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Identification and fungicide screening of *Phyllosticta capitalensis* causing leaf spot on sweet viburnum in China

Wen LI[1], Yixuan RUAN[1], Zhengping BIAN[2], Yueqiu HE[1], Kai FENG[1], Liang LIU[1], Zhilong WANG[1,*], Feng HUANG[3,*]

[1] *Ningbo City College of Vocational Technology, Ningbo Zhejiang, China*
[2] *Ningbo Forest Farm, Ningbo Zhejiang, China*
[3] *Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, Key Laboratory of Green Prevention and Control on Fruits and Vegetables in South China Ministry of Agriculture and Rural Affairs, Guangdong Provincial Key Laboratory of High, Guangzhou, Guangdong, China*
*Corresponding authors. E-mail: wangzhl01@163.com, rm12407@126.com

Summary. Sweet viburnum (*Viburnum odoratissimum* Ker-Gawl.) is a widely used ornamental plant, which has dense branches and leaves, and fast spreading and evergreen habit. In October 2022, leaf spot symptoms were observed in a hedge of sweet viburnum in Yuanshi Garden, Ningbo, China. Fungi were isolated from symptomatic leaves, and were identified using morphological characteristics and phylogenetic analyses of partial sequences of internal transcribed spacer (ITS), actin (*act*), and translation elongation factor 1-alpha (*tef*1-α), and were evaluated in pathogenicity tests. The causal agent of sweet viburnum leaf spot was identified to be *Phyllosticta capitalensis*. Effects of seven fungicides on *P. capitalensis* were assessed *in vitro*. Fungicide EC_{50}s (mg L^{-1}) against *P. capitalensis* were: 270.77 for 75% chlorothalonil (WP); 0.02 for 250 g L^{-1} azoxystrobin SC; 0.27 for 10% difenconazole WDG; 0.02 for 75% trifloxystrobin + tebuconazole WDG, 9.03 for 35% fluopyram + tebuconazole SC, 5.90 for 500 g L^{-1} fluazinam SC, and 89.11 for 10% prothioconazole SC. Among these, azoxystrobin SC and trifloxystrobin + tebuconazole WDG could be used for control of viburnum leaf spot. This is the first report of *P. capitalensis* causing leaf spot of sweet viburnum, and this study provides guidance for chemical management sweet viburnum leaf spot, and on other host plants.

Keywords. Chemical control, new disease, pathogen identification, phylogeny.

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ORCID:
WL: 0000-0002-0983-7910
YR: 0009-0004-1956-1833
ZB: 0009-0008-7439-175X
YH: 0009-0002-8988-9012
KF: 0009-0000-4501-7883
LL: 0000-0001-8044-1006
ZW: 0000-0001-8734-1729
FH: 0000-0002-8406-5068

INTRODUCTION

Viburnum, including almost 200 species, is a large genus of flowering shrubs (Landis *et al.*, 2021), of which sweet viburnum (*Viburnum odoratissimum* Ker-Gawl.) is a shrub native to the Himalayas and Japan. Due to its attributes of fast and spreading growth, dense branches, evergreen leaves, and attractive flowers, sweet viburnum has been widely used as ornamental plants (Shober *et al.*, 2017).

Several fungi and viruses have been reported causing diseases on sweet viburnum. The positive-sense RNA virus designated as 'Viburnum-like virus'

was recently isolated from sweet viburnum, and siRNA sequencing indicated that this virus actively replicates within the host and elicits antiviral defence mechanisms (Gao *et al*., 2024). *Erysiphe hedwigii* causes powdery mildew, and *Colletotrichum gloeosporioides* causes anthracnose of sweet viburnum (Yang *et al*., 2015; Cho *et al*., 2016; Michael *et al*., 2022), and economically important leaf spot diseases are caused by *Diaporthe eres*, *Corynespora cassiicola*, *Alternaria* spp., and *Neofusicoccum parvum* (Qiu *et al*., 2021; Ma *et al*., 2022; Zhang *et al*., 2022a; Wan *et al*., 2023). Occasionally, these leaf spot diseases can kill their host plants. Thus, it is important to identify the causative pathogens, and assessment potential management methods for these diseases.

The present study outlines observation, isolation and description of the pathogen responsible for leaf spot on sweet viburnum leaves. Assessments were also made of the activity of different fungicides as potential agents for control of this disease.

MATERIALS AND METHODS

Pathogen isolation and morphology

To identify the pathogen associated with the disease, 20 symptomatic leaves were collected from ten different sweet viburnum plants. Pathogen isolation was carried out using methods described by Li *et al*. (2021a). Leaf tissue was excised from lesion margins, and was surface sterilized with 75% ethanol for 30 sec. rinsed three times in sterile distilled water, then immersed in 10% sodium hypochlorite for 5 min, and again washed three times in distilled water, followed by drying on sterile filter paper. The excised leaf pieces were then cut into small segments (1 cm^2) and transferred to Petri plates containing potato dextrose agar (PDA: 20% diced potato, 2% glucose, 1.5% agar, and distilled water.). The plates were then incubated at 28°C for 2 d under a 12 h light, 12 h dark regime. Nine individual resulting colonies were selected and transferred to fresh PDA plates, and after incubation were preserved in 10% glycerol at -80°C for future use. Morphology of the colonies on PDA was observed from the upper and lower sides of the plates after 5 d incubation. Conidium morphology was examined using a Leica DM3000 microscope system, and the morphology and sizes of 50 conidia were assessed.

Pathogenicity testing

To confirm the pathogenicity of the isolated fungus, Koch's postulates were assessed for one of the isolates ("F2") obtained. Wounds were created on leaf surfaces of healthy sweet viburnum plants by pricking. Mycelium plugs from fungal colonies in PDA cultures were then placed onto the wounded sites. PDA without fungi was used for inoculation controls. The plants were then maintained in a growth chamber at 25°C and 85% relative humidity. Photographs of developing disease symptoms were taken at 10 to 20 d post-inoculation. Subsequently, the fungi were re-isolated from the inoculated leaf tissues and identified using the methods described above.

DNA extraction, amplification, and sequencing

For accurate identification, genomic DNA was extracted from the samples using the TIANamp Genomic DNA Kit (Cat. No 4992254, Tiangen Biotech (Beijing) Co. Ltd), following the manufacturer's instructions. Partial sequences of the ribosomal internal transcribed spacer (ITS), actin (*act*) and translation elongation factor 1-alpha (*tef*1) were amplified and sequenced, as described by Wikee *et al*., (2013) and Li *et al.* (2021a), and the nucleotide sequences were deposited in GenBank. Multiple alignments of concatenated ITS, *act* and *tef1*-α sequences were carried out using MEGA 7 with default settings. A concatenated phylogenetic tree was constructed using the Maximum Likelihood method (ML) with JTT model, as implemented in MEGA 7 (Jones *et al*., 1992). The fungus strains used in this study are listed in Table 1.

Fungicide tests

In vitro fungicide activity against *P. capitalensis* was assessed following the method of Li *et al.* (2021b). Seven fungicides were selected, including 75% chlorothalonil WP, 250 g L^{-1} azoxystrobin SC, 10% difenconazole WDG, 75% trifloxystrobin + tebuconazole WDG (25% trifloxystrobin, 50% tebuconazole), 35% fluopyram + tebuconazole SC (17.5% fluopyram, 17.5% tebuconazole), 500 g L^{-1} fluazinam SC, and 10% prothioconazole SC. The concentrations of each fungicide used in PDA plates for the tests are shown in Table 2. Disks were excised from the edges of colonies on PDA plates at 5 d post inoculation using a 6 mm diam. sterilized punch. The side of each disk containing fungal hyphae was positioned at the center of each PDA plate. The inoculated plates were incubated at 28°C in the dark. PDA plates without fungicide were used as the negative experimental controls. Diameters of resulting colonies were measured at 5 d post-inoculation. Each treatment was con-

Table 1. Fungus strains used in this study.

Species	GenBank No.		
	ITS	*tef*	*act*
Phyllosticta ampelicida CBS 111645	JN692542	EU683653	JN692518
Phyllosticta brazilianiae LGMF 333	JF343574	JF343595	JF343658
Phyllosticta capitalensis CPC 20251	KC291333	KC342553	KC342530
Phyllosticta capitalensis CPC 20256	KC291337	KC342557	KC342534
Phyllosticta capitalensis CPC 20257	KC291338	KC342558	KC342535
Phyllosticta capitalensis F2	PP785331	PP788704	PP788703
Phyllosticta capitalensis JFRL-03-40	ON076573	ON081651	ON081650
Phyllosticta capitalensis LC 0002	KC291350	KC342570	KC342547
Phyllosticta citriasiana CBS 123370	FJ538355	FJ538413	FJ538471
Phyllosticta citribraziliensis LGMF09	JF261436	JF261478	JF343618
Phyllosticta citricarpa CBS 102374	FJ538313	GU349053	FJ538429
Phyllosticta citrichinaensis ZJUCC 2010152	JN791664	JN791515	JN791589
Phyllosticta hypoglossi CBS 101.72	FJ538365	FJ538423	FJ538481
Phyllosticta kerriae MUCC 17	AB454266	KC342576	AB704209
Guignardia mangiferae IMI 260576	JF261459	JF261501	JF343641
Phyllosticta owaniana CBS 776.97	FJ538368	FJ538426	FJ538484
Phyllosticta paracapitalensis CPC 26517	KY855622	KY855951	KY855677
Phyllosticta paracitricarpa CPC 27169	KY855635	KY855964	KY855690
Phyllosticta podocarpi CBS 111646	AF312013	KC357671	KC357670
Phyllosticta spinarum CBS 292.90	JF343585	JF343606	JF343669
Phyllosticta ampelicida CBS 111645	JN692542	EU683653	JN692518

ducted at least three times. Toxicity regression equations and EC_{50} values were calculated using Data Processing System (DPS 7.05).

RESULTS

Disease symptoms and pathogen isolation

In October 2022, sweet viburnum plants cultivated in Ningbo Yuanshi Park showed extensive necroses on the leaf tips or edges. The necroses developed from light to dark yellow, eventually drying to scorched appearance. During the subsequent infection stage, minute black spots emerged, which later developed into conidiomata (Figures 1, a and b). As the disease lesions expanded across entire leaves the leaves abscised from the tree. Approx. 30% of the sweet viburnum plants in the Park exhibited these symptoms, which significantly impeded their growth. After 7 d of incubation, isolated fungal colonies each had a white margin surrounding a dark green centre (Figure 1c). Conidia in the cultures were hyaline, oval-shaped and tapered at the ends, each with a hyaline, unstable apical appendage, measuring 2.7 to 5.2 µm in length. Conidia were of mean dimensions 6.6 to 14.3 × 5.2 to 8.1 µm (n=50) (Figure 1 d). Based on the colony morphology, the fungus was determined to be a member of the *Phyllosticta* species (Damm *et al.*, 2009; Wikee *et al.*, 2013).

Pathogenicity test

To determine if the isolates could induce the symptoms observed in the field, Koch's postulates were assessed on 20 1-year-old sweet viburnum plants. At 14 d after inoculation, leaf spot symptoms appeared on the leaves inoculated with isolate F2, while the control plants showed no symptoms. By 21 d post-inoculation, 80% of the inoculated plants displayed typical leaf spots accompanied by conidiomata within areas of necroses (Figure 2, a and b). The inoculated fungus was subsequently re-isolated from the inoculated leaf tissues, confirming that isolate F2 represented the pathogen responsible for the leaf spot in sweet viburnum plants.

Phylogenetic analysis

For accurate identification of the pathogen, a multilocus phylogenetic analysis was conducted using concat-

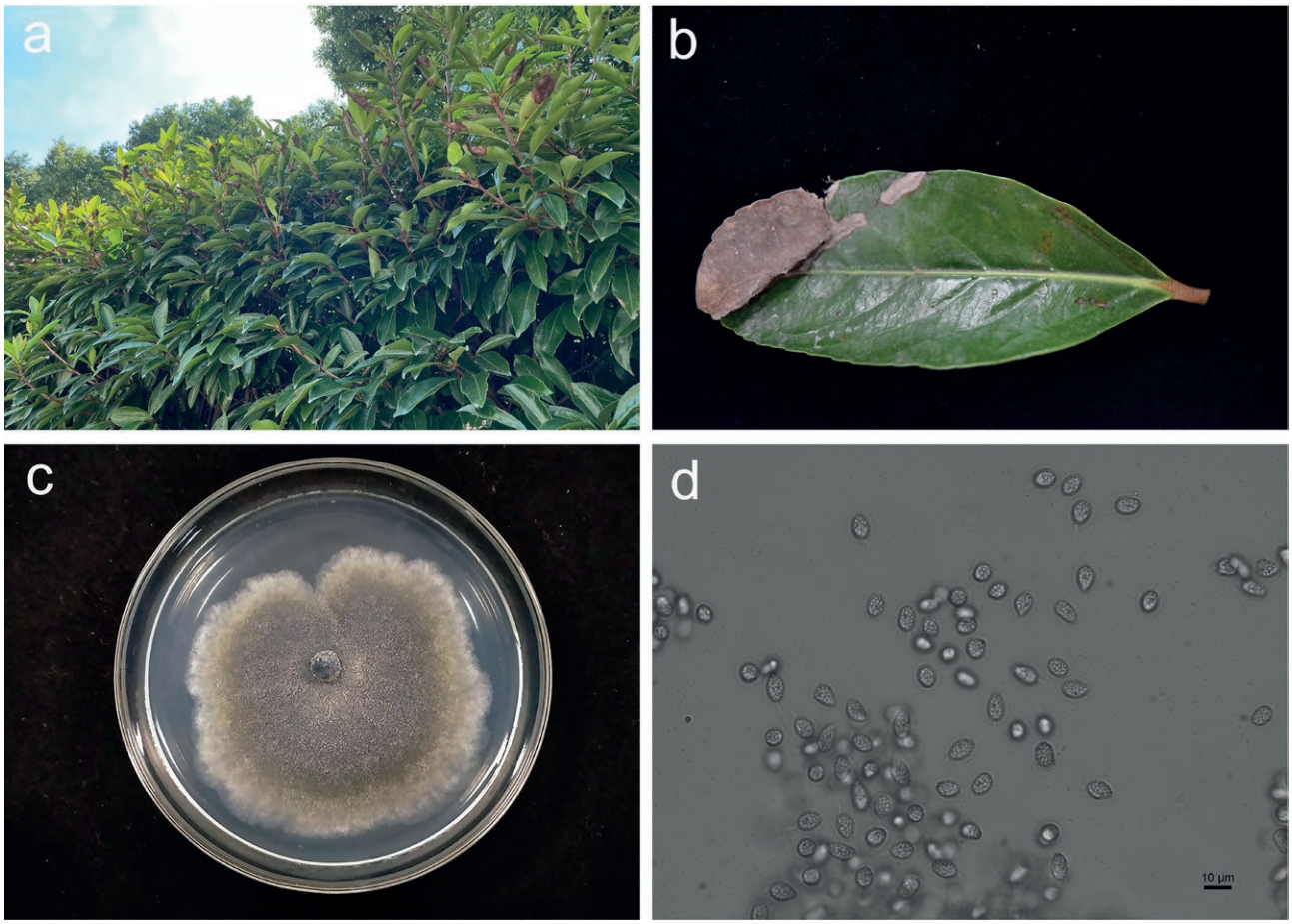

Figure 1. Disease symptoms and pathogen isolation. a and b, Disease symptoms on affected sweet viburnum leaves. c, Colony of *Phyllosticta capitalensis* grown on a PDA plate for 7 days at 28°C. d, Conidia of *P. capitalensis* observed under a light microscope at 200× magnification.

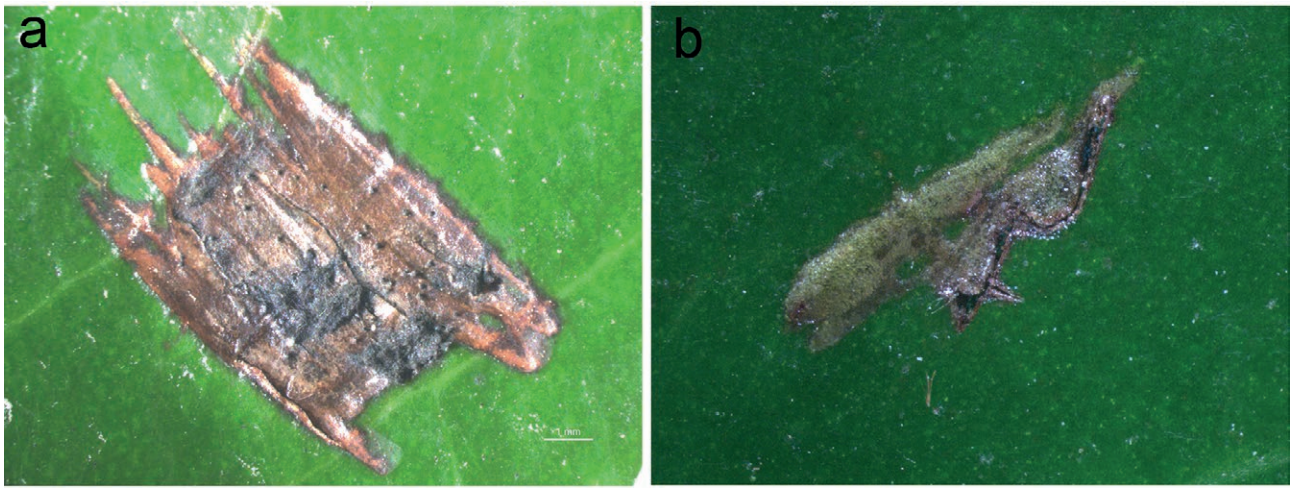

Figure 2. Pathogenicity test. Koch's postulates were conducted by inoculating healthy plants with fungal mycelium PDA plugs (2 a) or PDA plug inoculation controls (Figure 2 b). These photographs were taken at 21 d post inoculation.

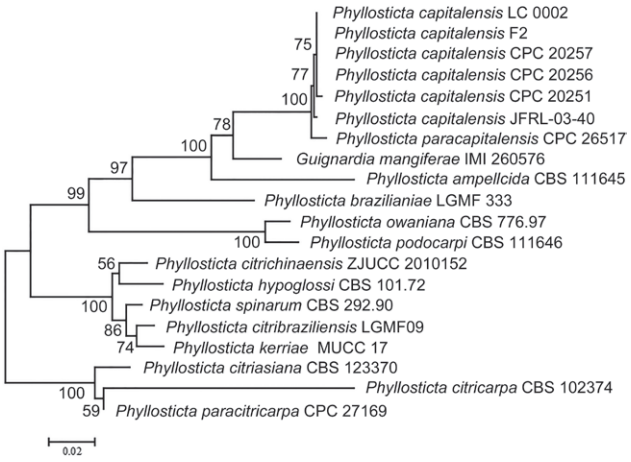

Figure 3. Phylogenetic tree for *Phyllosticta capitalensis* isolate F2 and closely related *Phyllosticta* species. Results were inferred from the concatenated sequences of ITS, *act* and *tef*1-α.

enated sequences of ITS, *act* and *tef*1-α genes from the 20 representative isolates, including isolate F2. The phylogenetic tree obtained (Figure 3) showed that isolate F2 is within the *P. capitalensis* clade, which is supported by moderate bootstrap support value of 77 %.

In vitro *fungicide tests*

The sensitivity of *P. capitalensis* to seven common fungicides was assessed. The results indicated that the EC_{50} values for the seven fungicides assessed against *P. capitalensis* were: 270.77 for 75% chlorothalonil WP, 0.02 for 250 g L^{-1} azoxystrobin SC, 0.27 for 10% difenconazole WDG, 0.02 for 75% trifloxystrobin + tebuconazole WDG, 9.03 for 35% fluopyram + tebuconazole SC, 5.90 for 500 g L^{-1} fluazinam SC, and 89.11 for 10% prothioconazole SC (Table 2). Among the tested fungicides, azoxystrobin and trifloxystrobin + tebuconazole exhibited the greatest antifungal activity against *P. capitalensis*. In contrast, chlorothalonil and prothioconazole exhibited limited efficacy against this pathogen.

DISCUSSION

Phyllosticta capitalensis has been recognized as an endophyte with a broad host range (Glienke-Blanco *et al*., 2002; Silva and Pereira 2007; Silva *et al*., 2008; Wikee *et al*., 2013; Bogas *et al*., 2022). Additionally, it has also been identified as an important pathogen responsible for leaf spot and black spot diseases on many plant species (Glienke *et al*., 2011; Wang *et al*., 2012; Zhang *et al*., 2022b; Jiang *et al*., 2023). While sweet viburnum leaf spot diseases caused by *Alternaria* (Qiu *et al*., 2021) and leaf blotch caused by *C. siamense* (Li *et al*., 2023) have been documented, the present report is the first of *P. capitalensis* as a pathogen affecting sweet viburnum.

Plant pathogen identification and pesticide screening for disease management assessments are important for developing effective disease control. For example, two microbial pathogens, *Streptomyces* sp. and *C. gloeosporioides*, cause leaf spot diseases on sweet cherry in Beijing, China (Ji *et al*., 2022). Upon pathogen identification, 10% difenconazole had the greatest inhibitory effect on mycelium growth of *P. capitalensis*, followed by 250 g L^{-1} of pyrazole ether ester, 50% imidacloprid, and 2% pyrimidine nucleoside antibiotics. These pesticides are the first choice for the chemical control the leaf spot of sweet cherry (Wu *et al*., 2015). *Phyllosticta capitalensis* isolates from leaf spots of *Polygonatum cyrtonema* were best targeted by triadimefon (Cui *et al*., 2023). Together with the present study, these fungicides plus 10% difenconazle WDG are likely to be good choices for control of leaf spot diseases caused by *P. capitalensis*.

Table 2. Growth inhibition activity of seven fungicides against *Phyllosticta capitalensis* F2

Fungicide	Concentrations assessed $(mg\ L)^{-1}$	Toxicity regression equation	EC_{50} $(mg\ L^{-1})$	Correlation coefficient *r*
75% chlorothalonil	1, 2, 4, 8, 16	$y = 4.4005 + 1.3858x$	270.77	0.9131
250g/L azoxystrobin	1, 2, 4, 8, 16	$y = 5.6516 + 0.1769x$	0.02	0.8963
10% difenconazole	0.125, 0.25, 0.5, 1, 2	$y = 6.0058 + 0.3913x$	0.27	0.8526
75% trifloxystrobin + tebuconazole	0.5, 1, 2 4, 8	$y = 6.8191 + 0.4820x$	0.02	0.8952
35% fluopyram + tebuconazole	0.5, 1, 2, 4 8	$y = 6.0194 + 0.9760x$	9.03	0.9158
500g/L fluazinam	0.125, 0.25, 0.5, 1, 2	$y = 6.8488 + 1.5041x$	5.90	0.9857
10% prothioconazole	0.125, 0.25, 0.5, 1, 2	$y = 5.1066 + 2.1279x$	89.11	0.9759

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