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Post-print of Pathogen Identification, Biological Characteristics, and In Vitro Fungicide Screening for Guangxi Curcuma zedoaria Leaf Blight

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Abstract

To identify the pathogen causing leaf blight of Guangxi Curcuma kwangsiensis in Qinzhou City, Guangxi, investigate its biological characteristics, and screen effective control agents, this study employed conventional tissue isolation for pathogen isolation from diseased leaves, conducted pathogenicity determination based on Koch's postulates, and determined its taxonomic position through morphological characterization combined with ITS and TUB gene sequence analysis. Simultaneously, the biological characteristics of the pathogen and its sensitivity to four fungicides were investigated using the mycelial growth rate method. The results demonstrated: (1) Based on colony morphology, spore morphology, and multi-gene concatenated phylogenetic analysis, the pathogen causing leaf blight of Guangxi C. kwangsiensis was identified as the Diaporthe fungus Diaporthe phaseolorum. (2) The optimal culture medium for this pathogen was PDA, with glucose and peptone serving as the best carbon and nitrogen sources, respectively; temperatures of 25~28 ℃ were conducive to mycelial growth, with a lethal temperature of 56 ℃; the optimal pH range of 5~7 favored mycelial growth; full light exposure was beneficial for mycelial growth. (3) Toxicity assay results indicated that all four tested fungicides exhibited strong inhibitory activity against the leaf blight pathogen of Guangxi C. kwangsiensis, among which 250 $g \cdot L^{-1}$ pyraclostrobin EC and 75% trifloxystrobin · tebuconazole WG demonstrated superior antifungal efficacy, with EC_{50} values of 0.0550 and 0.1216 $g \cdot mL^{-1}$, respectively. In summary, the leaf blight pathogen of Guangxi C. kwangsiensis is the Diaporthe fungus D. phaseolorum, whose mycelial growth is significantly influenced by temperature, light, pH, and carbon/nitrogen source conditions. 250 $g \cdot L^{-1}$ pyraclostrobin EC and 75% trifloxystrobin · tebuconazole WG can serve as candidate agents for leaf blight control. These findings provide theoretical guidance for the effective prevention and management of leaf blight in Guangxi C. kwangsiensis.

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Full Text

Identification, Biological Characteristics, and Indoor Fungicide Screening of the Pathogen Causing Leaf Blight of *Curcuma kwangsiensis* in Guangxi

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Abstract

To identify the pathogen causing leaf blight of *Curcuma kwangsiensis* in Qinzhou, Guangxi, investigate its biological characteristics, and screen effective fungicides, we isolated pathogens from infected leaves using conventional tissue separation methods. Pathogenicity was confirmed based on Koch's postulates, and the taxonomic status was determined through morphological characterization combined with ITS and TUB gene sequence analysis. Biological characteristics and sensitivity to four fungicides were evaluated using the mycelial growth rate method. The results showed that: (1) Based on colony and spore morphology characteristics and multi-gene phylogenetic analysis, the pathogen was identified as *Diaporthe phaseolorum* (Diaporthaceae). (2) The optimal culture medium was PDA, with glucose and peptone serving as the best carbon and nitrogen sources, respectively. The optimal temperature range for mycelial growth was 25–28 °C, with a lethal temperature of 56 °C. The optimal pH range was 5–7, and full-light conditions promoted mycelial growth. (3) All four tested fungicides exhibited strong inhibitory activity against the pathogen, with 250 $g \cdot L^{-1}$ pyraclostrobin EC and 75% trifloxystrobin · tebuconazole WG showing the best efficacy, with EC_{50} values of 0.0550 and 0.1216 $\mu g \cdot mL^{-1}$, respectively. In conclusion, the pathogen causing leaf blight of *C. kwangsiensis* is *D. phaseolorum*, whose mycelial growth is significantly affected by temperature, light, pH, and carbon/nitrogen sources. The fungicides 250 $g \cdot L^{-1}$ pyraclostrobin EC and 75% trifloxystrobin · tebuconazole WG are promising candidates for field control. These findings provide theoretical guidance for effective management of leaf blight in *C. kwangsiensis*.

Keywords: *Curcuma kwangsiensis*; leaf blight; pathogen identification; biological characteristics; fungicide screening

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Curcuma kwangsiensis, belonging to the family Zingiberaceae and genus *Cur-*

cuma L., is known as "Mao Ezhu" or "Gui Ezhu" and is one of the botanical sources of the traditional Chinese medicine "Ezhu" alongside *C. phaeocaulis* and *C. wenyujin* (Flora of China, 1981; Chinese Pharmacopoeia, 2020). As a recorded species in successive editions of the Chinese Pharmacopoeia and one of the "Top Ten" authentic medicinal materials from Guangxi, *C. kwangsiensis* possesses therapeutic effects such as promoting qi circulation, breaking blood stasis, eliminating accumulation, relieving pain, and clearing heart heat. Modern research has demonstrated that its volatile oils and curcuminoids exhibit antitumor, anticoagulant, and antiplatelet aggregation activities (Li et al., 2021). With increasing demand driven by expanding medicinal applications, cultivation areas in Guangxi have grown from 2,333 ha in 2022 to 3,333 ha in 2023, primarily distributed across Lingshan County in Qinzhou, Bobai County in Yulin, Babu District in Hezhou, and Long'an County in Nanning. Lingshan County, the authentic core production area, accounts for 40% of the total cultivation area in Guangxi.

Leaf diseases represent a significant constraint on *C. kwangsiensis* production. In Luwu Town, Lingshan County, leaf blight occurred severely during 2022–2023, with incidence rates of 30–50% and up to 80% in heavily affected fields. Field symptoms typically begin as yellow spots at leaf tips, margins, or centers, which progressively expand toward the midrib, causing extensive yellowing and eventual whole-plant wilting and death. Previous studies on *C. kwangsiensis* diseases have been limited. Jiang et al. (2016) reported a leaf spot disease caused by *Phomopsis* sp. in Long'an County, which produces distinct V-shaped lesions with brown margins, dark brown centers, and visible black pycnidia—symptoms that differ from those observed in our study despite superficial similarities. Wang (2023) also described leaf blight caused by a *Diaporthe* species with comparable symptoms but identified a different pathogen species. Studies on other *Curcuma* species include Li (2021) on *Neopestalotiopsis asiatica* causing leaf blight in *C. phaeocaulis*, and Feng (2012) and Ma (2018) on leaf spot diseases in *C. wenyujin* caused by *Colletotrichum* species. While these studies employed morphological identification combined with multi-gene phylogenetic analysis, previous work on *C. kwangsiensis* only identified pathogens to the genus level via ITS sequencing and used samples from Long'an County rather than the core production area of Lingshan County. Whether the pathogen causing leaf blight in Lingshan, its biological characteristics, and effective fungicides align with previous reports remains unclear.

Given these knowledge gaps, identifying the pathogen from the main production area of Lingshan, screening effective control agents, and characterizing its biological properties are essential for developing targeted management strategies. This study isolated and purified the leaf blight pathogen from Lingshan County, Qinzhou, confirmed pathogenicity, determined its taxonomic status through morphological and multi-gene sequence analysis, characterized its biological traits, and evaluated the inhibitory activity of four fungicides to provide a theoretical basis for field management of this disease.

1.1 Experimental Materials

Diseased leaf samples were collected in July 2022 from Lingshan County, Qinzhou, Guangxi (109°17 27.60 E, 22°24 59.36 N) from one-year-old *C. kwangsiensis* plants established in March. Culture media including potato dextrose agar (PDA), potato sucrose agar (PSA), oatmeal agar (OMA), cornmeal agar (CMA), and Sabouraud dextrose agar (SDA) were prepared following protocols described by Cao (2021). The four fungicides tested for inhibitory activity are listed in Table 1 .

1.2 Methods

1.2.1 Pathogen Isolation and Purification Pathogen isolation followed conventional tissue separation methods described by Fang (1998). Diseased tissue sections (5 mm × 5 mm) were excised from the lesion margins, surface-sterilized in 75% ethanol for 30 s and 2.5% sodium hypochlorite for 3 min, rinsed three times in sterile water, air-dried, and transferred to PDA plates. After incubation at 28 °C, emerging mycelial tips were subcultured onto fresh PDA plates and purified through five days of culture.

1.2.2 Pathogenicity Testing Pathogenicity was evaluated using detached leaf inoculation. Healthy *C. kwangsiensis* leaves were washed with tap water, air-dried, surface-sterilized with 75% ethanol, and wounded symmetrically on both sides of the midrib using a sterile needle. Mycelial plugs (8 mm diameter) were placed on the wounds with the mycelial side contacting the leaf surface, with PDA plugs serving as controls. Inoculated leaves were incubated at 28 °C with three replicates per treatment. Disease development was recorded daily, and the pathogen was re-isolated from symptomatic tissue to fulfill Koch's postulates (Xie, 2006).

1.2.3 Pathogen Identification Colony morphology was documented following Fang (1998), and preliminary identification was performed using Wei (1979) and the Index Fungorum database (http://www.indexfungorum.org). For molecular identification, genomic DNA was extracted using the MightyAmp DNA Polymerase Ver.3 kit (Takara Bio Inc., Japan, cat. no. R076A). The internal transcribed spacer (ITS) region and β-tubulin (TUB) gene were amplified using primers ITS1/ITS4 (White et al., 1990) and T1/Bt-2b (Dong et al., 2021), respectively. PCR reactions contained 25 L 2×MightyAmp Buffer, 5 L 10×Additive for High Specificity, 1.5 L each primer (15 pmol), and template DNA in a 50 L volume. Cycling conditions were: 98 °C for 2 min; 40 cycles of 98 °C for 10 s, 60 °C for 15 s, 68 °C for 60 s; and final extension at 68 °C for 10 min. PCR products were visualized on 1.0% agarose gels, and positive amplicons were sequenced by Sangon Biotech (Shanghai). Sequences were

BLAST-searched against NCBI GenBank, and related sequences were downloaded for phylogenetic analysis using MEGA 7.0 software with the neighbor-joining method (Kumar et al., 2016). A multi-gene phylogenetic tree was constructed using combined ITS and TUB sequences (Table 2).

1.2.4 Biological Characteristics of the Pathogen Biological characteristics were evaluated using strain E-10 with modifications to protocols by Cao (2021) and Dan (2023). Six variables were tested: culture medium, temperature, light, pH, carbon source, and nitrogen source.

(1) Effect of Culture Media: Mycelial plugs (6 mm diameter) were inoculated onto the center of PDA, PSA, OMA, CMA, and SDA plates (one plug per plate, three replicates) and incubated at 28 °C in darkness for 5 days. Colony diameters were measured using the cross-cross method to determine the optimal medium.

(2) Effects of Temperature, Light, and pH: Mycelial plugs were cultured on PDA at temperatures of 5, 10, 15, 20, 25, 28, 30, 35, and 40 °C in darkness. For light effects, cultures were exposed to three photoperiods (L/D = 24 h/0 h, 0 h/24 h, and 12 h/12 h) at 28 °C. For pH effects, PDA was adjusted to pH 2–10 using 1 $mol \cdot L^{-1}$ HCl or NaOH. All treatments had three replicates, and colony diameters were measured after 5 days.

(3) Lethal Temperature: Lethal temperature was determined following methods by Cao (2021) and Dan (2023).

(4) Effects of Carbon and Nitrogen Sources: For carbon source tests, glucose in PDA was replaced with equal amounts of sucrose, α-lactose, soluble starch, mannitol, or inositol. For nitrogen source tests, sodium nitrate in Czapek medium was replaced with ammonium sulfate, cysteine, peptone, or potassium nitrate. One mycelial plug per plate was incubated at 28 °C in darkness for 5 days, after which colony diameters were measured.

1.2.5 Indoor Fungicide Screening Mycelial growth rate method was used to determine toxicity. Five-day-old mycelial plugs (6 mm) were transferred to PDA medium containing serial dilutions of each fungicide (prepared by adding stock solutions to molten PDA cooled to ~50 °C). Control plates received no fungicide. After 6 days of incubation at 28 °C (three replicates per treatment), colony diameters were measured and inhibition rates were calculated as:

Inhibition rate = [(Control colony diameter − Plug diameter) − (Treated colony diameter − Plug diameter)] / (Control colony diameter − Plug diameter) × 100%

EC_{50} values were calculated using SPSS 19.0, along with slope ± standard error, chi-square values, degrees of freedom, and P-values.

1.2.6 Statistical Analysis All data were analyzed using SPSS 19.0 with one-way ANOVA. Multiple comparisons were performed using LSD and Duncan's

new multiple range tests, with different lowercase letters indicating significant differences ($P < 0.05$). Biological characteristic data were visualized using Origin 2018 software.

2.1 Disease Symptoms and Pathogenicity Determination

Field surveys revealed that leaf blight affects both old and young leaves of *C. kwangsiensis*, typically initiating at leaf tips or margins with incidence rates of 30–50%. Early symptoms appear as light yellow spots surrounded by yellow halos, which progressively expand inward from the leaf margin, eventually causing whole-leaf yellowing and plant death (Fig. 1 [Figure 1: see original paper]: A, B). Eight strains were isolated from five diseased leaf samples, with white, fluffy colony morphology. Strain E-10, showing the highest isolation frequency (87.5%), was selected for pathogenicity testing. Inoculation of healthy leaves with strain E-10 produced water-soaked lesions within 48 h, distinct yellow halos by 4 days, and complete yellowing by 6 days, mimicking natural field symptoms (Fig. 1: C–E). Control leaves remained symptomless (Fig. 1: F). Re-isolation from symptomatic tissue yielded colonies identical to the inoculated strain, fulfilling Koch's postulates and confirming strain E-10 as the pathogen.

2.2 Morphological Identification

Colonies of strain E-10 exhibited white, fluffy, radial mycelial growth on the upper surface and beige, non-zonate reverse sides (Fig. 2 [Figure 2: see original paper]: A, B). Microscopic examination revealed α-conidia that were oblong with flattened ends, unicellular, measuring (3.05–3.35) m × (0.81–1.16) m (Fig. 2: C). β-conidia were filamentous with a hooked, pointed tip and a flat, rounded base, measuring (16.87–24.98) m × (0.89–1.68) m (Fig. 2: D). These morphological features preliminarily identified strain E-10 as a *Diaporthe* sp. fungus.

2.3 Biological Characteristics

2.3.1 Temperature Effects on Mycelial Growth Strain E-10 grew at temperatures from 10 to 35 °C, with optimal growth at 25–30 °C. No growth occurred at 5 °C or 40 °C. After 5 days, colony diameters reached 8.50 cm and 8.32 cm at 25 °C and 28 °C, respectively, with no significant difference between these temperatures ($P > 0.05$) but significant differences from other treatments ($P < 0.05$). Mycelial growth was sensitive to temperatures above 30 °C. The lethal temperature was determined to be 56 °C for 20 min, as mycelial growth ceased at this temperature.

2.3.2 Light Effects on Mycelial Growth Strain E-10 grew under all three light regimes (L/D = 24 h/0 h, 12 h/12 h, and 0 h/24 h), producing yellow pigments in each case. Colony diameters were 8.35 cm, 6.2 cm, and 4.97 cm, respectively, with full light (24 h/0 h) promoting significantly better growth than continuous darkness ($P < 0.05$), indicating a high light requirement for optimal mycelial development.

2.3.3 pH Effects on Mycelial Growth Mycelial growth occurred across pH 2–10, with optimal growth at pH 5–7. Colony diameters at pH 5, 6, and 7 were 5.85 cm, 5.83 cm, and 7.58 cm, respectively, showing no significant differences among them ($P > 0.05$) but significant differences from other pH levels ($P < 0.05$). These results indicate that the pathogen prefers slightly acidic to neutral environments.

2.3.4 Culture Medium Effects on Mycelial Growth Strain E-10 grew on all five tested media, with the fastest growth on PDA (colony diameter 8.45 cm after 5 days) producing dense mycelium. Growth on SDA, PSA, CMA, and OMA was not significantly different ($P > 0.05$), though all supported mycelial development.

2.3.5 Carbon and Nitrogen Source Effects on Mycelial Growth Among seven carbon sources tested, glucose supported the largest colony diameter (4.67 cm), significantly outperforming other sources ($P < 0.05$), followed by α-lactose, maltose, sucrose, inositol, mannitol, and soluble starch (3.47 cm). For nitrogen sources, peptone supported the most vigorous growth (colony diameter 6.77 cm) with dense aerial mycelium, significantly better than other sources ($P < 0.05$). Cysteine could not be utilized, and no growth occurred on the control agar medium. Thus, glucose and peptone were identified as the optimal carbon and nitrogen sources, respectively.

2.4 Molecular Biological Identification

ITS and TUB gene sequencing of strain E-10 yielded fragments of 547 bp and 741 bp, respectively. BLAST analysis revealed 99% homology with *Diaporthe phaseolorum*. Phylogenetic analysis using combined ITS and TUB sequences placed strain E-10 in a clade with *D. phaseolorum* with 99% bootstrap support (Fig. 4 [Figure 4: see original paper]). Based on morphological and molecular evidence, strain E-10 was identified as *Diaporthe phaseolorum*, with GenBank accession numbers OK175678 (ITS) and OK326872 (TUB).

2.5 Indoor Fungicide Screening

All four fungicides inhibited the pathogen, with 250 g·L^{-1} pyraclostrobin EC and 75% trifloxystrobin·tebuconazole WG showing the strongest activity (EC_{50} = 0.0550 and 0.1216 µg·mL^{-1}, respectively) (Table 3). The biological fungicide *Trichoderma harzianum* SC (10 bn·g^{-1}) and 98% oxamiline SP showed moderate activity (EC_{50} = 1.9986 and 4.1736 µg·mL^{-1}, respectively). These results suggest all four fungicides are potential candidates for field control of leaf blight in *C. kwangsiensis*.

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3 Discussion and Conclusion

The genus *Diaporthe* historically employed dual nomenclature for its anamorphic (*Phomopsis*) and teleomorphic stages. However, the 2011 Melbourne nomenclatural meeting adopted the “one fungus, one name” principle, establishing *Diaporthe* as the accepted name due to its priority (Taylor, 2011). *Diaporthe* species infect leaves, stems, and fruits, causing necrosis, leaf spots, cankers, wilting, and dieback, resulting in significant economic losses (Stantos et al., 2009; Manawasinghe et al., 2019; Yu, 2021; Chen, 2020). This study reports the first identification of *D. phaseolorum* causing leaf blight in *C. kwangsiensis*, with symptoms consistent with previous reports on *Curcuma* species (Jiang et al., 2016; Li, 2021; Wang, 2023), though pathogen species and biological characteristics differed, likely due to variations in sampling time, location, and environmental conditions.

Biological characteristics of *D. phaseolorum* have been less studied, with most research focusing on soybean stem diseases (Shen, 2015) and diseases in blueberry and kiwifruit (Li et al., 2017; Thomidis et al., 2019; Wang, 2022). Other *Diaporthe* species show variable biological traits. For example, *Phomopsis asparagi* causing leaf blight in *Dalbergia odorifera* and *P. mangiferae* causing mango branch blight both grow optimally at 25–30 °C and pH 6–7, with no significant light effects (Fu et al., 2017; Chen, 2020), matching our temperature and pH results. *P. amygdali* causing peach branch blight prefers PDA, PSA, and oatmeal media at 20–25 °C, with sucrose and potassium nitrate/alanine as optimal carbon/nitrogen sources and pH 6–7 (Gu et al., 2013), showing some differences from our findings, possibly due to host and geographic variations (He, 2011). Indeed, the same pathogen can exhibit different biological characteristics on different hosts, as demonstrated with *P. eucommicola* causing optimal growth at 22 °C and pH 5.5 on poplar versus 30 °C and pH 7 on *Eucommia* (Zheng et al., 2011). Similarly, *P. vexans* shows variable optimal conditions even on the same host (Ma, 2005; Ye et al., 2021).

Our fungicide screening revealed that pyraclostrobin and trifloxystrobin·tebuconazole, both strobilurin fungicides, exhibited strong toxicity against *D. phaseolorum* by inhibiting mitochondrial respiration. These fungicides demonstrate strong penetrative and systemic activity, providing preventive, protective, cu-

rative, and eradicative effects. Previous studies reported >80% control efficacy of 75% trifloxystrobin · tebuconazole WG and 32.5% benzovindiflupyr · azoxystrobin SC against *Pseudostellaria heterophylla* leaf spot (Wang, 2014). Li (2021) found that 97% azoxystrobin strongly inhibited *C. phaeocaulis* conidial germination ($EC_{50} = 0.0277$ μg · mL^{-1}). Ma (2018) reported strong inhibition of *C. wenyujin* southern blight by pyraclostrobin ($EC_{50} < 1$ μg · mL^{-1}). Tao et al. (2020) observed high sensitivity of *Diaporthe eres* causing leaf blight in *Polygonatum sibiricum* to prochloraz, tebuconazole, and pyraclostrobin. These results align with our findings, confirming that pyraclostrobin and tebuconazole are effective against *Diaporthe* pathogens. Additionally, the biological fungicide *T. harzianum* showed promising efficacy, providing an alternative for sustainable disease management. However, as these results were obtained under controlled conditions, further field validation is required.

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