Characterization of Causal Agents of a Novel Disease Inducing Brown-Black Spots on Tender Tea Leaves in China

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Abstract

A novel disease characterized by small brown-black spots (1 to 2 mm in diameter) on tender tea leaves (*Camellia sinensis*) has been observed in many regions of Hubei Province, China, which severely affects the yield and quality of tea. Tea leaf samples with typical symptoms were collected from three major tea-cultivation regions of Hubei, and were subjected to pathogen isolation for etiological analysis. As a result, 34 *Pestalotiopsis* isolates were obtained from 20 samples, and they were identified as *Pestalotiopsis theae* (14 isolates), *P. camelliae* (12), and *P. clavispora* (8), determined by morphologies and phylogenetic analysis based on internal transcribed spacer, and partial β -tubulin and translation elongation factor 1- α genes. Pathogenicity tests on detached tea leaves showed that no matter what mycelial discs or conidium suspensions were used, inoculation of the *Pestalotiopsis* fungi could result in small brown-black spots (1 to 2 mm in diameter) on wounded leaves, similar

to those observed in the field in the sizes and colors. It also revealed that only *P. theae* had pathogenicity on unwounded tea leaves, and *P. theae* and *P. clavispora* showed significantly higher virulence than *P. camelliae*. Inoculation test with conidium suspension on intact tea leaves in the field further confirmed that *P. theae* as the pathogen of brown-black spots. Reisolation of the pathogens from diseased leaves confirmed that the symptom was caused by the inoculation of *Pestalotiopsis* fungi. The *P. theae* isolates responsible for brown-black spots were also compared with those for tea gray blight disease in growth rate, pathogenicity, and molecular characteristics in parallel. To our knowledge, this is the first report that the *Pestalotiopsis* fungi cause brown-black spot disease on tender tea leaves. The results provide important implications for the prevention and management of this economically important disease.

Tea is one of the three main nonalcoholic beverages (tea, coffee, and cocoa) worldwide, and is the most widely consumed drink after water in the world (Bond 2012). Tea contains some 4,000 bioactive compounds (Mahmood et al. 2010), many of which are beneficial for human health, such as polyphenols, caffeine, vitamins, amino acids, proteins, polysaccharides, and trace elements, which are helpful for the prevention and treatment of cancer, cardiovascular and cerebrovascular diseases, and improvement of immunity (Bhatt et al. 2010; Namita et al. 2012). Tea (*Camellia sinensis* [L.] O. Kuntze) originated from southwestern China and is currently planted in more than 1,000 counties in 20 provinces of China (Z. M. Chen and X. L. Sun 2013). For a long time, tea yield in China has been ranked first in the world, and tea is still crucial in local economies and people's daily life (Ma et al. 2014).

However, diseases pose serious threats to tea production around the world. More than 100 diseases have been found in China, about 15 of which occur on buds and tender leaves (Z. M. Chen and X. L. Sun 2013). Among these diseases, blister blight, net blister blight, white scab, bud blight, leaf blight, gray blight, and brown round spot can be epidemic and/or affect the quality of the product (Z. M. Chen and X. L. Sun 2013). In recent years, a novel disease characterized by small brown-black spots (1 to 2 mm in diameter) has been observed in many tea gardens of Hubei Province, China. The disease poses a big potential threat for tea production in Hubei, since many varieties susceptible to this disease are widely cultivated, e.g., *C. sinensis* var.

Enshitaizicha, susceptible to this disease, is widely cultivated in Enshi prefecture (over 60,000 ha).

Although several tea leaf diseases have been reported in China, only white scab and brown round spot diseases can cause brown-black spots on tender leaves (Z. M. Chen and X. L. Sun 2013; Yang et al. 2012). Typically, the spots caused by white scab have brown edge and a large grayish white center, and brown round spot causes wholly brown-black spots with a small grayish white center (Z. M. Chen and X. L. Sun 2013). For the disease in the present study, the spots are wholly dark brown without any white part in the center. Hence, this disease might be a novel tea leaf disease and is here designated as tea leaf brown-black spot disease. Due to the economic importance of tea in China, it is imperative to develop proper strategies to prevent or mitigate the impact of this new disease. For this purpose, we have investigated for the first time the etiology of this novel disease.

Materials and Methods

Collection of samples. In July and August of 2013, 20 leaf samples showing typical brown-black spots 1 to 2 mm in diameter were collected from Xuan'en, Yichang and Yingshan counties, Hubei Province, China. Simultaneously, 12 leaf samples showing the typical symptoms of tea gray blight disease were collected from Yichang, Yingshan, and Zhuxi counties, Hubei Province, and kept in a refrigeration house at 4°C until use.

Isolation of fungi. For leaf samples showing brown-black spots, four to five leaf blocks (4 to 5 mm²) containing at least one lesion were excised from each diseased leaf after they were surface-sterilized with 0.1% HgCl₂ for 1 min and 75% ethanol for 1 min, rinsed twice with sterilized water, and cultured on potato dextrose agar (PDA, 20% diced potatoes, 2% glucose, and 1.5% agar) plates for fungal colony formulation as previously reported (Bai et al. 2015). For leaf samples showing gray blight disease symptoms, pathogen isolation was conducted by culturing the conidia picked from acervuli over the lesions. The resulting colonies were further purified by culturing a single conidium according to the previous report (Bai et al. 2015). The isolates that did not sporulate

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were purified by the single-mycelium tipping method (Yaegashi et al. 2012).

Morphology characterization of fungi. Mycelial discs taken from active growth areas were inoculated on PDA plates and incubated in the dark at 25°C for 4 days. Typical colony morphology, mycelial density, and aerial hypha growth were observed under a microscope (Nikon Eclipse E600 FN, Nikon, Japan). Fifty conidia of each isolate were measured and photographed with SPOT 4.6 (Diagnostic Instruments, Inc., U.S.A.). Morphological characterization of the fungi was conducted according to the standards described by Ge et al. (2009) and Guba (1961).

Determination of growth rate. PDA discs (5 mm in diameter) covered with mycelia were taken from the edge of a growing colony with a sterile puncher. One disc was placed in the center of PDA plate and incubated in the dark at 25°C. The colony diameter was measured using the cross intersect method and recorded daily during 4 days of incubation. The growth rate was calculated with the subtraction of the diameter of initial disc. The experiment was performed in three replicates for each isolate.

Molecular characterization of fungi. Genomic DNA of fungi was extracted according to the cetyltrimethylammonium bromide (CTAB) method as previously described (Freeman et al. 1996). The extracted DNA was subjected to an amplification in a PCR Thermal Cycler (Model PTC-100, MJ Research, U.S.A.) of three regions including the ribosomal internal transcribed spacer (ITS), including a partial 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and partial 28S ribosomal RNA gene), the partial β -tubulin (*TUB*), and partial translation elongation factor 1- α (*TEF*) genes using the primer pairs ITS1/ITS4 (5'-TCCGTA GGTGAACCTGCGG-3'/5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), and Bt2a/Bt2b 5'-GGTAACCAAATCGGTGCTGCTTTC-3'/5'-ACCCCTAGGTGATGACCCCTTGGC-3') (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997), and EF1-728F/EF-2 (5'-CATCGAGAAGTTCGAGAAGG-3'/5'-TACTTGAAGG AACCCCTACC-3') (Carbone and Kohn 1999; O'Donnell et al. 1998), respectively. Amplifying conditions were programmed as pre-denaturing at 94°C for 4 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min, and finally complementary extension at 72°C for 7 min. The PCR products were detected in agarose gel electrophoresis (1%), and the target DNA bands were recovered using DNA gel extraction kit (Wuhan More Biotechnology Corporation, Wuhan, China), and subjected to sequencing by Sangon Biotech (Shanghai) Co., Ltd, China.

The sequences were compared with the best matches available in GenBank with BLASTn (<http://blast.ncbi.nlm.nih.gov>). Multiple alignments of DNA sequences was conducted using MAFFT 6.85 (<http://mafft.cbrc.jp/alignment/software/>) with the default settings. DNA sequences were edited and annotated with GeneDoc (Nicholas et al. 1997; <http://www.nrbcs.org/gfx/genedoc/ebinet.htm>). A phylogenetic tree was constructed using MEGA 5.2.2 (Tamura et al. 2011; <http://megasoftware.net/>) with neighbor-joining (NJ) or maximum likelihood (ML) method, with the default parameters and bootstrap values of 1,000 replicates.

Pathogenicity analysis. Pathogenicity analysis was conducted according to a previous reports (Wang et al. 2016; Zhou and Xu 2014). Tender leaves were collected from *C. sinensis* var. Taicha No. 12. Before inoculation, leaves were disinfected in alcohol (75%), rinsed with sterile water several times, and air-dried. Colonized agar discs were prepared from fresh colony as described above and put on the upside of detached tea leaves that were wounded six times with a needle (insect pin, 0.45 mm in diameter) or left unwounded. Uncolonized discs were used as controls. For inoculation of conidia, 50 μ l of a suspension at the concentration of 10^5 conidia/ml was inoculated onto the upside of each tea leaf (over the six needle-piercing holes) and covered with sterilized cotton on wounded or unwounded leaves. Control leaves were inoculated in parallel using sterile water. The inoculated leaves were incubated in a climate chamber at 25°C under 12 h of intermittent light. Diameter of disease spot was measured 3 days after inoculation. The experiment was performed in four replicates for each treatment.

Intact leaves inoculations were performed on newly developed leaves on tea trees (*C. sinensis* var. Enshitaizicha) that were cultivated in Wujiatai, Xuan'en County, Enshi, Hubei Province, China, from August to September of 2016. A 100 ml aliquot of spore suspension (10^5 conidia/ml) was sprayed onto approximately 20 leaves. Potato dextrose (PD, 20% diced potatoes and 2% glucose) solutions containing no conidia were used in parallel as controls. Three replicates were performed for the inoculation. The symptoms were monitored and recorded every 10 days until the experiment was complete.

Data statistics and analysis. Data were analyzed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way ANOVA, and means were compared using Tukey's test at a significance level of $P = 0.05$. Homogeneity of variance was tested before analysis.

Results

Field symptom and loss survey. This disease occurs on the tender leaves of many tea varieties, including Fuyun No. 6, Fuyun No. 1, Taicha No. 2, and Enshitaizicha, in Enshi prefecture, Yichang and Huanggang, Hubei Province, China. In the early stage, pinpoint-sized and light yellow spots are observed on the surface of infected leaves, and part of the spots are surrounded by yellowish or reddish-brown halos; in the late stage, the spots gradually turn blackish or reddish brown, and the spot sizes grow up to 1 to 2 mm in diameter (Fig. 1A and B). During the outbreak of this disease, numerous spots can be formed on a single leaf and finally fuse into a large lesion, resulting in a necrosis on the leaf. A survey in Ma'an village of Xuan'en county, Enshi prefecture, Hubei, revealed that this disease breaks out annually and the affected area is usually over 20 ha, leading to a 50% economic loss. Additionally, this disease causes apparent deterioration of tea quality, as the prepared tea is very brittle and tastes bitter and astringent, like raw persimmon.

Culture properties of the isolates from tea leaf samples showing typical brown-black spots. Twenty leaf samples showing typical brown-black spots (round, sunken, and 1 to 2 mm in diameter) (Fig. 1A and B) were collected for pathogen isolation, and a total of 44 purified fungal isolates were obtained, among which 34 showed typical colony characteristics of *Pestalotiopsis* spp. from the collected samples. All colonies were wholly white at the beginning of incubation and were classified into three different groups (I to III) at the end of incubation (Fig. 2A).

Group I contained 14 isolates (Table 1) such as TP1-2O and TP2-2W. Mycelia were white in the early stage and turned grayish white later. Typically, mycelia were flocculence-like and formed three to four concentric aerial rings in each colony. No pigments were produced (Fig. 2A, group I).

Group II included 12 isolates (Table 1) such as the representative LT3-2O, LT4-1O, and LT4-2B. Mycelia were originally snow-white, and then turned grayish white in the late stage, producing light- to yellow-brown pigments on the undersides of the colonies. Mycelia grew along the agar surface, and the aerial hyphae were sparse (Fig. 2A, group II).

Group III included the final 18 isolates (Table 1), such as TP2-1A and PF2-8O. Mycelia were also white in early growth and later changed to grayish white and flocculence-like appearance, and no pigments were produced, similar to the isolates of group I. Noticeably, concentric aerial hyphal rings could also be formed, while the rings were less apparent and the number was smaller than that of group I (Fig. 2A, group III).

Microscopic characteristics of the *Pestalotiopsis* isolates. For isolates in group I, the conidia were fusoid, straight, or a bit curved. Each conidium had five cells, among which the three median cells were dark brown, and the two end cells were hyaline. Conidia had lengths of 20.1 to 30.9 μ m and widths of 3.7 to 8.3 μ m. Each conidium formed two to four apical appendages and one basal appendage. The apical and basal appendages had lengths of 19.22 to 41.41 μ m and 3.6 to 8.7 μ m, respectively (Table 2, Fig. 2B, group I).

For the isolates in group II, except for LT3-2O, which could not produce conidia under the tested conditions, the conidia were also fusoid. Each conidium contained five cells, among which the top one was hyaline, the middle three were light brown, and the basal

one was a bit paler than the brown cells. Conidia showed lengths of 28.8 to 34.3 μm and widths of 5.6 to 8.8 μm . Each conidium formed two to five but mainly three to four apical appendages with lengths of 15.5 to 38.7 μm , while no basal appendage was formed (Table 2, Fig. 2B, group II).

For the isolates in group III, the fusoid conidia also had five cells, among which the first two median cells were dark brown, the third median cell was light brown, and the two end cells were hyaline. Conidia were straight or a bit curved, 19.8 to 26.3 μm in length and 4.9 to 8.7 μm in width. Each conidium produced two to four apical appendages and one basal appendage. The apical and basal appendages had lengths of 15.2 to 29.3 μm and 4.1 to 9.3 μm , respectively (Table 2, Fig. 2B, group III).

The group I, II, and III isolates were tentatively identified as *P. theae*, *P. camelliae*, and *P. clavispora* according to their colony and microscopic characteristics, respectively, in comparison with type species (Table 2). Some representative isolates (TP1-2O and TP2-2W from group I, LT4-1O and LT4-2B from group II, and TP2-1A and PF2-8O from group III) were used for most further analyses.

Growth rate of the representative *Pestalotiopsis* isolates. The isolates showed various growth rates that displayed significant differences even within the same group (Fig. 3A). LT3-2O of *P. camelliae* grew at a rate of only 5.6 mm/day, which was the lowest, while TP1-2O (*P. theae*) and TP-2-1A (*P. clavispora*) had the same rate of 15.0 mm/day. The other isolates displayed higher growth rates, varying between 16.5 and 17.7 mm/day (Fig. 3A).

Molecular characterization and phylogenetic analysis of the *Pestalotiopsis* isolates. The ITS sequences of the group I, II, and III isolates were amplified by PCR and the target bands with lengths of 506 to 586 bp were purified, sequenced, and submitted to the NCBI database (Table 1). BLASTn searches showed that they shared the highest similarities with those of *P. theae* (group I isolates), *P. camelliae* (group II isolates), and *P. clavispora* (group III isolates), respectively (Table 1).

Sequence alignment showed that 34 ITS sequences were classified into three different groups, which was consistent with the classification of the isolates by culturing features (Supplementary Fig. S1). Nucleotide differences were primarily observed in the positions of

Fig. 1. Typical symptoms of brown-black spot and gray blight diseases on leaves of *Camellia sinensis* (L.) O. Kuntze. **A**, tender leaves seriously infected by brown-black spot disease in the field in Xuan'en county; **B**, typical symptoms of brown-black spot disease on the leaves collected from Xuan'en, Yichang, and Yingshan counties, Hubei Province, P. R. China, respectively; **C**, typical symptoms of gray blight disease on the tea leaves collected from Yingshan, Yichang, and Zhuxi counties, Hubei Province, respectively.

Fig. 2. Morphologies of the representative isolates belonging to *Pestalotiopsis* spp. obtained in this study. **A**, views of the surface and the bottom of each PDA plate of representative isolates. **B**, conidia (scale bar = 10 μm) were produced by these isolates on PDA at a prolonged culture time (over 10 days). "N/A" means no production of conidium under the tested conditions.

Table 1. Information of the *Pestalotiopsis* isolates obtained in this study and their BLASTn results

Isolate ^a	Species	Region ^b	Sequence ^c	Accession no. ^d	Total score	Coverage (%)	E value	Identity (%)	Accession no. ^e	Isolate ^f
TP1-1O	<i>P. theae</i>	Yichang	ITS	MF115525	913	100	0	99	KM513607	JS-2
TP1-2O	<i>P. theae</i>	Yichang	ITS	MF115526	907	100	0	99	KM513607	JS-2
TP1-1C	<i>P. theae</i>	Yichang	ITS	MF115527	920	100	0	100	KM513607	JS-2
TP1-2G	<i>P. theae</i>	Yichang	ITS	MF115528	920	100	0	100	KM513607	JS-2
TP1-2-E	<i>P. theae</i>	Yichang	ITS	MF115529	920	100	0	100	KM513607	JS-2
TP2-1W	<i>P. theae</i>	Yichang	ITS	MF115530	920	100	0	100	KM513607	JS-2
TP2-2W	<i>P. theae</i>	Yichang	ITS	MF115531	920	100	0	100	KM513607	JS-2
TP2-2W	<i>P. theae</i>	Yichang	<i>TUB</i>	MF115571	773	93	0	99	DQ137868	PSH2000I-099
TP2-2W	<i>P. theae</i>	Yichang	<i>TEF</i>	MF115580	435	100	4e ⁻¹¹⁸	93	AB453847	TAP36O105
YS4-1	<i>P. theae</i>	Yingshan	ITS	MF115532	920	100	0	100	KM513607	JS-2
YS5-1	<i>P. theae</i>	Yingshan	ITS	MF115533	915	100	0	99	KM513607	JS-2
ZX4-2	<i>P. theae</i>	Zhuxi	ITS	MF115534	915	100	0	99	KM513607	JS-2
TP3-2A	<i>P. theae</i>	Yichang	ITS	MF115535	915	100	0	99	KM513607	JS-2
TP3-2A	<i>P. theae</i>	Yichang	<i>TUB</i>	MF115572	778	93	0	100	DQ137868	PSH2000I-099
TP3-2A	<i>P. theae</i>	Yichang	<i>TEF</i>	MF115581	440	100	9e ⁻¹²⁰	93	AB453847	TAP36O105
WS3-2	<i>P. theae</i>	Yingshan	ITS	MF115536	902	99	0	99	KM513607	JS-2
WS3-12	<i>P. theae</i>	Yingshan	ITS	MF115537	902	99	0	99	KM513607	JS-2
TP1-1E	<i>P. theae</i>	Yichang	ITS	MF115538	913	100	0	99	KM513607	JS-2
YS1-1	<i>P. theae</i>	Yingshan	ITS	MF115539	913	100	0	99	KM513607	JS-2
YS2-1	<i>P. theae</i>	Yingshan	ITS	MF115540	913	100	0	99	KM513607	JS-2
PF3-1	<i>P. theae</i>	Xuan'en	ITS	MF115541	909	100	0	99	JN943624	3.9188
YC5-1	<i>P. theae</i>	Yichang	ITS	MF115542	922	100	0	100	HQ832793	LH13
YC5-1	<i>P. theae</i>	Yichang	<i>TUB</i>	MF115573	774	94	0	99	DQ137872	PSHI2002Endo312
YC5-1	<i>P. theae</i>	Yichang	<i>TEF</i>	MF115582	435	100	4e ⁻¹¹⁸	93	AB453847	TAP36O105
YC6-3	<i>P. theae</i>	Yichang	ITS	MF115543	922	100	0	100	HQ832793	LH13
YC6-3	<i>P. theae</i>	Yichang	<i>TUB</i>	MF115574	778	93	0	100	DQ137872	PSHI2002Endo312
YC6-3	<i>P. theae</i>	Yichang	<i>TEF</i>	MF115583	435	100	4e ⁻¹¹⁸	93	AB453847	TAP36O105
ZX1-1	<i>P. theae</i>	Zhuxi	ITS	MF115544	922	100	0	100	HQ832793	LH13
ZX5-2	<i>P. theae</i>	Zhuxi	ITS	MF115545	922	100	0	100	JN943624	3.9188
ZX1-2	<i>P. theae</i>	Zhuxi	ITS	MF115546	917	100	0	99	HQ832793	LH13
YC2-1	<i>P. theae</i>	Yichang	ITS	MF115547	922	99	0	100	JN943624	3.9188
YC2-1	<i>P. theae</i>	Yichang	<i>TUB</i>	MF115575	749	96	0	98	DQ137868	PSH2000I-099
YC2-1	<i>P. theae</i>	Yichang	<i>TEF</i>	MF115584	435	100	4e ⁻¹¹⁸	93	AB453847	TAP36O105
YC1-1	<i>P. theae</i>	Yichang	ITS	MF115548	905	100	0	99	HQ832793	LH13
WS1-2	<i>P. theae</i>	Yingshan	ITS	MF115549	863	98	0	98	KM513607	JS-2
WS1-10	<i>P. theae</i>	Yingshan	ITS	MF115550	863	98	0	98	KM513607	JS-2
JF12-3	<i>P. camelliae</i>	Xuan'en	ITS	MF115551	822	100	0	94	KY244076	INTA-IMC1359
LT3-2O	<i>P. camelliae</i>	Xuan'en	ITS	MF115552	1000	99	0	99	KM199336	CBS 443.62
LT3-2A	<i>P. camelliae</i>	Xuan'en	ITS	MF115553	1000	99	0	99	KM199336	CBS 443.62
LT4-1G	<i>P. camelliae</i>	Xuan'en	ITS	MF115554	1009	100	0	100	KM199336	CBS 443.62
LT4-3B	<i>P. camelliae</i>	Xuan'en	ITS	MF115555	1003	100	0	99	KM199336	CBS 443.62
LT4-3B	<i>P. camelliae</i>	Xuan'en	<i>TUB</i>	MF115576	754	94	0	99	KX895303	LC3348
LT4-3B	<i>P. camelliae</i>	Xuan'en	<i>TEF</i>	MF115585	527	100	7e ⁻¹⁴⁶	99	KX895176	LC3154
LT4-2B	<i>P. camelliae</i>	Xuan'en	ITS	MF115556	1003	99	0	100	KM199336	CBS 443.62
LT4-2C	<i>P. camelliae</i>	Xuan'en	ITS	MF115557	1003	99	0	100	KM199336	CBS 443.62
LT4-1	<i>P. camelliae</i>	Xuan'en	ITS	MF115558	1000	99	0	99	KM199336	CBS 443.62
LT4-2	<i>P. camelliae</i>	Xuan'en	ITS	MF115559	937	100	0	98	KM199336	CBS 443.62
LT4-2A	<i>P. camelliae</i>	Xuan'en	ITS	MF115560	937	100	0	98	KM199336	CBS 443.62
LT4-1O	<i>P. camelliae</i>	Xuan'en	ITS	MF115561	769	98	0	92	KX894934	LC3003
LT4-1O	<i>P. camelliae</i>	Xuan'en	<i>TUB</i>	MF115577	617	94	6e ⁻¹⁷³	93	KX895303	LC3348
LT4-1O	<i>P. camelliae</i>	Xuan'en	<i>TEF</i>	MF115586	538	100	3e ⁻¹⁴⁹	99	KX895176	LC3154
LT4-1B	<i>P. camelliae</i>	Xuan'en	ITS	MF115562	769	98	0	92	KX894934	LC3003
TP2-1N	<i>P. clavispora</i>	Yichang	ITS	MF115563	1778	98	0	100	KX866908	KFRD-68
TP2-1N	<i>P. clavispora</i>	Yichang	<i>TUB</i>	MF115578	771	95	0	99	KX721073	MAC-01
TP2-1N	<i>P. clavispora</i>	Yichang	<i>TEF</i>	MF115587	532	100	1e ⁻¹⁴⁷	99	KM402034	ZJXJ-PA1
TP2-1A	<i>P. clavispora</i>	Yichang	ITS	MF115564	1778	98	0	100	KX866908	KFRD-68
TP2-4C	<i>P. clavispora</i>	Yichang	ITS	MF115565	909	100	0	100	KX866908	KFRD-68
PF2-1	<i>P. clavispora</i>	Xuan'en	ITS	MF115566	909	100	0	100	KX866908	KFRD-68
PF2-1	<i>P. clavispora</i>	Xuan'en	<i>TUB</i>	MF115579	773	96	0	99	KX721073	MAC-01
PF2-1	<i>P. clavispora</i>	Xuan'en	<i>TEF</i>	MF115588	527	100	6e ⁻¹⁴⁶	99	KM402034	ZJXJ-PA1
PF2-8A	<i>P. clavispora</i>	Xuan'en	ITS	MF115567	902	100	0	99	KX866908	KFRD-68
PF2-8O	<i>P. clavispora</i>	Xuan'en	ITS	MF115568	857	100	0	98	KP689177	FL21
MA1-2	<i>P. clavispora</i>	Xuan'en	ITS	MF115569	739	98	0	94	KM438014	UMT85-K
MA3-1	<i>P. clavispora</i>	Xuan'en	ITS	MF115570	739	98	0	94	KM438014	UMT85-K

^a The isolates obtained in this study.^b Regions where the samples were collected in Hubei Province.^c Sequences of ITS, *TUB*, and *TEF*, respectively.^d GenBank accession numbers for the isolates obtained in this study.^e GenBank accession numbers for the isolates obtained in NCBI.^f The isolates registered in NCBI with the best matches with those obtained in this study.

47 to 118, 372 to 396, and 461 to 480 nt. Specifically, sequences in the group of *P. theae* had an 8 nt deletion in the position of 377 to 385 nt, in the group of *P. camelliae* a 34 to 36 nt insertion in 58 to 92 nt, and in the group of *P. clavispora* a 51 to 52 nt deletion in 47 to 118 nt. The nucleotide differences corresponded to the specific species *P. theae*, *P. camelliae*, and *P. clavispora*, respectively.

In the phylogenetic tree constructed by NJ method (Fig. 4), 34 ITS sequences formed three major clusters with related strains available in GenBank. Within each cluster, the sequences were highly divergent and as a result, most of them formed discrete branches (Fig. 4). For example, five isolates (LT4-2, LT4-2A, LT4-10, LT4-1B, and JF12-3) were distantly separated from other *P. camelliae* isolates, and three isolates (TP2-1A, TP2-4C, and PF2-1) were clearly separated from other *P. clavispora* isolates. Generally, each cluster corresponded to one species of *P. theae*, *P. camelliae*, or *P. clavispora* matching to groups I, II, and III, respectively (Figs. 2 and 4). Six representative isolates (TP2-2W, TP3-2A; TP2-1 N, PF2-1; and LT4-3B, LT4-10) belonging to the three species were further subjected to sequencing their partial *TUB* and *TEF* sequences. BLASTn searches also indicated that they shared the highest similarities with those of *P. theae*, *P. camelliae*, and *P. clavispora*, respectively (Table 1). Phylogenetic analysis based on the concatenated sequences including ITS, *TUB*, and *TEF* sequences confirmed the identification of the species (Fig. 5B).

Pathogenicity tests of the *Pestalotiopsis* isolates. Pathogenicity analysis of the *Pestalotiopsis* isolates were initially tested on detached leaves of *C. sinensis* var. Taicha No. 12. Under the condition of needling, inoculation of agar discs without mycelia did not result in any symptoms on tea leaves; in contrast, inoculation of mycelial discs caused apparent lesions for the isolates with high virulence at 1 day post inoculation (dpi). The lesions were brown, and the lengths varied significantly among three species (Figs. 3B and 5A). At 3 dpi on wounded leaves, representative isolates of *P. theae* and *P. clavispora* almost all spread to the leaf side edges and caused larger lesions (12.3 to 16.2 mm in length) than the representative isolates of *P. camelliae*, which resulted in much smaller lesions (0.7 to 3.7 mm in length) (Figs. 3B and 5A). Noticeably, brown-black spots with diameters of 1 to 2 mm were also formed at the edge of all lesions (Fig. 5A and C), which was similar to the symptoms observed in the field (Figs. 1 and 5CI, II). However, for the unwounded leaves, only TP1-2O and TP2-2W of *P. theae* were able to induce lesions that were generally small (6.7 and 7.5 mm in length, respectively) and accompanied by brown-black spots with sizes of 1 to 2 mm (Fig. 5A). Like the control, five isolates belonging to *P. camelliae* and *P. clavispora* could not cause any disease symptoms on unwounded leaves (Fig. 5A).

The conidium suspension of TP1-2O and TP2-2W (*P. theae*) did not induce any disease symptoms on unwounded leaves, while after wounding they caused brown lesions (Fig. 5B) of 4.4 and 12.4 mm, respectively. Such lesions were surrounded by brown-black spots (Fig. 5CIII), which was similar to the symptoms of natural infection (Figs. 1A and B) or those obtained with mycelial discs (Figs. 5CI and II).

Pathogenicity analysis of TP2-2W of *P. theae*, PF2-8 of *P. clavispora*, and LT4-2B of *P. camelliae* were further tested on intact leaves with a conidium suspension in the field. At 20 dpi with TP2-2W of *P. theae*, small brown-black spots clearly appeared on the leaves and remained small until the experiments ended at 30 dpi (Fig. 5E), matching well with the original ones that had been observed. However, no obvious symptoms were observed for the PF2-8 of *P. clavispora*, LT4-2B of *P. camelliae*, and the control (data not shown).

The reobtained isolates from the inoculation-induced diseased leaves matched well with the ones for inoculation, as indicated by the colony morphology, microscopic observation, and their identical ITS sequences.

Characterization of isolates related to tea gray blight disease.

To compare the molecular and biological features of the isolates responsible for the brown-black spot disease (designated as BSD isolates) and those for the gray blight disease (designated as GBD isolates), 12 leaf samples showing gray blight disease symptoms (Fig. 1C) were collected from the same (Yichang and Yingshan counties) and different (Zhuxi county) regions at almost the same time (within the same week) with the samples showing the brown-black spot disease symptoms, and were subjected to pathogen isolation by culturing the conidia picked from acervuli over the lesions. As expected, *P. theae* colonies, which were identified by the morphologies and ITS sequences (see below), were abundantly isolated. A *P. theae* isolate was taken from each leaf sample, and three to four isolates were selected for the samples from each county (12 in total, designated as YC-, YS-, and ZX-isolates according to their origins; Table 1) for further characterization. After culturing on PDA in the dark at 25°C, all the GBD isolates showed the macro-morphology, conidium conformation, and color similar to those of BSD isolates (Fig. 2A and B). However, the conidia of the GBD isolates were obviously longer than those of the BSD isolates (30.6 to 32.3 versus 24.3 to 24.6 µm in average length) (Table 2). The GBD isolates showed significantly varying growth rates ranging from 9.2 to 18.0 mm/day, which covered the growth rate range of the BSD isolates (Supplementary Fig. S2). On the ITS phylogenetic tree, YC- and ZX-isolates (except for ZX4-2) were clearly separated from BSD isolates, and were closely related to the tea endophytic isolates (PSHI20002Endo310 and PSHI20002Endo312) collected from Yunnan

Table 2. Morphological characteristics of the representative isolates of *Pestalotiopsis* spp. obtained in this study and the referenced ones

Species and group	Isolate	Conidium size ranges ^a (µm)		Number of apical appendages	Length of apical appendages ^b (µm)	Morphology of apical appendages	Color of median cells	Length of basal appendages ^c (µm)
		Length	Width					
<i>P. theae</i> (group I)	TP1-2O	20.1–24.6–27.8	3.7–5.6–7.7	2–3	19.2–29.7–39.8	Knobbed	Concolorous, dark brown	3.6–7.1–11.3
	TP2-2W	20.5–24.3–30.9	5.2–6.9–8.3	2–4	15.8–29.8–41.4			5.0–6.9–8.7
<i>P. theae</i> (GLD ^d)	YS-2-1	22.8–30.6–35.6	3.5–6.2–8.2	2–3	14.8–28.2–37.6			2.1–6.5–9.1
	YC-6-3	21.9–31.8–37.0	4.9–7.4–10.3	2–4	4.4–21.7–50.2			3.7–7.5–10.9
	ZX-5-1	26.0–32.3–42.7	4.5–6.6–8.3	2–4	16.3–31.2–45.6			4.5–7.9–9.9
<i>P. theae</i> (ref ^e)		24.8–29.5	6.6–8.3	2–3	23.6–42.5			5.9–9.4
		23–35	5.5–8	2–4	25–60			
<i>P. camelliae</i> (group II)	LT4-10	28.8–33.0–38.0	5.6–7.1–8.4	2–5	15.5–24.9–44.8	Tubular	Concolorous, light brown	0
	LT4-2B	24.2–30.0–34.3	6.4–7.8–8.8	2–5	16.0–27.5–38.7			0
<i>P. camelliae</i> (ref ^f)		27–28.5–33	7.5–8.5–9.3	3–6	13.5–23.8–34			0
<i>P. clavispora</i> (group III)	TP2-1A	19.8–22.8–26.4	4.9–6.6–7.8	2–3	15.2–22.2–34.1	Tubular	Two dark brown, one light brown	4.1–6.3–8.3
	PF2-8O	19.0–23.1–26.312	5.4–6.9–8.7	2–4	14.4–22.8–29.3			4.7–7.0–9.3
<i>P. clavispora</i> (ref ^f)		21.2–27.1	5.2–7.1	2–3	16.5–26.5			1.8–4.2

^a Minimum, average, and maximum length and width of 50 conidia recorded from each selected *Pestalotiopsis* spp. from China.

^b Minimum, average, and maximum length of apical appendages of 50 conidia.

^c Minimum, average, and maximum length of basal appendages of 50 conidia.

^d Isolates related to tea gray blight disease.

^e The referenced species according to Ge et al. 2009.

^f The referenced species according to Zhang et al. 2012.

Province and GBD isolates (PSH20001-083 and PSH20001-099) collected from Guangxi and Zhejiang provinces, respectively (Ge et al. 2009). However, YS-isolates were clustered together with BSD isolates (Fig. 4; Table 1). Unlike BSD isolates, GBD isolates showed no virulence on detached tender leaves of *C. sinensis* var. Taicha No. 12 under unwounded conditions, whereas they induced lesions with lengths ranging from 3.5 to 29.8 mm after wounding. Unlike BSD isolates, GBD isolates did not result in the appearance of brown-black spots along with the induction of brown lesions on tender tea leaves (Fig. 5D).

Discussion

In this study, brown-black spot disease was described as a novel tea leaf disease as it causes apparent symptoms on tender tea leaves that are quite different from those previously reported (Z. M. Chen and X. L. Sun 2013; Maharachchikumbura et al. 2014). Typical diseased samples were collected from three main tea cultivation regions of Hubei, China, and the related pathogens were isolated and characterized. The results showed that *Pestalotiopsis* fungi (34 isolates, see the typical conidia of a *Pestalotiopsis* species in Fig. 2B: fusiform,

5-celled, brown median cells, and hyaline end cells, appendages) were dominant among all fungi isolated from the diseased samples, though *Phomopsis* (12 isolates) and *Colletotrichum* fungi (eight isolates) were also isolated simultaneously. The colony and microscopic characteristics suggested that the *Pestalotiopsis* isolates might belong to *P. theae*, *P. camelliae*, and *P. clavispora*, respectively, according to the standards described by Ge et al. (2009) and Guba (1961). The results of phylogenetic analysis confirmed those of morphological characterization. Hence, the isolates in groups I, II, and III were identified to belong to *P. theae*, *P. camelliae*, and *P. clavispora*, respectively (Fig. 2). No matter what mycelial discs or conidium suspensions were used, inoculation of the *Pestalotiopsis* fungi could result in brown-black spots (1 to 2 mm in diameter), which were similar to the symptoms observed in the field. In contrast, inoculation of the *Phomopsis* and *Colletotrichum* fungi could not cause any disease symptoms, though they were found to be the causal agents of tea leaf brown blight disease in China (Y. J. Chen et al. 2016; Guo et al. 2014). The results obtained through the identification of these pathogens in microscopy and using various molecular tools, as well all the assays conducted to fulfill Koch's postulates, clearly show that *P. theae*,

Fig. 3. Growth rates and virulence of the representative isolates of *P. theae*, *P. camelliae*, and *P. clavispora*. **A**, growth rates were accessed by culturing a mycelium plugs on PDA plates in the dark at 25°C at 4 days post inoculation (dpi). **B**, lesion lengths on wounded tender leaves of var. Taicha No. 2 induced by inoculation of their mycelial discs at 25°C for a light period of 12 h at 3 dpi. Data were analyzed with SPSS Statistics 21.0 (WinWrap Basic; <http://www.winwrap.com>) by one-way ANOVA, and means were compared using Tukey's test at a significance level of $P = 0.05$. Letters over the bars indicate the significant difference at $P = 0.05$. Replicates for the experiments are indicated in the panels.

P. camelliae, and *P. clavispora* were the dominant fungi associated with brown-black spots on tea tender leaves. Although *P. camelliae* and *P. clavispora* showed no virulence on tea leaves on unwounded leaves, we concluded that they were still closely associated with the disease, considering their high incidence in the diseased plants. Both species might induce severe disease by invading tea plants via wounds with assistance from insects, mechanical damage, or other unknown factors, which had been exemplified by *Phomopsis* species (Bai et al. 2015). The results also confirmed that this disease is quite different from the two tea diseases reported previously, namely white scab disease and brown round spot disease. The latter two always cause lesions with a grayish appearance in the center on tender tea leaves, while this gray part is always absent on leaves affected by small brown-black spot. It has also been shown that white scab is caused by *Phyllosticta theae* of Sphaeriales, Ascomycota, and the brown round spot disease by *Cercospora theae* of Moniliales, Deuteromycotina (Z. M. Chen and X. L. Sun 2013).

Morphological characteristics, such as conidium sizes and apical appendage length of *Pestalotiopsis*, may vary responsively with the changes of culture conditions. In many cases, simple observation of morphology is not sufficient to distinguish the members of genus *Pestalotiopsis* (Jeewon et al. 2003). Molecular characterization of fungal genes, such as ITS, *TUB*, and *TEF*, can reveal more specific differences between the *Pestalotiopsis* fungi (Maharachchikumbura et al. 2014). In this study, 34 isolates were firstly subjected to phylogenetic analysis of ITS sequences using NJ method. The results revealed that these isolates clustered together with the species of *P. theae*, *P. camelliae*, and *P. clavispora*, respectively (Fig. 4A), which was also confirmed by the results of ML method (Supplementary Fig. S3). In both phylogenetic trees, some isolates (LT4-2, LT4-2A, LT4-10, LT4-1B, JF12-3) were located between species *P. camelliae* and *P. karstenii* (possibly a synonym of *P. maculans*, Jeewon et al. 2003), but they could be easily distinguished from *P. karstenii* according to

Fig. 5. Typical symptoms on tender leaves of var. Taicha No. 2 induced by inoculation of the representative isolates of *P. theae*, *P. camelliae*, and *P. clavispora*. **A**, symptoms induced by inoculations of mycelial discs of three *Pestalotiopsis* species on wounded (upper) and unwounded (below) leaves, respectively. **B**, symptoms induced by conidia of isolate TP2-2W under the both conditions, respectively. **C**, close view of the symptoms induced by mycelial discs of isolate TP2-2W on wounded (I) and unwounded (II) leaves, and by conidia sprayed on wounded leaves (III). **D**, symptoms induced by mycelial discs of ZX5-1 isolate on wounded and unwounded leaves, respectively, at 3 days post inoculation, as representative symptoms for GBD isolates. **E**, symptoms on intact tea leaves (*C. sinensis* var. Enshitaizicha) inoculated with conidium suspension (10^5 /ml) of TP2-2W of *P. theae*.

their morphological properties, such as conidium size, apical appendage number, and basal appendage-forming ability (Z. M. Chen and X. L. Sun 2013; Maharachchikumbura et al. 2014) (Table 2). The taxonomic status was further confirmed by phylogenetic analysis with *TUB* and *TEF* sequences for the isolates located far from the major branch of each species (Fig. 4B). Consistent with the results of the phylogenetic analysis, the virulence of the tested isolates was also clearly delimited among different species. For example, three isolates of *P. camelliae* (LT3-20, LT4-10, and LT4-2B) caused similarly long but much shorter lesions than those induced by the isolates belonging to the other two species. All these results clearly demonstrate that the obtained isolates belong to *P. theae*, *P. camelliae*, and *P. clavispora*, respectively. However, these isolates showed considerably high divergence from each other within each species as shown by the NJ or ML phylogenetic tree analysis of ITS sequences (Fig. 4), suggesting that they might have complicated origins in the tea cultivation regions of Hubei.

In previous reports, the three *Pestalotiopsis* species had not been found to be capable of causing apparent symptoms on tender tea leaves, but *P. theae* was well characterized as a causal agent of tea gray blight disease, which attacks mature and old leaves and leads to large brown spots with the formation of apparent concentric rings in the late period of disease (see Fig. 1C and Z. M. Chen and X. L. Sun 2013). Here, we revealed that *P. theae* can also induce visible symptoms on tender tea leaves. It was reasonable to suspect that brown-black spot disease might be the early symptoms of gray blight disease. For this reason, we characterized the *P. theae* isolates responsible for tea gray blight disease (GBD isolates) that occurred simultaneously, and revealed that GBD isolates were distinctly different from BSD isolates in conidium sizes and pathogenicity on unwounded leaves. The BSD isolates of *P. theae* in this study are also quite different in morphology from the GBD isolates characterized in previous studies, as the conidium sizes and the lengths of apical appendages for the former exceed the bottom line of those of the latter (conidium sizes: 23 to 35 × 5.5 to 8 µm; apical appendage lengths: 25 to 60 µm) (Z. M. Chen and X. L. Sun 2013). Additionally, BSD isolates were located in the clusters distinct from the known *P. theae* isolates previously identified from China (including tea endophytic *P. theae* isolates and previously identified BSD isolates) and most of the newly identified GBD isolates (including YC- and ZX-isolates) in this study. Moreover, during a several-month observation, we found that on tender leaves of Fuyun No. 6, the small brown-black spots caused by brown-black spot disease could not grow to large lesions as always observed with gray blight disease. More importantly, GBD isolates induced no brown-black spots on the inoculated leaves. It can be concluded that BSD isolates are possibly novel strains different from GBD isolates according to the morphologies, pathogenicity, and phylogenetic analysis, and the brown-black spot disease caused by the three *Pestalotiopsis* fungi is not the early stage of the gray blight disease; instead, it is a novel tea leaf disease considering the differences in symptoms and pathogens.

Pestalotiopsis fungi are commonly asexual and are extensively distributed in nature. They are classified into pathogenic, saprophytic, and endogenous types according to their pathogenicity and living habits (Ge et al. 2009). The pathogenic variants of *Pestalotiopsis* are considered to be economically important plant pathogens. They have a broad range of hosts and can cause plant diseases all over the world, especially in tropic and subtropic areas (Z. M. Chen and X. L. Sun 2013; Maharachchikumbura et al. 2014). *P. theae* can infect tea, loquat (*Eriobotrya japonica*), and sweet persimmon (*Diospyros kaki*), causing symptoms of greenish brown lesions and large grayish brown circular ringspots on leaves, respectively (Y. Chen et al. 2013; Tuset et al. 1999). This fungus was also reported to be capable of infecting camellia (*C. japonica*), while the pathological mechanism still remains unknown (Ge et al. 2009). Unlike *P. theae*, *P. camelliae* has been little known and seems to be a new species associated with gray blight of camellia in China (Zhang et al. 2012). *P. clavispora* has a broader range of hosts than *P. theae* and *P. camelliae*, and causes leaf diseases on various plants, but is not pathogenic to tea leaf and is an endophyte in tea plants (Ge et al. 2009). To our knowledge, this is

the first report that *P. camelliae* and *P. clavispora* can cause apparent symptoms on tender leaves.

In summary, a novel disease designated as brown-black disease, which occurs on tea tender leaves in many tea cultivation regions of Hubei, China, was characterized and distinguished from other known diseases in this study. The causal agents were identified to be closely associated with three *Pestalotiopsis* species. According to the morphological characteristics and phylogenetic relationships, the main pathogenic species was identified as *P. theae*. Our results provide diagnostic tools that should help improve the prevention and management of this economically important disease.

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