Postprint: Identification and Inhibitory Effects of Bletilla striata Root Rot Pathogen

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Abstract

To identify the pathogen causing Bletilla striata tuber rot and screen Chinese medicinal herb extracts that inhibit the pathogen, this study employed conventional tissue isolation methods to isolate the pathogen, identified the pathogenic strains through morphological and molecular biological techniques, and observed the antifungal activity of seven Chinese medicinal herb extracts against the pathogen. The results showed: (1) A total of 14 fungal strains and 4 bacterial strains were isolated from diseased leaves, leaf sheaths, and tubers. Pathogenicity tests conducted both indoors and outdoors demonstrated that strain GF-1 produced symptoms consistent with those observed in the field, with a pathogenicity rate of 100%. (2) Based on morphological identification, strain GF-1 was identified as a pathogen belonging to the genus Epicoccum. The colony was white, fluffy, and circular; hyphae were prostrate, growing outward and upward, aerial, colorless, septate, and branched, and produced conidia and chlamydospores. (3) The ITS sequence of strain GF-1 (full length 522 bp) showed the highest similarity (99.62%) to the sequence of Epicoccum sorghinum (MN493119.1) from sugarcane deposited in GenBank, and 98.88% similarity to the previously reported E. sorghinum (MF948994.1) causing Bletilla striata leaf spot. (4) Culture media supplemented with 0.1~0.2 g · mL-1 of extracts from seven Chinese medicinal herbs including Cyclocarya paliurus could completely inhibit the growth of GF-1 colonies; when the culture medium contained 0.05 g · mL-1 of extracts, cinnamon and clove extracts could still completely inhibit colony growth. In summary, it is concluded that the pathogen causing Bletilla striata root rot is Epicoccum sorghinum, and culture media supplemented with 0.1~0.2 g · mL-1 of extracts from seven Chinese medicinal herbs including Cyclocarya paliurus can completely inhibit the growth of the pathogen.

Full Text

Identification and Inhibitory Effect of Pathogens Causing Tuber Rot of *Bletilla striata*

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Abstract

To identify the pathogens causing tuber rot in *Bletilla striata* and evaluate the inhibitory effects of herbal extracts on these pathogens, we isolated pathogens using conventional tissue isolation methods and identified pathogenic strains through morphological and molecular biological techniques. We also examined the antimicrobial effects of seven herbal extracts. The results showed: (1) A total of 14 fungal and 4 bacterial strains were isolated from diseased leaves, leaf sheaths, and tubers. Pathogenicity tests both in vitro and in vivo demonstrated that strain GF-1 produced symptoms consistent with field observations, with a pathogenicity rate of 100%. (2) Morphological identification revealed GF-1 as an *Epicoccum* pathogen, forming white, fluffy, circular colonies with aerial, colorless, septate, branched hyphae, and producing both conidia and chlamydospores. (3) The ITS sequence of strain GF-1 (522 bp) showed highest similarity (99.62%) to *Epicoccum sorghinum* (MN493119.1) from sugarcane in GenBank, and 98.88% similarity to the reported *E. sorghinum* (MF948994.1) causing leaf spot in *B. striata*. (4) Seven herbal extracts, including *Cyclocarya paliurus*, at concentrations of 0.1–0.2 $g \cdot mL^{-1}$ completely inhibited GF-1 colony growth in culture medium. At 0.05 $g \cdot mL^{-1}$, cinnamon and clove extracts still achieved complete inhibition. In summary, the pathogen causing tuber rot in *Bletilla striata* was identified as *Epicoccum sorghinum*, and seven herbal extracts at 0.1–0.2 $g \cdot mL^{-1}$ can completely inhibit its growth.

Keywords: *Bletilla striata*, tuber rot, pathogen isolation and identification, *Epicoccum sorghinum*, herbal extracts

Introduction

Bletilla striata, a perennial herb in the Orchidaceae family, is known by various names including Baiji, Lianjicao, Lizhizi, and Gangen. The genus *Bletilla* comprises six species worldwide, all distributed in Asia, with four species found in

China: *B. striata*, *B. formosana*, *B. sinensis*, and *B. ochracea* (Flora of China, 1999). *Bletilla striata* possesses hemostatic, lung-tonifying, tissue-regenerating, and pain-relieving properties, and is used to treat conditions such as hemoptysis, hematemesis, chronic gastric ulcers, tumors, traumatic bleeding, carbuncles, and skin fissures (Chinese Pharmacopoeia Commission, 2020). With its elegant foliage, large colorful flowers, and extended flowering period, it is also valued in landscape design for flower beds, borders, and mass plantings (Shi, 2010; Zhu et al., 2020). Additionally, *B. striata* serves industrial purposes in adhesives, silk sizing, facial masks, coatings, and wine production (Liu et al., 2005; Gouvg et al., 2009). Thus, it is a versatile plant with medicinal, ornamental, and industrial value.

In recent years, increasing market demand has expanded cultivation scale, but various diseases have frequently emerged, including leaf spot (Ke et al., 2018; Zhou et al., 2018), brown leaf spot, and rust (Song, 2019), severely affecting yield, quality, and ornamental value. Tuber rot is a common disease in *B. striata*, typically occurring from June to August, characterized by brown lesions on underground tubers that become dark brown and soft-rot internally with a foul odor. The root vascular system is destroyed, losing water transport capacity. Above-ground symptoms begin as brown, dried, elongated lesions on stems, leaf bases, and leaf sheaths with yellowish halos, eventually causing complete wilting and death. Diseased plants can be easily pulled from the soil with the roots attached, and affected tubers become lignified and fibrous. The overall incidence is approximately 15%, causing significant damage that severely impacts medicinal quality and yield. This disease is easily confused with leaf spot (Zhou et al., 2018). Current reports indicate that *Fusarium oxysporum* and *F. solani* are the main pathogens causing *B. striata* tuber rot (Sun et al., 2013; Song, 2019), though these studies did not report above-ground symptoms. Therefore, it remains unclear whether the pathogen causing tuber rot in Guangxi is consistent with those causing leaf spot or previously reported tuber rot pathogens. Herbal extracts, characterized by low pollution, toxicity, and residue, are widely used for disease control in medicinal plants; for example, extracts of *Houpoea officinalis* and *Cnidium monnieri* effectively control root rot in astragalus and schisandra (Liu et al., 2009; Ma et al., 2010), but their efficacy against *B. striata* diseases requires verification. This study investigated tuber rot in *B. striata* by isolating pathogens from different tissues using conventional methods, identifying them through morphological and molecular techniques, and determining the inhibitory effects of herbal extracts. The objectives were to definitively identify the pathogen causing tuber rot in Guangxi and screen effective herbal extracts to provide theoretical guidance for disease management.

Materials and Methods

1.2.1 Pathogen Isolation

In June 2021, diseased *B. striata* plants were collected from Yanshan District, Guilin City (longitude 110.309, latitude 25.0604), initially diagnosed as either leaf spot or tuber rot. The disease typically occurs from mid-June to early August with approximately 15% incidence. Initially, above-ground symptoms are absent while underground roots and tubers develop brown lesions; cut tubers show dark brown soft rot with a foul odor. As the disease progresses and one-third to one-half of the tuber rots, the root system completely decays, above-ground stems turn brown, and irregular lesions appear on leaf tips and bases with dark brown centers and yellowish-brown to light yellow margins, eventually causing wilting. Diseased stems can be easily pulled from the soil [Figure 1: see original paper].

Pathogen isolation followed conventional tissue isolation methods (Fang, 1998). Diseased leaves, leaf sheaths, and underground tubers were collected from infected plants, washed clean, and cut into 5 mm × 5 mm pieces at the junction of healthy and diseased tissue. These pieces were surface-sterilized with 75% ethanol for 30 seconds, then 0.1% mercuric chloride solution for 60 seconds, rinsed with sterile water, air-dried, and placed on PDA medium. Each Petri dish (Φ = 90 mm) contained 3–4 tissue pieces and was incubated at 25°C for 3 days, after which isolates were observed and purified. Pure cultures were obtained using the streak plate method, where mycelium was picked with an inoculation loop and streaked on PDA plates, numbered, and incubated at 25°C until single colonies appeared.

1.2.2 Pathogenicity Testing

(1) Inoculation in controlled conditions: Three-year-old healthy tissue-cultured *B. striata* plants were brought to the laboratory for wound inoculation. Leaves and tubers were rinsed with sterile water, surface moisture absorbed with sterile filter paper, and inoculated in a laminar flow hood. The procedure involved gently scraping the abaxial leaf surface with a scalpel (approximately 5 mm × 5 mm), while tubers were stab-inoculated. Mycelial plugs (5 mm diameter) were taken from the colony edge using a cork borer and placed mycelium-side down on wounds. Healthy *B. striata* leaves and tubers inoculated with PDA plugs served as controls. Each leaf received 3 wounds (3 replicates), and each tuber was cut longitudinally with one wound per half (3 replicates). Samples were placed in trays with moist absorbent paper for humidity maintenance. Disease incidence was recorded after 6 days, calculated as: incidence = (number of infected sites / total inoculated sites) × 100%.

(2) Field inoculation: Strains showing high pathogenicity in controlled conditions were further tested in the field. Healthy plant leaves were wounded on the adaxial surface with a scalpel (approximately 5 mm × 5 mm). Mycelial plugs (5 mm diameter) from actively growing cultures were placed mycelium-side down

on wounds and secured with transparent tape; sterile PDA plugs served as controls. Each treatment used 3 plants with 1–2 leaves each, and 3 plugs per leaf (9 inoculation sites total). Plants were covered with plastic bags for 24 hours, and disease incidence was recorded after 6 days using the same formula.

(3) Re-isolation of pathogens: Pathogens were re-isolated from diseased tissue from field inoculations to verify Koch's postulates, using the same method described in section 1.2.1.

1.2.3 Morphological Identification

For the pathogenic strain from field inoculations, actively growing colonies were selected and examined using the slide culture method on PDA medium in disposable Petri dishes at 25°C. After 3–5 days, mycelial and conidial morphology were observed and photographed using a Leica DM2500 upright microscope. Conidial dimensions (length and width) and hyphal diameter were measured. Literature was consulted for preliminary identification.

1.2.4 Molecular Identification

Mycelium was scraped from cultures, cell walls disrupted using liquid nitrogen grinding, and DNA extracted using the CTAB method. The fungal universal primer pair ITS1/ITS4 was used for PCR amplification, and products were verified by 1% agarose gel electrophoresis. PCR product purification and sequencing were performed by Wuhan Qingke Innovation Biotechnology Co., Ltd. The obtained sequences were subjected to BLAST analysis against GenBank (http://www.ncbi.nlm.nih.gov), aligned using Vector NTI Advance 11, and a phylogenetic tree was constructed using UPGMA analysis in MEGA 5.0. Molecular identification was based on homology analysis (Li et al., 2002).

1.2.5 Inhibitory Effect of Herbal Extracts on the Pathogen

Seven test herbs were dried at 60°C, powdered, and sieved through a 40-mesh screen (0.45 mm aperture), then sealed and stored at -20°C. Ethanol was used as solvent for triple shaking extraction (Han et al., 2002). The three filtrates were combined and concentrated under reduced pressure to a final concentration of 1 $g \cdot mL^{-1}$, sealed, and stored at 4°C. The mycelial growth rate method (Wu, 1988) was used to determine inhibitory effects. Herbal extracts at 1 $g \cdot mL^{-1}$ were added to PDA medium, mixed thoroughly, and prepared at concentrations of 0.05, 0.1, and 0.2 $g \cdot mL^{-1}$ based on previous studies (Ma et al., 2010; Liu et al., 2019). PDA with sterile water served as blank control. Mycelial plugs (5 mm diameter) from colony edges were inoculated onto medicated plates with three replicates per treatment and incubated at 25°C. Colony diameters were measured every 24 hours after 48 hours using the cross method for seven consecutive recordings. Inhibition rate was calculated as: inhibition rate (%) = [(control colony diameter - treatment colony diameter) / (control colony diameter - plug diameter 5.0 mm)] × 100%.

Results

2.1 Pathogen Isolation

Based on field symptoms, the disease was preliminarily diagnosed as tuber rot or leaf spot. Pathogens were isolated from the junction of healthy and diseased tissue of leaves, leaf sheaths, and tubers from infected plants, yielding 18 isolates (14 fungi, 4 bacteria). Specifically, 2 fungi and 1 bacterium were isolated from leaves; 9 fungi and 2 bacteria from leaf sheaths; and 3 fungi and 1 bacterium from tubers. This indicated relatively diverse and complex microbial communities, though no molds were isolated from any tissue. Fungi were designated GF-1 through GF-14, and bacteria GB-1 through GB-4. Notably, strains GF-1 and GF-7 were isolated from all three tissue types. [Figure 2: see original paper]A shows some of the isolated strains.

2.2 Pathogenicity Testing

All 18 isolates were inoculated in controlled conditions. After 3 days, fungi GF-1, GF-4, GF-5, and GF-7 showed obvious disease symptoms [Figure 2: see original paper]B. Disease incidence was recorded after 6 days (Table 1). Strains with high incidence in controlled conditions (GF-1, GF-3, GF-4, GF-5, and GF-7) were further tested in field inoculations. Only GF-1 produced black lesions with scorched appearance and central desiccation [Figure 3: see original paper]A, matching field symptoms. In controlled inoculations, GF-1 caused distinct yellow halos [Figure 2: see original paper]B:GF-1 and tuber rot upon cutting [Figure 2: see original paper]C, consistent with field-collected disease symptoms. Other strains showed no lesion expansion or desiccation without disease symptoms [Figure 3: see original paper]B-E. Pathogens re-isolated from GF-1-inoculated diseased tissue were identical to the original inoculum, fulfilling Koch' s postulates and confirming GF-1 as the causal agent.

2.3 Morphological Identification

On PDA medium at 25°C, strain GF-1 produced white, fluffy colonies after 5 days. Mycelia were aerial, prostrate, and grew outward and upward, forming circular, fluffy, white colonies with brown to dark brown centers and greenish-brown to yellowish-brown margins on the reverse side [Figure 4: see original paper]:A,B. Hyphae were colorless, fast-growing, septate, 3.2–6.9 m in diameter, with branched conidiophores. Chlamydospores were rectangular or elliptical, measuring (6.5–9.6) m × (6.5–7.1) m. Conidia were solitary, ovate, approximately (5.3–8.9) m × (1.4–3.6) m [Figure 4: see original paper]:C,D. Colony diameter reached 5.2–7.0 cm after 5 days (Φ = 90 mm Petri dish). While no similar species was found in Chinese mycological records, literature review (Aveskamp et al., 2010) indicated GF-1 belonged to the genus *Epicoccum*.

2.4 ITS Sequence Phylogenetic Analysis

The ITS rDNA PCR product of strain GF-1 was sequenced, edited, and assembled into a 552 bp sequence. BLAST analysis against GenBank revealed that GF-1' s ITS sequence (TSB1G643013) showed highest similarity (99.62%) to *E. sorghinum* (MN493119.1) from sugarcane, and 98.88% similarity to *E. sorghinum* (MF948994.1) isolated from *B. striata*. Phylogenetic tree construction using MEGA 5.1 grouped GF-1 with *E. sorghinum* from sugarcane in the same clade, showing the closest relationship, while the *E. sorghinum* from *B. striata* was more distantly related, clustering with tobacco *E. sorghinum* (KJ767080.1). This may reflect natural variation in the pathogen, indicating some divergence in molecular evolution [Figure 5: see original paper].

2.5 Inhibitory Effect of Herbal Extracts on the Pathogen

The inhibitory effects of herbal extracts are shown in [Figure 6: see original paper] and Table 2. Compared to the sterile water control, all seven herbal extracts at 0.1 and 0.2 $g \cdot mL^{-1}$ completely inhibited GF-1 colony growth. At the lower concentration of 0.05 $g \cdot mL^{-1}$, cinnamon and clove extracts maintained complete inhibition, while star anise extract achieved 99.6% inhibition. However, at this concentration, *Cyclocarya paliurus*, *B. striata*, *Houpoea officinalis*, and *Cnidium monnieri* extracts did not achieve complete inhibition, with colony diameters of 13.8 mm, 25.6 mm, 6.6 mm, and 9.9 mm, and inhibition rates of 82.4%, 58.8%, 96.8%, and 90.2%, respectively.

Discussion

3.1 Pathogen Identification of *Bletilla striata* Tuber Rot

Tuber rot is a disease caused by various pathogens including *Pythium*, *Fusarium*, and *Phytophthora* species, characterized by root decay that impairs water and nutrient absorption, leading to yellowing, wilting, and plant death. In addition to these typical features, the tuber rot observed in this study also produced brown, elongated lesions on stem bases and leaf sheaths, similar to symptoms reported for tuber rot caused by *Rhizoctonia* spp. in *B. striata* (Zeng et al., 2012). Tuber rot has high incidence and severely impacts medicinal yield and quality, becoming a major production constraint for many medicinal plants including *Panax notoginseng*, *Atractylodes macrocephala*, and *Astragalus membranaceus*, with *Fusarium* species as primary pathogens (Shen et al., 2014). Previous studies identified *F. oxysporum* and *F. solani* as causal agents of *B. striata* tuber rot in Yunnan (Sun et al., 2013) and Guizhou (Song, 2019), respectively, though these reports did not describe above-ground symptoms. Through field investigation, pathogen isolation, and combined morphological and molecular identification, this study identified the pathogen causing tuber rot in Guilin, Guangxi as *Epicoccum sorghinum*, differing from previous reports. This pathogen was

identified relatively recently, with domestic reports emerging after 2018 documenting its role in leaf spot, brown spot, and ring spot diseases in various horticultural crops (Yu et al., 2019; Laurel et al., 2021). *E. sorghinum* causing leaf spot in *B. striata* produces brown lesions with yellow halos (Zhou et al., 2018), whereas the strain in this study initially caused underground tuber rot with foul odor, later producing elongated gray-brown lesions on stem bases and leaf sheaths with desiccated centers and yellow halos. The molecular phylogenetic analysis placed these strains in different clades, suggesting they may represent different physiological races causing distinct diseases.

3.2 Inhibitory Effect of Herbal Extracts on *Bletilla striata* Tuber Rot Pathogen

Chemical pesticides are commonly used for medicinal plant tuber rot control due to their rapid efficacy, convenient application, and low cost, but they often cause excessive pesticide residues that affect medicinal quality. Botanical fungicides are safer and non-toxic but slower acting and more susceptible to environmental influences (Mu et al., 2014). Eugenol, the main component of clove extract, exerts antimicrobial effects by disrupting microbial cell membrane structure (Yu, 2020). The primary chemical constituents of *Asarum heterotropoides* are terpenoids with immunomodulatory and antibacterial properties, while osthole from *Cnidium monnieri* shows good rapid efficacy (Ma et al., 2010; Xiao, 2019). Liu et al. (2009) found that magnolol, the active component of *Houpoea officinalis*, has anti-inflammatory and antibacterial effects. Zhao et al. (2011) reported that acetone solutions of the same substance showed significantly better antibacterial activity than aqueous solutions. *E. sorghinum* secretes a toxin similar to phenylacetic acid with strong pathogenicity (Zhu, 2018) and has a broad host range, infecting plants in Cucurbitaceae, Fabaceae, Brassicaceae, Salicaceae, Poaceae, Portulacaceae, Liliaceae, and Araceae families (Li et al., 2020). However, no biological control studies for this pathogen have been reported. This study extracted seven herbal medicines using ethanol and evaluated their inhibitory effects against the pathogen. Extracts of *Cyclocarya paliurus*, *B. striata*, *H. officinalis*, star anise, cinnamon, *C. monnieri*, and clove at 0.1 and 0.2 $g \cdot mL^{-1}$ completely inhibited *E. sorghinum* growth. At 0.05 $g \cdot mL^{-1}$, star anise, cinnamon, and clove extracts also achieved nearly complete inhibition (99.6–100%). These herbal extracts demonstrated significant in vitro inhibitory activity, though their field efficacy and optimal application concentrations require further investigation.

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Note: Figure translations are in progress. See original paper for figures.