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Isolation and Identification of the Pathogen Causing Polygonatum Leaf Spot and Screening of Botanical Fungicides

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Abstract: In order to investigate the incidence of Polygonatum leaf spot disease in Enshi City, Hubei Province, identify its pathogenic fungi, and screen plant extracts with effective antibacterial activity against the pathogen, this study systematically investigated the occurrence of leaf spot disease in the Polygonatum cultivation base of Laifeng County, Enshi City. Leaves of Polygonatum seedlings with typical leaf spot symptoms were collected for pathogen isolation. The pathogen was identified through morphological characteristics and molecular analysis, and its pathogenicity was verified by seedling inoculation. The mycelial growth rate method was employed to evaluate the antibacterial activity of nine plant extracts. Results showed that a highly pathogenic strain FR-1 was isolated and identified as Fusarium incarnatum. Among the nine plant extracts, only Coptis chinensis water extract and Berberis julianae water extract exhibited significant inhibitory effects. The 50 mg/mL Coptis chinensis water extract demonstrated 82.43% inhibition rate against F. incarnatum with EC50 of 6.249 mg/mL, and 77.50% spore inhibition rate with EC50 of 8.236 mg/mL. In conclusion, Coptis chinensis shows potential for development as a botanical fungicide.

Keywords: Polygonatum; Leaf Spot Disease; Fusarium; Plant Extracts; Antibacterial Activity.

1. Introduction

Polygonatum sibiricum (commonly known as "yam polygonatum"), a perennial herbaceous plant of the Liliaceae family, possesses significant medicinal value. Widely distributed in Hunan, Hubei, Guizhou, and other regions of China, it is a traditional medicinal and edible plant renowned for its antioxidant, anti-aging, cytoprotective, spleen-tonifying, and immune-enhancing properties[1, 2]. Due to its medicinal importance, P. sibiricum holds a prominent position in the traditional Chinese medicine industry. With growing consumer demand for health-oriented products, its market potential continues to expand. To ensure the stable development of P. sibiricumcultivation, scientifically effective integrated pest management strategies must be implemented during cultivation to mitigate the adverse impacts of diseases and pests on yield and quality[3].

Leaf spot disease, a prevalent fungal disease in P. sibiricum, is primarily caused by pathogens from genera such as Alternaria, Colletotrichum, or Fusarium [4, 5]. This soil-borne fungal disease occurs multiple times annually, with peak incidence in July and October, exacerbated by rainy conditions. Severe infections lead to extensive leaf necrosis, significantly threatening both yield and quality of P. sibiricum. Chemical pesticides, though cost-effective and rapid-acting, pose ecological risks, environmental pollution, and residue accumulation that compromise the commercial value of *P. sibiricum. In contrast, plant-derived biopesticides offer notable advantages [6, 7]. These natural agents exhibit low toxicity to mammals and beneficial insects, preserve ecological balance, and delay resistance development in target pathogens due to their unique modes of action. From an agricultural perspective, they ensure crop safety without disrupting normal growth. Furthermore, plant-based pesticides are widely sourced, biodegradable, and sustainably produced, aligning with green agriculture principles and fulfilling the demand for environmentally friendly farming practices.

In this study, the dominant pathogen causing leaf spot disease was isolated from infected P. sibiricum leaves in Enshi City, Hubei Province. The pathogen was identified through traditional morphological characterization and multigene phylogenetic analysis. Using the isolated strain (Fusarium incarnatum) as the target, the inhibitory activities of nine plant extracts against F. incarnatum were evaluated via the mycelial growth rate method. The most effective plant extract was screened to provide a theoretical basis for the scientific diagnosis and field control of P. sibiricum leaf spot disease.

2. Materials and Methods

2.1. Materials

Samples: Polygonatum sibiricum specimens exhibiting typical leaf spot disease symptoms were collected from the Polygonatum cultivation base in Laifeng County, Enshi City, Hubei Province.

The primary chemicals and reagents: Potato Dextrose Agar (PDA), DNA Marker, primers ITS1 and ITS4, Fungal Genomic DNA Extraction Kit, dNTP, and Taq DNA Polymerase.

Key instruments comprised:

PCR Amplifier (Hangzhou Longji Scientific Instrument Co., Ltd.)

High-Speed Refrigerated Centrifuge (Anhui Zhongke Zhongjia Scientific Instrument Co., Ltd.)

Electrophoresis Apparatus (Shanghai Peiqing Technology Co., Ltd.)

Electronic Balance (Mettler Toledo)

Laminar Flow Cabinet (Jiangsu Tongjing Purification Instrument Co., Ltd.)

Constant Temperature Incubator (Shanghai Jinghong Laboratory Equipment Co., Ltd.)

Optical Microscope (BX43, OLYMPUS Corporation)

Constant Temperature Shaking Incubator (Shanghai Jinghong Laboratory Equipment Co., Ltd.).

2.2. Methods

2.2.1. Isolation, Purification, and Preservation of the Pathogen Causing Polygonatum Leaf Spot

Plant samples: Polygonatum sibiricum specimens exhibiting typical leaf spot symptoms were collected on July 9, 2023, from the Polygonatum cultivation base in Laifeng County, Enshi City, Hubei Province. The samples were transported to the laboratory for pathogen isolation and identification, following established protocols [8]

Diseased leaves were rinsed under running water, immersed in 75% ethanol for 1–2 minutes, followed by treatment with 14% sodium hypochlorite solution for 15 seconds, and then washed 2–3 times with sterile distilled water. Excess moisture was removed using sterilized filter paper. Tissue preparation: Leaf fragments (5 mm × 5 mm) from the junction between diseased and healthy tissues were excised and placed on Potato Dextrose Agar (PDA) medium. Fungal cultivation: Inoculated PDA plates were incubated at 28°C for 7 days. Strain purification: Pure cultures were obtained via the streak plate method. Plates were incubated at 28°C until isolated colonies formed, after which cultivation was terminated.

2.2.2. Pathogenicity Assay of Polygonatum Leaf Spot

Leaf inoculation: The reactivated fungal strain was inoculated onto PDA plates and incubated at 28°C for 5–7 days until colony maturation. Mycelial plugs (6 mm diameter) were excised from the colony margins using a sterile cork borer. Leaf preparation: Healthy Polygonatum sibiricum leaves were surface-sterilized with 75% ethanol, rinsed three times with sterile deionized water, and air-dried.

Inoculation: Wounds were created on leaves using sterile needles, followed by placement of mycelial plugs. Inoculated leaves were maintained in an intelligent illuminated incubator (>90% humidity) for 7 days, with daily observation of lesion development.

Root irrigation inoculation: Fungal culture: A 6 mm mycelial plug from 5–7-day-old PDA cultures was transferred to 100 mL Potato Dextrose Broth (PDB) and incubated at 28°C, 180 rpm for 3 days. The fungal suspension was diluted to 200 mL and irrigated into the rhizosphere of potted P. sibiricum plants. Three parallel control groups were established, and plants were incubated at 25°C for 7 days with daily disease monitoring[9, 10].

Control group: Agar plugs from uninoculated PDA medium were used as inoculum. Each treatment group included three biological replicates (one leaf per replicate) to ensure experimental reliability.

Pathogen re-isolation: Pathogens were re-isolated from symptomatic tissues of artificially infected leaves and compared morphologically and molecularly with the original inoculated strain to validate Koch's postulates.

2.2.3. Morphological Identification of the Pathogen Causing Polygonatum Leaf Spot

The experiment followed standard microbiological protocols: The activated fungal strain was inoculated onto PDA plates and purified by incubation at 28°C for 5–7 days. Mycelial plugs (6 mm diameter) from colony margins were transferred to fresh PDA plates and incubated at 26°C in darkness for 5 days. Hyphal samples were treated with a modified lactophenol cotton blue staining method. After fixation and staining, mycelial growth was observed and recorded under an optical microscope. Hyphal morphological characteristics, including colony morphology, spore dimensions, and structural features, were documented using an optical microscopic imaging system.

2.2.4. Molecular Identification and Phylogenetic Analysis of the Pathogen Causing Polygonatum Leaf Spot

Identified colonies were inoculated into 200 mL Potato Dextrose Broth (PDB) and incubated at 28°C, 180 rpm for 48 h. Mycelia were collected via sterile filtration, flash-frozen in liquid nitrogen, and ground to a fine powder using a sterilized mortar and pestle.then Approximately 0.5 g of powdered mycelia was transferred to a sterile EP tube. Genomic DNA was extracted from the pathogen FR-1 using a Fungal Genomic DNA Extraction Kit (Sangon Biotech) following the protocol described by Lousie et al.The ITS region was amplified using primers ITS1 (TCCGTAGGTGA ACCT GCGC) and ITS4(TCCTCCGCTTATTGATATGC). The thermal cycling conditions were: initial denaturation at 94°C for 4 min; 30 cycles of denaturation (94°C, 50 s), annealing (55°C, 45 s), and extension (72°C, 10 min). Each 50 μL reaction contained 25 μL PCR Mix, 2 μL DNA template, 1 μL each of forward and reverse primers (10 μmol/L), and ddH_2O to adjust the total volume. Electrophoresis: 5–8 μL of PCR product was mixed with 3 μL 10× DNA loading buffer and electrophoresed on a 1% agarose gel at 120 V. Target bands were visualized using a gel imaging system. Sequencing: Purified PCR products were sent to Wuhan Yuanxi Biotechnology Co., Ltd. for bidirectional sequencing.

2.2.5. Preparation of Plant Extracts

Dried plant materials, including Coptis chinensis, Berberis julianae, Portulaca oleracea, and Andrographis paniculata and so on were pulverized using a grinder. Sieved plant powder (10 g) was weighed for extraction. The powder was mixed with deionized water at a ratio of 1:20 (w/v). Initially, 100 mL of deionized water was added, and the mixture was shaken in a constant temperature incubator shaker at 36°C, 180 rpm for 24 h. The extract was filtered and collected.The residue was re-extracted with another 100 mL of deionized water under identical conditions (36°C, 180 rpm, 24 h). The filtrate was combined with the primary extract.The pooled extract was subjected to high-speed centrifugation at 8,000 rpm for 15 min, and the supernatant was stored for subsequent experiments.

2.2.6. Screening of Antibacterial Activity of Plant Extracts

Antibacterial Activity Assay via Mycelial Growth Rate Method; Appropriate amounts of glucose, potato infusion powder, and agar were dissolved in plant aqueous extracts (replacing deionized water) to prepare PDA medium. The medium was sterilized by autoclaving and poured into 9 cm Petri dishes. After solidification, drug-containing plates with a final extract concentration of 50 mg/mL were obtained. Mycelial plugs (5 mm diameter) were aseptically excised from the margins of pre-cultured Fusarium incarnatum colonies using a sterile cork borer. Each plug was inoculated at the center of a drug-containing PDA plate.Three biological replicates per treatment were incubated in darkness at 28 ± 0.5°C for 7 days. Colony expansion diameters were measured post-incubation.

The antibacterial activity of water extracts from various traditional Chinese medicinal herbs was determined using the growth rate method. The calculation formula is as follows:

$$\text{Inhibition rate (\%)} = \frac{\textit{Colony diameter in control} - \textit{Colony diameter in treatment}}{\textit{Colony diameter in control}} \times 100\%$$

2.2.7. Analysis of the Virulence of Pathogenic Bacteria Against Coptis Chinensis Water Extract

Antibacterial Spectrum Determination of Coptis chinensis Aqueous Extract:The growth rate method was employed to evaluate the inhibitory activity of Coptis chinensis aqueous extract against Polygonatum leaf spot pathogen FR-1. PDA media containing the extract at concentrations of 0, 10, 20, 30, 40, and 50 mg/mL were prepared. Experimental procedures followed those described in Section 2.2.6. The colony diameter was measured to quantitatively analyze the dose-response relationship between the extract concentration and mycelial growth inhibition. A toxicity regression model was constructed using Probit analysis, and the EC50 value was calculated.

Inhibition of Spore Germination by Coptis chinensis Aqueous Extract: The method was adapted from Dikec et al[11]. with modifications. Under sterile conditions, the Polygonatum leaf spot pathogen was inoculated onto PDA medium via the streak plate method and incubated at 28 ± 0.5°C for 7 days to promote conidial formation and maturation.

The spore suspension was prepared according to Guan Lijie[12]with slight modifications: Spores were washed from the culture using 1% sterile glucose solution (containing 0.5% Tween 80), filtered through sterile gauze, and adjusted to a concentration of 10^6 spores/mL with sterile water. Aliquots (10 μL) of Coptis chinensis aqueous extract at inhibitory concentrations (20, 40, 60, 80, and 100 mg/mL) were added to the spore suspension. A 10 μL aliquot of the spore suspension was then placed onto a concave slide, with 10 μL of 1% sterile glucose solution (containing 0.5% Tween 80) used as the control. Samples were incubated at 28 ± 0.5°C for 12 hours, and spore germination was observed under an optical microscope (400× magnification). The germination rate was calculated, and the inhibition efficacy was determined. A toxicity regression model was established using Probit analysis to derive the EC_{50} value.

Calculation Formula:

Spore Inhibition Rate (%) = [(Control Germination Rate − Treatment Germination Rate) / Control Germination Rate] × 100%

3. Results and Analysis

3.1. Isolation, Purification, and Preservation of Pathogenic Fungi Causing Polygonatum Cyrtonema Leaf Spot Disease

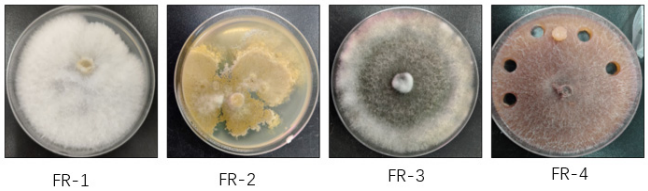

Fig 1. The Morphology of four pathog

Single colonies were isolated using the streak plate method and identified based on morphological characteristics, including colony morphology, hyphal structure, and growth rate. Four fungal strains exhibiting distinct morphological traits were successfully isolated and purified. These strains showed significant differences in colony morphology and growth rate on PDA medium, as shown in Figure 1. The strains were designated as FR-1, FR-2, FR-3, and FR-4.

3.2. Pathogenicity Test of Polygonatum Cyrtonema Leaf Spot Pathogen

Verification via Koch's Postulates: The four fungal strains isolated and purified in Section 2.2.1 were reinoculated onto healthy Polygonatum leaves. As shown in Figure 2, the results demonstrated that strain FR-1 induced disease symptoms in healthy leaves with strong infectivity, consistent with the symptoms observed in field-infected plants. Leaves inoculated with FR-1 exhibited yellowing along the midrib, followed by gradual expansion of lesions. Irregularly shaped necrotic spots with blurred margins, often accompanied by yellow halos or water-soaked appearances, developed on the inoculated leaves. Maximum lesion formation, characterized by tan-brown spots and wilting, occurred between days 5 and 14. To confirm pathogenicity, diseased leaf tissues were subjected to re-isolation using the tissue isolation method. Morphological comparison confirmed that the re-isolated pathogen exhibited identical colony morphology, hyphal structure, and sporulation characteristics to the original inoculated strain (FR-1). This confirms that the FR-1 strain is indeed the pathogen responsible for the Polygonatum leaf spot disease.

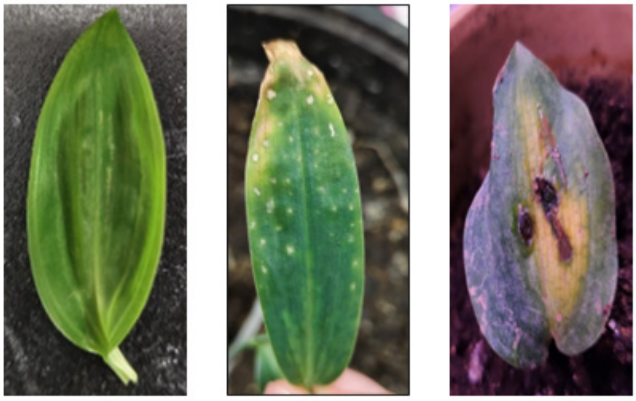

Fig 2. Pathogenicity detection of Polygonatum sibiricum by FR-1 A: Healthy Polygonatum leaves B: Polygonatum leaves sprayed with FR-1 spores C: Polygonatum leaves inoculated directly through a cut wound

3.3. Morphological Identification of Polyporus Yellow Spot Disease Pathogen

Systematic Morphological Characterization of the Most Pathogenic Strain FR-1: The highly pathogenic strain FR-1 was selected for systematic morphological analysis. As shown in Figure 3, after 5 days of incubation on PDA medium at 28°C, the colony morphology stabilized, exhibiting a typical cotton-like growth pattern. The colony surface displayed dense, powdery-white aerial hyphae, while the reverse side showed pale yellow pigmentation. Microscopic examination revealed the following features: macroconidia were sickle-shaped (fusiform), microconidia were oval to reniform (kidney-shaped), and hyphae exhibited distinct septation. These morphological characteristics align closely with descriptions of Fusarium incarnatum. This confirms that the pathogenic fungus isolated from Polygonatum plants in Laifeng County, Enshi City, Hubei Province, belongs to Fusarium incarnatum.

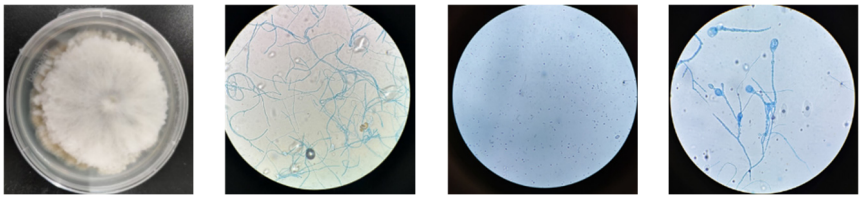

Fig 3. Fungal morphology under microscope
A: Top view of colony morphology B: mycelium C: spores D: aerial hyphae

3.4. Molecular Biological Identification and Phylogenetic Analysis of the Pathogen Causing Polygonatum Cyrtonema Leaf Spot Disease

The conserved gene sequences of strain FR-1 were amplified via PCR, sequenced, and subjected to homology comparison with related strains in the GenBank database using the BLAST program. Sequences including MT446117.1 Fusarium sp. strain ZMXR12, MN882828.1 Fusarium incarnatumstrain DTO 417-F2, MH884156.1 Fusarium sp. strain AQ15, OQ422663.1 Fusarium incarnatumisolate Sample-189, OQ422646.1 Fusarium incarnatum isolate Sample-172, OQ421771.1 Fusarium equiseti isolate Sample-261, and HQ332532.1 Fusarium equiseti were retrieved from GenBank. A phylogenetic tree was constructed using the neighbor-joining method (Figure 4). Phylogenetic analysis confirmed that the pathogen belongs to Fusarium incarnatum.

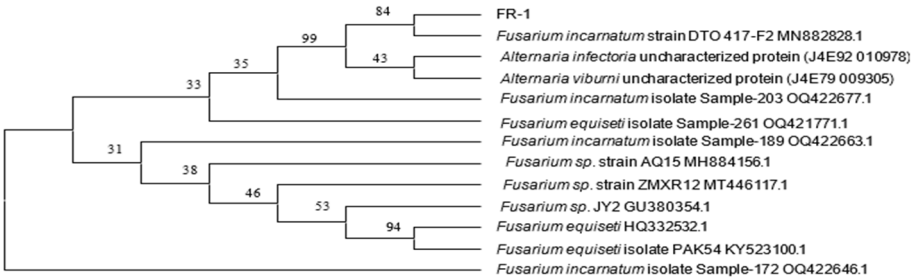

Fig 4. Phylogenetic tree of pathogenic fungi

3.5. Antibacterial Activity of Plant Extracts

Table 1. Antifungil activity of extracts from various plants

Extract	inhibition rate (%)	95% confidence interval	5% significant level
Berberis julianae	81.89±0.56	81.15-86.40	a
Coptis chinensis	82.43±0.86	81.51-85.27	a
Andrographis paniculata	22.41±2.85	15.33-29.49	b
Purslane	2.04±1.70	1.98-6.25	d
dandelion	16.30±3.35	7.68-24.62	c
Perilla leaf	5.56±0.00	5.56-7.56	d
Dried Tangerine Peel	24.07±3.21	16.11-32.02	b
Astragalusmem branaceus	15.00±8.73	14.69-9.69	c
Datura stramonium	6.9±1.00	6.65-7.15	d

Inhibitory Effects of Plant Extracts on FR-1:As shown in Table 1, at a concentration of 50 mg/mL, the inhibitory effects of plant extracts on the mycelial growth of Polygonatum leaf spot pathogen FR-1, ranked from strongest to weakest, were as follows: Coptis chinensis (Chinese goldthread), Berberis julianae (Juliana barberry), Citrus reticulata (tangerine peel), Andrographis paniculata (green chireta), Taraxacum officinale(dandelion), Astragalus membranaceus (milkvetch), Datura stramonium (jimsonweed), Perilla frutescens (perilla leaf), and Portulaca oleracea (purslane).Among these, the aqueous extracts of Coptis chinensis and Berberis julianae exhibited the strongest inhibition, with mycelial growth inhibition rates exceeding 80%, significantly higher than those of other plant extracts. In contrast, the extracts of Citrus reticulata, Andrographis paniculata, and Taraxacum officinale showed moderate antifungal activity, with inhibition rates of approximately 20%. Notably, the aqueous extracts of Datura stramonium, Perilla frutescens, and Portulaca oleracea exhibited negligible inhibitory effects on mycelial growth, with inhibition rates below 10%. These results indicate that Coptis chinensis and Berberis julianae aqueous extracts possess superior antifungal efficacy against FR-1.

3.6. Analysis of the Toxicity of Coptis Chinensis Water Extract

Further Analysis of the Antimicrobial Virulence of Coptis chinensis Aqueous Extract: Based on the experimental results, the antimicrobial potency of Coptis chinensis aqueous extract was further investigated. Data from Table 2 indicate that Coptis chinensis aqueous extract exhibited the strongest antifungal activity, with its inhibitory effect on the mycelial growth of Fusarium incarnatum (the causal agent of Polygonatum leaf spot) demonstrating a pronounced concentration-dependent manner. Experimental data revealed that at a concentration of 10 mg/mL, the extract achieved a mycelial growth inhibition rate of 59.66%. The inhibition rate increased progressively with higher concentrations, reaching a maximum of 82.43% at 50 mg/mL. The calculated half-maximal effective concentration (EC50) for mycelial growth inhibition was 6.249 mg/mL, confirming the significant inhibitory efficacy of Coptis chinensis aqueous extract against the pathogen.

Inhibition of Spore Germination by Coptis chinensis Aqueous Extract:

As shown in Table 3, within the tested concentration range, the antifungal activity of Coptis chinensis aqueous extract exhibited a strong positive correlation with its concentration. Specifically, the extract demonstrated dose-dependent suppression of conidial germination in the pathogen. In the untreated control group, the natural spore germination rate was 91.32%. After treatment with 10 mg/mL and 50 mg/mL of the extract, the corresponding spore germination inhibition rates were 55.40% ± 0.53 and 77.50% ± 0.53, respectively. The calculated EC50 value for spore germination inhibition was 8.236 mg/mL, further validating the potent inhibitory effect of Coptis chinensis aqueous extract on spore germination.

Conclusion: Collectively, these results demonstrate that Coptis chinensis aqueous extract significantly inhibits both mycelial growth and spore germination of Fusarium incarnatum, the causal agent of Polygonatum leaf spot, in a concentration-dependent manner.

Table 2. The effect of different concentrations Coptis water extract on the growth rate of pathogenic fungi

Mass concentration (mg/mL)	Hypha diameter(cm)	inhibition rate (%)	Significance level	Virulence regression equation	correlation coefficient	EC_{50} (mg/mL)
0	88.00	-	-	Y = 1.385X-1.102	0.979	6.249
10	31.33	59.66±2.34	e			
20	35.50	64.39±0.61	d			
30	27.50	68.75±1.25	c			
40	23.17	73.67±1.40	b			
50	12.67	82.43±0.86	a			

Table 3. The effect of different concentrations Coptis water extract on spore growth

mass concentration (mg/mL)	inhibition rate (%)	Significance level	Virulence regression equation	correlation coefficient	EC_{50}(mg/mL)
50	77.50±0.530	a	Y = 1.275X-1.167	0.989	8.236
40	69.16±0.548	b			
30	64.22±0.282	c			
20	59.25±0.450	d			
10	55.40±0.271	e			

4. Discussion and Conclusion

This study identified that strain FR-1 induces typical leaf spot symptoms in Polygonatum leaves, consistent with the disease manifestations observed in Polygonatum cultivation bases in Laifeng County, Enshi City, Hubei Province. As a significant plant pathogenic fungus, Fusarium incarnatumcan cause various tissue pathologies, including stem rot, leaf blight, and seedling damping-off. Multi-locus phylogenetic analysis confirmed that strain FR-1 isolated from Laifeng County belongs to F. incarnatum.

Following pathogen identification, the inhibitory effects of nine plant aqueous extracts on F. incarnatum were tested using the growth rate method. Plate assay results demonstrated that at 50 mg/mL, Berberis julianae (Juliana barberry) and Coptis chinensis (Chinese goldthread) exhibited the highest inhibition rates of 81.89% ± 0.56% and 82.43% ± 0.86%, respectively, with other extracts showing inhibition rates below 70%. Subsequent virulence assays focused on Coptis chinensis aqueous extract, revealing an EC50 value of 6.249 mg/mL for mycelial growth inhibition and 8.236 mg/mL for spore germination inhibition.

Numerous studies have reported the antifungal potential of plant extracts against Fusariumspecies. Neela et al[13]. demonstrated that extracts from pepper, guava, papaya, moringa, mimosa, periwinkle, violet, and Andrographis paniculata partially inhibit Fusarium oxysporummycelial growth. Kursa et al[14]. evaluated leaf extracts of Achillea millefolium L. (yarrow), Tanacetum vulgare L. (tansy), Salvia officinalis L. (sage), and Artemisia absinthium L. (wormwood) against Fusarium incarnatum-equiseti species complex (major cereal pathogens). At 5%, 10%, and 20% concentrations, these extracts suppressed mycelial growth in a species- and concentration-dependent manner. Sage (S20) and tansy (T20) extracts at 20% concentration showed strong inhibition (83.53% and 72.58%, respectively), while wormwood (W20) and yarrow (Y20) extracts achieved moderate inhibition (63.82% and 67.57%, respectively).

This study not only validates the antimicrobial properties of Coptis chinensis but also provides scientific evidence for its potential application as a botanical fungicide. Future efforts to optimize extraction methods and application strategies could position Coptis chinensis aqueous extract as an efficient and safe phytoprotective agent for agricultural disease management.

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