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FULL PAPER

Botrytis sinoallii: a new species of the grey mould pathogen on *Allium* crops in China

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Abstract A study was conducted to identify *Botrytis* spp. isolated from bulb onion, green onions, garlic, and garlic chives grown in Hubei Province of China. Based on colony morphology and conidial characteristics, 367 strains of *Botrytis* spp. were identified as five distinct species, namely, *B. cinerea*, *B. squamosa*, *B. porri*, *B. byssoidea*, and an undescribed *Botrytis* species (*Botrytis* sp.), which accounted for 64.3, 29.9, 3.3, 0.3, and 2.2%, respectively. The previously undescribed species is herein described as a new species, *B. sinoallii* sp. nov., which is characterized by production of numerous small sclerotia on potato dextrose agar. Phylogenetic analysis using partial sequences of three nuclear genes (*G3PDH*, *HSP60*, and *RPB2*) showed that *B. sinoallii* forms a unique lineage, which is closely related to *B. squamosa*, a well-known species on *Allium* crops, but distantly related to the other species of *Botrytis* on *Allium* crops, including *B. cinerea*, *B. porri*, *B. aclada*, *B. allii*, *B. byssoidea*, *B. globosa*, and *B. sphaerosperma*. Results of inoculation tests showed that *B. sinoallii* is a newly identified agent that can cause leaf blight of green onion, garlic, and garlic chives. Potential impact of *B. sinoallii* on production of *Allium* crops in China is discussed.

Keywords Leaf blight · Morphology · Phylogeny · Taxonomy

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Introduction

Allium crops, including bulb onions (*Allium cepa* L.), green onions (*A. fistulosum* L.), garlic (*A. sativum* L.), and garlic chives (*A. tuberosum* Rottl. ex Spreng.), are important vegetables in China and in many other countries. In China, the history of cultivation of green onions, garlic, and garlic chives can be traced back more than 2000 years, whereas bulb onions were introduced only in the early part of the twentieth century. In 2009, the planting area for these four *Allium* crops in China reached approximately 1.45 Mha, accounting for 60% of the world total (Chen et al. 2009).

Diseases, including grey mould caused by *Botrytis* species, are major factors limiting production of *Allium* crops (McDonald et al. 2004). Hennebert (1963) reported seven species of grey mould pathogens on plants belonging to the genus *Allium*. They are *B. aclada* Fresen., *B. byssoidea* Walker, *B. cinerea* Pers., *B. globosa* Raabe, *B. porri* Buchw., *B. squamosa* Walker, and *B. sphaerosperma* Buchw. Yohalem et al. (2003) reported that *B. allii*, which was previously regarded as the synonym of *B. aclada* (Hennebert 1973), is a hybrid species of *B. aclada* × *B. byssoidea*. Symptoms of diseases caused by *Botrytis* spp. on *Allium* crops include flower/blossom blight (Ellerbrock and Lorbeer 1977), neck/bulb rot (Chilvers et al.

2004; Zhang et al. 2008), leaf blight (Presly 1985; Delhey et al. 2006; Zhang et al. 2009), and garlic clove rot (Zhang and Wang 1993; Dugan et al. 2007).

Morphological characteristics of macroconidia, conidiophores, and sclerotia are the primary criteria for identifying *Botrytis* species. So far, 26 species of *Botrytis* have been described based on these morphological characteristics (Hennebert 1973; Yohalem et al. 2003; Wang et al. 1996; O'Gorman et al. 2008; Zhang et al. 2010). Recently, DNA-based molecular techiniques have been widely used to identify fungi, including *Botrytis* species. Phylogenetic analyses of *Botrytis* species using three nuclear gene sequences of *G3PDH* encoding glyceraldehyde-3-phosphate dehydrogenase, *HSP60* encoding heat-shock protein 60, and *RPB2* encoding DNA-dependent RNA polymerase subunit II (Staats et al. 2005) corroborated the morphological delimitation of *Botrytis* species by Hennebert (1973) and Yohalem et al. (2003). O'Gorman et al. (2008) indicated that phylogenetic analysis based on the partial sequences of β-tubulin gene and *G3PDH* supported the previous work by Ruehle (1931) on the classification of *B. mali* Ruehle, the causal agent of postharvest decay of apple, as a unique species. Moreover, information on the sequence of *NEP2* coding for necrosis and ethylene-inducing protein 2 can be used for designing polymerase chain reaction (PCR) primers to distinguish different species of *Botrytis* (Mirzaei et al. 2008).

In 2006, a strain of *Botrytis* designated as OnionBc-23 was isolated from a diseased leaf of green onion grown in Xianning City of Hubei Province, China (Zhang et al. 2007). The colony morphology of strain OnionBC-23 grown on potato dextrose agar (PDA) (Zhang et al. 2007) differed from other species of *Botrytis* on *Allium* crops described by Chilvers and du Toit (2006). This preliminary finding suggests the possibility of existence of a new species of *Botrytis* on green onion in Hubei Province. Therefore, a study was conducted to: (1) determine the identity of strain OnionBC-23 as a new species of *Botrytis* by characterizing its cultural, morphological, and molecular features; and (2) determine the potential impact of this new species of *Botrytis* on *Allium* crops by surveying the natural distribution of this pathogen in Hubei Province and comparing its pathogenicity with other species of *Botrytis* from *Allium* crops.

Materials and methods

Collection and isolation of *Botrytis* spp.

Samples of diseased leaves of bulb onion, green onion, garlic, or garlic chives showing symptoms of leaf blight with the sign of grey mould were collected from 59 counties/cities (Fig. 1) in Hubei Province of China in

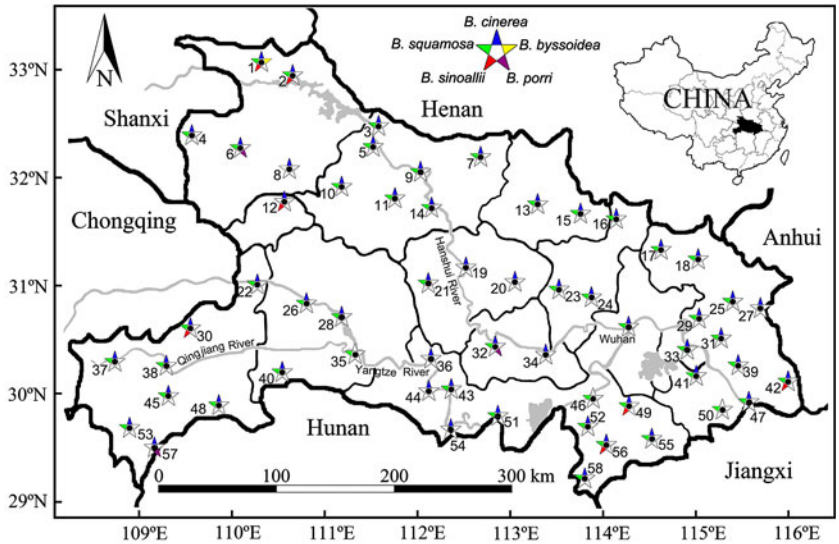

Fig. 1 Geographic distribution of *Botrytis sinoallii*, *B. cinerea*, *B. squamosa*, *B. porri,* and *B. byssoidea* on *Allium* crops (garlic, garlic chives, green onion, bulb onion) grown in Hubei Province, China. The location of Hubei Province is defined as a *dark patch* in the map of China. *Numbers* on the map of Hubei Province represent counties/cities where diseased samples were collected: *1* Yun Xi; *2* Yun Xian; *3* Lao Hekou; *4* Zhu Xi; *5* Gu Cheng; *6* Zhu Shan; *7* Zao Yang; *8* Fang Xian; *9* Xiang Fan; *10* Bao Kang; *11* Nan Zhang; *12* Shen Nong Jia; *13* Sui Zhou; *14* Yi Cheng; *15* Guang Shui; *16* Da Wu; *17* Hong An; *18* Ma Cheng; *19* Zhong Xiang; *20* Jing Shan; *21* Jing Men; *22* Ba Dong; *23* Ying Cheng; *24* Xiao Gan; *25* Luo Tian; *26* Zi Gui; *27* Ying Shan; *28* Yi Chang; *29* Tuan Feng; *30* Jian Shi; *31* Xi Shui; *32* Qian Jiang; *33* E Zhou; *34* Xian Tao; *35* Yi Du; *36* Jing Zhou; *37* Li Chuan; *38* En Shi; *39* Qi Chun; *40* Wu Feng; *41* Da Ye; *42* Huang Mei; *43* Jiang Ling; *44* Gong An; *45* Xuan En; *46* Jia Yu; *47* Wu Xue; *48* He Feng; *49* Xian Ning; *50* Yang Xin; *51* Jian Li; *52* Chi Bi; *53* Xian Feng; *54* Shi Shou; *55* Tong Shan; *56* Chong Yang; *57* Lai Feng; *58* Tong Chen

spring (March–May) 2005–2009. In each county/city, at least five commercial fields for each *Allium* crop were surveyed. In each field, five samples were collected: three to five diseased leaves from two to five plants for each sample. Leaves of each sample were kept in a paper bag, air-dried at room temperature (20–25°C) for 2 days, and stored at 4°C. To isolate pathogens, one diseased leaf was selected from each sample and cut into small pieces (approximately 5 × 5 mm). Three leaf pieces for each sample were soaked in 1 ml of sterile distilled water (SDW) in an autoclaved microcentrifuge tube (1.5 ml). The tube was capped and vortexed for 30 s to wash off spores from the tissues. Spore suspension in each tube was serially diluted with SDW. An aliquot of 100 µl of each diluted spore suspension was pipetted onto PDA in a Petri dish. The suspension was then spread using a flame-sterilized glass rod. After incubation at 20°C in the dark for 48 h, ten single colonies for each sample were individually transferred onto PDA in Petri dishes, one colony per dish. The dishes were incubated at 20°C in the dark for 15 days, and the resulting cultures were identified on the basis of colony morphological characteristics described by Chilvers and du Toit (2006).

Cultures representing different *Botrytis* species occurring in each surveyed county/city were selected, and a mycelial agar plug from each representative culture was transferred to a PDA slant in a glass tube (18 × 2 cm), incubated at 20°C in the dark for 12 days, and stored at 4°C until use. Working cultures for each *Botrytis* strain were established by transferring mycelial agar plugs from each stock culture onto PDA in Petri dishes, which were then incubated at 20°C in the dark for 1 week. A total of 367 strains of *Botrytis* spp. were isolated from bulb onion, green onion, garlic, and garlic chives. Five species of *Botrytis*, namely *B. cinerea*, *B. squamosa*, *B. porri*, *B. byssoidea*, and a previously undescribed *Botrytis* species (*Botrytis* sp.) were identified based on colony morphological characteristics (Chilvers and du Toit 2006). Thirteen strains (Table 1) representing *B. cinerea* (strains OnionBC-1, GarlicBC-5, and LeekBC-8), *B. squamosa* (strains OnionBC-19, GarlicBC-2, and LeekBC-2), *B. porri* (strains OnionBC-95, GarlicBC-16, and GarlicBC-38), an unidentified *Botrytis* sp. (strains OnionBC-23, OnionBC-59, and LeekBC-18), and *B. byssoidea* (strain OnionBC-76) were selected for further characterization of the colony morphology and production of conidia and sclerotia on PDA and for testing pathogenicity on garlic, garlic chives, and green onion. Three strains of *B. aclada* (strains OnionBC-7, OnionBC-15, and OnionBC-18) isolated from diseased onion bulbs (Zhang et al. 2008) were also included in this study (Table 1).

Strain characterization

To determine mycelial growth rate and colony morphology of the representative strains of *Botrytis* spp., mycelial agar plugs (6 mm diameter) were removed from the colony margin of a 2-day-old PDA culture (20°C) of each strain using a cork borer and inoculated onto PDA in five Petri dishes, one plug per dish. The dishes were incubated at

Table 1 The origin and GenBank accession numbers of DNA sequences for strains of *Botrytis* spp. investigated in this study

Species	Strain	Origin (location, time)[a]	GenBank Acc. No.			
			ITS	*RPB2*	*HSP60*	*G3PDH*
B. sinoallii	OnionBC-23	Xianning, 2006	EU519203	EU514479	EU514488	EU519217
	OnionBC-59	Jianshi, 2007	FJ169664	FJ169678	FJ169658	FJ169646
	LeekBC-18	Changyang, 2008	FJ169673	FJ169679	FJ169660	FJ169651
B. cinerea	OnionBC-1	Shennongjia, 2004	FJ169667	FJ169677	FJ169656	FJ169649
	GarlicBC-5	Wuhan, 2005	EU519210	EU514475	EU514485	EU519212
	LeekBC-8	Jianshi, 2007	FJ169666	FJ169676	FJ169655	FJ169648
B. squamosa	OnionBC-19	Wuhan, 2007	EU519208	EU514478	EU514487	EU519216
	GarlicBC-2	Wuhan, 2005	EU519205	EU514474	EU514484	EU519214
	LeekBC-2	Wuhan, 2006	FJ169668	FJ169682	FJ169659	FJ169647
B. aclada	OnionBC-7	Ezhou, 2006	EU000196	EU514476	EU514483	EU519213
	OnionBC-15	Wuhan, 2006	EU093077	EU093078	EU100387	EU100386
	OnionBC-18	Wuhan, 2006	FJ169669	FJ169674	FJ169657	FJ169645
B. porri	OnionBC-95	Qianjiang, 2008	FJ169672	FJ169683	FJ169663	FJ169653
	GarlicBC-16	Zhushan, 2007	EU519206	EU514481	EU514490	EU519219
	GarlicBC-38	Laifeng, 2007	FJ169665	FJ169675	FJ169654	FJ169644
B. byssoidea	OnionBC-76	Yunxi, 2008	FJ169671	FJ169681	FJ169661	FJ169652

[a] Location indicates counties/cities where the diseased leaf samples were collected (Fig. 1)

20°C in the dark, and the colony diameter in each dish was measured after incubation for 24 and 48 h to determine the radial growth rate (Wu et al. 2007). Colony morphology and production of conidia and sclerotia by different strains of *Botrytis* spp. were observed after incubation for 3–30 days. Fifty sclerotia for each representative strain of *Botrytis* sp. were randomly chosen in the five-dish PDA cultures and measured for length and width.

To assess the conidial morphology of the representative strains of *B. aclada*, *B. cinerea*, and *B. porri*, conidia formed at 20°C in the dark on 12-day-old PDA cultures were examined for shape and size under a compound light microscope. For strains of *B. squamosa*, sclerotia formed at 20°C in the dark on 20-day-old PDA cultures were placed on moistened sand (autoclaved) in Petri dishes, 15 sclerotia per dish and 5 dishes per strain. The dishes were sealed individually using Parafilm® M (Menasha, WI, USA) and incubated at 4°C in the dark for 30 days. Conidia produced on sclerotia were examined for shape and size. For strains of the unidentified *Botrytis* sp., mycelial agar plugs (6 mm diameter) were removed from 2-day-old PDA cultures of each strain and inoculated on five young leaves of green onion (cultivar Ruo Ye) detached from 60-day-old plants, one plug per leaf. Leaves of green onion inoculated with PDA alone were used as control. The inoculated green onion leaves were placed on moist paper towels in a plastic tray (15 × 12 × 4 cm, L × W × H). The trays were individually sealed with 0.1-mm-thick transparent plastic film (Gold Mine Plastic Industry Ltd, Jiangmen, China) and incubated at 20°C under the regime of 12-h light/12-h dark for 5 days. Conidia formed on the necrotic lesions at the inoculation sites were examined for shape and size. For each representative *Botrytis* strain investigated, 50 randomly chosen conidia were measured for length and width. Strain OnionBC-76 of *B. byssoidea* failed to produce sclerotia or conidia on PDA or on leaves of green onion. Therefore, it was not included in this experiment for conidial characterization.

Scanning electron microscopy was used to examine the morphological feature of conidia of strain OnionBC-23. Conidia of this strain were mounted on a glass cover slip (3 × 3 mm, L × W) by gently pressing the cover slip on the sporulated area of a grey mould lesion on a diseased green onion leaf. Conidia on the cover slip were vapor-fixed for 48 h in a Petri dish containing a drop of 2% (w/v) aqueous solution of osmium tetroxide. The cover slip was then immersed in 2.5% (w/v) glutaraldehyde solution in sodium phosphate buffer (0.05 M, pH 7.0) at 4°C for 16 h, washed three times in the sodium phosphate buffer for 10 min each time, and dehydrated in a graded ethanol series using the method described by Giesbert et al. (1998). After critical point drying in a dryer (13200-AB, SPI Supplies, PA, USA) and gold coating in a sputter coater (JFC-1600, NTC, Japan), conidia on the cover slip were examined under a scanning electron microscope (JSM-6390/LV, NTC, Japan).

DNA extraction, amplification, and sequencing

Mycelia of each representative *Botrytis* strain (Table 1) were collected from 2-day-old cultures grown at 20°C on autoclaved cellophane films placed on PDA in Petri dishes. Genomic DNA was extracted using the mini-preparation procedure described by Möller et al. (1992). Extracted DNA was dissolved in TE buffer [10 mM Tris–hydrochloric acid (HCl), 1 mM ethylenediaminetetraacetate (EDTA), pH 8.0] in a 1.5-ml centrifuge tube and stored at −20°C as stock solution until use. Concentration of the stock DNA solution was diluted and roughly estimated on agarose gels after electrophoresis by comparison of brightness of DNA bands with that of the DNA marker (TAKARA Biotechnology Co., Ltd, Dalian, China) at 10–30 ng/μl. The stock DNA solution was diluted with double-distilled water to the final concentration of about 50 ng DNA/μl. An aliquot of 1 μl of the diluted DNA solution was added to each PCR reaction mixture in a 0.25-ml PCR tube to amplify the internal transcribed spacer (ITS) region (White et al. 1990) or the partial DNA sequences of the three nuclear genes, *G3PDH*, *HSP60*, and *RPB2* (Staats et al. 2005). All PCR amplifications were performed in PTC-100TM Peltier Thermal Cycler (Hercules, CA, USA) with the programs described by Zhang et al. (2010). The PCR product (ITS, *G3PDH*, *HSP60*, or *RPB2*) was separated by 1% (w/v) agarose gel electrophoresis. The target DNA was purified from the gel using the AxyPrep™ DNA Gel Extraction Kit (Axygen Scientific Inc., Union City, NJ, USA), A-tailed and ligated into the pMD18-T vector (TaKaRa Biotechnology Co., Ltd, Dalian, China), and then transformed into competent cells of *Escherichia coli* DH5α. Positive *E. coli* clones grown on Luria–Bertani agar medium containing ampicillin (50 μg/ml) were selected and individually tested for size of the DNA insert. Three positive clones containing the sequence of ITS or each nuclear gene (*G3PDH*, *HSP60*, and *RPB2*) were individually sequenced in both directions with the dideoxynucleotide termination method using a Big Dye Terminator v2.0 Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) on an ABI PRISM 377-96 automatic sequencer (Beijing AuGCT Biotechnol. Co., Ltd, Beijing, China). The complementary sequences for ITS or each nuclear gene for each representative *Botrytis* strain were aligned with DNAMAN sequence analysis software (version 5.2.2, Lynnon Corporation, Vaudreuil, Quebec, Canada) to get a consensus sequence, which was then submitted to GenBank (Table 1).

Phylogenetic analysis

The representative strains of *Botrytis* spp. under this investigation (Table 1) and reference strains representing 23 recognized species of *Botrytis* (Staats et al. 2005) were included in the phylogenetic analysis. Strain 9201 of *Monilinia fructigena* Honey and strain 484 of *Sclerotinia sclerotiorum* de Bary (Staats et al. 2005) were used as outgroups. A combined gene sequence data set (*G3PDH* + *HSP60* + *RPB2*) and three separate gene sequence data sets (*G3PDH*, *HSP60*, *RPB2*) were established using the partial gene sequences of *G3PDH*, *HSP60,* and *RPB2* cloned in this study (Table 1) and in a previous study (Staats et al. 2005). Phylogenetic analyses were conducted using sequences from the Clustal W multiple alignment output by the neighbor-joining (NJ) method or by the maximum parsimony (MP) method (Saitou and Nei 1987) in the MEGA 4.0 interface: pairwise alignment parameters: gap opening penalty 15, gap extension penalty 6.66; multiple alignment parameters: gap opening penalty 15, gap extension penalty 6.66, delay divergent cutoff 30%, DNA transition weight 30%; weight matrix IUB with 1,000 bootstrap replicates. Nucleotide gaps/missing data in the sequences for each gene were completely deleted (Tamura et al. 2007).

Pathogenicity test

Pathogenicity of 16 representative strains of *Botrytis* spp. was tested on green onion cultivar Ruo Ye, garlic cultivar Feng Suan No. 1, and garlic chives cultivar Ping Feng No. 6. The three *Allium* crops were planted in the fall of 2008 in a field near the campus of Huazhong Agricultural University, Wuhan, China. In early March of 2009, young healthy leaves were excised from these crops, washed, and placed on moist paper towels in plastic trays (30 × 25 × 6 cm, L × W × H), with 17 leaves per tray spaced at about 0.5 cm between leaves. Mycelial agar plugs (6 mm diameter) of each *Botrytis* strain were removed from the colony margin of a 2-day-old PDA culture (20°C), and two plugs were inoculated face down on each leaf spaced about 6 cm between the two agar plugs. For the control treatment (CK), two uncolonized PDA plugs were inoculated on each leaf. The trays were sealed individually using 0.1-mm-thick transparent plastic film (Gold Mine Plastic Industrial Ltd). There were five trays (replicates) for each crop. After incubation at 20°C under the regime of 12-h light/12-h dark for 3 days, lesion length that developed at each inoculation site was measured. The experiment was performed twice.

Data analysis

Data on the radial growth rate of each *Botrytis* strain on PDA and leaf lesion length caused by each *Botrytis* strain on green onion, garlic, and garlic chives were analyzed using the analysis of variance (ANOVA) program in SAS software (SAS Institute, Cary, NC, USA, Version 8.0, 1999). Data on leaf lesion length collected from the two independent tests were pooled together for ANOVA, as there were no strain × experiment interaction effects. Means of radial growth rate or leaf lesion length for different strains of *Botrytis* spp. were separated using the least significant difference test at $\alpha = 0.05$.

Results

Taxonomy

Based on colony morphology of 15-day-old PDA cultures (20°C), 367 *Botrytis* strains collected from bulb onion, green onion, garlic, and garlic chives in 59 counties/cities of Hubei Province, China, were identified as five distinct species, namely, *B. cinerea*, *B. squamosa*, *B. porri*, *B. byssoidea* and an undescribed *Botrytis* species (*Botrytis* sp.). Among these *Botrytis* species, *B. cinerea* was the most common (64.3%), distributed in 56 counties/cities, followed by *B. squamosa* (29.9%) in 50 counties/cities (Fig. 1; Table 2). Three other species occurred sporadically in Hubei Province, *B. porri* (3.3%) in three counties/cities, *B. byssoidea* (0.3%) in one county, and *Botrytis* sp. (2.2%) in seven counties/cities.

Cultures of strains OnionBC-23, OnionBC-59, and LeekBC-18 of *Botrytis* sp. on PDA did not produce fluffy aerial mycelia and conidia, but they formed numerous

Table 2 Number and percentage of strains of *Botrytis* species collected from four *Allium* crops grown in Hubei Province of China

Host plant	Bc	Bsq	Bp	Bb	Bsi	Total
Green onion (*A. fistulosum*)	93	36	1	1	7	138
Onion (*A. cepa*)	16	3	0	0	0	19
Garlic (*A. sativum*)	104	44	11	0	0	159
Garlic chives (*A. tuberosum*)	23	27	0	0	1	51
Total (%)	236 (64.3)	110 (29.9)	12 (3.3)	1 (0.3)	8 (2.2)	367

Bc, *B. cinerea*; Bsq, *B. squamosa*; Bp, *B. porri*; Bsi, *B. sinoallii*; Bb, *B. byssoidea*

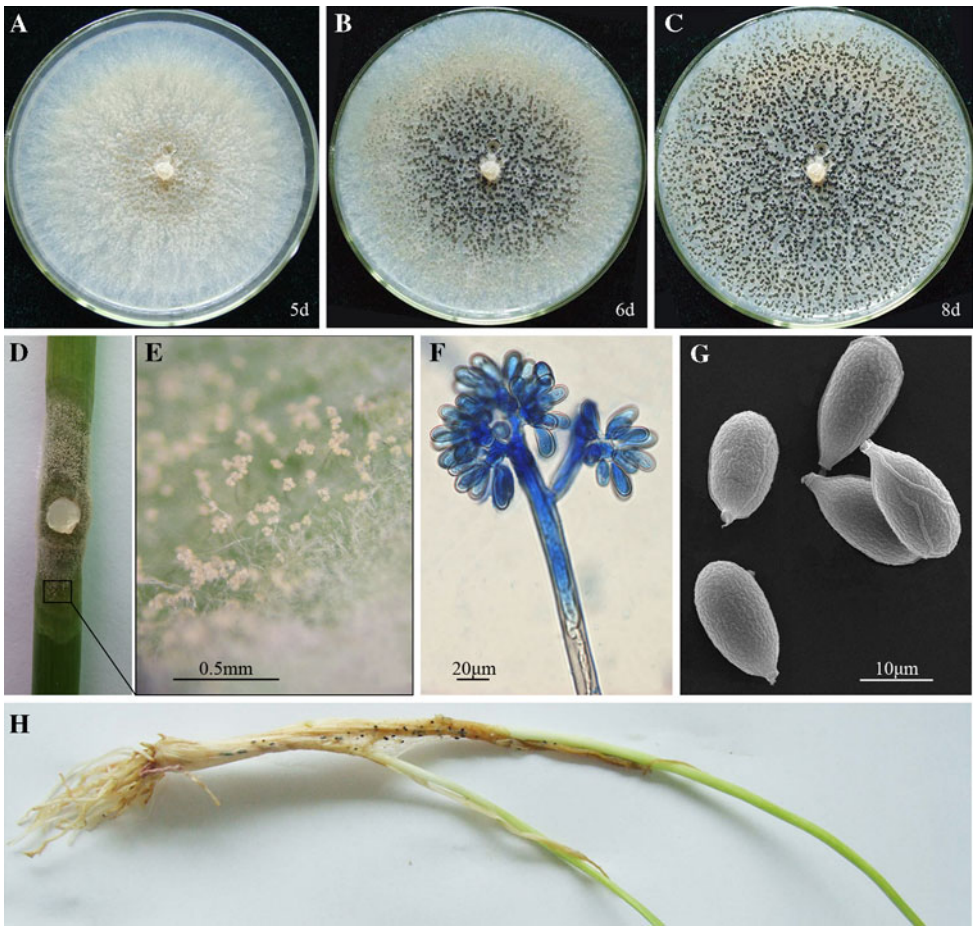

Fig. 2 Morphological characteristics of colonies, sclerotia, conidiophores, and conidia produced by strain OnionBC-23 of *Botrytis sinoallii*. **a–c** A colony of *B. sinoallii* on potato dextrose agar (PDA) at 20°C in the dark for 5, 6, and 8 days. Note development of numerous small sclerotia from the center of the colony. **d**, **e** Sporulation of *B. sinoallii* on a lesion of a green onion leaf (at 20°C for 5 days). **f** A conidiophore of *B. sinoallii* (stained with lactophenol cotton blue) bearing clusters of botryose conidia. **g** A scanning electron micrograph showing conidial morphology of *B. sinoallii*. **h** Numerous small sclerotia produced by strain OnionBC-23 of *B. sinoallii* on a plant of green onion (at 20°C for 10 days)

small sclerotia (Fig. 2a–c; Table 3). In contrast, cultures of strains of *B. cinerea*, *B. squamosa* and *B. porri* on PDA produced fewer but larger sclerotia, compared to strains of *Botrytis* sp. Cultures of *B. byssoidea* strain OnionBC-76 did not produce sclerotia on PDA. Conidial formation was frequently observed on PDA cultures of strains of *B. cinerea* and *B. porri*, but was not observed on PDA cultures of strains of *Botrytis* sp., *B. squamosa*, and *B. byssoidea*. Based on these results, strains of *Botrytis* sp. from green onion and garlic chives were assigned to a new species, named *B. sinoallii* sp. nov.

Botrytis sinoallii J. Zhang, G.Q. Li & W.Y. Zhuang, sp. nov. Figs. 2a–h

MycoBank no.: 516747.

Etym. "*sinoallii*" refers to the place ("*sino-*" = China) and the host plant ("*-allii*" = of *Allium*), from which the fungus was originally isolated.

Coloniis in PDA ad 13.9 mm/day (20°C) crescentibus, pallide griseis; myceliis aeriis velutinis nullis; *sclerotiis* abundis, irregularibus, sphaericis vel ellipsoideis, 0.1–1.2–4.5 × 0.1–0.8–2.1 mm; *conidiophoris* apice alternatim ramosis, erectis, septatis, 544–885–1256 × 11.2–14–15.4 μm, cellulis condiogenis ad apicem parum inflates; *conidiis* ellipsoideis vel ovoideis, unicellularibus, hyalinis vel brunnis, porcate rugosis, 15.7–20.0–25.1 × 8.4–11.9–13.3 μm.

Holotypus HMAS 250008 (M), dried culture specimens (from strain OnionBC-23) grown on PDA is deposited in the China General Microbiological Culture Collection Center (CGMCC) in the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China (HMAS).

Ex-holotype cultures 3.13896 (CGMCC), OnionBC-23 (Huazhong Agricultural University). This fungal strain was collected by Ms. L. Zhang from a diseased leaf of *A. fistulosum* in Xianning City, Hubei Province, China, on 2 May 2006.

Additional specimens HMAS 250009 (M), dried culture specimens (from strain OninBC-59) grown on PDA is deposited in CGMCC.

Additional cultures 3.13897 (CGMCC), strain OnionBC-59 (Huazhong Agricultural University). This fungal strain was collected by Ms. J. Zhang from a diseased leaf of *A. fistulosum* in Jianshi County, Hubei Province, China, on 6 May 2007.

Mycelial growth at 13.9 mm/day on PDA at 20°C. Colonies white to pale grey; sclerotia abundant on PDA

Table 3 Radial growth rate (RGR), colony characteristics, and morphology of conidia and sclerotia formed by *Botrytis sinoallii* and other *Botrytis* species

Species	Colony		Conidia[b]			Sclerotia		
	RGR (mm day^{-1})	Characteristics	Shape	Size (μm)	L/W	No. per dish	Shape	Size (mm)
B. sinoallii (3 strains)	13.9b[a]	Mycelia white, sclerotia abundant and small, no conidia	Elliptical to oblong	16–25 × 8–13	1.68	1220 ± 79	Spherical, elliptical	0.1–4.5 × 0.1–2.1
B. aclada (3 strains)	11.3b	Gray powdery appearance conidia and no sclerotia	Oblong	6–14 × 4–7	1.81	–	–	–
B. byssoidea (1 strain)	12.6b	Mycelia white to gray, no conidia, no sclerotia	–	–	–	–	–	–
B. cinerea (3 strains)	17.8a	Mycelia fluffy and gray, sclerotia scattered, conidia brown	Elliptical to ovoid	7–14 × 6–13	1.28	84 ± 27	Irregular, spherical	1.0–12.9 × 0.7–6.1
B. porri (3 strains)	12.6b	Mycelia ropy, conidia gray to brown, sclerotia convoluted	Obovate	10–18 × 7–14	1.36	19 ± 13	Cerebriform, convoluted	2.0–13.0 × 1.5–7.0
B. squamosa (3 strains)	10.8b	Mycelia white/cottony, collapsed, sclerotia scattered, no conidia	Oblong	12–30 × 8–20	1.44	239 ± 123	Elliptical, spherical	0.6–12.9 × 0.5–5.2

"–" indicates no formation of conidia or sclerotia on PDA or on leaves of green onion after incubation at 20°C for 30 days

L/W Length to width ratio

[a] Mean values followed by the same letters within the column indicate no significant difference ($P > 0.05$) according to least significant difference (LSD) test

[b] Conidia produced on PDA for 12 days at 20°C by strains of *B. aclada*, *B. cinerea*, or *B. porri*, on leaves of green onion inoculated with strains of *B. sinoallii* for 5 days at 20°C, or on germinated sclerotia of strains of *B. squamosa* for 30 days at 4°C

and onion leaves, black in color, spherical to elliptical in shape, discrete or aggregated, small in size (0.1–1.2–4.5 × 0.1–0.8–2.1 mm); conidiophores and conidia did not form on PDA. Conidiophores on infected leaves of green onion erect, septate, alternately branched at the top, pale grey, 544–885–1256 × 11.2–14–15.4 μm in size. Conidiogenous cells slightly inflated at the apex. Conidia in botryose clusters, unicellular, hyaline to pale brown in color, elliptical to oblong in shape, wrinkled on the surface with ridges, 15.7–20.0–25.1 × 8.4–11.9–13.3 μm in size, length/width ratio 1.68.

Analysis of ITS sequences

The ITS and flanking regions of rDNA amplified from all representative strains of *Botrytis* spp. comprised 539 bp. Without the flanking regions (the partial 18S rDNA and the partial 28S rDNA), the sequence of ITS (ITS1 + 5.8S rDNA + ITS2) for each *Botrytis* strain was composed of only 453 bp. The 453-bp ITS region of three strains of *B. sinoallii* (OnionBC-23, OnionBC-59, and LeekBC-18) showed 99.8–100% identity. Meanwhile, the 453-bp ITS region of strains of *B. sinoallii* showed 98.9–100% identity to that of the representative strains of *B. porri*, *B. aclada*, *B. cinerea*, *B. squamosa*, and *B. byssoidea* listed in Table 1 (data not shown). Therefore, the sequence information of the ITS region was not used to infer the phylogenetic status of *B. sinoallii* in the genus *Botrytis* in this study.

Phylogenetic analysis

The data set of the combined partial sequences of *G3PDH* + *HSP60* + *RPB2* for 68 fungal taxa contained 2969 characters, of which 2216 were conserved and 747 were variable, and the number of the parsimony-informative characters was 517. NJ and MP analyses of the combined DNA sequences generated two phylogenetic trees with a similar topological structure (data not shown). In the NJ tree, strains of 23 well-recognized species of *Botrytis* and *B. sinoallii* formed a clade with 100% bootstrap support, compared with the outgroup fungi (*M. fructigena* and *S. sclerotiorum*) (Fig. 3). Within the *Botrytis* clade, strains of *B. sinoallii* (OnionBC-23, OnionBC-59, and LeekBC-18) were grouped together, forming a monophyletic lineage with 100% bootstrap support. Meanwhile, strains of *B. cinerea*, *B. squamosa*, *B. aclada*, *B. porri*, and *B. byssoidea* investigated in this study were grouped together, with respective reference strains representing these species of *Botrytis* (Staats et al. 2005) to form respective lineages with the bootstrap support of 90–100% (Fig. 3). The lineage of *B. sinoallii* was closely related to *B. ranunculi*, *B. ficariarum*, *B. elliptica*, and *B. squamosa* but was distantly related to *B. aclada*/*B. allii*, *B. byssoidea*, *B. cinerea*,

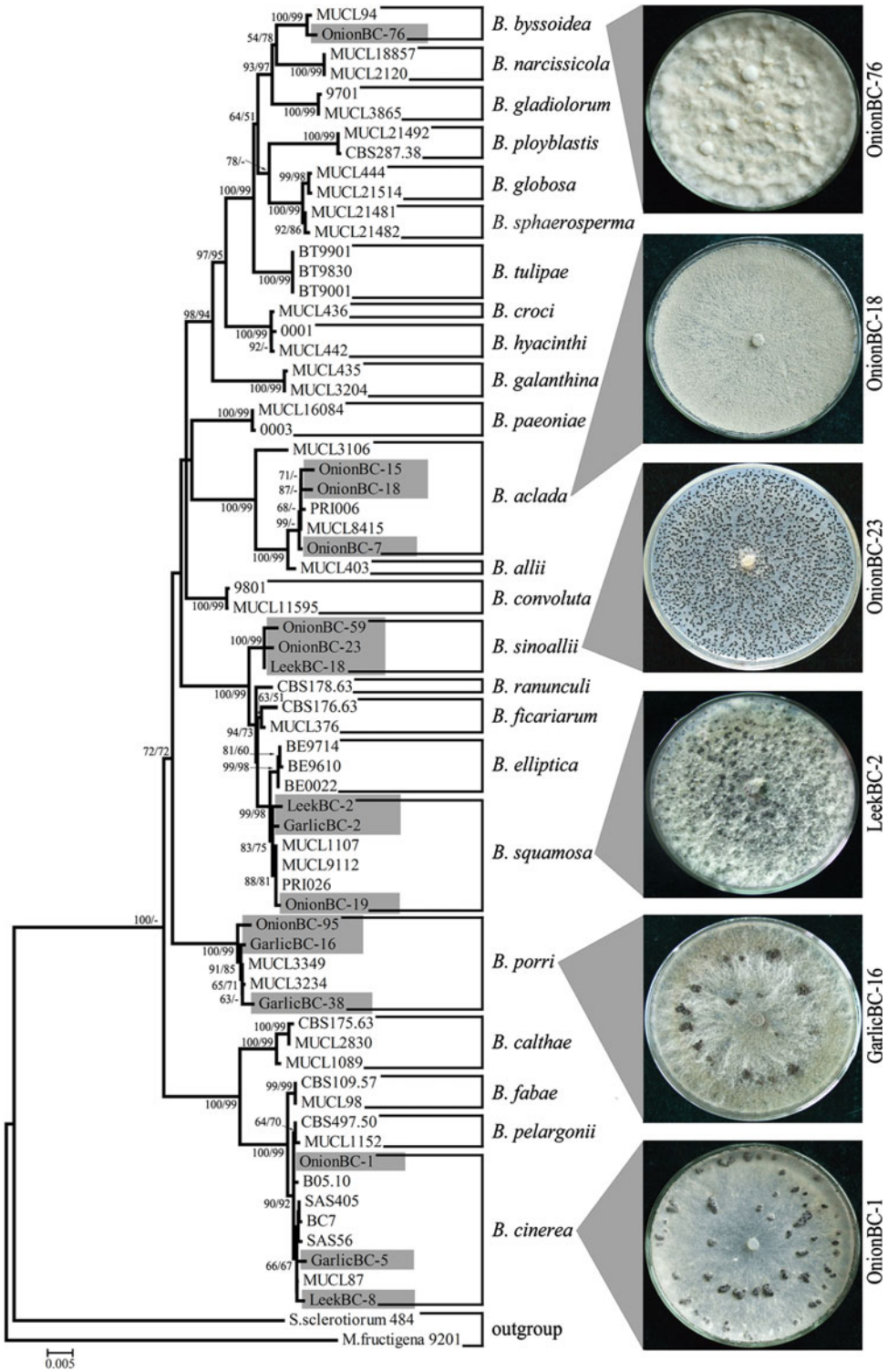

Fig. 3 Molecular phylogeny of 68 taxa of *Botrytis* spp., *Sclerotinia sclerotiorum*, and *Monilinia fructigena* presented by a neighbor-joining (NJ) tree inferred from the data set containing the combined DNA sequences of *G3PDH*, *RPB2*, and *HSP60*. The *numbers* labeled at each node indicate the bootstrap (*BS*) percentage ($N = 1000$): BS value from the NJ analysis/BS value from the maximum parsimony (MP) analysis. *Shaded areas* in the graph highlight strains of *B. aclada*, *B. byssoidea*, *B. cinerea*, *B. porri*, *B. sinoallii,* and *B. squamosa* investigated in this study. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured by the *scale bar* (0.5% of sequence divergence). Colony morphology of potato dextrose agar (PDA) cultures of the six *Botrytis* strains representing *B. aclada*, *B. byssoidea*, *B. cinerea*, *B. porri B. sinoallii*, and *B. squamosa* is shown beside the phylogenetic tree

B. globosa, *B. porri,* and *B. sphaerosperma*. Results from phylogenetic analyses using the three separate data sets of *G3PDH*, *HSP60*, and *RPB2* are similar to the result of the phylogenetic analysis using the combined data set (data not shown).

Pathogenicity

Whereas PDA-inoculated control leaves of green onion, the garlic and garlic chive leaves remained healthy at 20°C for 72 h; leaves of these crops inoculated with *B. sinoallii*, *B. cinerea, B. squamosa, B. aclada, B. porri*, or *B. byssoidea* developed water-soaked lesions. The average lesion length on each crop varied with different species of *Botrytis* and even with different strains within each *Botrytis* species (Table 4). For example, leaf lesion length caused by strains of *B. sinoallii* varied from 24.5 to 26.4 mm (mean 25.3 mm) on green onion, from 13.0 to 15.7 mm (mean 14.3 mm) on garlic, and from 19.4 to 24 mm (mean 21.8 mm) on garlic chives. However, leaf lesion length caused by strains of *B. squamosa* varied from 11.4 to 15.6 mm (mean 13.7 mm) on green onion, from 14.9 to 18.7 mm (mean 17.0 mm) on garlic, and from 14.3 to 17.0 mm (mean 15.2 mm) on garlic chives (Table 4). After incubation for 96 h, sporulation was profuse on lesions caused by strains of *B. sinoallii* (Fig. 2d, e), *B. aclada*, *B. cinerea*, and *B. porri* (data not shown), but no sporulations were observed on lesions caused by *B. byssoidea* and *B. squamosa* (data not shown).

Discussion

Eight species of *Botrytis* on *Allium* spp., namely, *B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea*, *B. porri*, *B. squamosa*, *B. globosa*, and *B. sphaerosperma*, have been previously recorded in the literature (Hennebert 1963, 1973; Yohalem et al. 2003). The study reported here showed that four of these *Botrytis* species, namely, *B. cinerea*, *B. squamosa*, *B. porri*, and *B. byssoidea*, were found on *Allium* crops grown in Hubei Province, China, whereas the other four species, namely *B. aclada*, *B. allii*, *B. globosa*, and *B. sphaerosperma*, were not found on *Allium* crops grown in this province. Zhang et al. (2008) reported that *B. aclada* frequently occurs on onion bulbs (*A. cepa*) in storage or on sale in supermarkets in Wuhan, the capital city of Hubei Province, causing the onion bulb rot. No occurrence of *B. aclada* on bulb onions and on other *Allium* crops grown in Hubei Province under field conditions suggests that *B. aclada* occurring on onion bulbs in Wuhan might be bulb-borne from the north of China, where bulb onions are massively cultivated and commercially imported to Wuhan for consumption as a vegetable.

A new species of *Botrytis*, *B. sinoallii*, isolated from green onion and garlic chive grown in Hubei Province is described in this study. It has distinct morphological characteristics of sclerotia (yield, shape, and size) and conidia (shape and size) different from those of *B. aclada*, *B. cinerea*, *B. squamosa*, *B. porri*, and *B. byssoidea* according to this study, as well as different from *B. allii*,

Table 4 Lesion length (mm) on leaves of green onion, garlic, and garlic chives caused by different species of *Botrytis* (at 20°C for 72 h)

Treatment[a]	Strain	Lesion length (mm) ($n = 34$)		
		Green onion	Garlic	Garlic chives
B. sinoallii	OnionBC-23	24.5 ± 6.5de[b]	14.3 ± 2.5gh[b]	24.0 ± 3.8cd[b]
	OnionBC-59	25.0 ± 3.5d	13.0 ± 2.4h	22.1 ± 5.2def
	LeekBC-18	26.4 ± 4.8cd	15.7 ± 5.3efgh	19.4 ± 3.0fgh
B. cinerea	OnionBC-1	25.9 ± 7.2cd	15.7 ± 5.4efgh	26.7 ± 4.9ab
	GarlicBC-5	30.3 ± 6.3ab	18.0 ± 3.8def	25.8 ± 5.6bc
	LeekBC-8	31.4 ± 6.0ab	16.6 ± 5.5efg	28.7 ± 5.2a
B. squamosa	OnionBC-19	14.2 ± 3.7hi	14.9 ± 4.4fgh	14.4 ± 3.5ij
	GarlicBC-2	15.6 ± 3.1h	18.7 ± 2.9de	17.0 ± 2.0hi
	LeekBC-2	11.4 ± 4.4i	17.4 ± 5.1efg	14.3 ± 4.6j
B. aclada	OnionBC-7	24.5 ± 3.6de	26.1 ± 5.9a	22.1 ± 3.1de
	OnionBC-15	21.8 ± 3.6efg	24.9 ± 8.2ab	16.9 ± 3.2hij
	OnionBC-18	19.6 ± 5.6g	22.8 ± 8.9bc	17.1 ± 4.9h
B. porri	OnionBC-95	28.7 ± 4.5bc	17.6 ± 3.5ef	20.1 ± 1.6efg
	GarlicBC-16	21.4 ± 3.5fg	22.5 ± 6.0bc	21.2 ± 6.0efg
	GarlicBC-38	23.7 ± 4.2def	21.1 ± 2.6cd	19.3 ± 2.3gh
B. byssoidea	OnionBC-76	32.6 ± 4.5a	22.7 ± 2.5bc	28.4 ± 6.1ab
CK		0j	0i	0k

[a] Leaves of green onion, garlic, or garlic chives were inoculated with potato dextrose agar (PDA) cultures of each *Botrytis* strain or uncolonized PDA (CK)

[b] Means within each column followed by the same letters are not significantly different at 5% level according to the least significant difference test

B. globosa, and *B. sphaerosperma* reported in previous studies (Hennebert 1963, 1973; Yohalem et al. 2003; Chilvers and du Toit 2006). The morphological delimitation of *B. sinoallii* as a unique species is further supported by phylogenetic analyses of the three nuclear genes (*G3PDH*, *HSP60*, and *RPB2*). Whether *B. sinoallii* occurs on *Allium* crops grown in other provinces in China or in other countries remains unknown and needs further investigation.

Phylogenetic analyses of this study showed that *B. sinoallii* was closely related to *B. elliptica*, *B. ficariarum*, *B. ranunculi*, and *B. squamosa*. However, *B. sinoallii* is different from these species in morphological features of sclerotia and conidia. Sclerotia of *B. sinoallii* (1.1 × 0.7 mm) are smaller than sclerotia of *B. elliptica* (1–5 mm diameter) (Furukawa et al. 2005), *B. ficariarum*, and *B. ranunculi* (3–10 × 3–5 mm) (Hennebert and Groves 1963). Conidia of *B. sinoallii* (15.7–25.1 × 8.4–13.3 μm) are smaller in size than conidia of *B. elliptica* (21.5–30.0 × 12.8–20.0 μm) (Furukawa et al. 2005) but larger than conidia of *B. ficariarum* (8.8–18 × 4.5–9.5 μm) and *B. ranunculi* (11–15 × 6–10 μm) (Hennebert and Groves 1963). Besides morphological features, the host range of *B. sinoallii* is also different from that of *B. elliptica*, *B. ficariarum*, and *B. ranunculi*. According to this study, *B. sinoallii* can infect green onion, garlic, and garlic chives belonging to Alliaceae, whereas *B. elliptica* was found on *Lilium* spp. and *Fritillaria ussuriensis* Maxim. belonging to Liliceae (Furukawa et al. 2005). *B. ficariarum* and *B. ranunculi* were found on *Ficaria verna* Huds. and *Ranunculus* spp. belonging to Ranunculaceae (Staats et al. 2005).

Although *B. sinoallii* was isolated from *Allium* crops less frequently than *B. cinerea* and *B. squamosa* in Hubei Province, potential threat of this pathogen on production of *Allium* crops in this province still exists. First, the presence of *B. sinoallii* in the northwest, southwest, and southeast counties/cities of Hubei Province suggests that it is adaptive to the climatic conditions of this province, which is humid and cool in spring, humid and hot in summer, dry and cool in autumn, and dry and cold in winter. Second, the massive numbers of conidia produced on infected leaves of green onion, garlic, and garlic chive may serve as the inoculum source for the secondary spread of the disease in fields. Third, the disease caused by *B. sinoallii* is of particular importance on garlic, because garlic is one of the most important *Allium* crops in Hubei Province. On the other hand, *B. sinoallii* might pose no potential threat to broad bean (*Vicia faba* L.), pea (*Pisium sativum* L.), oilseed rape (*Brassica napus* L.), and wheat (*Triticum aestivum* L.) in Hubei Province, as *B. sinoallii* was not found in fields of these winter crops grown near *Allium* crops during our surveys in 2006–2009 (Zhang et al. 2007, 2008, 2009, 2010).

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