Pak. J. Bot., 55(6): 2403-2408, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-6(13)

FIRST REPORT OF *PHYLLOSTICTA CAPITALENSIS* CAUSING LEAF BROWN SPOT OF *LIGUSTRUM JAPONICUM* IN CHINA

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Abstract

In 2021, the Leaf brown spot of the Japanese privet (*Ligustrum japonicum*) was observed in Changxi Avenue, Nanchang City, Jiangxi Province, China. 16 isolates from diseased leaves were obtained, and their pathogenicity was confirmed. The pathogen was identified as *Phyllosticta capitalensis* based on morphological characteristics, cultural appearance, and molecular characteristics. To our knowledge, this is the first report of *P. capitalensis* causing leaf brown spot of *L. japonicum* in China.

Key words: *Phyllosticta capitalensi*s; *Ligustrum japonicum*; Leaf brown spot; China.

Nomenclature: PDA: Potato dextrose agar; ITS: Internal transcribed spacer; ACT: Actin; TEF1-a: Translation elongation factor; GPD: Glyceradehyde-3-phosphate dehydrogenase.

Introduction

Japanese privet (*Ligustrum japonicum* Thunb. fam. Oleaceae) is a well-known evergreen shrub or small tree. As it is easy to grow, Japanese privet is now usually grown as an evergreen ornamental shrub and hedge around the world (Baek *et al*., 2015). In addition to its importance as an ornamental, the plant also has medicinal value (Ngo *et al*., 2017; Kim *et al*., 2019). Japanese privet is also widely planted throughout China and is an important ornamental species but various fungal species can damage its ornamental feature. Some examples of the damage are leaf spots caused by *Pseudocercospora ligustri, Pseudocercospora rizhaoensis, Diaporthe eres*, *Alternaria alternata* and *Phyllosticta capitalensis*, as well as anthracnose caused by *Colletotrichum gloeosporioides* and *C. siamense* (Shen *et al*., 2017; Wang & Liu, 2019; Li *et al*., 2022; Liu *et al*., 2022; Sabahi *et al*., 2022; Fang *et al*., 2023; Liao *et al*., 2023). Leaf spot disease can lead to premature defoliation or even death of the plant, hence, it is critical to be aware of and control the leaf spot disease on *L. japonicum*. Therefore, identification of the causative agent of the leaf spots is important to determine the best protection strategy.

In October 2021, a new fungal infection was observed on the leaves of Japanese privet growing in Changxi Avenue, Nanchang City, Jiangxi Province, China. Brown spot lesions with yellow margins were formed on the edge or middle of the leaf, with these symptoms observed on nearly 30% of the leaves. The diseased plants grew poorly and appeared stunted. To date, similar disease symptoms on Japanese privet have never been reported in China. In this study, the fungal pathogen was identified based on its morphological and molecular characteristics.

Materials and Methods

Pathogen isolation and morphological identification: In October 2021, typical leaf brown spot symptoms were observed on roughly 30% of Japanese privet leaves at a location in Changxi Avenue, Nanchang city (115°82'91″ N, 28°76'37″ E), and brought back to the laboratory for pathogen isolation. Ten symptomatic leaves were randomly collected from the field for a fungal isolation experiment. Their infected leaf tissue edges were cut into small pieces (4×4 mm), surface-sterilized in 70% ethanol for 10 s and 1% NaClO for 30 s, and then rinsed thrice in sterile distilled water. Leaf pieces were then placed onto potato dextrose agar (PDA), incubated at 25°C for 3 to 4 days, and observed daily. Mycelial fragments emerging from leaves tissues on the agar were then cut out and transferred to fresh PDA dishes to obtain pure cultures. All isolates were cultured on PDA dishes for 15 days and examined periodically until sporulation. At least 20–50 pycnidia and conidia were examined and measured using an optical microscope (Nikon Eclipse Ni-U). The morphological characters were recorded and compared with previous descriptions (Wikee *et al*., 2013a; Zhang *et al*., 2015).

Pathogenicity assays: To confirm pathogenicity, six healthy detached leaves from unaffected *L. japonicum* plants were collected. Each leaf was slightly wounded with a sterilized needle, and then 50 µl of conidial suspension (1×10^6 conidia/mL) was inoculated onto the punctured areas of the leaves. For the control treatment, wounded leaves were inoculated with sterilized distilled water. Inoculated leaves were incubated at 28°C and 80% relative humidity for seven days and observed daily. After seven days, if positive, the fungus was re-isolated from the artificially infected leaves using the technique described previously.

Molecular identification of the pathogen: For molecular identification. Each isolate was cultured in potato dextrose broth at 25℃ for seven days. The mycelium was collected by sterilized tips, and genomic DNA was extracted by using the CTAB method (Murray *et al*., 1980). The internal transcribed spacer (ITS) region, the actin (*ACT*), translation elongation factor (*TEF1-a*), and glyceraldehyde-3-phosphate dehydrogenase (*GPD*) genes were amplified using primers ITS1/ITS4, ACT-512F/ ACT-783R, EF1/EF2 and Gpd1-LM/ Gpd2-LM, respectively (Carbone & Kohn, 1999; Myllys *et al*., 2002; Wikee *et al*., 2013a). Amplification reactions were performed in a 25 µL reaction volume, including12.5 µL 2 × Phanta Max Master Mix (Vazyme, Nanjing, China), 0.1 µM of each forward and reverse primer, and 1–10 ng genomic DNA in a Gen Amp PCR system T100 thermal cycler (Bio-Rad, FosterCity, USA). The PCR cycle conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 30 s and a final elongation step at 72°C for 5 min. Sequencing was performed by Tsingke Biotechnology Co., Ltd., Changsha, China. The obtained gene sequences were assembled by the SeqMan program (Lasergene, DNASTAR, Inc.). The sequences were deposited in the GenBank (Table 1). Additional sequences were retrieved from GenBank (Table 1). All the sequences were aligned by using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/). Phylogenetic analyses were conducted using the concatenation of multiple sequences (ITS, *ACT*, *TEF1-a,* and *GPD*) with maximum likelihood in IQtree v1.5.6 (Nguyen *et al*., 2015). *Botryosphaeria obtusa* was chosen as the out-group for the phylogenetic tree.

Results and Discussion

Brown spot disease can infect both new and old leaves of Japanese privet. Initially, brown lesions with yellow margins occurred on the edge or middle of the leaf, then the lesions gradually expanded and merged, to finally become dry and then died. Moreover, infected leaves often turned yellow (Fig. 1A).

A total of 16 isolates were obtained from the affected leaves, and all displayed the same colony characteristics. The fungal colonies were gray and uneven, with a granular and white edge, and then turned black with black spherical conidia (Fig. 1B, C). Pycnidia were black brown, globose, and 52-107 µm in diameter (Fig. 1D). Conidia were one-celled, nearly elliptical, each with a hyaline, ranging from 8 to 14 × 6 to 8 µm (n=50)(Fig. 1E). Morphological characteristics were consistent with those of *Phyllosticta* species.

Results of pathogenicity experiments showed that isolate JFRL 03-40 was highly pathogenic to detached leaves of *L. japonicum*. The initial symptoms on leaves were small, light brown spots. And 12 days after inoculation, infected leaves developed similar symptoms to those observed on naturally diseased leaves (Fig. 1F). Furthermore, *P. capitalensis* was successfully re-isolated, and the control leaves remained asymptomatic, fulfilling Koch's postulates.

For further identification, a multigene phylogenetic analysis was conducted. The ITS, *ACT, TEF*, *GPD* genes of isolate JFRL, 03-40, were amplified. The sequences were deposited in GenBank (Accession No. ON076573 for ITS; ON81650 for *ACT*; ON81651 for *TEF1-a,* and ON81652 for *GPD*). BLAST search analysis of GenBank (NCBI) showed that the sequences had 100% similarity with those of *Phyllosticta capitalensis* (sexual type: *Guignardia mangiferae*) (GenBank accession no. ITS, MN635751; *ACT*, FJ538448; *TEF1-a,* KU306117 and *GPD*, KM816630). Phylogenetic analyses were conducted using a concatenation of multiple sequences (ITS, *ACT*, *TEF1-a,* and *GPD*) with maximum likelihood in IQtree v1.5.6. Phylogenetic analysis showed that strain JFRL 03-40 was the closest relative to *P. capitalensis* and clustered in one clade (Fig. 2). Based on morphological and phylogenetic characters, the isolates were identified as *Phyllosticta capitalensis* Henn.

P. capitalensis is an important plant pathogen that produces leaf spots on more than 20 species of plants worldwide, including reports of *P. capitalensis* causing leaf spot disease on Japanese privet in Iran (Wikee *et al*., 2013b; Esmaeilzadeh *et al*., 2020; Liao *et al*., 2020; Jiang *et al*., 2022; Li *et al*., 2022; Sabahi *et al*., 2022; Zhang *et al*., 2022). However, to the best of our knowledge, this is the first report of leaf brown spots caused by *P. capitalensis* on *L. japonicum* in China. *P. capitalensis* has also been reported as an endophyte with a wide range of hosts such as *Magnoliaceae*, *Citrus* spp., *Calophyllum* spp., *Mangifera indica* and *Punica granatum* (Wikee *et al*., 2013b). Endophytic *Phyllosticta* species could become pathogenic under environmental pressure (Wikee *et al*., 2013a; Wikee *et al*., 2013b). Therefore, *P. capitalensis* is expected to pose a serious threat to the production of ornamental plants in this area. Thus, it is necessary to increase awareness, attention and the need to control the disease to ensure sustainability of the Japanese privet.

Acknowledgment

This work was supported by the Youth Fund of the Jiangxi Provincial Department of Education (GJJ180210).

Table 1. Sources of isolates and GenBank accession numbers used in this study.

Species	Code[1]	Host	Country	GenBank no.[2]			
				ITS	*TEF1-a*	*ACT*	*GPD*
Botryosphaeria obtusa	CMW 8232	Conifers	South Africa	AY972105	DQ280419	AY972111	-
P. aloeicola	CPC 21020	*Aloe ferox*	South Africa	KF154280	KF289193	KF289311	KF289124
	CPC 21021	*Aloe ferox*	South Africa	KF154281	KF289194	KF289312	KF289125
P. bifrenariae	CBS 128855	*Bifrenaria harrisoniae*	Brazil	JF343565	JF343586	JF343649	JF343744
	CPC 17467	*Bifrenaria harrisoniae*	Brazil	KF170299	KF289207	KF289283	KF289138
P. brazillianiae	CBS 126270	*Mangifera indica*	Brazil	JF343572	JF343593	JF343656	JF343758
	LGMF 333	*Mangifera indica*	Brazil	JF343574	JF343595	JF343658	JF343760
P. capitalensis	CBS 123404	*Musa paradisiaca*	Thailand	FJ538333	FJ538391	FJ538449	KF289095
	CPC 17748	*Heliconia* sp.	Thailand	KF206190	KF289180	KF289286	KF289096
	JFRL-03-40	***Ligustrum japonicum***	**China**	**ON076573**	**ON81651**	**ON81650**	**ON81652**
P. citriasiana	CBS 120486	*Citrus maxima*	Thailand	FJ538360	FJ538418	FJ538476	JF343686
	CBS 120487	*Citrus maxima*	China	FJ538361	FJ538419	FJ538477	JF343687
P. citribraziliensis	CBS 100098	*Citrus limon*	Brazil	FJ538352	FJ538410	FJ538468	JF343691
	CPC 17464	*Citrus* sp.	Brazil	KF170300	KF289224	KF289280	KF289159
P. citricarpa	CBS 120489	*Citrus sinensis*	Brazil	FJ538315	FJ538373	FJ538431	KF289150
	CBS 127454	*Citrus limon*	Australia	JF343583	JF343604	JF343667	JF343771
P. citrichinaensis	ZJUCC 200956	*Citrus reticulata*	China	JN791620	JN791459	JN791533	-
	ZJUCC 200964	*Citrus maxima*	China	JN791611	JN791461	JN791535	-
P. citrimaxima	CBS 136059	*Citrus maxima*	Thailand	KF170304	KF289222	KF289300	KF289157
P. concentrica	CBS 937.70	*Hedera helix*	Italy	FJ538350	FJ538408	KF289257	JF411745
	CBS 134749	*Hedera* sp.	Spain	KF170310	KF289228	KF289288	KF289163
P. cussonia	CPC 13812	*Cussonia* sp.	South Africa	KF170311	KF289223	KF289262	KF289158
	CPC 14873	*Cussonia* sp.	South Africa	JF343578	JF343599	JF343662	JF343764
P. elongata	CBS 126.22	*Oxycoccus macrocarpos*	USA	FJ538353	FJ538411	FJ538469	KF289164
P. foliorum	CBS 174.77	*Cryptomeria japonica*	USA	KF170308	KF289200	KF289245	KF289131
	CBS 447.68	*Taxus baccata*	Netherlands	KF170309	KF289201	KF289247	KF289132

Table 1. (Cont'd.).

Species	Code[1]	Host	Country	GenBank no.[2]			
				ITS	*TEF1-a*	*ACT*	*GPD*
P. gaultheriae	CBS 447.70	*Gaultheria humifusa*	USA	JN692543	JN692531	KF289248	JN692508
P. hostae	CGMCC 3.14355	*Hosta plantaginea*	China	JN692535	JN692523	JN692511	JN692503
	CGMCC 3.14356	*Hosta plantaginea*	China	JN692536	JN692524	JN692512	JN692504
P. hubeiensis	CGMCC 3.14986	*Viburnum odoratissimim*	China	JX025037	JX025042	JX025032	JX025027
	CGMCC 3.14987	*Viburnum odoratissimim*	China	JX025038	JX025043	JX025033	JX025028
P. hymenocallidicola	CBS 131309	*Hymenocallis littoralis*	Australia	JQ044423	KF289211	KF289242	KF289142
	CPC 19331	*Hymenocallis littoralis*	Australia	KF170303	KF289212	KF289290	KF289143
P. hypoglossi	CBS 101.72	*Ruscus aculeatus*	Italy	FJ538365	FJ538423	FJ538481	JF343694
	CBS 167.85	*Ruscus hypoglossum*	Italy	FJ538366	FJ538424	FJ538482	JF343696
P. ilicis-aquifolii	CGMCC 3.14358	*Ilex aquifolium*	China	JN692538	JN692526	JN692514	-
	CGMCC 3.14359	*Ilex aquifolium*	China	JN692539	JN692527	JN692515	-
P. leucothoicola	CBS 136073	*Leucothoe catesbaei*	Japan	AB454370	-	KF289310	-
P. mangifera-indica	CPC 20274	*Mangifera indica*	Thailand	KF170305	KF289190	KF289296	KF289121
P. owaniana	CBS 776.97	*Brabejum stellatifolium*	South Africa	FJ538368	FJ538426	KF289254	JF343767
	CPC 14901	*Brabejum stellatifolium*	South Africa	JF261462	JF261504	KF289243	JF343766
P. paxistimae	CBS 112527	*Paxistima mysinites*	USA	KF206172	KF289209	KF289239	KF289140
P. podocarpicola	CBS 728.79	*Podocarpus maki*	USA	KF206173	KF289203	KF289252	KF289134
P. pseudotsugae	CBS 111649	*Pseudotsuga menziesii*	USA	KF154277	KF289231	KF289236	KF289167
P. spinarum	CBS 292.90	*Chamaecyparis pisifera*	France	JF343585	JF343606	JF343669	JF343773
P. vacciniicola	CPC 18590	*Vaccinium macrocarpum*	USA	KF170312	KF289229	KF289287	KF289165
Phyllosticta sp.	CPC 17454	*Mangifera indica*	Brazil	KF206206	KF289192	KF289278	KF289123
	CPC 17455	*Mangifera indica*	Brazil	KF206207	KF289191	KF289279	KF289122

[1]CPC: Culture collection of P.W. Crous, housed at CBS; IFO: Institute for Fermentation, Osaka, Japan; LGMF: Culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; ZJUCC: Zhejiang University Culture Collection, China; CGMCC: China, General Microbiological Culture Collection, Beijing, China

[2]ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *TEF1-a*: Partial translation elongation factor 1-α gene; *ACT*: Partial actin gene; GPD: Partial glyceraldehyde-3-phosphate dehydrogenase gene. The new sequences generated in this study are shown in bold

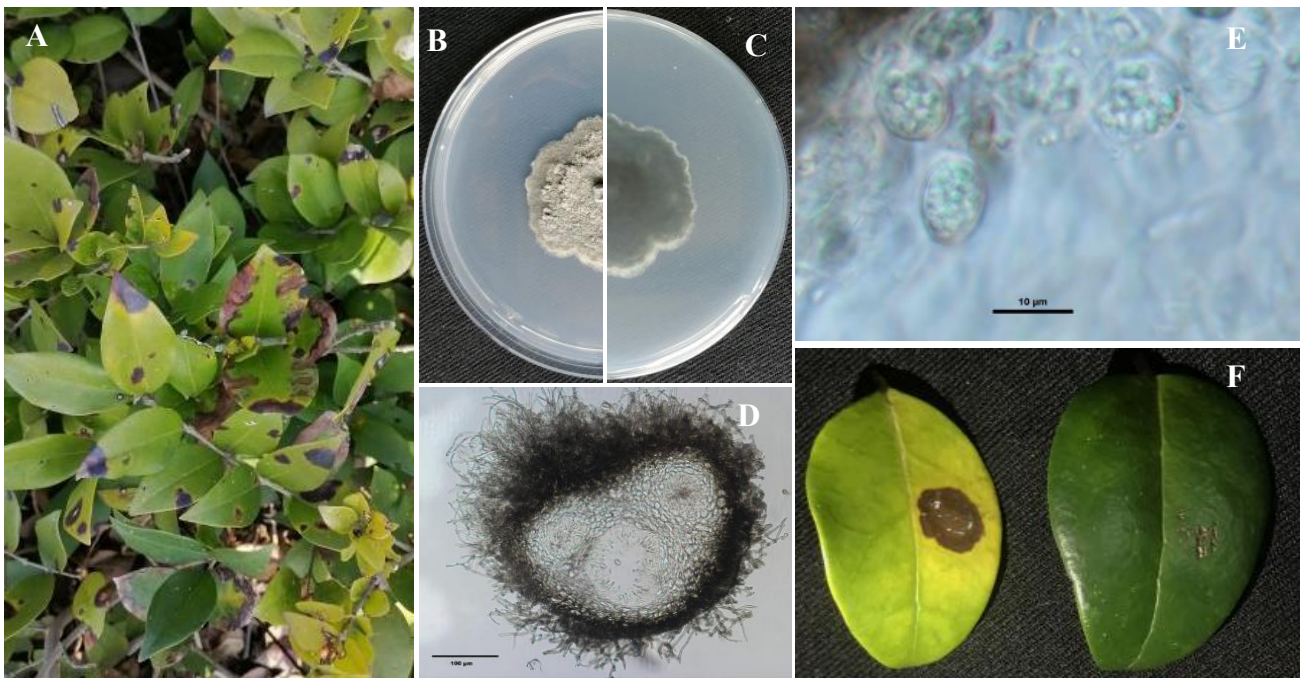

Fig. 1. (A) Leaf brown spot disease on *Ligustrum quihoui*; (B-C) Cultural characters of *Phyllosticta capitalensis*, isolate JFRL-03-40 on PDA at 28°C for seven days; (D) pycnidium; (E) conidia; (F) Pathogenicity test. Scale bars: D = 100 µm, E = 10 µm.

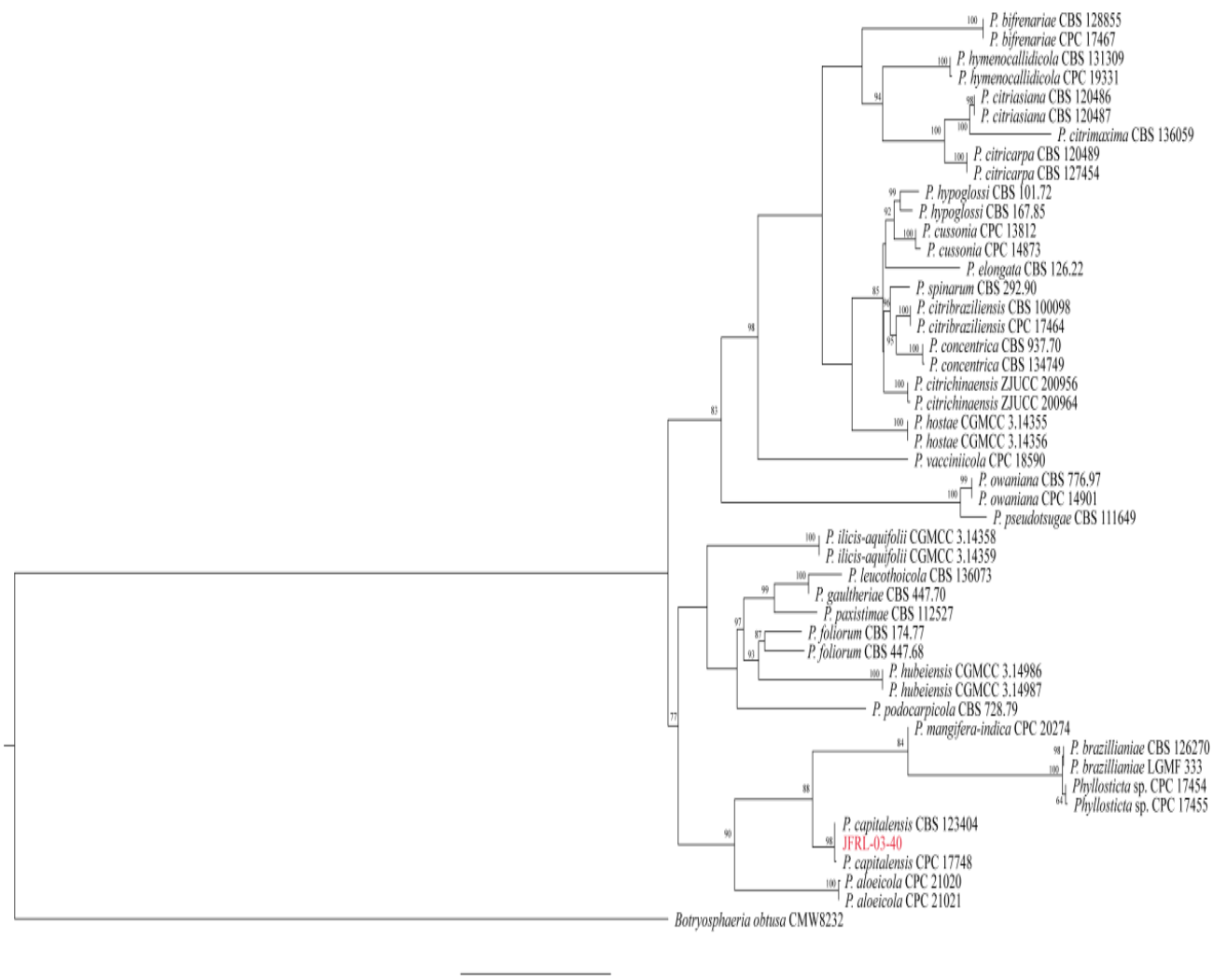

Fig. 2. Phylogenetic tree constructed using IQtree v1.5.6 software showing the relationship of *Phyllosticta capitalensis* with other *Phyllosticta* species.

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(Received for publication 11 August 2022)