

Article

Identification and Pathogenicity of Fungal Pathogens Associated with Leaf Disease of *Fallopia multiflora* in China

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Abstract

Fallopia multiflora (Thunb.) Harald is a valuable medicinal plant with substantial economic and therapeutic value, widely cultivated in southern China. In 2025, a leaf disease outbreak occurred in an *F. multiflora* plantation in Tongnan District, Chongqing, China. Diseased samples were collected for pathogen isolation, and seven representative strains were selected from 50 pure isolates via preliminary pathogenicity tests. Species identification was performed using a combination of morphological characterization and multi-locus phylogenetic analysis, targeting the Internal Transcribed Spacer (ITS), Translation Elongation Factor 1-alpha (*TEF1*), RNA Polymerase II Second Largest Subunit (*RPB2*), Beta-tubulin (β -*TUB2*), Heat Shock Protein 60 (*HSP60*), and Glyceraldehyde-3-Phosphate Dehydrogenase (*G3PDH*) gene regions. The isolates were identified as *Alternaria alternata*, *Botrytis cinerea*, *Botrytis polygoni*, *Epicoccum mackenziei*, and *Lasiodiplodia citricola*. Pathogenicity assays on living *F. multiflora* leaves confirmed that all identified species could induce disease symptoms, with distinct interspecific differences. This study verifies that multiple pathogenic fungi can infect *F. multiflora*, with potential co-infection. It improves our understanding of the pathogenic fungal community associated with this medicinal plant and lays a foundation for subsequent disease management in its cultivation.

Keywords: horticultural plants; pathogenic fungi; morphology; multi-locus phylogenetic analysis



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1. Introduction

Fallopia multiflora (Thunb.) Moldenke. (Syn. *Polygonum multiflorum* Thunb.), commonly known as “He Shou Wu” or “Fo-Ti”, is a perennial twining vine belonging to the family Polygonaceae. The plant is characterized by its opposite leaves with ochreae at the nodes, small greenish flowers in axillary racemes, and laterally compressed achenes [1,2]. It thrives across a broad altitudinal range (200–2200 m), often climbing among shrubs or

sprawling over rocks in mixed broadleaf forests, native to central and southern China. As a significant herbal medicine in Chinese tradition, it has been utilized for nearly a thousand years. Recent studies have demonstrated its tuberous root antioxidative properties [3,4], containing abundant anthraquinones possessing anti-cancer effects [5]. The leaves, rich in lecithin, derivatives, and flavonoids such as myricitrin, quercitrin, and afzelin, can inhibit cholesterol absorption, promote metabolism, lower blood lipid levels, prevent atherosclerosis, and offer significant antioxidant activity, making them ideal for use in health-promoting tea [6].

Fallopia multiflora holds long-standing cultural and medicinal significance, and its commercial value has grown steadily as a natural source of bioactive stilbenes and other secondary metabolites; recently developed genomic resources have further advanced molecular research to support disease diagnostics and future resistance-breeding initiatives [7,8]. Despite its considerable economic importance, published records documenting foliar and root diseases of *F. multiflora* remain sparse, with only a handful of case reports available to date. In 2012, *Alternaria astragali* was identified as the causal agent of leaf spot on *F. multiflora* in Kunming, China [9]. In 2014, *Fusarium oxysporum*, the pathogen responsible for Fusarium wilt, was isolated from greenhouse-cultivated *F. multiflora* in Yeongju-si, Korea [10]. These findings underscore the critical need for systematic surveys and comprehensive identification of causal pathogens in commercial *F. multiflora* plantations [11].

In the spring of 2025, severe leaf diseases with various symptoms were frequently encountered in a *Fallopia multiflora* plantation, located in Tongnan District, Chongqing, China. To discover their causal pathogens, samples were collected twice from the same plantation in February and April. A preliminary pathogenicity assay was performed on the fungi isolated from diseased leaves of *F. multiflora*, and six fungal species were ultimately confirmed as pathogenic fungi. Their phenotypic and molecular characteristics were further characterized through colony morphology observation and multi-gene phylogenetic analysis.

2. Materials and Methods

2.1. Sample Collection and Fungal Isolation

Diseased leaves of *F. multiflora* were randomly collected from a *F. multiflora* plantation in Tongnan District (30°12'51.26" N, 105°44'01.74" E, 249 m above sea level), Chongqing, China, in February and April of 2025. Leaf samples with different symptoms were collected from the plantation, totaling 46 samples (Figure 1). The collected samples were placed in sterile plastic bags and transported to the laboratory for fungal isolation. A typical lesion representing a kind of symptom was cut into a square (diameter: 1 cm) containing both diseased and health tissue; divided into several pieces (≥ 4) along with the central axis of the square's center point; surface disinfected with 75% ethanol solution for 1 min, 2% sodium hypochlorite for 30 s, and rinsed with distilled water for 3 times; and then put onto Petri-dish with potato dextrose agar (PDA; Difco, Montreal, QC, Canada). Fungal hyphae from the edge of the upcoming colonies were collected and transferred to PDA Petri dishes. Meanwhile, the previously mentioned tissues containing both diseased and healthy parts were put onto two moist filter papers placed in a 90 mm Petri dish and incubated at 25 °C to promote sporulation. Individual conidia were carefully picked using sterile glass needles under a stereomicroscope and inoculated on PDA media. Purified single-spore cultures ($n = 50$) were preserved in test-tube slants at 4 °C and glycerol stocks at −80 °C, and deposited in the Fungi Herbarium of Yangtze University (YzU), Jingzhou, Hubei Province, China.

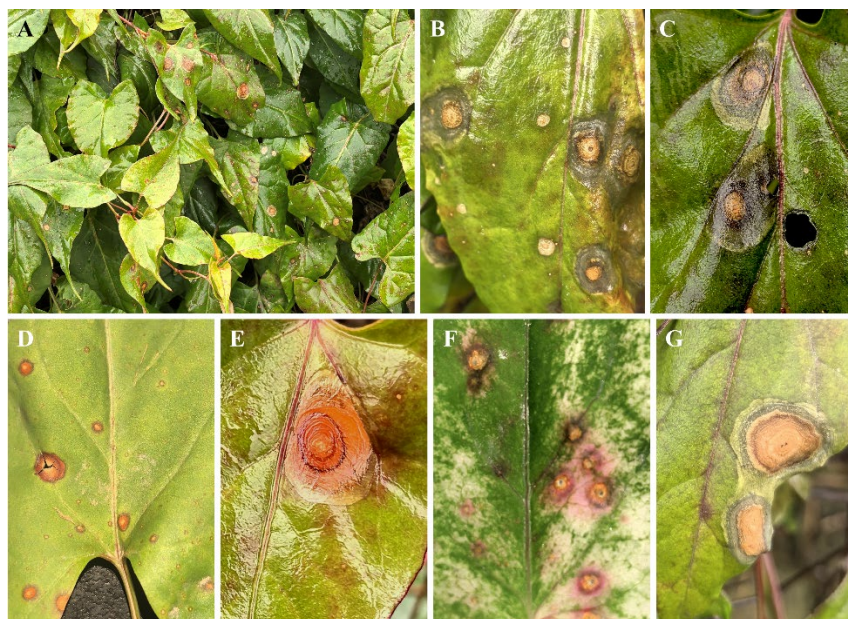


Figure 1. Various leaf symptoms on *Fallopia multiflora* in the field. (A) Overall disease situation; (B–G) diverse lesion morphologies.

2.2. Morphological Observations

The growth rate, color, and texture of colonies on PDA or Oatmeal agar (OA; Difco, Montreal, QC, Canada) were determined [12]. Mycelial plugs (6 mm in diameter) were taken from the edge of colonies and inoculated at 25 °C in darkness. The cultural characteristics were recorded after 7 days.

To observe the conidial morphology (sporulation patterns, color, shape, septa, size, etc.), the following culture conditions were used for different fungal genera:

- *Alternaria* spp.: Mycelia were cultured on potato carrot agar (PCA) and V8 juice agar (V8A) at 22 °C under an 8 h light/16 h dark cycle. The conidia and sporulation patterns were determined after 7 days [13].
- *Botrytis* spp.: Conidia and sporulation phenotypes were observed after 7 days of incubation on water agar (WA) [14] and *Fallopia multiflora* leaf tissue-amended water agar (FM-WA) at 25 °C under a 12 h light/dark photoperiod.
- *Lasiodiplodia* spp. and *Epicoccum* spp.: Conidia, conidiomata, and pycnidia developed after 14 days grown on PDA at 25 °C with a 12 h light/12 h dark cycle were investigated [15,16].

All the structures were mounted in Lactophenol Picric Acid solution (LPA) or stained with Lactophenol Cotton Blue solution (LPCB) and photographed with a Nikon ECLIPSE Ni-U microscope (Nikon, Tokyo, Japan). It was measured and described for each characteristic (n = 30).

2.3. DNA Extraction and PCR Amplification

Genomic DNA was extracted from fresh mycelia collected from colonies grown on PDA using the CTAB method [17,18]. For PCR amplification, a 25 µL reaction mixture was prepared, consisting of 21 µL of 1.1 × Taq PCR Star Mix (TSINGKE, Beijing, China), 2 µL of template DNA, and 1 µL of each primer. It was conducted in a Bio-Rad T100 thermocycler (Hercules, CA, USA). The details for PCR primers and reaction conditions for ITS, *TEF1*, *RPB2*, β -*TUB2*, *HSP60*, and *G3PDH* gene regions are listed in Table 1. The resulting products are sent to the TSINGKE company for bidirectional sequencing.

Table 1. PCR primer pairs used in the study and their related references.

Gene Region	Primer Pair	Sequence (5'-3')	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al. 1990 [19]
	ITS4	TCCTCCGCTTATTGATATGC	
RPB2	RPB2-5F	GAYGAYMGWGATCAYTTYGG	Sung et al. 2007 [20]
	RPB2-7CR	CCCATRGCTTGTYRCCCAT	
TEF1	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn et al. 1999 [21]
	EF1-986R	TACTTGAAGGAACCCTTACC	
β -TUB	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Glass et al. 1995 [22]
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
GAPDH	GAPDH-F	GGTTCAACGGATTTAGGATGTTGG	Staats M et al. 2005 [23]
	GAPDH-R	ACCTTGTCACAACGCTTCAGACCA	
HSP60	HSP60-for	CAACAATTGAGATTTGCCCAAG	Staats M et al. 2007 [24]
	HSP60-rev	GATGGATCCAGTGGTACCGAGCAT	

2.4. Phylogenetic Analyses

The resulting sequences were analyzed using BioEdit v.7.0.9 [25] and assembled with PHYLIT3.2 [26]. The relevant sequences were retrieved from the NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 10 March 2025) database based on BLAST search results (Table S1). The concatenated dataset of multiple loci (ITS, *TEF1*, *RPB2*, β -*TUB2*, *HSP60*, and *GAPDH*) was aligned using MEGA v.11.0.11 and OFPT v.1.9.0 [27,28]. The phylogenetic analysis of each alignment was conducted using maximum likelihood (ML) and Bayesian inference (BI) methods. For the ML analysis, bootstrapping with 1000 replicates was performed based on the nucleotide substitution model selected by MrModeltest v.2.3 [29]. For the BI analysis, a Markov chain Monte Carlo (MCMC) algorithm was run with 1,000,000 generations using two independent chains initiated from random trees, with the best-fit nucleotide substitution model determined by MrModeltest v.2.3 according to the Akaike Information Criterion (AIC). Trees were sampled every 100 generations; the first 25% of samples were discarded as burn-in, and a 50% majority-rule consensus tree with corresponding posterior probability values was subsequently calculated. Finally, the phylogenetic trees were edited with FigTree v.1.3.1 [30], and branch supports for each clade (ML bootstrap value > 60% and Bayesian posterior probability > 0.6) were indicated in the phylograms.

2.5. Pathogenicity Tests

Pathogenicity assays were performed following the guidelines of Bhunjun et al. [12] to determine the pathogenicity of the obtained strains on leaves of *Fallopia multiflora*. Seven representative strains were used after preliminary determination of all strains' pathogenicity. Plant samples collected from the plantation were taken and transplanted into pots and grown in a greenhouse (25 °C, 12 h light cycle) for two weeks. Mycelial discs (6 mm in diameter) from the edge of growing colonies on PDA or empty media (controls) were inoculated onto healthy leaves after surface sterilization with 70% ethanol solution. For each strain, three similar living leaves per plant (n = 3) were tested, and the experiment was performed in triplicate [31]. Disease progression was observed daily. Because of the primary medicinal and economic values of *Fallopia multiflora* tuberous root, it was also treated with all the previous strains. The fresh tubers were purchased from the local market; surface-sterilized by 70% ethanol solution for 2 min, 1% sodium hypochlorite for 3 min;

rinsed with sterile distilled water three times; and air-dried in a clean bench [32]. Two holes (6 mm) per one tuberous root were inoculated with previously mentioned mycelial discs, and controls were treated with aseptic PDA discs. All the roots were transferred into moistened clean boxes kept in a dark condition at 25 °C and evaluated after 7 days. One strain was tested on two tubers, and the experiment was also performed three times [33,34].

The disease severity was evaluated after 7 days. To fulfill Koch's postulates, pathogens were re-isolated from symptomatic tissues and confirmed by morphology and *RPB2* sequencing [35]. To determine the disease severity, lesion size (LS) is measured and analyzed using mean $\pm$ standard deviation (SD). Before intergroup comparisons, normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) were assessed [36]. When these assumptions were satisfied, one-way analysis of variance (one-way ANOVA) was used to compare LS among strains, followed by Tukey's HSD post hoc test for pairwise comparisons [37]. All tests were two-tailed with a significance level of $p < 0.05$, and statistical analyses were conducted using IBM SPSS Statistics 24.0.0 [38].

3. Results

Based on morphological and multi-locus phylogenetic analyses, five fungal pathogenic species were identified from leaf symptoms of *Fallopia multiflora*, including *Alternaria alternata* (Figure 1B,C), *Botrytis cinerea* (Figure 1D,E), *B. polygoni* (Figure 1E), *Epicoccum mackenziei* (Figure 1F), and *Lasiodiplodia citricola* (Figure 1G). The morphology, phylogenetic position, and pathogenicity of each species were described and illustrated in this study.

3.1. Taxonomy

- *Alternaria alternata* (Fr.) Keissl, Beih. Bot. Centralbl. 29: 434 (1912) (Table 2, Figure 2)

Cultural characteristics (on PDA, 25 °C, 7 d)

YzU 251134 formed a 51 mm colony with radial dense velvety mycelia containing loose mycelial ring near the irregular margin, off-white to brown in color and reverse off-white to pale luteous (Figure 2a); **YzU 251148** colonies were 51–54 mm circularly, diffuse dense in texture, cottony or fluffy, white to sulfur yellow (centrally), reverse white to straw yellow, with distinct wedge-shaped sectors at the periphery (Figure 2b); **YzU 251155** produced fluffy aerial mycelia, dense in the center, then gradually turning to sparse, 47–49 mm in size, with regular margin, white to saffron, sienna to orange in reverse (Figure 2c).

Conidial morphology

On PCA (22 °C, 7 d): **YzU 251134** produced conidia in chains of 2–5, yellow-brown to light olive-green, muriform, $19.1\text{--}37.3 \times 6.7\text{--}9.4$, with 2–7 transverse and 0–2 longitudinal septa; beak $2.3\text{--}5.9$ (–15.3) $\times 2.4\text{--}4.0$ μm ; **YzU 251148** conidia were normally 3–8 units per chain, yellow-brown to dark brown, variable (ovate, spindle, elliptic, obpyriform), $18\text{--}35.6 \times 7.3\text{--}14.9$ μm , 1–6 transverse and 0–3 longitudinal septa, beak $2\text{--}5 \times 2\text{--}4$ μm ; **YzU 251155** formed conidia 2–6 in a chain, yellow-brown to dark brown, ovate to spindle, $(10.9\text{--}) 16.4\text{--}20.8$ (–26.3) $\times (2.6\text{--}) 3.4\text{--}10.3$ μm , 3–6 transverse and 0–2 longitudinal septa; beak $2\text{--}12.1$ (–19.2) $\times 2.3\text{--}3.9$ μm (Figure 2B, 2D).

On V8A (22 °C, 7 d): **YzU 251134** produced conidia 3–5 units in a chain, medium yellow-brown to isabelline, nearly smooth ellipsoid or ovoid, $14\text{--}41 \times 8.4\text{--}8.9$ μm , 0–6 transverse and 0–2 longitudinal septa, obtuse beak $2.5\text{--}10.8$ (–19.7) $\times 1.7\text{--}3.9$ μm ; **YzU 251148** conidia were in chains of 3–5, brown to dark brown, ovate, elliptic or bpyriform, $18.5\text{--}42.1 \times 9\text{--}16.4$ μm , 0–6 transverse and 0–3 longitudinal septa; beak $2.2\text{--}5.1$ (–11) $\times 2.1\text{--}3.8$ μm ; **YzU 251155** formed conidia 2–5 per chain, citrine yellow-brown, ovate-spindle or muriform/elliptic, $18.1\text{--}22.4 \times 7.4\text{--}11.1$ μm , 3–5 transverse and 0–3 longitudinal septa, beak $2.7\text{--}8.5$ (–14.4) $\times 2.2\text{--}4.1$ μm (Figure 2C,E).

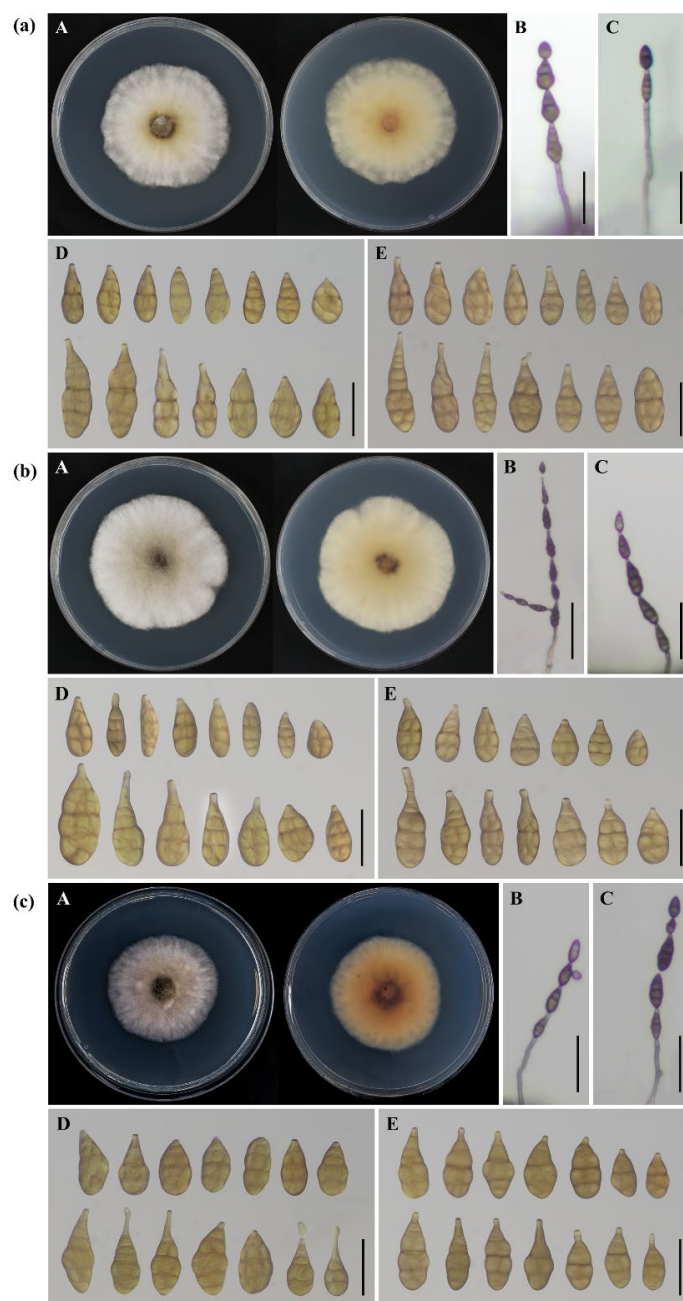


Figure 2. Morphology of three representative strains of *Alternaria alternata*. ((a), YzU 251134; (b), YzU 251148; (c), YzU251155); (A) Colony phenotype (on PDA for 7 days at 25 °C); (B,C) Sporulation patterns (on PCA and V8A at 22 °C); (D,E) Conidia (on PCA and V8A at 22 °C). Scale bars: (B,C) = 50 µm; (D,E) = 25 µm.

Note: The morphology of all three *A. alternata* strains matched the description of Simmons (2007), which also fell into section *Alternata*, phylogenetically (Woudenberg et al. 2015). YzU 251148 formed wider conidia in longer chains among the three strains (Table 2), while YzU 251134 and YzU 251148 produced conidia with longer bodies when compared with YzU 251155. The strain YzU 251134 grouped with the type strain CBS 127672 of morpho-species *A. alternata* (*Astragalus bisulcatus*), supported by high BS (86%) and PP (0.93) values, YzU 251148 fell into a clade with representative strain CBS 918.96 of *A. tenuissima* (BS/PP = 96%/0.73), and YzU 251155 was with the type strain (CBS 916.96) of *A. alternata* (BS/PP = 96%/1.0) (Figure 3).

- *Botrytis cinerea* Pers., Neues Magazin für die Botanik 1: 127 (1794). (Table 2, Figure 4)

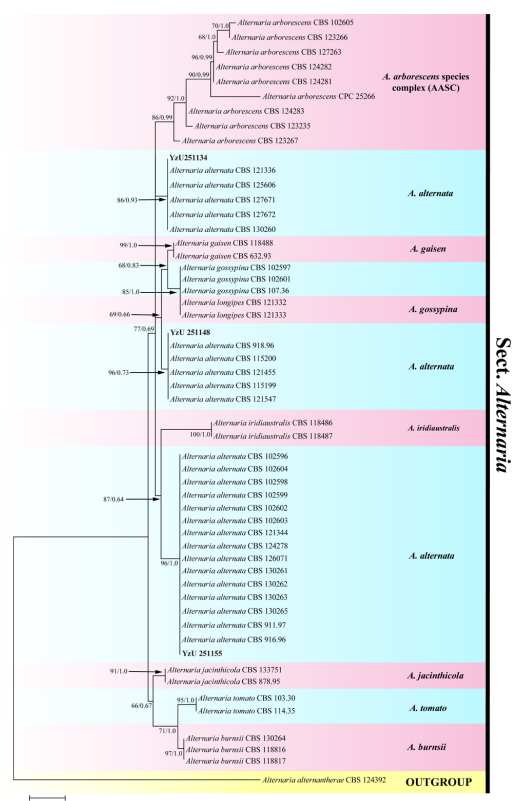


Figure 3. Phylogenetic tree based on the combined gene sequences of ITS, *TEF1*, and *RPB2* generated from *Alternaria* spp. on *Fallopia multiflora*. The maximum likelihood bootstrap values (BS > 60%) and Bayesian posterior probabilities (PP > 0.60) are provided for the nodes (PP/BS). The current strains are in bold.

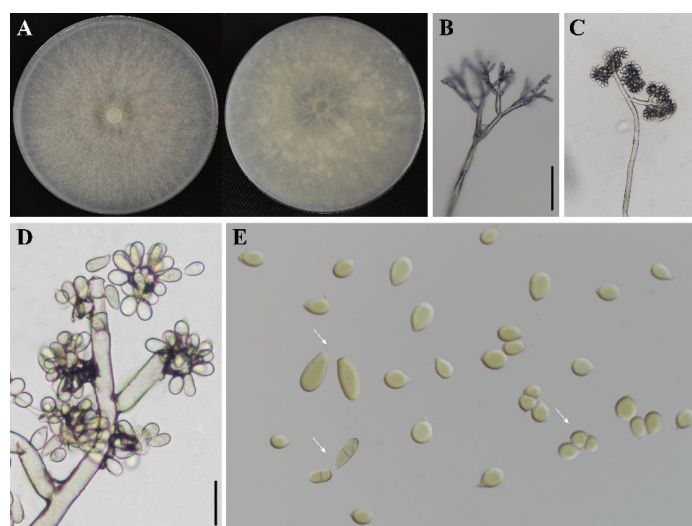


Figure 4. Morphological characteristics of *Botrytis cinerea* (YzU 251127); (A) colony phenotype (on PDA for 7 days at 25 °C); (B–D) sporulation patterns; (E) conidia (elongated, septate, and microconidia). Scale bars: (B,C) = 50 µm; (D,E) = 20 µm. Arrows indicate spores of different morphotypes.

Cultural Characteristics (on PDA, at 25 °C)

Colonies of YzU 251127 covered 90 mm plates on 6 days, radial growth, floccose, with a ring of abundant, elevated aerial mycelium present near the center, initially white to creamy, later turning gray to gray-brown, the reverse pale to gray-brown.

Conidial morphology (on WA, at 25 °C, 7 d)

The conidiophore is erect, arising singly from aerial hyphae, slender, and branched, hyaline with a smooth surface (Figure 4B). The conidia, hyaline to light brown, were often produced in clusters on short stalks or at conidiophore apices, ovate, ellipsoidal, pyriform, or globose, sometimes flat in one part, generally unicellular, sporadically septate, $8.3\text{--}12.9 \times 6.1\text{--}8.9 \mu\text{m}$ in size (Figure 4E, Table 2). Seldom sclerotia were produced after 14-day inoculation.

Note: A multi-locus analysis (*RPB2*, *HSP60*, *GAPDH*) placed YzU 251127 in an individual clade with *Botrytis cinerea* (BS/PP = 100%/1.0) (Figure 4). In terms of morphology, the strain exhibits no significant differences from reported strains of *Botrytis cinerea* (Table 2).

- *Botrytis polygoni* He, Wen, Bai, Jing, and Wang, Mycologia 113: 78–91 (2021). (Table 2, Figure 5)

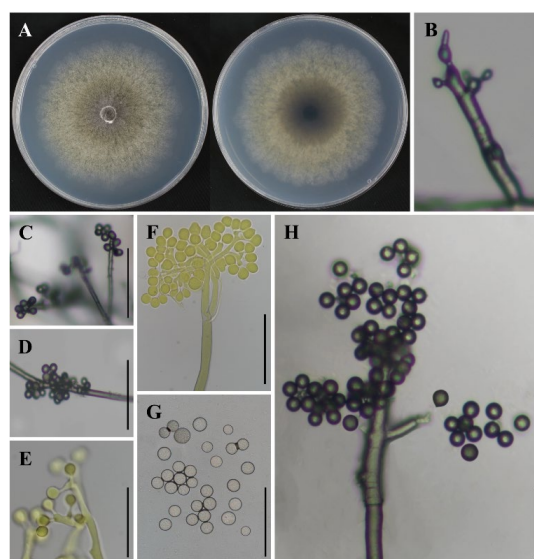


Figure 5. Morphological characteristics of *Botrytis polygoni* (YzU 251144); (A) colony phenotype (on PDA for 7 days at 25 °C); (B–D) sporulation patterns (on FM-WA at 25 °C); (E) bearing structures of conidia; (F,H) conidiophore with conidial clusters at the ends; (G) conidia of *B. polygoni*. Scale bars: (B,E–H) = 50 μm , (C,D) = 75 μm .

Cultural Characteristics (on PDA, 25 °C, 7 d)

Colonies of YzU 251144 covered 65–66 mm, radially growing with sparse aerial mycelia, irregular margin, edge light yellow-green gradually to olivaceous in the center, and darker color in the same reverse areas (Figure 5A), showing with clear color rings.

Conidial morphology (on FM-WA, 25 °C for 7 d under a 12 h light/dark cycle)

Conidiophores were hyaline, solitary, and branched with little or undifferentiated hyphae at the end serving as the bearing structures of conidia, which aggregated to form viscous spore masses (Figure 5B–F). Sometimes, conidia are directly formed inside hyphae and pile up around the hyphae (Figure 5D). The conidia are hyaline, unicellular, spherical, and sub-spherical, $15.16\text{--}21.15 \mu\text{m}$ (av.: $17.68 \pm 0.47 \mu\text{m}$) (Figure 5G; Table 2). Sclerotia appeared after 14 days.

Note: Multi-locus phylogenetic analyses (*GAPDH*, *HSP60*, and *RPB2*) demonstrated that YzU 251144 and *Botrytis polygoni* formed an independent clade (BS/PP = 100%/1.0) (Figure 6). Morphologically, our strains show similar characteristics to the first report strains (Poly-2, Poly-3) collected from three plants in the Polygonaceae, in Gansu, China, but the microconidia were not observed [39] (Table 2). This study is the first record of the species from this plant in southwest China.

- *Epicoecum mackenziei* Jayasiri, Camporesi, and K.D. Hyde, Mycosphere 8(8): 1093 (2017). (Table 2, Figure 7)

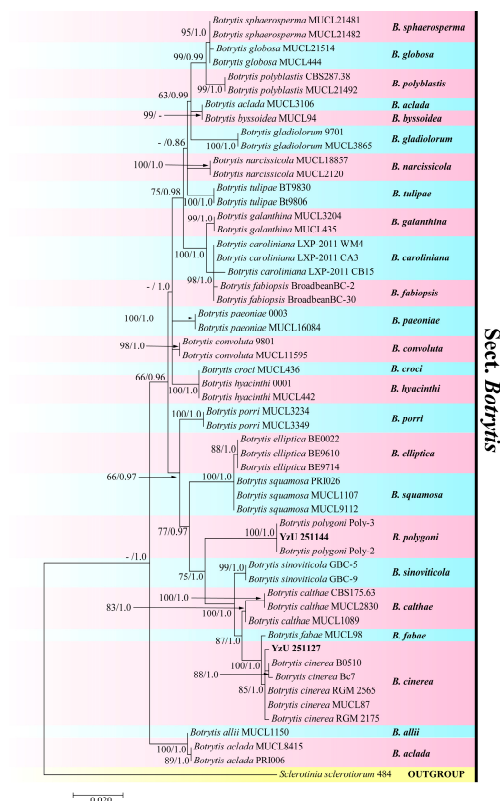


Figure 6. Maximum likelihood (ML) phylogram of *Botrytis* spp. from *Fallopia multiflora* based on a combined dataset of *HSP60*, *GAPDH*, and *RPB2* gene sequences. The maximum likelihood bootstrap support values > 60% (ML) and Bayesian posterior probabilities > 0.60 (PP) are given at the nodes (ML/PP). The current strains are in bold.

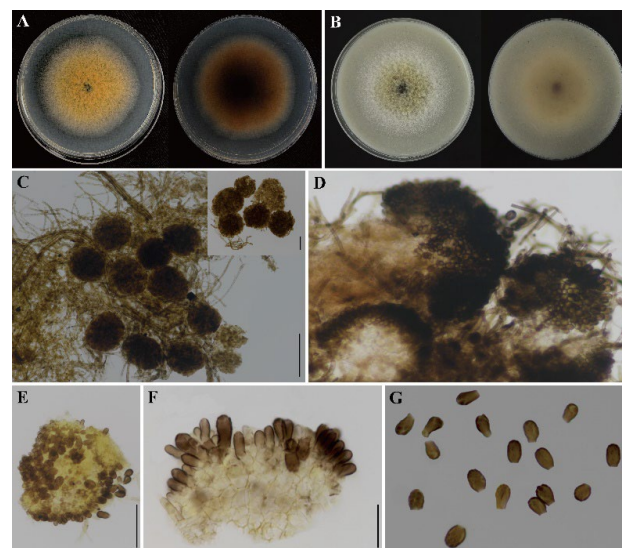


Figure 7. Morphology of *Epicoccum mackenziei* (YzU 251158). (A) Colony phenotype (on PDA for 7 days at 25 °C); (B) colony phenotype (on OA for 7 days at 25 °C); (C–E) conidiomata (on PDA at 25 °C); (F) conidia produced on conidiophores; (G) conidia (on PDA at 22 °C). Scale bars: (C–G) = 50 µm.

Cultural Characteristics (YzU 251158; on PDA and OA at 25 °C, 7 d):

On PDA, colonies were loose and villous with a relatively smooth margin, 52–53 mm, buff to luteous, saffron to chestnut in reverse; On OA, colonies were 56–57 mm in size, sparse, fluffy, and woolly, white to amber on both sides.

Conidial morphology (On PDA at 25 °C, 12 h light/12 h dark cycle, 10 d):

Conidiomata comprised numerous short, colorless conidiophores or conidiogenous cell aggregates. It was surrounded by conidia, dark brown, later developed into spherical or subspherical clusters consistent with sporodochia, $15.68\text{--}83.30 \times 20.40\text{--}36.30 \mu\text{m}$ (Figure 7C), which were mostly superficial on PDA and immersed on OA. For 14 days, solitary conidia apically produced from densely packed conidiophores, variable in shape—subglobose, broadly ellipsoid, or Cucurbitaceae seed-like—pale to dark brown, with finely granular to verrucose ornamentation, $3.93\text{--}9.43 \times 2.36\text{--}5.40 \mu\text{m}$ (Figure 7G, Table 2).

Note: Phylogenetic analysis of the ITS, *RPB2*, and *β -TUB2* gene loci revealed that YzU 251158 fell into a clade of *Epicoccum mackenziei* containing the type strain MFLUCC 16-0335 (BS/PP = 98%/1.0) (Figure 8). Morphologically, it was firstly reported by producing ascocarps (teleomorph) with hyaline, 1–3-septate ascospores ($21\text{--}25 \times 2\text{--}6 \mu\text{m}$) from the host, and documented its anamorph only forming shape-variable, irregular, unicellular or multicellular, intercalary or terminal, solitary or in chains, smooth, occasionally tuberculate, pale to dark brown, multicellular globose chlamydospores ($9\text{--}16 \times 7\text{--}15 \mu\text{m}$) in culture [40]. Later, it was noted that *Epicoccum* spp. have hyphomycetous synanamorphs, characterized by dark conidiomata, branched conidiophores, and unicellular to multicellular hyaline conidiogenous cells that produce pigmented conidia, dictyoseptate, occasionally verruculose [41]. In this study, no dictyospores were observed.

- *Lasiodiplodia citricola* Abdollahzadeh, Javadi and Phillips, Persoonia 25: 1 (2010) (Table 2, Figure 9)

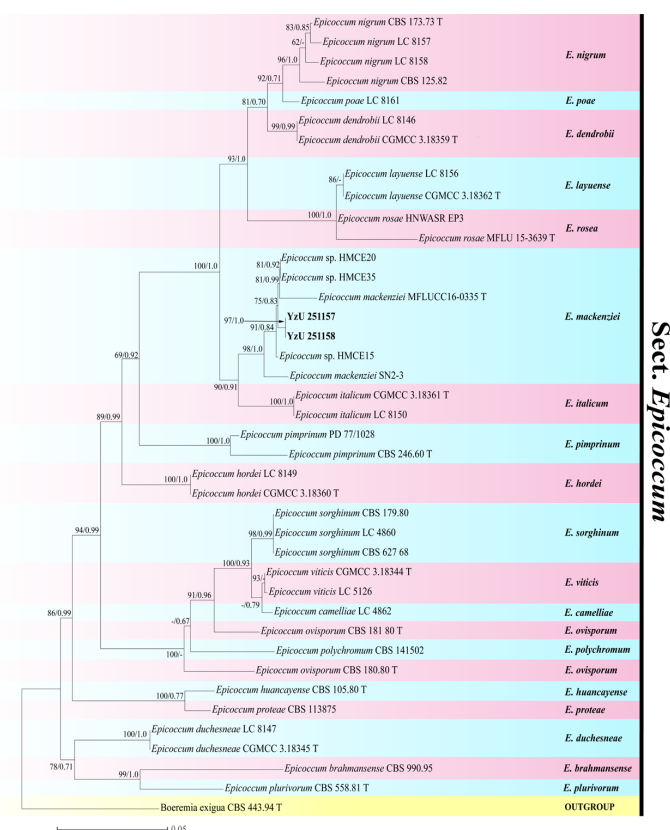


Figure 8. Maximum likelihood (ML) phylogram of *Epicoccum mackenziei* from the *Fallopia multiflora* based on a combined dataset of ITS, *β -TUB2*, and *RPB2* gene sequences. The maximum likelihood bootstrap support values > 60% (ML) and Bayesian posterior probabilities > 0.60 (PP) are given at the nodes (ML/PP). The current strains are in bold.

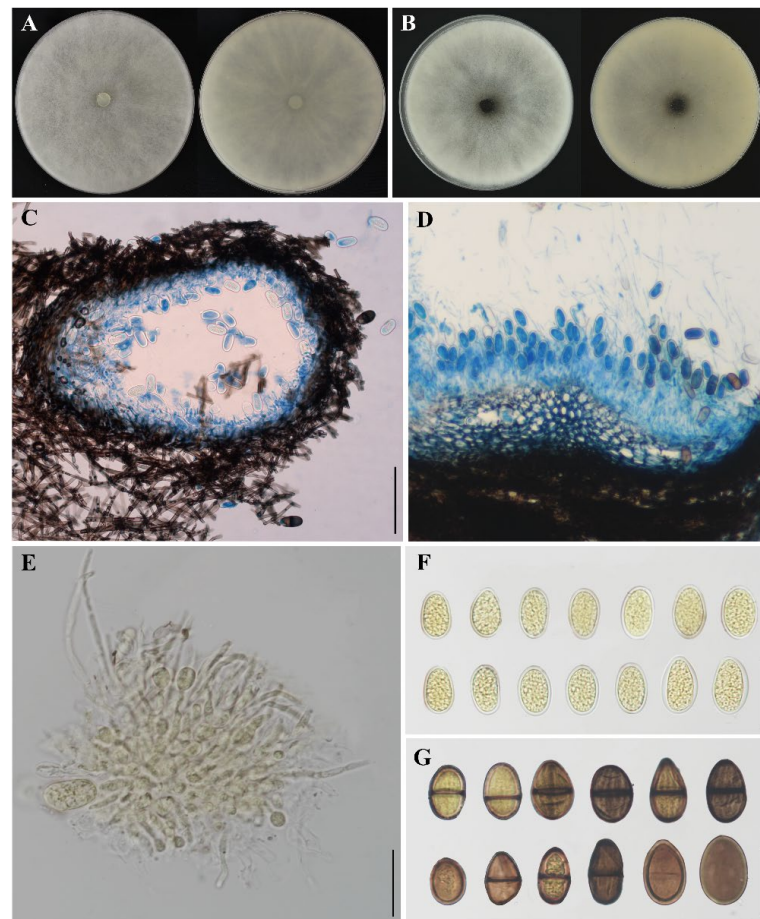


Figure 9. Morphological characteristics of *Lasiodiplodia citricola* (YzU 251163); (A) colony phenotype (on PDA for 7 days at 25 °C); (B) colony phenotype (on OA for 7 days at 25 °C); (C,D) vertical sections through pycnidia; (E) conidiogenous cells and paraphyses; (F) immature conidia; (G) mature conidia. Scale bars: (C,D) = 75 µm; (E–G) = 25 µm.

Cultural characteristics (on PDA and OA at 25 °C, 7 d):

On PDA, colonies of YzU 251163 covered 90 mm plates in 5 days; woolly, convex, with abundant aerial mycelia, white to pale greenish-gray, gradually darkening to a grayish hue, reverse the same as surface. On OA, it reached 90 mm in 6 days; smooth surface, velvety, dense aerial hyphae, grayish-green to olive green, with consistent reverse (Figure 9A,B).

Conidial morphology (on PDA at 25 °C, 12 d):

Pycnidia were solitary, semi-immersed, uniloculate, globose to subglobose, appearing as black dots. Conidiogenous cells lining the pycnidial cavity—holoblastic, annellidic, hyaline, cylindrical (Figure 9D,E). Conidia displayed a sub-ovoid to ellipsoid shape, sometimes wider in the middle, pumpkin seed-shaped, occasionally verruculose, with a rounded apex that tapered to a truncated base, double-layered, and 1-septate with longitudinal striations, yellowish-brown to brown or coffee colored, $21.5\text{--}31.1 \times 15.1\text{--}20.9$ µm; immature conidia were lacking septa and were transparent after staining with LCBS (Figure 9D) and mounted by LPAS (Figure 9E,F), measuring $21.8\text{--}27.1 \times 12.9\text{--}16.4$ µm (Figure 9F,G, Table 2).

Note: Based on phylogenetic analysis of ITS, *RBP2*, and *β-TUB2* gene regions, YzU 251163 clustered with two strains of *L. citricola* (CBS 124706 and CBS 124707), supported with high BS/PP values of 88%/0.98 (Figure 10). Morphologically, YzU 251163 exhibits typical morphological characteristics of *Lasiodiplodia citricola*: pumpkin seed-shaped, dark-brown, mature spores (Table 2).

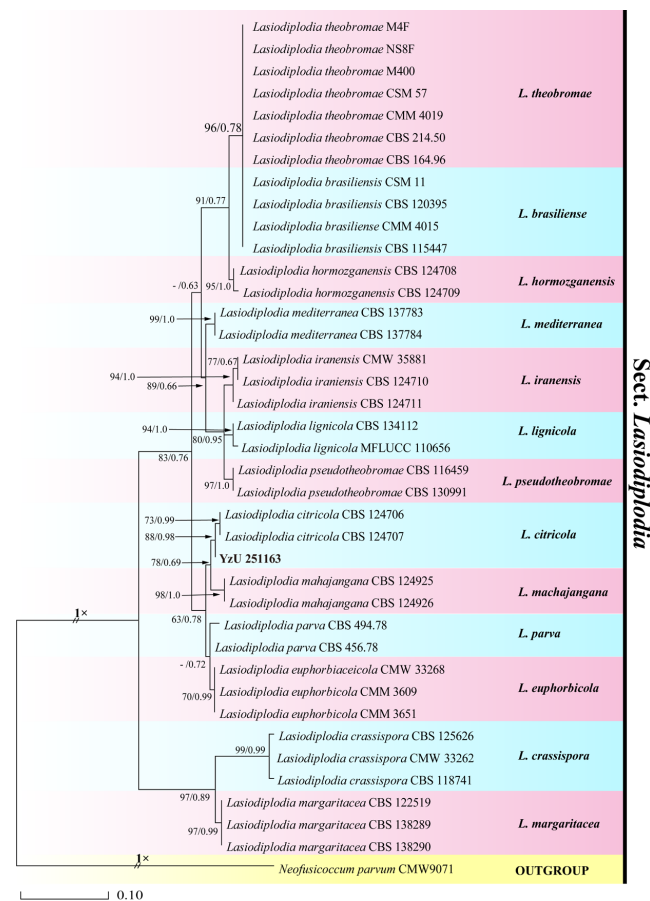


Figure 10. Phylogenetic tree constructed using the maximum likelihood method based on concatenated sequences of ITS, *RPB2*, and β -*TUB2* sequences of *Lasiodiplodia citricola*, isolated from *Fallopia multiflora*. The bootstrap support (BS > 60%) and Bayesian posterior probability (PP > 0.6) are given at the nodes (BS/PP). The current strains are in bold.

Table 2. Morphological comparison of the present strains and their closely related species.

Species	Strain	Conidia		Sporulation Pattern (Conidia per Chain)	Medium	Reference
		Body (μm)	Septa			
<i>Alternaria alternata</i>	CBS 127672 T	10–35 $\times$ 5–7	0–7	4–10 (unbranched)	PCA	[14]
	YzU 251134	19.1–37.3 $\times$ 6.7–9.4	2–7	2–5 (unbranched)	PCA	This study
		14.3–41 $\times$ 8.41–8.8	0–6	2–4 (unbranched)	V8A	
	CBS 918.96 T	32–45 $\times$ 11–13	1–8	3–12 (branched)	PCA	[14]
	YzU 251148	18–35.6 $\times$ 7.3–14.9	1–6	3–8 (branched)	PCA	This study
		18.5–42.1 $\times$ 9–16.4	0–6	2–6 (branched)	V8A	
	CBS 916.96 T	7–25 (–40) $\times$ 5–12	4–7	1–7 (1–3 branched)	PCA	[14]
	YzU 251155	11–26.3 $\times$ 2.7–10.3	3–6	2–6 (few branched)	PCA	This study
		18.1–22.4 $\times$ 7.4–11.1	3–5	2–4 (few branched)	V8A	
<i>Botrytis cinerea</i>	COAD 3406	8–13 $\times$ 6–8			PDA	[42]
	YzU 251127	(3.6)–8.3–12.9 (–21.3) (9.93 $\times$ 7.44)			WA	This study
<i>Botrytis polygoni</i>	Poly–1	10.2–18.36 (13.09 $\pm$ 1.75)			PDA	[39]
	Poly–2	12.24–19.38 (14.62 $\pm$ 2.47)			PDA	
	Poly–3	11.22–18.87 (15.34 $\pm$ 1.54)			PDA	
	YzU 251144	16.67–17.68 (17.18 $\pm$ 0.47)			FM-WA	This study

Table 2. Cont.

Species	Strain	Conidia		Sporulation Pattern (Conidia per Chain)	Medium	Reference
		Body (µm)	Septa			
<i>Epicoccum mackenziei</i>	YzU 251158	18.81–7.87 × 10.8–4.73 (13.72–8.37)			PDA	This study
<i>Lasiodiplodia citricola</i>	IRAN 1521C	20–31 × 10.9–19 (24.5 × 15.4)			WA	[43]
	YzU 251163	21.5–31.1 × 15.1–20.9 (26.8–17.5)			PDA	This study

Note: Full names and their corresponding abbreviations of all culture media used in this experiment. FM—*Fallopia multiflora* leaf tissue-amended water agar; PDA—potato dextrose agar; PCA—potato carrot agar; OA—Oatmeal agar; V8A—V8 juice agar; WA—water agar.

3.2. Pathogenicity Assay

The representative strains were determined their pathogenicity on the living leaves and tuber roots of *Fallopia multiflora* (Figures 11 and 12). Among them, *Lasiodiplodia citricola* was the most destructive foliar and tuberous root pathogen.

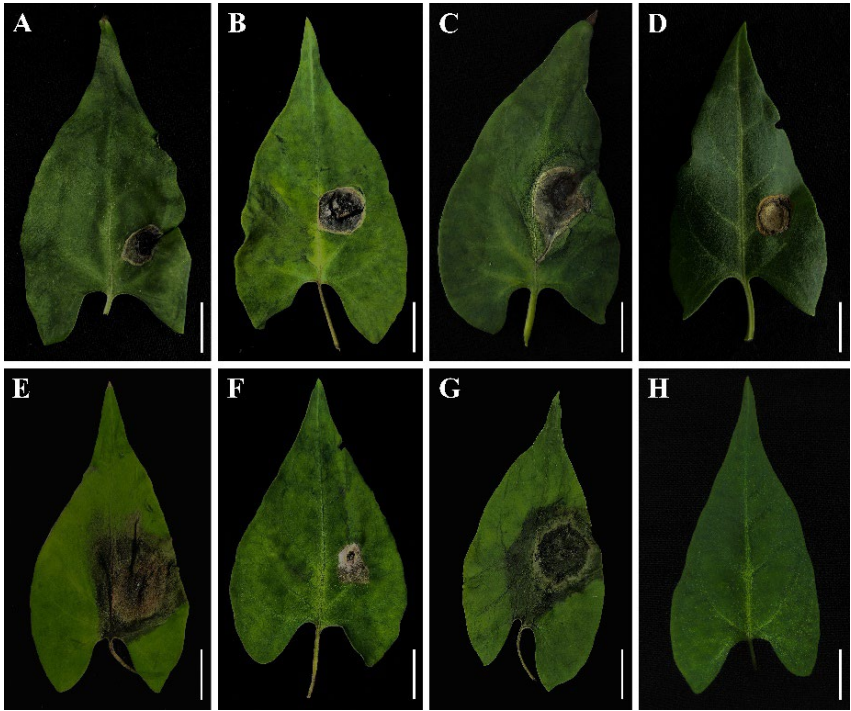


Figure 11. Pathogenicity on leaves of *Fallopia multiflora*. (A) *Alternaria alternata* YzU 251134; (B) *A. alternata* YzU 251148; (C) *A. alternata* YzU 251155; (D) *Botrytis cinerea* YzU 251127; (E) *B. polygoni* YzU 251144; (F) *Epicoccum mackenziei* YzU 251158; (G) *Lasiodiplodia citricola* YzU 251163; (H) control. Scale bar = 10 mm.

3.2.1. Pathogenicity on Leaves

The present fungal species induced various symptoms with different virulence on leaves (Figure 13). *Alternaria alternata* strains caused a similar symptom, which was a dark brown spot with a yellow circle at the edge. The strain YzU 251155 (in a clade with the type morpho-strain *A. alternata* CBS 916.96) was the most pathogenic strain among them (Figure 11A–C). *Botrytis cinerea* induced yellow-brown lesions (Figure 11D). *B. polygoni* showed strong pathogenicity, causing leaf blight with a reddish-brown area in the center for its reproduction (Figure 11E). *Epicoccum mackenziei* resulted in relatively weak pathogenicity with yellowish necrotic symptoms (Figure 11F). *Lasiodiplodia citricola* was the most pathogenic species, causing a blackish necrotic symptom, darker in the center

surrounded by a yellowish circle and lighter outside of the circle (Figure 11G). The controls remained symptomless (Figure 11H).

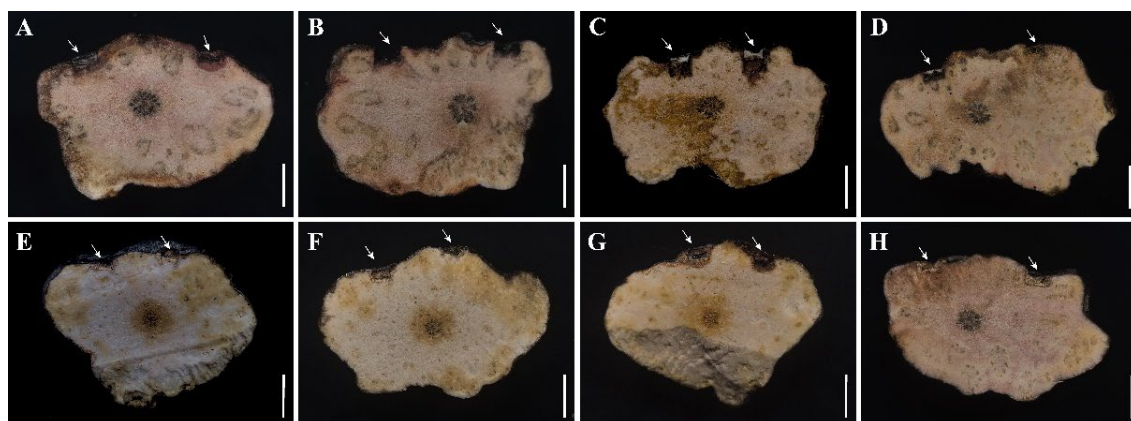


Figure 12. Pathogenicity on the tuberous root of *Fallopia multiflora*. (A) *Alternaria alternata* YzU 251134; (B) *A. alternata* YzU 251148; (C) *A. alternata* YzU 251155; (D) *Botrytis cinerea* YzU 251127; (E) *B. polygoni* YzU 251144; (F) *Epicoccum mackenziei* YzU 251158; (G) *Lasiodiplodia citricola* YzU 251163; (H) control. Scale bar = 10 mm. The arrow indicates the site of pathogenic treatment.

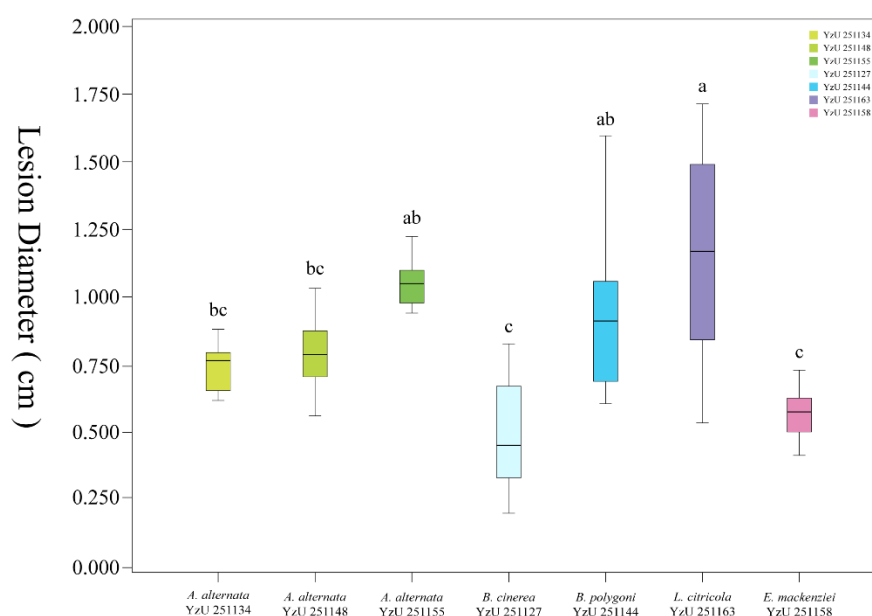


Figure 13. Box plot showing lesion size (cm) caused by the present strains on *Fallopia multiflora*. Different lowercase letters (a, b, c) indicate significant differences in pathogenicity among strains at $p < 0.05$ (one-way ANOVA followed by Tukey's test).

3.2.2. Pathogenicity Test on Tuberous Root of *Fallopia multiflora*

Among these seven pathogens, *Alternaria alternata* strain YzU 251155 and *Lasiodiplodia citricola* showed the highest pathogenicity on the tuberous root of *F. multiflora* (Figure 13). By 7 days, *A. alternata* YzU 251155 induced an infection of 6.73 ± 1.74 mm in depth, followed by YzU 251134 (1.80 ± 1.31 mm). However, *L. citricola* reached 4.25 ± 1.11 mm necrotic symptoms in depth. The other strains showed no further infections, consistent with asymptomatic controls. By day 12, the mycelia of *L. citricola* covered the entire tuberous root, resulting in severe rotting.

4. Discussion

Fallopia multiflora (Polygonaceae), a high-value medicinal horticultural plant, is widely cultivated globally with well-established propagation techniques [44]. However, reports on its foliar diseases remain limited and often lack systematic etiological data, hindering the development of effective disease diagnosis and control strategies [45]. To date, only *Alternaria astragali* and *Fusarium oxysporum* have been documented as the causal agents of leaf spot and wilt diseases on *F. multiflora*, respectively [9,10]. Owing to the scarcity of prior research on these foliar diseases and insufficient sample sizes in existing studies, their impacts on plant survival and yield have long been overlooked. In the present survey, plant mortality caused by leaf diseases was observed in the *F. multiflora* plantations investigated by our team—a phenomenon linked to the lack of comprehensive and instructive research on this disease. Accordingly, this study performed a systematic identification of the causal pathogens via multilocus phylogenetic analysis, morphological characterization, and pathogenicity assays, leading to the identification and characterization of five fungal pathogens associated with *F. multiflora* foliar diseases: *Alternaria alternata*, *Botrytis cinerea*, *B. polygoni*, *Epicoccum mackenziei*, and *Lasiodiplodia citricola*. This constitutes the first global report of all five fungal species as pathogens of *F. multiflora*, and the first report of a foliar disease on this medicinal horticultural plant caused by multiple fungal pathogens in China, thus expanding the known pathogen spectrum of *F. multiflora*.

Alternaria species are ubiquitous and frequently isolated from a diverse range of plant hosts, functioning as saprophytes, endophytes, and pathogens [46]. To date, only *Alternaria astragali* has been reported to cause disease in *F. multiflora*. *Alternaria alternata*, one of the fungal pathogens identified herein, possesses an extremely broad host range, being capable of infecting nearly all plant species [47]. Its infection of *F. multiflora* leaves (Figure 1B,C) is therefore not unexpected. An interesting finding was additionally noted in the present study: the three *Alternaria* isolates identified exhibited significant intraspecific variation in pathogenicity. This further demonstrates pronounced intraspecific differentiation within the species [48], supporting its classification as a species complex [49].

Botrytis species have a worldwide distribution and a broad host range encompassing both cultivated and wild plants [50]. In this study, both *B. polygoni* (Figure 1D,E) and *B. cinerea* were isolated from rufous-brown, concentric ring lesions on *F. multiflora* (Figure 1E), and their co-infection under favorable environmental conditions may exacerbate disease severity. *Botrytis* species, particularly *B. cinerea*, are necrotrophic generalist pathogens that secrete a diverse arsenal of cell wall-degrading enzymes, toxins, and other virulence factors—molecules that enable them to colonize a wide range of host plants and their tissues [51]. Co-infection by multiple pathogens, including different *Botrytis* species or *Botrytis* in combination with other fungal pathogens, can alter disease progression and typically results in larger lesions or enhanced tissue maceration under permissive environmental conditions, due to additive or synergistic effects in suppressing host defense responses and degrading host tissues [52]. Given that *Botrytis* species vary in host range, pathogenic aggressiveness, and responses to disease management strategies, the concurrent isolation of *B. polygoni* and *B. cinerea* from the same lesion (Figure 1E) may complicate epidemiological investigations of this disease. It should be taken into consideration when designing pathogenicity assays and formulating disease control measures [53].

Epicoccum is widely distributed in agricultural and natural ecosystems, commonly isolated from leaves, stems, fruits, soil, and decaying plant matter, where they act as an occasional opportunistic pathogen [54]. *Epicoccum* species frequently display a facultative endophytic or saprobic lifestyle. They are also noted for producing diverse secondary metabolites with antagonistic activity against other plant pathogens, which has led to their evaluation as biological-control agents in some systems [55,56]. Pathogenicity is

highly species and strain-dependent: many *Epicoccum* isolates are weakly pathogenic or nonpathogenic on tested hosts, while others can cause distinct leaf-spot diseases under favorable conditions. For example, *E. mackenziei* has recently been reported causing dark-brown leaf spot on tea [57]. Although aggressiveness appears to vary among isolates and hosts (*E. mackenziei* caused symptoms on *Fallopia multiflora*, Figure 1G), so the pathogenicity of local isolates should be quantified experimentally [58].

Lasiodiplodia are normally distributed in tropical and subtropical regions and temperate areas, infecting cashew, coconut, mango, mulberry, grapevines, etc. [59]. Members of this genus commonly behave as endophytes or saprobes but readily switch to an opportunistic pathogenic lifestyle under host stress or wounding, causing gummosis, canker, dieback, fruit and root/tuber rots in many woody and fruit crops [16,60,61]. Reports of *Lasiodiplodia* on Polygonaceae are uncommon in the literature, so the isolation of *Lasiodiplodia* from severe foliar lesions on *F. multiflora* (Figure 1G) and its apparent association with tuberous root rot represents a noteworthy host expansion that should be confirmed with pathogenicity tests and monitored for epidemiological significance [62].

5. Conclusions

This study represents the first report of a leaf disease on *Fallopia multiflora* (a valuable medicinal horticultural plant) in China caused by multiple fungal pathogens. Morphological characterization, multilocus phylogenetic analysis, and pathogenicity assays collectively expand the known pathogen spectrum of this plant. Notably, *Lasiodiplodia citricola*, *Botrytis polygoni*, and *Alternaria alternata* exhibited strong pathogenicity to the leaves of *F. multiflora* under experimental conditions, among which *A. alternata* and *L. citricola* also displayed strong pathogenicity to the tuberous roots of this plant. The co-occurrence of these pathogens, coupled with the ability of certain isolates to infect tuberous roots, highlights the urgency of implementing integrated disease monitoring and management strategies and raises concerns about potential adverse impacts on the medicinal quality and safety of *F. multiflora*-derived products.

Future research should prioritize investigating interspecific pathogen interactions, the molecular mechanisms governing fungal virulence, and the accumulation of fungal secondary metabolites in infected host tissues. These efforts will lay a scientific foundation for developing targeted disease control measures, thereby safeguarding the quality and yield of *F. multiflora* in horticultural production.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12020204/s1>, Table S1: Strains used in this study and their GenBank accession numbers.

Author Contributions: Conceptualization, J.D. and S.C.; methodology, S.C. and L.H.; software, S.C. and Q.S.; validation, S.C. and L.H.; formal analysis, L.H.; investigation, S.C.; resources, J.D.; data curation, S.C. and M.L.; writing—original draft preparation, S.C.; writing—review and editing, J.D. and P.B.G.; visualization, X.M.; supervision, J.D.; project administration, J.D. and L.H.; funding acquisition, J.D. All authors have read and agreed to the published version of the manuscript.

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