Genera *Acremonium* and *Sarocladium* Cause Brown Spot on Bagged Apple Fruit in China

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Abstract

Apple fruit spot disease has caused serious economic losses for years in China since the widespread application of fruit bagging in production. Although the three genera *Trichothecium*, *Alternaria*, and *Acremonium* have been reported to be the causal agents, studies on the disease etiology and pathogen biology are still sparse. Here, we report characterization of eight fungal isolates from lesions on 126 symptomatic fruit samples collected in Shaanxi Province, China. Pathogenicity of the isolates was assessed. DNA sequences were obtained at four loci, including D1/D2 domains of the large-subunit nrRNA gene, internal transcribed spacer regions 1 and 2, 5.8S nrDNA gene, a fragment of the actin gene, and a fragment of the β -tubulin. Based on phylogenetic analysis and morphological features, three new species were found: *Acremonium mali*, *Sarocladium*

liquanensis, and *Sarocladium mali*. In addition, we made the first report of *Sarocladium terricola* as a plant pathogen. Temperature and moisture significantly affected in vitro conidial germination of five *Acremonium*-like species, and their impact on infection of apple fruit was tested using *Acremonium sclerotigenum*. Conidia of five species germinated from 15 to 35°C in free water; four of the species had optimum temperature around 25°C, whereas conidia of *S. terricola* had an optimum temperature of 30°C. Conidial germination rate increased as relative humidity (RH) increased. The five isolates had relatively high conidial germination rates at RH > 97%, with a significant decline at 95% RH. Incidence of infection also increased in proportion to RH. In free water, conidial germination was relatively unaffected by temperature.

In China, covering apples with fruit bags from May to October is a widely used production technology. The bags can protect apple fruit from pests and rain-dispersed pathogens, and they can also reduce pesticide residues at harvest. However, this change of fruit microenvironment has led to the emergence of fruit spot disease in China. Black spot disease on apple fruit was first observed in 2000 in Tianshui and Longnan districts of Gansu Province (Xu et al. 2000), and it was later discovered in most of the production areas in Shaanxi, Shandong, and Hebei Provinces (Wang et al. 2007). Incidence levels ranged from 3 to 60% in these areas, and in one case, they reached 90% (Guo et al. 2005), causing serious economic losses. A brown spot disease affecting apple in Shandong Province caused 1 to 30% annual yield losses in most orchards from 2010 to 2012 (Li et al. 2014).

Several factors seem to influence incidence of fruit spot disease. The widely grown cultivar Fuji was the most susceptible. Rainfall showed a significant positive correlation with fruit spot incidence (Li et al. 2015). Disease incidence dropped significantly when air-permeable fruit bags were used. All of these studies indicated that temperature and moisture inside the fruit bags may have significant impact on disease incidence (Dai et al. 2019; Li et al. 2015; Wang et al. 2007).

Apple fruit spot can be caused by a wide range of fungal species, and the symptoms also vary with the predominant causal agents and apple cultivars. *Cylindrosporium pomi* and *Phoma pomi*, which cause green brown spots with red haloes, were detected in Gansu Province (Xu et al. 2000), whereas *Trichothecium roseum*,

Acremonium stictum, and *Alternaria* spp. caused black or brown spots on apple fruit in Shaanxi Province (Guo et al. 2005). In Shandong Province, *Acremonium sclerotigenum* was reported as the causal agent of brown spot disease (Li et al. 2014).

The objective of this study was to characterize the diversity of brown spot pathogens in Shaanxi Province. Orchard surveys were carried out from 2014 to 2016. The impact of temperature and relative humidity (RH) on conidial germination and disease incidence was also characterized.

Materials and Methods

Sampling areas and isolation technique. One hundred twenty-six symptomatic apple fruits were collected from 19 orchards in Liquan, Qianxian, Wugong, and Yangling districts of Shaanxi Province, China, from 2014 to 2016. Sampled fruit were rinsed with sterile water, air dried, and surface sterilized with 75% ethyl alcohol for 30 s followed by rinsing with sterile water and air drying under aseptic conditions. Tissue samples (0.5 cm³) from the margins of brown spot lesions were transferred to potato dextrose agar (PDA) in a sterile environment and incubated at 25°C for 1 week in darkness, and pure cultures were obtained by single-spore transfer (Wiseman et al. 2015). Pure isolates were stored in 20% glycerol at –80°C.

The representative isolates were deposited in the Agricultural Culture Collection of China (ACCC; Beijing, China), and dried cultures were deposited in the Herbarium Mycologicum Academiae Sinicae (HMAS; Beijing, China). New species names and descriptions were deposited in Fungal Names (<http://fungalinform.im.ac.cn/fungalname/fungalname.html>).

Culture characteristics and morphological observation. After 2 weeks of growth on PDA and oatmeal agar (OA) plates at 25°C, colony color was described, and colony diameter was measured. The effect of temperature on mycelial growth on PDA was assessed at temperatures of 5, 10, 15, 20, 25, 30, 35, and 40°C. Microscopic examination was carried out after 2 weeks of growth on PDA at 25°C in darkness. Isolates were allowed to grow onto an adjacent sterile coverslip that had been partially inserted into the agar surface at a 60° angle to measure and observe fungal structures (Li et al. 2011), and sterile water was used as a mounting medium for microscopy. Wherever possible, measurements were made on 30 spores per isolate (Batzer et al. 2005).

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Genomic DNA extraction, polymerase chain reaction, and sequencing. Genomic DNA for polymerase chain reaction (PCR) was extracted from mycelium that had grown on PDA petri dishes at 25°C for 14 days according to the protocol of Li et al. (2011). Primer pairs ITS1-F/ITS4 (White et al. 1990), LSU1Fd/LR5 (Cubeta et al. 1991; Vilgalys and Hester 1990), ACT-512F/ACT1Rd (Carbone and Kohn 1999; Groenewald et al. 2013), and Tub-F/Tub-R (Cruse et al. 2002) were used to amplify the internal transcribed spacer (ITS) regions 1, 2, and 5.8S rDNA gene, the D1/D2 domains of the large subunit (LSU) nucleus ribosome RNA (nrRNA) gene, a fragment of the actin gene (*ACT1*), and a fragment of β -tubulin gene (*BT2*), respectively. The PCR amplification mixture consisted of 1 unit of Taq polymerase (Thermo Scientific), 1× Taq buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.4 μ M forward and reverse primers, and 2 μ l of template DNA, with sterile water making up the total volume of 25 μ l. Reactions were performed on a PCR System S1000 Thermal Cycler (Bio-Rad). The cycling conditions for ITS and LSU were an initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s and a final 10-min extension step at 72°C. The same cycling conditions were used for amplifying *ACT1* and *BT2*, except that the annealing temperatures were changed to 58°C and 63°C, respectively. The PCR products were sequenced at Organism Technology, Shanghai, China. Sequences were deposited in the National Center for Biotechnology Information's GenBank (Table 1).

Sequence alignment and phylogenetic analysis. Reference sequences used for phylogenetic analysis were downloaded from the

National Center for Biotechnology Information's GenBank sequence database (Table 1). Sequences were aligned with MAFFT v. 7 (<http://mafft.cbrc.jp/alignment/server/index.html>), manually inspected, and adjusted using BioEdit v. 5.0.9.1 (Hall 1999) to avoid comparing non-homologous positions. Maximum parsimony (MP) analysis, maximum likelihood (ML) analysis, and Bayesian analysis were performed with PAUP v. 4.0b 10 (Swofford 2003), RAXML v. 7.0.3 (Stamatakis et al. 2005, 2008), and MrBayes v. 3.1.2 (Altekar et al. 2004), respectively. For MP analysis, heuristic searches were performed with 1,000 random sequence additions. Clade reproducibility was assessed by 1,000 bootstrap replications. For ML analysis, GTRGAMMA was used as the optimal nucleotide substitution model. Clade reproducibility was assessed by 1,000 bootstrap replications. For Bayesian analysis, the optimal nucleotide substitution models for each gene region and the combined data were estimated using MrModeltest v. 2.2 (Nylander 2004). The sequences described in this study were deposited in GenBank. Trees were visualized using TreeView v. 1.6.6 (Page 1996), and alignments and representative trees were deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S21591>).

Pathogenicity test. Field inoculation. The experiments were performed in an apple orchard located in Yangling, Shaanxi Province, China from June to September 2015. Asymptomatic apple fruit (cv. Fuji), similar in size and lacking blemishes, was selected for inoculation. Fruit surfaces were sanitized with 75% ethanol for 30 s, rinsed with sterile distilled water, and air dried. A conidial suspension (1×10^6 ml⁻¹) of the test isolates was misted on the surface with

Table 1. Species and GenBank accession numbers included in *Acremonium* and *Sarocladium* phylogenetic analyses^y

Species	Strain	Origin	GenBank accession number ^z			
			LSU/D1D2	ITS	<i>ACT1</i>	β -tubulin
<i>Acremonium alternatum</i>	CBS 407.66 ^T	<i>Hypoxyylon deustum</i> , Austria	HQ231988	—	—	—
<i>Acremonium bodinense</i>	CBS 101148 ^T	Soil in sugarcane field, Japan	HQ232003	—	—	—
<i>Acremonium brachypenium</i>	CBS 866.73 ^T	<i>Cocos nucifera</i> , Sri Lanka	HQ232004	—	—	—
<i>Acremonium campiosporum</i>	CBS 756.69 ^T	Soil, parasitic on nematodes, Germany	HQ232008	—	—	—
<i>Acremonium cavaeaeum</i>	CBS 101149 ^T	Outer wall of wooden house, Japan	HF680202	HF680220	—	HF680240
<i>Acremonium chrysogenum</i>	CBS 144.62 ^T	Soil, Maharashtra	HQ232017	—	—	—
<i>Acremonium citrinum</i>	CBS 384.96 ^T	Decaying fruit, Papua New Guinea	HF680217	HF680236	—	HF680257
<i>Acremonium curvulum</i>	CBS 430.66 ^T	Wheat field soil, Germany	HQ232026	—	—	—
<i>Acremonium exiguum</i>	CBS 587.73 ^T	<i>Tubulicium dussii</i> on <i>Dicksonia antarctica</i> , Sri Lanka	HQ232035	—	—	—
<i>Acremonium flavum</i>	CBS 596.70 ^T	Agricultural soil, Germany	HQ232037	—	—	—
<i>Acremonium fusidioides</i>	CBS 840.68 ^T	Dung of antelope, Central African Republic	HQ232039	FN706542	—	HF680257
<i>Acremonium fusidioides</i>	CBS 266.89	Agricultural soil, Germany	HF680205	HF680224	—	HF680245
<i>Acremonium fusidioides</i>	CBS 109069	Barley seeds, Ethiopia	HF680204	HF680223	—	HF680244
<i>Acremonium hennebertii</i>	CBS 768.69 ^T	<i>Elaeis guineensis</i> , Zaire	HQ232044	HF680238	—	HF680260
<i>Acremonium inflatum</i>	CBS 212.69 ^T	Intertidal salt marsh mud, the United Kingdom	HQ232050	—	—	—
<i>Acremonium mali</i>	ACCC 39305 ^T	<i>Malus × domestica</i> , China	MF993114	MF987658	—	MF987667
<i>Acremonium parvum</i>	CBS 381.70A ^T	<i>Tubercularia vulgaris</i> , The Netherlands	HQ231986	HF680219	—	HF680239
<i>Acremonium pilosum</i>	CBS 124.70 ^T	Agricultural soil, The Netherlands	HF680209	HF680228	—	HF680249
<i>Acremonium pilosum</i>	CBS 410.70	Agricultural soil, The Netherlands	HF680208	HF680227	—	HF680248
<i>Acremonium pilosum</i>	CBS 125.70	Agricultural soil, The Netherlands	HF680210	HF680229	—	HF680250
<i>Acremonium pilosum</i>	CBS 390.73	<i>Periconia cookie</i> on dead bamboo, India	HQ232043	AB540578	—	HF680258
<i>Acremonium persicinum</i>	CBS 310.59 ^T	<i>Tectona grandis</i> , India	HQ232077	—	—	—
<i>Acremonium pinkertoniae</i>	CBS 157.70 ^T	Soil, Italy	HQ232089	—	—	—
<i>Acremonium psammosporum</i>	CBS 590.63 ^T	Bark of <i>Populus canadensis</i> , Belgium	HQ232100	—	—	—
<i>Acremonium pseudozeilanicum</i>	CBS 560.73 ^T	Decaying leaf of <i>Cocos nucifera</i> , Sri Lanka	HQ232101	—	—	—
<i>Acremonium recifei</i>	CBS 137.35 ^T	Mycetoma of human, Brazil	HQ232106	—	—	—
<i>Acremonium roseolum</i>	CBS 289.62 ^T	Dead stems of <i>Dactylis glomerata</i> , Sheffield	HQ232123	—	—	—
<i>Acremonium salmoneum</i>	CBS 721.71 ^T	Dung of deer, Rajasthan	HQ232125	—	—	—
<i>Acremonium sclerotigenum</i>	CBS 124.42 ^T	Dune sand, under <i>Ammophila</i> and <i>Convolvulus</i> , France	HQ232126	—	—	—
<i>Acremonium sclerotigenum</i>	ACCC 39310	<i>Malus × domestica</i> , China	MF987655	—	—	—

(Continued on next page)

^y ACCC, Agricultural Culture Collection of China, Beijing, China; CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; ET, epitype strain; ITS, internal transcribed spacer; LSU, large subunit; MUCL, Mycothe'que de l'Universite' Catholique de Louvain, Louvain-la Neuve, Belgium; NT, neotype strain; T, type strain; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas.

^z Accession numbers of sequences newly determined in this study are indicated in bold.

a handheld spray bottle until runoff. Fruit bags were used to enclose fruit after the conidial suspension was almost air dry. For the control, sterile water was used instead of a conidial suspension. Lesion symptoms were assessed at 30 days after inoculation, when spots were >1 mm in diameter, and when reisolations were done. Four fruits were inoculated for each treatment. The experiment was performed three times on different dates (25 June, 10 August, and 12 September).

In vitro inoculation. Healthy mature apple fruits (cv. Fuji) purchased from a local grocery store were used for laboratory detached fruit inoculation in Yangling, Shaanxi. The fruits were washed with sterile water and surface disinfected with 75% ethyl alcohol for 30 s; then, they were rinsed with sterile distilled water. After the surface had air dried, a sterile inoculating needle was used to make about 30 wounds (0.3-mm deep and 0.3 mm in diameter) on the surface, after which conidial suspensions (1×10^6 ml⁻¹) of test isolates ACCC 39311, ACCC 39305, ACCC 39306, ACCC 39309, and ACCC 39308 were misted on the fruit surface until runoff. For the control, sterile water was used instead of a conidial suspension. For each isolate, four fruits were inoculated. Inoculated fruits were transported to a moisture chamber with 100% RH at 25°C. Ten days after inoculation, symptom development was assessed. The experiment was conducted three times on different dates. Ten days after inoculation, when lesions surrounding the inoculation site were >1 mm in diameter, reisolations of the causal agent were attempted, and the ITS regions of these isolates were PCR amplified and sequenced.

Effects of temperature and RH on conidial germination. The effect of temperature on conidial germination was tested at 5, 10,

15, 20, 25, 30, 35, and 40°C in distilled water. After a droplet (10 µl) of conidia suspension (1×10^6 ml⁻¹) was placed at each end of a sterile glass slide with a micropipette, the slide was placed in a 9-cm-diameter petri dish sealed with Parafilm containing 30 ml of 2% water agar to maintain the moisture of conidia suspension. The effect of RH was tested at RHs of 100, 99, 97, and 95% as well as a distilled water control. A droplet (10 µl) of conidial suspension (1×10^6 ml⁻¹) was placed at each end of a slide and air dried, after which the slide was placed inside a 9-cm-diameter petri dish containing 30 ml 2% water agar amended with different amounts of NaCl. The petri dish was sealed with Parafilm. RH levels of 100, 99, 97, and 95% were achieved with 0, 0.3, 0.9, or 1.5 M NaCl, respectively (Harris et al. 1970; Lang 1967; Wang et al. 2015). The petri dishes were placed in incubators at 25°C. The experiment was performed four times.

After 12 or 16 h of inoculation, a drop (10 µl) of lactophenol (lactic acid/phenol/glycerin/distilled water = 1:1:2:1 [vol/vol/vol/vol]) was added to each conidia suspension droplet to stop germination (Wang et al. 2015). Percentage conidial germination was measured by observing 500 conidia wherever possible with microscopy (Olympus BX51); conidia were considered to be germinated when the germ tube was longer than one-half the total length of a conidium.

Effects of temperature and RH on infection. The effects of temperature on infection by *A. sclerotigenum* were examined at seven temperature levels (10, 15, 20, 25, 30, 35, and 40°C). The inoculation method was the same as in the in vitro trials with wound inoculation

Table 1. (Continued from previous page)

Species	Strain	Origin	GenBank accession number ^z			
			LSU/D1D2	ITS	ACT1	β-tubulin
<i>Acremonium sclerotigenum</i>	ACCC 39311	<i>Malus × domestica</i> , China	MF987656	—	—	—
<i>Acremonium sclerotigenum</i>	ACCC 39312	<i>Malus × domestica</i> , China	MF987657	—	—	—
<i>Acremonium spinosum</i>	CBS 136.33 ^T	Toe nail of man, Argentina	HQ232137	—	—	—
<i>Acremonium tubakii</i>	CBS 790.69 ^T	Marine sediment, Japan	HQ232148	—	—	—
<i>Cosmospora khandalensis</i>	CBS 356.65 ^T	Decaying stem of the genus <i>Bambusa</i> , Maharashtra	HQ231996	—	—	—
<i>Cosmospora lavitskiae</i>	CBS 530.68 ^T	Plant debris from rhizosphere soil of <i>Zea mays</i> , Ukraine	HQ231997	—	—	—
<i>Gliomastix masseei</i>	CBS 794.69	<i>Morus indica</i> , Punjab	HQ232060	—	—	—
<i>Gliomastix murorum</i>	CBS 157.72	Culture of <i>Camarophyllus niveus</i> , The Netherlands	HQ232067	—	—	—
<i>Gliomastix polychroma</i>	CBS 181.27 ^T	Bark of <i>Hevea brasiliensis</i> , Sumatra	HQ232091	—	—	—
<i>Sarocladium bacillisporum</i>	CBS 425.67 ^T	Soil, Ontario, Canada	HE608658	HE608639	HE608633	—
<i>Sarocladium bactrocephalum</i>	CBS 749.69 ^T	<i>Ustilago</i> sp., Canada	HQ231994	HG965006	HG964952	—
<i>Sarocladium bactrocephalum</i>	UTHSC 09-384	Human eye, U.S.A.	HG965055	HG965007	HG964957	—
<i>Sarocladium bifurcatum</i>	UTHSC 05-3311 ^T	Bronchoalveolar lavage fluid, U.S.A.	HG965057	HG965009	HG964959	—
<i>Sarocladium gamsii</i>	CBS 707.73 ^T	Dead stem of <i>Pandanus lerrum</i> , Sri Lanka	HG965063	HG965015	HG964965	—
<i>Sarocladium glaucum</i>	CBS 796.69 ^T	Woolen overcoat, Solomon Islands	HE608657	FN691454	HE608631	—
<i>Sarocladium hominis</i>	UTHSC 04-1034 ^T	Right calf tissue, U.S.A.	HG965060	HG965012	HG964962	—
<i>Sarocladium implicatum</i>	CBS 959.73 ^{NT}	Desert soil, Egypt	HG965072	HG965023	HG964974	—
<i>Sarocladium kiliense</i>	CBS 122.29 ^T	Skin, Germany	HQ232052	FN691446	HG964975	—
<i>Sarocladium liquanensis</i>	ACCC 39306 ^T	<i>Malus × domestica</i> , China	MF987651	MF987659	MF987663	—
<i>Sarocladium mali</i>	ACCC 39308 ^T	<i>Malus × domestica</i> , China	MF987653	MF987662	MF987665	—
<i>Sarocladium mali</i>	ACCC 39307	<i>Malus × domestica</i> , China	MF987652	MF987661	MF987664	—
<i>Sarocladium ochraceum</i>	CBS 428.67 ^T	<i>Zea mays</i> , Kenya	HQ232070	HG965025	HG964977	—
<i>Sarocladium oryzae</i>	CBS 180.74 ^{ET}	<i>Oryza sativa</i> , India	HG965047	HG965026	HG964978	—
<i>Sarocladium pseudostrictum</i>	UTHSC 02-1892 ^T	Sputum, U.S.A.	HG965073	HG965029	HG964981	—
<i>Sarocladium strictum</i>	CBS 346.70 ^T	<i>Triticum aestivum</i> , Germany	HQ232141	FN691453	HG964982	—
<i>Sarocladium strictum</i>	CBS 640.75	Decaying wood, The Netherlands	HG965074	HG965030	HG964983	—
<i>Sarocladium subulatum</i>	MUCL 9939 ^T	Soil, Egypt	HG965075	HG965031	HG964984	—
<i>Sarocladium summerbellii</i>	CBS 430.70 ^T	Soil from greenhouse, The Netherlands	HG965078	HG965034	HG964987	—
<i>Sarocladium terricola</i>	CBS 243.59 ^T	Forest soil, U.S.A.	HE608659	FN706553	HE608632	—
<i>Sarocladium terricola</i>	MUCL 12011	Decaying leaf of <i>Milleta laurentii</i> , Democratic Republic of Congo	HG965083	HG965039	HG964991	—
<i>Sarocladium terricola</i>	UTHSC 03-2933	Bronchial wash fluid, U.S.A.	HG965086	HG965041	HG964994	—
<i>Sarocladium terricola</i>	ACCC 39309	<i>Malus × domestica</i> , China	MF987654	MF987660	MF987666	—
<i>Sarocladium zeae</i>	CBS 800.69 ^T	<i>Zea mays</i> stalk, U.S.A.	HQ232152	FN691451	HG965000	—

as described above. After inoculation, the apples were placed in sealed plastic bags, sterile water was misted on the inside surface of the plastic bags until runoff to maintain high RH, and bags containing inoculated fruit were placed in incubators at different temperatures. RH was controlled by saturated salt solution. Pure water, saturated potassium sulfate, potassium nitrate, potassium chloride, and sodium chloride solutions were placed in sealed glass jars to maintain RH at 100, 98, 95, 85, or 75%, respectively (Rockland

1960). Inoculated and control fruits used for RH experiments were air dried and then, transported to moist air cabinets with different RH levels. The experiment was performed four times, and four fruits were used per treatment. Symptom development was assessed 10 days after inoculation.

Data analysis. Conidial germination percentages obtained in four experiments performed on different dates were used to calculate treatment means and standard errors. Analysis of variance was

Fig. 1. The most likely tree based on LSU sequences. Maximum likelihood bootstrap (MLBS)>60%, maximum parsimony bootstrap (MPBS)>60%, and Bayesian posterior probabilities (BPP)>0.90 are given at the nodes. The scale bar represents the number of nucleotide substitutions per site. The tree is rooted to *Ceratocystis fimbriata*, *Ophiostoma piliferum*, and *Glomerella cingulata*. Strains generated in this study are shown in bold, and the type strains are indicated by superscript T.

Fig. 2. One of most parsimonious trees based on the combined dataset of LSU-ITS-BT2. Maximum likelihood bootstrap (MLBS)>60%, maximum parsimony bootstrap (MPBS)>60%, and Bayesian posterior probabilities (BPP)>0.90 are given at the nodes. The scale bar represents the expected changes per site. The tree is rooted to *Acremonium cavaraeanum*. Strains generated in this study are shown in bold, and the type strains are indicated by superscript T.

Fig. 3. Consensus phylogram resulting from a Bayesian analysis of the LSU-ITS-ACT1 dataset of the genus *Serocladium*. Maximum likelihood bootstrap (MLBS)>60%, maximum parsimony bootstrap (MPBS)>60%, and Bayesian posterior probabilities (BPP)>0.90 are given at the nodes. The scale bar represents the expected changes per site. The tree is rooted to *Acremonium varicolor*. Strains generated in this study are shown in bold, and the type strains are indicated by superscript T.

Fig. 4. *Acremonium mali* (strain ACCC 39305 (stored in Agricultural Culture Collection of China). **A and B**, Colonies on potato dextrose agar and oatmeal agar, respectively, after 14 days at 25°C. **C and D**, Conidia gathered in slimy head and long chain, respectively. **E**, Adelophialides. **F**, Globose conidia. **G**, Branched conidiophores. Scale bars = 10 μ m.

Fig. 5. *Sarocladium liquanensis* (strain ACCC 39306 (stored in Agricultural Culture Collection of China). **A and B**, Colonies on potato dextrose agar and oatmeal agar, respectively, after 14 days at 25°C. **C**, Adelophialides. **D**, Conidia gathered in slimy head. **E and F**, Branched conidiophores. Scale bars = 10 μ m.

conducted using generalized line models to assess the effects of temperature and RH on conidial germination.

Results

Phylogenetic analysis. In total, 60 fungal isolates that were similar to the genus *Acremonium* in morphology were isolated from apple brown spot lesions. Eight representative isolates were chosen for additional phylogenetic analysis with LSU markers (Table 1). The LSU dataset, including 38 reference species in Hypocreales, resulted in an alignment with 700 characters. Phylogenetic trees constructed with MP, ML, and Bayesian analyses were highly congruent (Fig. 1). The eight isolates fell into three clades (*Sclerotigenum* clade, *Fusidioides* clade, and *Sarocladium* clade) in Bionectriaceae (Summerbell et al. 2011). The *Sclerotigenum* clade contained three isolates (ACCC 39312, ACCC 39311, and ACCC 39310) that were phylogenetically indistinguishable from *A. sclerotigenum* type strain CBS 124.42. Based on morphological and phylogenetic characteristics, these isolates were identified as *A. sclerotigenum*.

The *Fusidioides* clade contained one isolate, ACCC 39305. An LSU-ITS-BT2 multigene phylogenetic analysis was performed to determine its phylogenetic position (Fig. 2). A total of 11 reference sequences were included in the alignment, which contained 1,545 characters (476/500/570 bp of LSU/ITS/BT2). The topologies of three methods were consistent, and isolate ACCC 39305 clustered together with *Acremonium fusidioides* and *Acremonium pilosum* with support of 100% maximum likelihood bootstrap (MLBS), 100% maximum parsimony bootstrap (MPBS), and 1.00 Bayesian posterior probabilities (BPP). Isolate ACCC 39305 formed an independent clade with 100% MLBS, 100% MPBS, and 1.00 BPP. All species in the *Fusidioides* clade can produce two kinds of conidia (Giraldo et al. 2014).

The *Sarocladium* clade contained four isolates: ACCC 39306, ACCC 39307, ACCC 39308, and ACCC 39309. An LSU-ITS-ACT1 multigene phylogenetic analysis was performed to determine their phylogenetic positions (Fig. 3). A total of 21 taxa were aligned in the genus *Sarocladium*, including one outgroup taxon, which contained 1,755 characters (505/520/730 bp of LSU/ITS/ACT1). The *Sarocladium* isolates formed two well-supported clades, with clade 1 producing conidia in chains and clade 2 producing conidia in slimy heads, except for *Sarocladium gamsii* (in clade 1) and *Sarocladium summerbellii* (in clade 2), which can produce conidia in both chains and slimy heads (Giraldo et al. 2015). Isolates ACCC 39307, ACCC 39308, and ACCC 39306 produced conidia in slimy heads and belonged to clade 1. Isolates ACCC 39307 and ACCC 39308 separated with *Sarocladium strictum* with strong support (80% MLBS, 75% MPBS, and 0.95 BPP). Isolate ACCC 39306 formed an independent clade. In clade 2, the isolate ACCC 39309 was indistinguishable from the type strain of *Sarocladium terricola* CBS 243.59.

Taxonomy. Based on the morphological and molecular data, the brown spot complex includes three new species.

***Acremonium mali* G. Y. Sun & Yanmin Hou, sp. nov. (Fig. 4): FN570590.** *Etymology.* It was named after the host (*Malus × domestica*) from which it was collected.

Holotype. China, Shaanxi Province: Xianyang City, on *Malus × domestica* Borkh., October 2013, Yanmin Hou, HMAS247717 (dried culture), ex type ACCC (Agricultural Culture Collection of China) 39305.

Colonies on OA at 25°C attained 44 mm in 14 days: white, flat, and membranous. On PDA at 25°C, they attained 43 mm in 14 days: white, flat, and powdery. Vegetative hyphae septate, hyaline, smooth and thin walled: (1.21) 1.53 to 2.67 (2.81)-µm wide. Conidiophores erect, usually simple, sometimes branched, straight or slightly bent

Fig. 6. *Sarocladium mali* (strain ACCC 39308, stored in Agricultural Culture Collection of China). **A** and **B**, Colonies on potato dextrose agar and oatmeal agar, respectively, after 14 days at 25°C. **C** and **F**, Conidia gathered in slimy head. **D**, Schizophialides. **E**, Branched conidiophores. **G**, Adelophialides. **H**, Chlamydospores. Scale bars = 10 µm.

up to (7.80) 10.13 to 26.16 (35.23)- μm long, hyaline, smooth walled. Phialides subulate, (8.51) 9.63 to 21.36 (22.77)- μm long, (1.56) 1.61 to 2.21 (2.76)- μm wide at the base, hyaline, thin and smooth walled; adelophialides present, and schizophialides not observed. Conidia unicellular of two types. I. Pyriform or fusiform with slightly truncate ends, (3.19) 3.66 to 5.46 (6.55) $\times$ (1.76) 1.92 to 2.48 (3.14) μm , hyaline, thin and smooth walled, adhering in long chains or slimy heads. II. Globose with warty surface, hyaline, arranged in chains. Chlamydospores and sexual morph were not observed.

Cardinal temperature for growth. Optimum 20 to 25°C, maximum 35°C, and minimum 15°C. No growth at 37°C.

Notes. In ML, MP, and Bayes trees, *A. mali* separated from *A. pilosum* with support values of 100/100/1.0. These species were distinguishable in both colony and conidia characters. The colony

of *A. mali* was white, whereas *A. pilosum* was grayish brown at the center with whitish periphery. The former can grow at 35°C, whereas the latter cannot. Both species produce two types of conidia. The globose conidia produced by *A. pilosum* were pale brown with filiform projections, whereas those produced by *A. mali* were hyaline and smooth.

***Sarocladium liquanensis* G. Y. Sun & Yanmin Hou, sp. nov.** (Fig. 5): FN570587. **Etymology.** It refers to the place (Liquan County, Shaanxi Province) where it was discovered.

Holotype. China, Shanxi Province: Xianyang City, Liquan County, on *Malus $\times$ domestica* Borkh., October 2013, Yanmin Hou, HMAS247718 (dried culture), ex type ACCC 39306.

Colonies on OA at 25°C attained 44 mm in 14 days: light orange, flat, and usually fasciculate at the center and glabrous toward the periphery. On PDA at 25°C, they attained 43 mm in 14 days: orange,

Fig. 7. Symptoms on apple fruit. **A.** *Acremonium sclerotigenum* ACCC 39311 (stored in Agricultural Culture Collection of China). **B.** *Acremonium mali* ACCC 39305. **C.** *Sarocladium liquanensis* ACCC 39306. **D.** *Sarocladium terricola* ACCC 39309. **E.** *Sarocladium mali* ACCC 39308. **A1, B1, C1, D1, and E1,** Symptoms on samples from field. **A2, B2, C2, D2, and E2,** Symptoms on field fruit inoculated on 25 June. **A3, B3, C3, D3, and E3,** Symptoms on field fruit inoculated on 10 August. **A4, B4, C4, D4, and E4,** Symptoms inoculated on detached fruit in laboratory with wounds. **A5, B5, C5, D5, and E5,** Symptoms inoculated on detached fruit in laboratory with no wound. Scale bars = 5 mm.

slightly wrinkled or cerebriform in the center, and producing red-orange pigment at 30°C. Vegetative hyphae septate, hyaline, smooth and thin walled, (1.10) 1.33 to 2.58 (2.71)- μm wide. Conidiophores erect, usually simple or branched, straight or slightly bent, up to (8.82) 10.03 to 27.16 (34.26)- μm long, hyaline, smooth walled. Phialides subulate, (8.91) 9.62 to 20.38 (21.07)- μm long, (1.48)

1.51 to 2.31 (2.36)- μm wide at the base, hyaline, thin and smooth walled; adelophialides present, and schizophialides not observed. Conidia unicellular, ellipsoidal to cylindrical, (3.19) 3.66 to 5.46 (6.55) $\times$ (1.76) 1.92 to 2.48 (3.14) μm , initially hyaline and smooth walled, arranged in slimy heads. Chlamydospores was present, and sexual morph was not observed.

Fig. 8. Effect of temperature on conidial germination. The average germination rate and the corresponding standard error (calculated based on the data in Table 2) at each temperature in the free water treatment are shown as a bar and a corresponding error bar, respectively. Different letters indicate that the germination rates were significantly different ($P < 0.05$) according to multiple comparisons of predicted values by the generalized linear model. **A,** *Ascremonium sclerotigenum* ACCC 39311 (stored in Agricultural Culture Collection of China). **B,** *Ascremonium mali* ACCC 39305. **C,** *Sarocladium liquanensis* ACCC 39306. **D,** *Sarocladium terricola* ACCC 39309. **E,** *Sarocladium mali* ACCC 39308.

Cardinal temperature for growth. Optimum 20 to 25°C, maximum 35°C, and minimum 15°C. No growth at 37°C.

Notes. In ML, MP, and Bayes trees, the new species gathered with the second large clade, which can produce conidia in slimy heads. In this clade, apart from *S. summerbellii*, which produces fusiform conidia in both chains and slimy heads, all other species produce ellipsoidal or cylindrical conidia. The new species can be distinguished from other species by producing orange pigment when cultivated at 30°C.

***Sarocladium mali* G. Y. Sun & Yanmin Hou, sp. nov. (Fig. 6): FN570588.** *Etymology.* It is named after the host (*Malus × domestica*) from which it was collected.

Holotype. China, Shanxi Province: Yangling, on *Malus × domestica* Borkh., October 2013, Yanmin Hou, HMAS247719 (dried culture), ex type ACCC 39308.

Colonies on OA at 25°C attained 50 to 60 mm in 14 days: orange white and membranous. On PDA at 25°C, they attained 45 mm in 14 days: light orange, at first membranous, and then becoming powdery. Vegetative hyphae septate, hyaline, smooth and thin walled, (1.16) 1.22 to 2.51-µm wide. Conidiophores erect, simple or branched, straight or slightly bent, up to (11.16) 14.67 to 27.73 (31.30)-µm long, hyaline, smooth walled. Phialides subulate, (11.62) 14.56 to 24.74 (31.50)-µm long, (1.32) 1.40 to 1.96 (2.13)-µm wide at the base, hyaline, thin and smooth walled; adelophaialides and schizophaialides are present. Conidia unicellular, ellipsoidal to cylindrical, (4.03) 4.08 to 4.44 (5.19) × (1.43) 1.54 to 2.04 µm, initially hyaline and smooth walled, arranged in slimy heads. Chlamydospores are present, and sexual morph was not observed.

Cardinal temperature for growth. Optimum 20 to 25°C, maximum 35°C, and minimum 15°C. No growth at 37°C.

Notes. The new species nested together with *S. strictum* and *Sarocladium bactrocephalum* in a well-supported clade; however, it is distinguished from the other two species by producing chlamydospores. The phialides of *S. strictum* (up to 56 × 2.5 µm) are larger than those of *S. mali*. *S. mali* can grow at 35°C, whereas *S. bactrocephalum* cannot, and *S. mali* cannot grow at 37°C, whereas *S. strictum* can.

Pathogenicity test. All five tested species (*A. sclerotigenum*, *A. mali*, *S. liquanensis*, *S. terricola*, and *S. mali*) caused pale to whitish spots, some with a pale to dark brown halo, on apple fruit in both field and laboratory experiments; these symptoms were similar to original symptoms (Fig. 7). The pathogens that were inoculated onto the fruit surface were reisolated from the spots, and their identity was confirmed by morphology and molecular identification, completing Koch's postulates.

For the field inoculation with no wounding, the size of spots differed with inoculation stage. Thirty days after inoculation, lesion diameter was 3 to 6 mm when inoculated on 10 August (Fig. 7 A3, B3, C3, D3, and E3) and 12 September (data not shown) and 1 to 2 mm when inoculated on 25 June (Fig. 7 A2, B2, C2, D2, and E2). For detached fruit inoculation in the laboratory accompanied by wounding, all isolates of *A. sclerotigenum*, *A. mali*, *S. liquanensis*, *S. terricola*, and *S. mali* caused dark brown spots 2 to 3 mm in diameter (Fig. 7 A4, B4, C4, D4, and E4). Only isolate

ACCC39311 of *A. sclerotigenum* caused spots without wounding at inoculation (Fig. 7 A5, B5, C5, D5, and E5).

Effects of temperature and RH on in vitro conidial germination. Temperature significantly affected conidial germination of all five tested species (*A. sclerotigenum*, *A. mali*, *S. liquanensis*, *S. terricola*, and *S. mali*) (Fig. 8 and Table 2). No germinated conidia were detected at 5°C (data not shown). Conidial germination rates differed among the tested species. *A. sclerotigenum* had the highest germination rate; more than two-thirds of the conidia of this species germinated from 20 to 30°C, and the highest germination rate was 92.2% at 25°C. *A. mali* reached its highest germination rate (48.8%) at 25°C, which was significantly higher than germination rates at the other tested temperatures ($P < 0.05$). Apart from *A. terricola*, which had its highest germination rate at 30°C, the other tested species reached their highest germination rates at 25°C.

Conidial germination rates of the five tested species increased as RH increased (Fig. 9 and Table 3). *A. sclerotigenum* had the highest conidia germination rates at all RH levels. In free water, it reached 92.2%, and it was 65.6% at 100% RH. When RH was <97%, germination rates declined significantly ($P < 0.05$) from 31.9 to 2.1% (Fig. 9A). Germination rate of *A. mali* reached the highest at 100% RH by 49.7%, which was similar to the rate in free water (48.8%). Additionally, the germination rate can still reach 16.6% at 97% RH followed by a significant ($P < 0.05$) decline at 95% RH (1.5%) (Fig. 9B). The same tendency can be seen in *S. mali* (Fig. 9E). For species *S. liquanensis* and *S. terricola*, the germination rates were above 20% when RH was >99% followed by a significant ($P < 0.05$) decline at 97% RH (Fig. 9C and D).

Effects of temperature and RH on infection of apple fruit. High temperature suppressed conidial germination (Table 4). Incidence of germination of *A. sclerotigenum* conidia was >75% from 10 to 35°C, reached 94.2% at 25°C, and declined to 7.5% at 40°C. Spots on non-wounded surfaces occurred only between 25 and 35°C.

The effect of RH was tested at 25°C (Table 5). Disease incidence was affected significantly by RH, rising as RH increased. Incidence was >50% at RH > 94%, falling to 21.4% at 85% RH. Incidence of germination at 10°C in free water (78.5%) exceeded that at 25°C and 100% RH (72.6%).

Discussion

T. roseum, *Alternaria* spp., and *A. sclerotigenum* have been reported to be the primary causal agents of fruit spot disease on bagged apples (Guo et al. 2005; Hao et al. 2004; Li et al. 2014). Our study, based on a survey of orchards in Shaanxi Province, was consistent with previous studies in that *T. roseum* typically caused small black spots surrounding the calyx, whereas *Alternaria* spp. caused larger black spots that were more widely scattered on the fruit surface. In this study, we focused primarily on *Acremonium*-like species, which cause brown spots on the fruit surface. In our survey, *A. sclerotigenum* was the primary causal agent of brown spot disease, which is consistent with Li et al. (2014). However, we found that four additional *Acremonium*-like species could also cause brown spot disease. Based on morphological characteristics and phylogenetic analysis, we identified one of these as a new species (*A. mali*) belonging to

Table 2. Average percentage of in vitro conidial germination and corresponding standard errors of five isolates (based on 500 conidia) at different temperatures 12 or 16 h after inoculation

Species	Temperature (°C)						
	10	15	20	25	30	35	40
<i>Acremonium sclerotigenum</i>	1.8 ± 0.2 e ^y	7.4 ± 0.5 d	68.6 ± 3.5 c	92.2 ± 1.9 a	80.0 ± 1.0 b	8.6 ± 0.6 d	0.2 ± 0.1 e
<i>Acremonium mali</i>	0.9 ± 0.4 cd	1.2 ± 0.5 cd	4.0 ± 1.4 c	48.8 ± 4.0 a	9.7 ± 2.7 b	4.2 ± 3.3 c	0.3 ± 0.3 d
<i>Sarocladium liquanensis</i>	1.4 ± 0.5 c	7.2 ± 4.4 bc	17.3 ± 5.0 b	45.57 ± 13.24 a	16.4 ± 1.4 b	1.6 ± 1.0 c	0.05 ± 0.1 c
<i>Sarocladium terricola</i>	2.3 ± 0.3 e	3.1 ± 0.7 e	19.3 ± 1.9 c	26.4 ± 3.4 b	50.5 ± 2.6 a	13.5 ± 0.7 d	0.3 ± 0.5 e
<i>Sarocladium mali</i> ^z	4.95 ± 1.1 d	12.3 ± 2.8 c	25.54 ± 4.3 b	47.17 ± 6.7 a	22.18 ± 4.0 b	12.53 ± 2.1 c	0.51 ± 0.4 d

^y Means and standard errors were calculated on the basis of four replications; means in a column followed by the same letter do not differ significantly ($P < 0.05$) according to multiple comparisons of predicted values by the generalized linear model.

^z Species was inoculated for 16 h.

the genus *Acremonium* and two as new species in the genus *Sarocladium* (*S. liquanensis* and *S. mali*) as well as one that was a newly recorded pathogen of brown spot in China (*S. terricola*). The genus *Sarocladium* was established by Gams and Hawksworth (1975). Its morphological features are similar to the genus *Acremonium*, which is a large and complex polyphyletic genus of Hypocreales (Giraldo

et al. 2015), and its taxonomy is chaotic based solely on its simple morphological features. The genus *Sarocladium* can be morphologically distinguished from the genus *Acremonium* by its commonly occurring adelophialides or repeatedly branched conidiophores (Summerbell et al. 2011). Based on phylogenetic analysis, the relationship between the genera *Acremonium* and *Sarocladium* was

Fig. 9. Effect of relative humidity on conidial germination. The average germination rate and the corresponding standard error (calculated based on the data in Table 3) in each relative humidity are shown as a bar and a corresponding error bar, respectively. Different letters indicate that the germination rates were significantly different ($P < 0.05$) according to multiple comparisons of predicted values by the generalized linear model. **A**, *Acremonium sclerotigenum* ACCC 39311 (stored in Agricultural Culture Collection of China). **B**, *Acremonium mali* ACCC 39305. **C**, *Sarocladium liquanensis* ACCC 39306. **D**, *Sarocladium terricola* ACCC 39309. **E**, *Sarocladium mali* ACCC 39308.

much clearer (Giraldo et al. 2014, 2015; Summerbell et al. 2011). In the study of Li et al. (2014), two types of strains were discovered among *A. sclerotigenum* that were causal agents of brown spot disease: strain 1 produced ellipsoidal to ovoid conidia assembling in slimy heads, and strain 2 produced spindle-shaped conidia in a long chain. All of our *A. sclerotigenum* isolates were coherent with strain 1, whereas the description of strain 2 was similar to the species that we identified as *S. terricola*.

Most species within the genera *Acremonium* and *Sarocladium* are either saprophytic fungi in soil or pathogens of plants (Perdomo et al. 2010). *Sarocladium oryzae*, the type species of the genus *Sarocladium*, is an important plant pathogen causing sheath rot of rice (Gams and Hawksworth 1975). It is also known to produce cerulenin, a secondary metabolite that can suppress leaf blast on rice (*Oryza sativa*) (Ayyadurai et al. 2005; Lanoiselet et al. 2012). Some species in the genera *Acremonium* and *Sarocladium* are considered opportunistic pathogens of humans or other mammals (De Hoog et al. 2000; Guarro et al. 1997; Summerbell 2003). An increasing number and diversity of infections caused by *Acremonium*-like species have been reported and constitute a wide spectrum of clinical diseases (Anadolu et al. 2001; Das et al. 2010; Lopes et al. 1995). The most common anatomical sites for *Acremonium* and *Sarocladium* isolates were the respiratory tract, nails, and eye. Among these species, *A. sclerotigenum* was commonly isolated from blood, tracheal aspirate, and nails (Guarro et al. 2009; Novicki et al. 2003; Perdomo et al. 2010). *S. terricola* was usually discovered from bronchoalveolar lavage fluid and sputum, but whether it is a human pathogen is not clear (Perdomo et al. 2010). The phylogenetic relationships between isolates discovered from brown spot disease on apple fruit and the causal

agents of human disease need additional study. The possibility that causal agents of brown spot disease on bagged apples may infect humans opportunistically suggests that these fungi could pose risks for immunocompromised individuals.

Brown spot disease has occurred mainly in China because of the widespread application of fruit bagging. The specialized microenvironment created by bagging seems to have played a role in inducing this disease (Li et al. 2015). In this study, we found that temperature and RH had significant impact on conidial germination. The bags can help the fruit surface to retain high levels of RH, particularly during prolonged rainy periods or wet growing seasons. From the pathogen testing in the field and laboratory, it is clear that occurrence of brown spot disease was influenced by fruit development stage and wounding of fruit. When inoculated on young fruit in June, spots were smaller (1- to 2-mm diameter); in contrast, spots were larger (3- to 6-mm diameter) when fruit were inoculated at the stage of fruit enlargement (in August and September). Similar phenomena were observed in *Trichotecium* black spot on bagged apples by Dai et al. (2019). They speculated that infection of younger fruit introduced host resistance responses that restricted lesion development, whereas these responses were weaker when inoculation occurred at later stages of fruit development (Dai et al. 2019; Hwang 1983; Kohn et al. 1983). Whether changes in the chemical composition of the fruit surface also contribute to the increased susceptibility to brown spot late in the season is a question that merits additional study. Reducing inoculum of the causal agents before covering the fruit with bags is a potentially effective approach for protecting apples from fruit spot disease; this is also a potential method to control moldy core and core rot, which are caused mainly by *Alternaria alternata* and *T. roseum* (Reuveni et al. 2002, 2003).

Table 3. Average percentage of in vitro conidial germination and corresponding standard errors of five isolates (based on 500 conidia) at different relative humidity at the temperature of 25°C 12 or 16 h after inoculation

Species	Relative humidity (%)				
	Free water	100	99	97	95
<i>Acremonium sclerotigenum</i>	92.2 ± 1.9 a ^y	65.6 ± 3.5 b	38.8 ± 1.1 c	31.9 ± 1.5 d	2.1 ± 0.6 e
<i>Acremonium mali</i>	48.8 ± 4.0 a	49.7 ± 10.9 a	35.6 ± 2.5 b	16.6 ± 7.0 c	1.5 ± 0.8 d
<i>Sarocladium liquanensis</i>	45.57 ± 13.24 a	23.3 ± 5.6 a	20.0 ± 3.2 a	2.5 ± 1.1 b	0.5 ± 0.4 b
<i>Sarocladium terricola</i>	26.4 ± 3.4 a	29.6 ± 6.3 a	27.6 ± 3.2 a	5.4 ± 3.4 b	0.6 ± 0.3 b
<i>Sarocladium mali</i> ^z	47.17 ± 6.7 a	45.28 ± 6.4 a	25.26 ± 2.9 b	7.82 ± 2.0 c	0.62 ± 0.4 d

^y Means and standard errors were calculated on the basis of four replications; means in a column followed by the same letter do not differ significantly ($P < 0.05$) according to multiple comparisons of predicted values by the generalized linear model.

^z Species was inoculated for 16 h.

Table 4. Effect of temperature on infection^x

	Temperature (°C)						
	10	15	20	25	30	35	40
Incidence (%)	78.5 ± 4.8 ^y	87.3 ± 5.0	90.8 ± 4	94.2 ± 3.4	88.3 ± 5.0	85.8 ± 3.9	7.5 ± 1.2
Diameter of fruit spots (mm)	1–3.3	1–3.1	1–2.2	1–3.5	1–2.1	1–3.4	1
Spots on nonwounded fruit surface	– ^z	–	–	+	+	+	–

^x Incidence rates and diameters of fruit spots were measured 10 days after in vitro inoculation of *Acremonium sclerotigenum*.

^y Means and standard errors were calculated on the basis of four replications.

^z No spot on nonwounded surface is indicated by –; spots happened on nonwounded surface is indicated by +.

Table 5. Effect of relative humidity on infection^x

	Relative humidity (%)					
	Free water	100	98	94	85	75
Incidence (%)	94.2 ± 3.4 ^y	72.6 ± 7.5	52.6 ± 4.8	51.1 ± 8.2	21.4 ± 5.5	9.8 ± 2.8
Diameter of fruit spots (mm)	1–3.5	1–3.1	1–2.2	1–2.1	1–1.8	1–2.5
Spots on nonwounded fruit surface	+ ^z	+	+	–	–	–

^x Incidence rates and diameters of fruit spots were measured 10 days after in vitro inoculation of *Acremonium sclerotigenum*.

^y Means and standard errors were calculated on the basis of four replications.

^z No spot on nonwounded surface is indicated by –; spots happened on nonwounded surface is indicated by +.

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