenuated in CanBc-1, whereas partially attenuated (by about 50%) in CCg425 (9), and in the size of dsRNA molecules, 3.0 kb in CanBc-1, whereas 6.8 kb in CCg425 (9). Therefore, the mycovirus or the dsRNA elements associated hypovirulence of CanBc-1 might be a novel species in *B. cinerea*. Further analysis of the sequence information about that specific dsRNA is warranted.

Table 4. Comparison of the yield and the size of conidia and sclerotia produced by virulent and hypovirulent strains of *Botrytis cinerea*.

Strain	PT[a]	Conidia		Sclerotia	
		Conidia/dish (*n*=5)	Size (μm) (*n*=30)	Sclerotia/dish (*n*=5)	Size (mm) (*n*=30)
CanBc-1	HV	5.0×10^4 d	10.8 × 8.6	14 b	1.1 × 1.0
CanBc-1c-66	V	2.5×10^8 a	10.7 × 7.5	0 c	NS
CanBc-1c-66a	HV	4.0×10^6 b	11.0 × 7.9	0 c	NS
CanBc-1c-66b	HV	5.0×10^6 b	11.0 × 7.8	0 c	NS
CanBc-1c-66c	HV	3.0×10^6 b	10.9 × 8.2	0 c	NS
CanBc-2	V	7.0×10^5 c	10.9 × 8.8	52 a	4.7 × 3.3
CanBc-2a	V	8.0×10^5 c	10.5 × 7.7	68 a	4.9 × 3.0
CanBc-2b	V	7.0×10^5 c	10.5 × 8.1	68 a	4.0 × 2.8
CanBc-2c	V	5.0×10^5 c	11.6 × 8.8	87a	3.9 × 2.8
$LSD_{0.05}$		0.3		35.8	

Note: PT=pathotype; NS=no sclerotial formation

Previous studies showed that mycoviruses or dsRNA elements can be transmitted vertically to asexual spores, including conidia (42, 44), and horizontally to other virulent fungal strains of the same or different taxona through hyphal fusions (anastomosis) or heterokaryon formation, which are regulated by vegetative incompatibility genes (*vic*) (1, 12, 35, 37, 40, 45). The incompatible hyphal interaction between strains of most ascomycetous fungal species containing different *vic* genes may inhibit or restrict movements of dsRNA elements or mycoviruses (30, 46). Death of hyphal cells was observed in intermingled areas of two interacting colonies of fungi, including *C. parasitica* (4) and *S. sclerotiorum* (28). On the other hand, Ihrmark et al. (23) 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, even belonging to different intersterility groups. Death of hyphal cells was not obviously observed in the interactions between isolates of *H. annosum* (17). This study demonstrates that about 80% of the single-conidium isolates of CanBc-1 were still hypovirulent to oilseed rape and the specific dsRNA (3.0 kb) was detected in four of these hypovirulent isolates. These results indicate that hypovirulence and the specific dsRNA in CanBc-1 can be vertically transmitted to its conidia. The horizontal transmission of hypovirulence and the specific dsRNA was successful from CanBc-1 to CanBc-1c-66, but unsuccessful from CanBc-1 to CanBc-2. This strain-specific hypovirulence transmission behavior might be regulated by the vegetative incompatibility system existing in *B. cinerea* (3), as CanBc-1c-66, a single-conidium isolate of CanBc-1, is closely related to CanBc-1, whereas CanBc-2 is unrelated to CanBc-1. Incompatible interactions between hyphal cells of CanBc-1 and CanBc-2, including cell death, might be one of the factors restricting the transmission of hypovirulence from CanBc-1 to CanBc-2.

Spontaneous appearance of genetically-distinct dsRNA elements has been reported in *R. solani* (7, 29). These dsRNAs were closely related to hypovirulence of this pathogenic fungus (7, 26). In the present study, a small dsRNA of about 2.3 kb in length was detected in CanBc-1c-78, one of the single-conidium isolates of CanBc-1 (Fig. 3). The effect of the presence of this dsRNA on pathogenicity of *B. cinerea* remains unknown, as this isolate was as hypovirulent as CanBc-1. Nuss and Koltin (28) indicated that small dsRNA species (about 1.0 kb) in the hypovirulent strain Ep713 of *C. parasitica* were derived from the large dsRNA species (12-13 kb) through the internal deletion mechanism. This mechanism may occur in our hypovirulent strain CanBc-1 of *B. cinerea*. Comparison of the sequence information of the two dsRNA species in that particular isolate of *B. cinerea* will be useful to validate this hypothesis.

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