FIRST REPORT OF CANKER DISEASE IN APPLE CAUSED BY *LASIODIPLODIA THEOBROMAE* IN CHINA

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ABSTRACT

Apple (Malus Domestica) is an economically important tree that is widely planted in the southern part of Xinjiang, China. This study described a disease in apple characterized by typical canker symptoms, occurring between March and May 2021in Aksu, Kashgar, and Hotan in the Xinjiang province. Three representative isolates (WL 3-1, WL 3-2, and WL 3-3) from the diseased tissues were identified as Lasiodiplodia theobromae using morphological and molecular characterization. Phylogenetic analyses of the combined ribosomal internal transcribed spacer region, β-tubulin, and translation elongation factor 1-a placed the three isolates in a well-supported cluster with L. theobromae. Pathogenicity tests demonstrated that L. theobromae was virulent to apple. To our knowledge, this is the first detailed report of L. theobromae infecting Apple in China.

Keywords: *Apple, Canker disease, Lasiodiplodia theobromae, Fungal identification.*

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Introduction

In recent years, Xinjiang high-quality apples (*Malus Domestica*) have become more influential throughout China and even in the East Asia. This variety of apple has greatly promoted and stimulated the regional economic development in China, making the fruit an important industry to improve the financial aspect of farmers . In Xinjiang, apples are the most important fruit in Aksu and Kashgar, covering an area of 600,000 hectares (http://tjj.xinjiang.gov.cn/tjj/nyyp). However, outbreaks of several diseases from time to time have been the main obstacle to achieving higher yields[1]. Apple production faces a huge threat from fungal diseases[2], of which *Lasiodiplodia theobromae* of the Botrytis family is the most important wood pathogen. The *L. theobromae* is related to wilt, stem-end rot, and ulceration in apple, resulting in the decline of fruit production[3]. *L. theobromae* is a fungus of the genus Botryosphaeria, with a sex type known as *Botryosphaeria rhodian*. The pathogen is a prevalent plant in tropical and subtropical regions, infecting more than 500 hosts. In recent years, there have been emerging reports of canker diseases, such as grape stem canker[4], olive canker[5], Nanyang Jacaranda canker[6], cocoa blight, grapevine canker[7], and peach tree gum disease[8]. From March to May 2021, a disease with typical ulcer symptoms was observed on the trunks of apple trees in orchards located in Aksu, Kashgar, and Hotan in Xinjiang province. The disease incidence rate in the survey area was about 22%. Therefore, this study aimed to characterize the species of *L. theobroma*e that caused an apple canker in Xinjiang province to provide detailed information about the pathogen.

Materials and methods

Sample collection and fungal isolation

The ulcer samples were taken from apple tree orchards in Aksu, Kashgar, and Hotan in the

Xinjiang province of China. The samples were stored in separate kraft paper bags at room temperature. Isolation of diseased fungi using conventional tissue separation method[9] was performed. In brief, a sterile scalpel was used to cut out the disease-health junction tissue (5×5 mm) with a typical diseased apple bark. A 75% ethanol was used to disinfect the surface for 30 seconds, followed by the addition of 1% sodium hypochlorite solution for 3 minutes and sterile water rinses for 3 times. The samples were blot dried on a sterile filter paper and inoculated into a potato dextrose agar (PDA), and incubated in the dark at 26°C as an inverted culture dish. After 2-3 days, the marginal hyphae of the colony were picked and transferred to PDA medium for purification. The purified samples were used for phylogenetic, morphological, and pathological analysis. The three strains with the same morphology were named WL 3-1, WL 3-2, and WL 3-3, which were stored in PDA slant medium.

Morphological characterization

Three strains, WL 3-1, WL 3-2 and WL 3-3, were inoculated on the PDA medium and cultured at 25°C to observe the morphological characteristics of the colonies. The strain was cultured on PDA medium to observe the sporulation structure and conidia morphology using the Nikon microscope (Nikon E200MV). The size of 100 conidia was measured, and the average value was obtained.

Virulence characterization

The virulence of 3 representative strains from WL 3-1, WL 3-2, and WL 3-3 were tested. In the field test, the pathogenicity was determined using 2-year-old branches (line: Red Fuji) of apple trees. In brief, the surface was cleaned with sterile water before inoculation and disinfected with 0.6% sodium hypochlorite. Using the scald method, the hyphae of the strain cultured were inoculated on the PDA medium for 4 days into the pores. The inoculation site was moistened with moist sterile absorbent cotton, and was wrapped with plastic wrap. A sterile PDA was used as the control group. All pathogenicity tests were repeated five times.

Molecular characterization

For molecular biology identification, the total DNA was extracted using an Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). The internal transcribed spacer (ITS) region was amplified using primers ITS1 and ITS4[10]. Primers Bt2a and Bt2b were used to amplify the β-tubulin (tub2)[11], and primers EF1-728F and EF1-986R were used to amplify a region of the transcription elongation factor (tef-1α). The amplification protocol was performed in a 25 μl volume, comprising of 2 μl (20 ng) template DNA, 1 μl each primer (10 μl mol/L), 12.5 μl Taq Master Mix (5 U/μl), and 9.5μl ddH_2O (Table 1).

Gene	Definition	Primers	Primer DNA sequence (5'–3')	Annealing temperature (°C)
ITS	internal transcribed spacer region	ITS1	TCCGTAGGTGAACCTGCGG	58°C
		ITS4	TCCTCCGCTTTTGATATGC	
tef-1α	translation elongation factor 1-alpha	EF1-728F	CATCGAGAAGTTCGAGAAGG	58°C
		EF1-986R	TACTTGAAGGAACCCTTACC	
tub2	portion of the beta-tubulin gene.	Bt2a	GGTAACCAAATCGGTGCTGCTTTG	61°C
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	

Table 1: Genes used in this study with PCR primers, primer DNA sequence, optimal annealing temperature and corresponding references.

DNA sequencing was performed at the Sangon Biological. Sequences were assembled using Seaman v.7.1.0. and compared with known sequences in GenBank by performing a BLAST search. *Spencermartinsia viticola* were included in the analysis as outgroups (Table 2). In the phylogenetic analysis, the maximum likelihood method was used to construct a joint evolutionary tree in IQTREE-1.6.12[12].

Species	host	Location	Strain no.	GenBank no.		
				ITS	EF	BT
Lasiodiplodia Parva	Nephelium lappaceum	Puerto Rico	97	MK282730	MK294148	MK294119
	Theobroma cacao	Sri Lanka	CBS 356.59	EF622082	EF622062	EU673113
Lasiodiplodia theobromae	Albizia falcataria	China	BL1331	KU712499	KU712500	KU712501
	Albizia falcataria	China	HD1332	KU712502	KU712503	KU712504
Lasiodiplodia gilanensis	pistachio	California	3K59	KP955690	KP955789	KP955888
	pistachio	California	2B89	KP955689	KP955788	KP955887
Lasiodiplodia citricola	Walnut	California	7E79	KC357299	KC357311	KC357305
	pistachio	California	3K62	KP955688	KP955787	KP955886
Lasiodiplodia egyptiacae	Jatropha curcas	Brazil	CMM3648	KF234549	KF226705	KF254933
	Syzygium cordatum	South Africa	CMM3611	KF234545	KF226691	KF254928
Lasiodiplodia crassispora	Eucalyptus urophylla	Venezuela	CMW13488	DQ103552	DQ103559	KU887507
	grapevine	California	UCD24Co	GU799456	GU799487	GU799479
Lasiodiplodia jatrophicola	Jatropha curcas	Brazil	CMM3610	KF234544	KF226690	KF254927
	Caryota mitis	China	DSYWK03	KJ885546	KM066118	KJ885547
Lasiodiplodia subglobosa	Jatropha curcas	Brazil	CMM4046	KF234560	KF226723	KF254944
	Citrus latifolia	Mexico	CMM3872	KF234558	KF226721	KF254942
Lasiodiplodia hormozganensis	Mangifera indica	Iran	IRAN1498C	GU945356	GU945344	KP872414
	Olea sp.	Iran	IRAN1500C	GU945355	GU945343	KP872413
Lasiodiplodia iraniensis	Juglans sp.	Iran	IRAN1502C	GU945347	GU945335	KP872416
	Salvadora persica	Iran	IRAN1520C	GU945348	GU945336	KP872415
Lasiodiplodia theobromae	Malus sp.	Xinjiang,China	WL3-1[a]	MZ379517	MZ382824	MZ382827
	Malus sp.	Xinjiang,China	WL3-2[a]	MZ379518	MZ382825	MZ382828
	Malus sp.	Xinjiang,China	WL3-3[a]	MZ379519	MZ382826	MZ382829
Spencermartinsia viticola	grapevine	French	GAR09	KT595694	KX09828	KT595695
	citrus	California	UCP105	JF271748	JF271784	JF271766

Table 2: Description of DNA sequences used in the phylogenetic analyses.

Results

The typical symptom of the apple canker was the formation of ulcerated trunk. In the early stage of infection, the bark tissue of the branches of the apple appeared sunken, resulting in gray-brown lesions.

The edges of ulcers were irregular in shape, while the size of ulcers varied. Within the progression of the disease, the sunken canker spread up and down along the trunk. The affected trees showed yellowing of leaves and premature deciduousness, and then the branches withered from the top, the xylem changed color, and finally the tree died (Figure 1 A, B).

Figure 1: Symptoms of bark canker caused by *L. theobromae* in an apple tree.
A, Symptoms on a naturally infected leaves in the field. B, Symptoms on a naturally infected stem in the field.
C, Necrotic lesion caused by inoculated L. theobromae strain WL 3-1 after 10 days on 2-year-old stems of apple tree branches

The colony on the PDA was initially white and fluffy, and the aerial hyphae were vigorous. After 5 days, the color of the hyphae gradually darkened to gray-black, with a loose and uniform texture and black fruiting bodies (Figure 2 A). The conidia are monospores, elliptical to ovoid, and the size is about (16.7 ~ 22.1)× (10.1 ~ 14.3) μm (n=20), aspect ratio at 1.65. From initially colorless and transparent (Figure 2 B), the color deepens to dark brown, with a septum in the center (Figure 2-C).

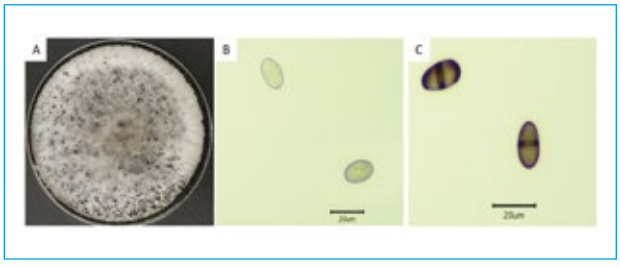

Figure 2: Morphology of *L. theobromae*. A, After 5 days of cultivation on PDA medium. B, Immature conidia. C, Mature conidia. Bar = 20μm.

These morphological features are consistent with the previous description of *L. theobromae* (Ariyawansa et al., 2015).

Using primers to amplify the ITS, tub2, and tef-1α sequences, More than 99.5% homology with *L. theobromae* was shown by the BLAST analysis, and the resulting sequences were deposited in the GenBank. The amplified ITS sequence (Accession No.MZ379517, MZ379517, and MZ379517), tub2 sequence (Accession No. MZ382827, MZ382828, and MZ382829) and tef-1α sequence (Accession No. MZ382824, MZ382825, and MZ382826). Phylogenetic analyses of combined ITS, tub2, and tef-1α sequences showed that the isolates are clustered with L. theobromae, which has a bootstrap support rate of more than 87% (Figure 3). Combining the sequencing results and morphological characteristics of the gene fragments, the three isolates were identified as *L. theobromae*.

In the pathogenicity experiments in the field, all branches inoculated with *L. theobromae* became ill and turned into dark brown or black with depressed ulcer lesions formed. The lesions extended longitudinally from the inoculation point, while the control remained healthy. When the discolored bark was scraped off, dark brown necrotic tissue was observed in the xylem of the branches (Figure 1C). L. theobromae was reisolated from each inoculated shoot, but not from the control injured tissue. In this way, Koch's hypothesis is fulfilled.

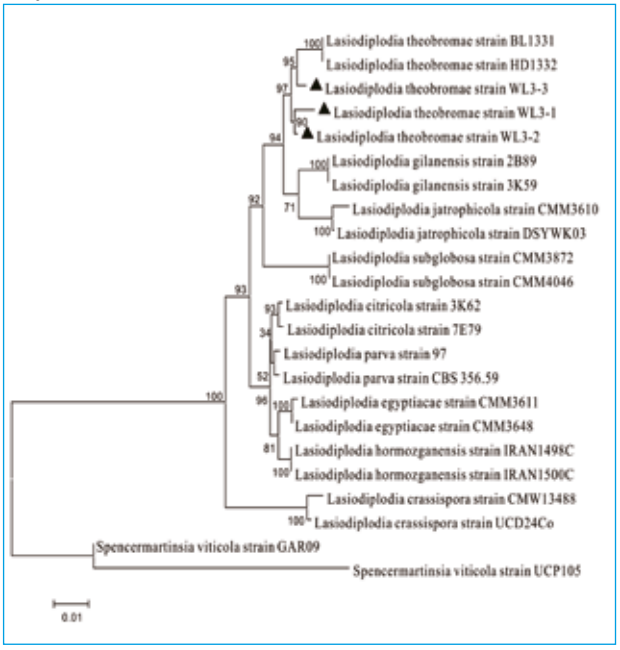

Figure 3: Phylogenetic tree based on maximum likelihood analysis of combined ITS, tub2, and tef-1α sequences data. Isolates marked with triangle were sequenced in this study.

Discussion

During the investigation, it was found that most apple growers regarded canker disease as Blackspot, and the wrong identification of canker disease resulted in deviations in disease prevention and control measures. This scenario has severely reduced the yield and quality of apples.

In this study, *L. theobromae* is a fungus isolated from the edge of apple tree bark with obvious active ulcer symptoms. It can cause the vertical expansion and ulceration of the branches and trunks of apple trees.This study is the first detailed study of the phylogeny, morphology and pathogenicity of L. theobromae on apple trees in China. The unique colony characteristics and morphological examination of this species support the identification of this species. Phylogenetic analysis of ITS, tub2, and tef-1α Sequence data also confirmed this.The results of the phylogenetic analysis also showed that the cocoa beans isolated from the apple tree are genetically very similar to the cocoa beans isolated from the Chinese Fusarium genus (Figure 3).

L. theobromae has been reported from a large number of woody plants around the world that it is related to the symptoms of Carya cathayensis stem canker in China[13], leading to canker and wood rot in Western Cape, South Africa[14], and rot lesions on mango fruits(15,16) and *Psidium guajava* L.[17]. In 2017, Ji[8] reported for the first time that *L. theobromae* can cause severe ulcer disease in Falcataria Moluccana in Guangdong Province, China. The colony phenotype and conidia morphology are consistent with those reported by Ji, but the conidia size (24.8±1.9×13.4±1.1 mm) is different. The differences may be a result of various factors, such as host, geographic origin, and climate or culture conditions.

The study found that the incidence of apple tree canker caused by *L. theobromae* has increased, indicating that the pathogen poses a new threat to the industry. Further research should investigate the occurrence and prevalence of the disease and formulate effective control measures to reduce the pathogen's impact on apple trees in Xinjiang, China.

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