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Identification of Pathogenic Fungi Causing Leaf Spot of *Urtica cannabina* and *Malus sieversii* in the Wild Fruit Forest of Tianshan Mountain, Xinjiang, China

(Pengenalpastian Kulat Patogen Menyebabkan Bintik Daun *Urtica cannabina* dan *Malus sieversii* di Hutan Buah Liar Gunung Tianshan, Xinjiang, China)

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ABSTRACT

Degradation of the wild apple trees in the wild fruit forest of Tianshan mountain of Xinjiang Province, China, has attracted great attention in recent years, and pathogens are believed to be an important responsible factor. We observed that *Malus sieversii* and its understory plant, *Urtica cannabina*, exhibited similar symptoms of leaf spot disease, and we suspect that they are caused by the same pathogens. DNA sequencing using ITS1 and ITS4 primers was applied to identify the pathogenic fungi from diseased leaves of *U. cannabina* and *M. sieversii*, which led to the identification of *Alternaria* sp. and *Fusarium* sp. as active pathogens causing same symptoms on leaves of both species. Our results implied that these two plants shared the same pathogenic fungi that cause leaf spot disease, and infection of the understory species *U. cannabina* might provide a reservoir of the pathogens which can attack *M. sieversii* and contribute at least in part, to the degradation of *M. sieversii.*

Keywords: Leaf spot; *Malus sieversii*; pathogenic fungi; rDNA-ITS; *Urtica cannabina*

ABSTRAK

Kemerosotan pokok epal liar di hutan buah liar gunung Tianshan di wilayah Xinjiang, China, telah menarik perhatian sejak beberapa tahun kebelakangan ini dan patogen dipercayai menjadi faktor utama yang terlibat. Kami memerhatikan bahawa *Malus sieversii* dan tumbuhan bawahnya, *Urtica cannabina*, menunjukkan simptom penyakit tompok daun yang sama dan kami mengesyaki bahawa ia disebabkan oleh patogen yang sama. Penjujukan DNA menggunakan pencetus ITS1 dan ITS4 digunakan untuk mengenal pasti kulat patogen daripada daun *U. cannabina* dan *M. sieversii* yang berpenyakit. Ini membawa kepada pengenalpastian *Alternaria* sp. dan *Fusarium* sp. sebagai patogen aktif yang menyebabkan gejala yang sama pada daun kedua-dua spesies. Hasil menunjukkan bahawa kedua-dua tumbuhan ini berkongsi kulat patogen yang sama yang menyebabkan penyakit bintik daun, dan jangkitan spesies *U. cannabina* mungkin menyediakan takungan patogen yang boleh menyerang *M. sieversii* dan sekurang-kurangnya menyumbang sebahagian kepada kemerosotan *M. sieversii*.

Kata kunci: Kulat patogen; *Malus sieversii*; rDNA-ITS; tompok daun; *Urtica cannabina*

INTRODUCTION

Tianshan wild fruit forest mainly distributes in the Yili Valley, Tacheng Basin and its low hilly areas in Xinjiang province of China, with an area of over 20,000 hm^2 (Cui et al. 2006). Tianshan wild fruit forest not only has the ecological functions of regulating climate, conserving water source, keeping wind-break and sand-fixation but also is an important apple germplasm resource bank in the world (Ling et al. 1998; Qi et al. 2021). It has an irreplaceable position in the study of the evolution of

plant species and the origin of cultivated fruit trees. However, Tianshan wild fruit forest has undergone serious degradation, and the actual distribution area of its main constructive species, *M. sieversii*, has reduced to only 30% of 50 years ago (Ding 2007; Zhang et al. 2021). Interestingly, our field survey showed that *U. cannabina* usually thrives under seriously diseased or dead *M. sieversii* trees, indicating a possible closely related interspecies interaction between these two plants.

Degradation of Tianshan wild fruit forest is speculated to be attributed to various factors including the damaging invasion of *Agrilus mali* Matsumura, apple rot disease, climate change, and overgrazing, which was the result of the combined action of the natural environment and human factors (Cui et al. 2018). Besides, our field survey indicated that leaf spot disease on *M. sieversii* is also very serious, infecting over 50% of the leaves of seriously degraded trees. Apple leaf spot was an early deciduous disease that can be found in apple-producing areas in the United States, India, Japan, Iran, and China, especially in the two major apple producing areas of the Bohai Bay and the Yellow River of China (Shao et al. 2014; Velho et al. 2014). Many studies have pointed out that most of the pathogens causing plant leaf spot diseases were Deuteromycotina and Ascomycotina species, including *Alternaria*, *Cercospora*, *Phyllosticta*, *Pestalotiopsis*, *Fusarium*, *Pseudocercospora*, *Cylindrocladium*, and *Myrothecium* (Carlier et al. 2000; Hayden et al. 2010; Kodsueb & Lumyong 2019; Shi et al. 2021). Lu et al. (1984) reported that apple leaf spot disease caused by *Alternaria mali* occurred for the first time in Shandong province of China. Liu et al. (2019) reported that the main pathogens of *M. sieversii* leaf spot in Tianshan wild fruit forest were *Alternaria* sp. This pathogenic fungus has strong pathogenicity, and usually has 10 to 20 spots on a diseased leaf, which is easy to cause leaf perforation or breakage, growth stagnation, and even coke shedding. When the disease was epidemic, the infection rate might reach 70%, and eventually leads to the decrease of apple yield or even causes the death of apple trees (Guo et al. 1995; Yang et al. 2020).

Recent studies have shown that forest pathogenic fungi can be widely spread through the soil, susceptible plant seedlings, insects and air, causing pathogen in other plants (Elad & Pertot 2014; Song et al. 2021). Study on the causes of the degradation of Tianshan wild fruit forest is still insufficient, and the importance of leaf spot disease on *M. sieversii* is underestimated. We speculated that the outbreak of *M. sieversii* leaf spot disease might be aggravated by infection of the same pathogens on *U. cannabina*, which might contribute to the degradation of the wild fruit forest in Tianshan Mountains. The objectives of our current study focus on: i) identification of fungal pathogens associated with *U. cannabina* and *M. sieversii* leaf spot disease; ii) evaluation of pathogenicity of identified pathogens on both *M. sieversii* and *U. cannabina*. Our findings will provide evidence to better understand the mechanism underlying the degradation of Tianshan wild fruit forest in China.

Materials and Methods

Collection of materials

Leaves of *U. cannabina* and *M. sieversii* infected with leaf spot were collected in Tianshan wild fruit forest in Xinyuan County, Xinjiang province, China (N43°34.149′, E 82°52.463′) in July 2016. Healthy *U. cannabina* and *M. sieversii* seeds were collected from the same and brought back to the lab for culture, and plant leaves were grown for the pathogenicity testing of identified fungi.

Isolation, purification and molecular identification of pathogenic fungi

Diseased leaves of *U. cannabina* and *M. sieversii* with typical leaf spot symptoms were selected for isolation of pathogenic fungi using conventional tissue isolation method. First, the leaves were rinsed with running tap water and then surface was disinfected with 75% alcohol for 1 min. The leaves were soaked in 1% sodium hypochlorite for 3 min before they were washed with sterile water for 3 times. After the moisture on the surface was absorbed with sterilized filter paper, the surface-sterilized diseased leaf fragments were transferred to PDA plates. Fungi strains were obtained by culturing in a constant temperature incubator at 25 °C for 5 days (Shi et al. 2020; Song et al. 2019).

For molecular identification, DNAs of isolated strains were extracted using the Rapid Fungal Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturers' instructions. Polymerase chain reactions (PCRs) using the fungi universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), which were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd (Shanghai, China). The PCR reaction mixture was 50 μL containing 5 μL (10 ×) PCR buffer, 0.5 μL (5 U/μL) Taq enzyme, 4 μL (10 mM) dNTPs, 2 μL DNA template, 3 μL (20 μM) primers and 35.5 μL ddH_2O. PCR was performed in the thermal cycler (Eppendorf, Germany) with the following program: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 60 s, 55 °C for 30 s, 72 °C for 1.5 min and the last extension at 72 °C for 7 min.

Negative control with no DNA added was included in each PCR run. The amplified products were separated by 1% agarose gels electrophoresis and sent to Shanghai Sangon Biological Engineering Technology & Service Co., Ltd (China) for sequencing. The complete internal transcribed spacer (ITS) sequences of the fungal strains (including 5.8S rDNA) were jointed and proofread with Seq-man II software, and then registered in the GenBank via Sequin 13.05 software (Al-Hammadi et al. 2018).

CONSTRUCTION OF PHYLOGENETIC TREE AND CLASSIFICATION OF STRAINS

Multiple alignments were performed using Clustal X 1.83 software. Based on Kimura2-parameter, model of the neighbor-joining method a phylogenetic tree was obtained using MEGA 4.1 software and the bootstrap confidence limits were derived by 1,000 replicates. The obtained strains were classified according to morphological observation (Simon & Clapp 2008) and ITS sequence analyses (Tang et al. 2020).

DETERMINATION OF THE PATHOGENICITY OF PATHOGENIC FUNGI

Healthy *U. cannabina* and *M. sieversii* leaves were first disinfected with 75% alcohol before inoculation of isolated strains. Isolated pathogenic strains were first cultured on PDA plates for 5 days, and then a fungal pie (φ5 mm) was obtained from the edge of the colony and inoculated onto a healthy leaf. Each treatment was repeated six times, with sterile PDA treatment as controls. Leaves were incubated in petri dishes and maintained at 25 °C in a growth chamber programmed for 12 h of fluorescent white light/day. Furthermore, the isolated fungi were verified following Koch's postulates, to confirm whether these fungi were original pathogens (Fredericks & Relman 1996).

RESULTS

HOMOLOGY AND CLUSTER ANALYSIS OF PATHOGENIC FUNGI

In total, 24 strains were isolated as potential pathogenic fungi, whose gene sequences were subsequently submitted to NCBI for registration. The accession numbers were MG214845-MG214868, with the rDNA-ITS sequences of these strains ranging from 479 to 538 bp in length. Strains Q9 (MG214859), CQ13 (MG214863), Q12 (MG214865), Q2 (MG214867), and BQ4 (MG214868) were 99% similar with reported gene sequences in GenBank, whereas other strains were 100% (Table 1).

TABLE 1. Homology analysis of the pathogenic fungi isolated from *U. cannabina* and reference strains

Strain	Accession no.	Reference Strain	Identity
A2-2	MG214845	*Fusarium equisetai* (KP721605.1)	100%
AQ18	MG214846	*Alternaria alternata* (KU324792.1)	100%
AQ16	MG214847	*Alternaria tenuissima* (KR912298.1)	100%
AQ17	MG214848	*Alternaria tenuissima* (KR912298.1)	100%
A1-1(1)	MG214849	*Alternaria alternata* (KU324782.1)	100%
Q1	MG214850	*Alternaria alternata* (KX179478.1)	100%
Q11	MG214851	*Alternaria alternata* (KX179478.1)	100%
Q14	MG214852	*Alternaria alternata* (KX179478.1)	100%
Q3	MG214853	*Alternaria tenuissima* (LT604490.1)	100%
BQ8	MG214854	*Alternaria tenuissima* (LT604490.1)	100%
BQ9	MG214855	*Alternaria tenuissima* (KR912298.1)	100%
BQ2	MG214856	*Alternaria tenuissima* (KX440627.1)	100%
Q5	MG214857	*Alternaria alternata* (KF380815.1)	100%
BQ19	MG214858	*Alternaria tenuissima* (FJ949086.1)	100%
Q9	MG214859	*Alternaria alternata* (KJ173524.1)	99%
AQ1	MG214860	*Alternaria tenuissima* (HQ647307.1)	100%
A2Q6	MG214861	*Alternaria tenuissima* (KJ008701.1)	100%
A1-1(2)	MG214862	*Alternaria tenuissima* (JX867219.1)	100%
CQ13	MG214863	*Alternaria alternata* (KF380821.1)	99%
Q7	MG214864	*Alternaria tenuissima* (JX860514.1)	100%
Q12	MG214865	*Alternaria alternata* (KF380815.1)	99%
CQ10	MG214866	*Alternaria alternata* (JF835813.1)	100%
Q2	MG214867	*Alternaria alternata* (KJ173524.1)	99%
BQ4	MG214868	*Alternaria alternata* (KP979753.1)	99%

The ITS sequences of 24 isolates were aligned and analyzed by BLAST in the GenBank database. The phylogenetic tree was constructed by neighbor-joining algorithms and the bootstrap value of fungi sequences clustering was 99%. According to the analysis of morphological observation and ITS sequence, there were three pathogenic fungi of *U. cannabina*, which corresponded to *Alternaria alternata*, *Alternaria tenuissima,* and *Fusarium equisetai* (Figure 1). Based on the results of homology alignment and cluster analysis, 24 isolates of *U. cannabina* can be classified into two genera, which belonged to the genus *Alternaria* and *Fusarium* of Deuteromycotina.

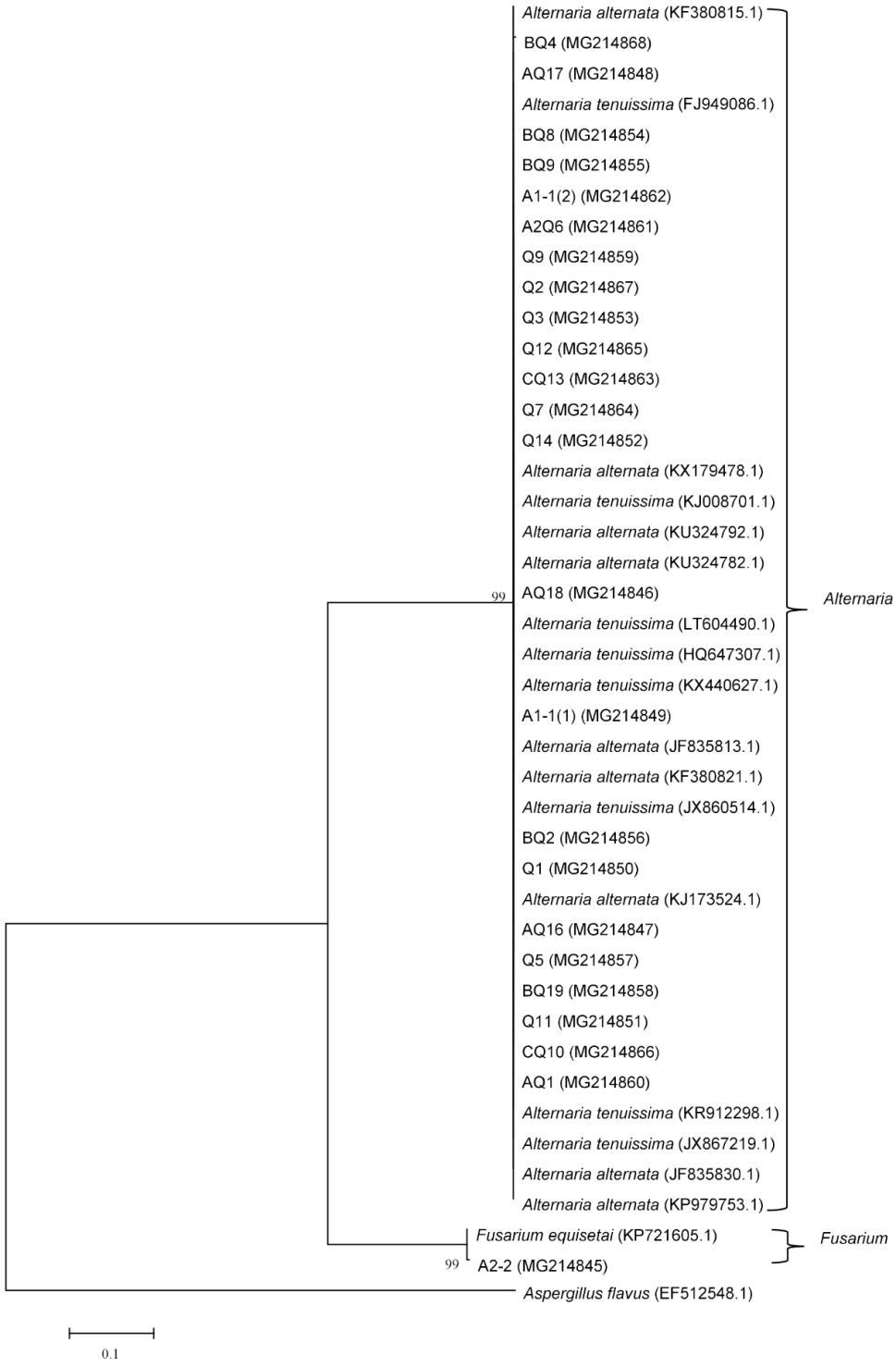

FIGURE 1. Neighbor-joining tree of the pathogenic fungi isolated from the leaves of *U. cannabina* and related strains based on aligned rDNA-ITS sequences

PATHOGENICITY DETECTION OF PATHOGENS

According to Koch's postulates, the pathogenicity of the purified strains was tested for re-inoculation. We selected typical and representative infected strains, i.e., strains AQ18, AQ1 and A2-2, representing *A. alternata*, *A. tenuissima,* and *F. equisetai*, respectively (Figure 2). The results indicated that all isolated pathogenic fungi had different degrees of infection with *U. cannabina* and *M. sieversii* leaves, and the percentage rate of leaf infection was shown in Table 2. The biggest lesions occurred after 5 days, brown to black lesions were observed in all inoculated leaves, whereas no symptoms developed on control leaves. Diseased tissue samples from lesion margins of inoculated leaf were transferred to fresh PDA plates, and the isolated fungi were re-identified by molecular analysis. The experiment was repeated three times.

TABLE 2. Pathogenicity level of all fungal pathogens to *U. cannabina* and *M. sieversii* leaves

Pathogen	Strain	Infection rate	
		U. cannabina	*M. sieversii*
Fusarium equisetai	A2-2	100%	100%
Alternaria tenuissima	AQ16	98%	100%
Alternaria tenuissima	AQ17	99%	99%
Alternaria tenuissima	Q3	100%	100%
Alternaria tenuissima	BQ8	98%	98%
Alternaria tenuissima	BQ9	97%	96%
Alternaria tenuissima	BQ2	100%	95%
Alternaria tenuissima	BQ19	99%	100%
Alternaria tenuissima	AQ1	100%	100%
Alternaria tenuissima	A2Q6	100%	100%
Alternaria tenuissima	A1-1(2)	99%	98%
Alternaria tenuissima	Q7	99%	98%
Alternaria alternata	AQ18	100%	100%
Alternaria alternata	A1-1(1)	98%	99%
Alternaria alternata	Q1	100%	99%
Alternaria alternata	Q11	99%	100%
Alternaria alternata	Q14	100%	99%
Alternaria alternata	Q5	98%	99%
Alternaria alternata	Q9	98%	100%
Alternaria alternata	CQ13	100%	98%
Alternaria alternata	Q12	99%	97%
Alternaria alternata	CQ10	98%	98%
Alternaria alternata	Q2	100%	99%
Alternaria alternata	BQ4	100%	100%
Control	—	0%	0%

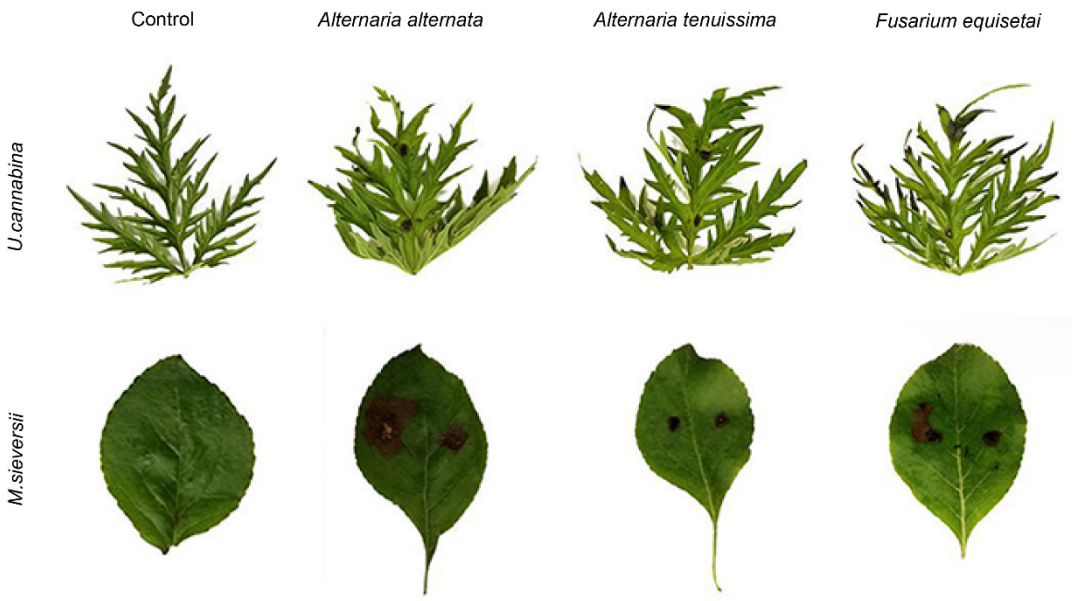

FIGURE 2. Symptoms of leaf spot on leaves of *U. cannabina* and *M. sieversii*

DISCUSSION

In this study, the pathogens of *U. cannabina* and *M. sieversii* causing leaf spot disease in Xinjiang Tianshan wild fruit forest were identified by rDNA-ITS, and we found the same pathogens on the diseased leaves of these two different plants. To the best of our knowledge, this is the first report of *A. alternata*, *A. tenuissima,* and *F. equisetai* causing leaf spot symptoms on *U. cannabina* in China. The results of the phylogenetic analysis showed that all 24 pathogenic fungi belonged to the genus *Alternaria* and *Fusarium* in deuteromycotina, with *Alternaria* sp. being more abundant than *Fusarium* sp. Our findings support the hypothesis that the degradation of *M. sieversii* growing in the Tianshan wild fruit forest might correlate to the fact that the dominant understory species *U. cannabina* may harbor pathogenic fungi that cause leaf spot disease on both plants to aggravate the symptoms on *M. sieversii*.

Menzel et al. (2016) pointed out that more than 80% of the companion plants of *Suaeda rigida* were identically infected by vesicular-arbuscular (VA) mycorrhizal fungi, which indicated that there was a high dependence between the companion species and the constructive species. In certain cases, one species can significantly affect the survival of other species (Johnson et al. 2010; Liu et al. 2020). *U. cannabina* is a dominant understory plant that is very beneficial to the reproduction and diffusion of pathogenic fungi (Lively et al. 1995). If the environmental conditions are suitable, the pathogens will propagate in large quantity, which leads to 'pathogen outbreak' (Dwyer et al. 2004; Le & Gregson 2019). After *U. cannabina* was infected with pathogens, spores may be transmitted to *M. sieversii* through air, soil or insects (Allison et al. 1986; Poletto et al. 2021). In this case, *U. cannabina* serves as the 'source' and 'bank' of incubating pathogenic fungi, which posed a great potential threat to the health of *M. sieversii* (Beckstead et al. 2010; Mansur et al. 2020; Power & Mitchell 2004).

Leaf spot caused by *Alternaria* species is common in all major apple producing areas of the world. Woudenberg et al. (2014) reported that the main pathogens causing leaf spot disease in Brazilian apple production areas were *A. iranica* and *A. alternata*. Rotondo et al. (2012) reported that the main pathogens causing apple leaf spot in Italy were *A. mali* and *A. alternata*. In addition, *A. mali*, *A. alternata,* and *A. tenuissima* are the major causes of apple leaf spot in Australia (Harteveld et al. 2014, 2013). In Japan and the United States, *A. alternata* and *A. tenuissima* are responsible for apple leaf spot disease (Filajdic & Sutton 1991; Sawamura 1962). The different pathogens of apple leaf spot in different producing areas may be caused by different climate and soil conditions.

CONCLUSION

In conclusion, our study confirms that *A. alternata*, *A. tenuissima,* and *F. equisetai* are the primary causal

species of leaf spot on *U. cannabina* and *M. sieversii*, and these pathogens need to be considered in restoration and conservation plans of Tianshan wild fruit forests in China. Therefore, it is necessary to further study the host transfer mechanism of pathogens between *U. cannabina* and *M. sieversii*, as well as and the prevention and control of these pathogens, which can provide theoretical basis for ameliorating the decline of Tianshan wild fruit forest in China.

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