Identification of Hyperparasitic Fungal Species Associated with Coffee Leaf Rust

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Abstract [**Objectives**] The paper was to elucidate the specific hyperparasitic fungal species that are associated with coffee leaf rust. [**Methods**] Tissue isolation, sample humidification, and three-point inoculation techniques were employed to isolate, culture, and purify the hyperparasitic fungi responsible for coffee leaf rust. The purified strains were identified using traditional morphological techniques and molecular biology methods. [**Results**] Four strains were isolated, specifically BS21 (*Cladosporium cladosporioides*), BS34 (*C. tenuissimum*), BS62 (*C. cladosporioides*), and BS75 (*C. colombiae*). [**Conclusions**] The findings of this research will contribute novel insights into the biological control of coffee leaf rust.

Key words Leaf rust; Hyperparasitic fungi; Isolation; Identification

1 Introduction

Coffee leaf rust is a significant disease affecting coffee crops on a global scale. Statistical data indicate that the annual loss in coffee production attributable to coffee leaf rust ranges from approximately 35% to 50% of total coffee output[1]. This disease consequently leads to economic losses estimated between 1 billion and 2 billion US dollars. Traditionally, the prevention and control of coffee leaf rust have been achieved through three primary methods. (i) The chemical control through the application of systemic fungicides is effective in managing coffee leaf rust[2]. However, it is associated with high costs and presents challenges in achieving optimal economic and ecological benefits. Furthermore, the persistent and repeated use of fungicides contributes to the development of resistance in pathogenic fungi. (ii) The selection and breeding of rust-resistant coffee varieties, such as S288, CIFC 7963, and Sarchimor, have been undertaken and these varieties have been disseminated. However, it is important to note that the quality of the coffee produced from these varieties is suboptimal, resulting in low market prices. (iii) Coffee leaf rust damage can be mitigated through the cultivation of coffee in sunny environments or by decreasing shade in coffee gardens. However, these practices have significant adverse effects on biodiversity[3-4], as well as a detrimental impact on coffee quality, which is characterized by a shorter maturation period and reduced sugar accumulation in comparison to shade-grown coffee. Consequently, the management of coffee leaf rust through biological control represents an effective strategy for mitigating crop disease risks in a sustainable, environmentally friendly, and safe manner.

In natural ecosystems, it is common for the coffee leaf rust fungus, *Hemileia vastatrix*, to be parasitized by other fungal species. Hyperparasitism of coffee leaf rust has been documented in Brazil, Mexico, Puerto Rico, Ethiopia, and Cameroon in Africa[5-8]. The phenotypic inconsistency of traits associated with the hyperparasitic phenomenon of coffee leaf rust across various production regions suggests that the coffee rust pathogen, *H. vastatrix*, is associated with a diverse array of hyperparasitic fungal species. Furthermore, the composition of these hyperparasitic fungi exhibits significant variation among different coffee production regions, highlighting a pronounced feature of regional differentiation. Furthermore, our pest surveys conducted in the coffee-producing regions of Yunnan Province revealed a significant prevalence of hyperparasitism, indicating the presence of local hyperparasitic fungal resources.

Yunnan Province, recognized as the foremost coffee production region in China, has over 80% of its cultivation area dedicated to the rust-resistant variety CIFC 7963. However, with the extension of the planting duration, the rust-resistant properties of this variety are gradually diminishing. Due to the low taste quality and poor efficiency of CIFC 7963, both companies and farmers are increasingly replacing varieties that lack resistance to rust. This trend has led to a rise in rust severity, and rust physiological minor species are evolving at an accelerated rate. Furthermore, the reliance on chemical control methods that utilize systemic fungicides is increasingly at odds with the expanding domestic coffee market[9]. This study aims to elucidate the hyperparasitic fungal species associated with coffee leaf rust by isolating and culturing diseased tissues. The identification of the isolated fungi was conducted through morphological and molecular biological methods. The findings are intended to support the collection and utilization of indigenous hyperparasitic fungal resources related to coffee leaf rust in Yunnan Province, thereby contributing to innovative strategies for the environmentally sustainable prevention and control of coffee leaf rust within the coffee industry in the region.

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2 Materials and methods

2.1 Materials

2.1.1 Test plants. Coffee leaves exhibiting symptoms of leaf rust and hyperparasitism were collected from the Guixia Planting Base of Baoshan Zuoyuan Coffee Co., Ltd., located in the Longyang District of Baoshan City, Yunnan Province [25°29′59″ N, 98°50′26″ E, altitude (1 247 ± 5) m]. These samples were subsequently preserved at the Institute of Tropical and Subtropical Cash Crops, Yunnan Academy of Agricultural Sciences (YAAS).

2.1.2 Test media and reagents. Potato Dextrose Agar (PDA) solid medium was utilized. A column-type fungal genomic DNA extraction kit, 2 × San*Taq* PCR Mix, a DNA molecular weight standard marker, 4S Green Plus nucleic acid dye, and fungal ITS fragment primers (ITS1: TCCGTAGGTGAACCTGCGG/ITS4: TCCTCCGCTTATTGATATGC) were employed. All reagents and primers were procured from Sangon Biotech (Shanghai) Co., Ltd.

2.2 Isolation of hyperparasitic fungi A conventional tissue isolation method was employed to isolate the pathogenic fungi. The procedure involved selecting aecium tissues exhibiting hyperparasitism, which were then cut into 5 mm × 5 mm tissue blocks. These blocks were rapidly sterilized using 75% ethanol for 5 sec, followed by immersion in a 0.1% $HgCl_2$ solution for 1 min. Subsequently, the tissues were thoroughly rinsed with sterile water five times. The sterilized leaf tissues were inoculated onto PDA medium plates and subsequently incubated at a constant temperature of 25 ℃ for a duration of 3 – 5 d. The mycelium was then transferred to a new PDA plate to repeat the purification process three times. The pure colonies were inoculated into PDA test tube slants and subsequently stored at 4 ℃ for prolonged periods after the mycelium had developed across the slants.

2.3 Molecular identification of test strains The total DNA from the test strains was extracted individually utilizing the fungal genomic DNA extraction kit, and subsequently stored at – 20 ℃ for preservation. This total DNA from the fungi served as a template for the amplification of the ITS sequence. The PCR was conducted in a total volume of 50 μL, which comprised 25 μL of 2 × San*Taq* PCR Mix, 1.5 μL of each primer (10 μmol/L), 2 μL of template DNA, and 20 μL of ddH_2O. The PCR amplification conditions were as follows: initial denaturation at 94 ℃ for 3 min, followed by 35 cycles of denaturation at 94 ℃ for 30 sec, annealing at 56 ℃ for 40 sec, and extension at 72 ℃ for 50 sec. A final extension step was performed at 72 ℃ for 10 min, after which the product was stored at 4 ℃. The products amplified by PCR were analyzed using 1% agarose gel electrophoresis. The excised gel was subsequently purified and submitted to Kunming Shuoqin Biotechnology Co., Ltd. for further testing. Sequences obtained from bidirectional mapping were spliced and subsequently submitted to the NCBI database for BLAST comparison utilizing DNAMAN version 8.

3 Results and analysis

3.1 Isolation and morphological characteristics of hyperparasitic fungi In the current study, four fungal strains, designated as BS21, BS34, BS62, and BS75, were isolated from aecium tissue blocks exhibiting hyperparasitism, as illustrated in Table 1.

Table 1 Morphological characteristics and collection information of hyperparasitic fungi

Strain	Morphological characteristics	Isolation site
BS21	Dark green, flocculent	ZY
BS34	Dark green, flocculent	ZY
BS62	Olive green, flocculent	ZY
BS75	Olive green, with central white aerial mycelia, flocculent	ZY

NOTE ZY represents the Guixia Planting Base of Baoshan Zuoyuan Coffee Co., Ltd., located in the Longyang District of Baoshan City.

The characteristics of the colony and its micromorphological characteristics are illustrated in Fig. 1.

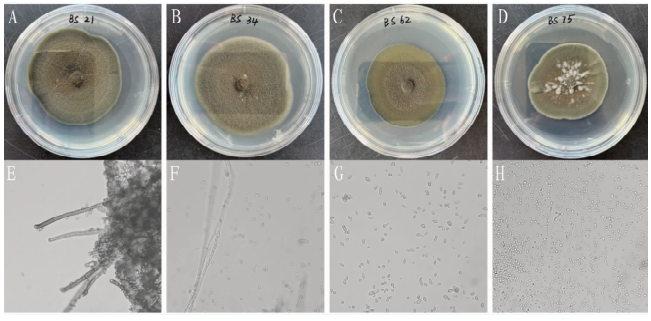

Fig. 1 Colony characteristics and micromorphological characteristics

3.2 Molecular identification of hyperparasitic fungi The total DNA extracted from the four pathogenic fungi served as a template for PCR amplification, resulting in the successful generation of a specific fragment measuring approximately 500 – 750 bp in length, as illustrated in Fig. 2. This fragment size aligns with the anticipated dimensions.

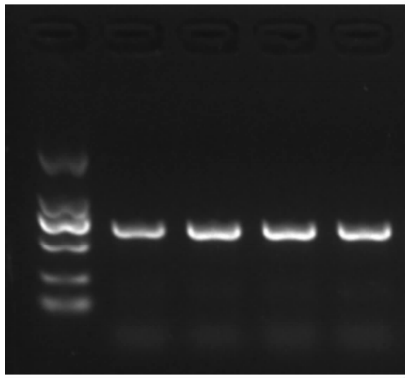

Fig. 2 Electropherogram of PCR amplified products of ITS fragments of the strain

The bi-directional sequences were submitted to the NCBI database for BLAST comparison. The results indicated that all four strains were identified as *Cladosporium* fungi. Specifically, strain BS21 exhibited a 99.82% similarity to *C. cladosporioides*, strain

BS34 demonstrated a 100% similarity to *C. tenuissimum*, strain BS62 showed a 99.81% similarity to *C. cladosporioides*, and strain BS75 also exhibited a 99.82% similarity to *C. colombiae* (Table 2).

Table 2 Comparison of ITS sequence of the strain in GenBank

Strain	Sequence size	Similar strain in GenBank	Similar strain registration number	Similarity rate//%
BS21	549	*Cladosporium cladosporioides*	MH425309	99.82
BS34	523	*C. tenuissimum*	MN700643	100.00
BS62	537	*C. cladosporioides*	KX639814	99.81
BS75	552	*C. colombiae*	OK510270	99.82

4 Conclusions and discussion

Rust hyperparasitism is prevalent among natural species. Live cell preparations derived from hyperparasitic fungi offer several advantages, including non-toxicity, safety for the target crop, minimal environmental impact, and sustained control efficacy. Consequently, these preparations have consistently represented a significant focus in the advancement of biological control methods[10]. Among the hyperparasitic fungi associated with rust, the genera *Tuberculina*, *Darluca filum*, *Fusarium*, *Acremonium*, *Cladosporium*, and *Trichoderma* are among the most prevalent taxa[11-12]. This investigation identified the phenomenon of hyperparasitism associated with coffee leaf rust for the first time in the coffee production region of Baoshan City in 2021. The fungi *C. cladosporioides*, *C. tenuissimum*, and *C. colombiae* were isolated, cultivated, and purified during this study. This report represents the inaugural documentation of hyperparasitic fungi affecting coffee leaf rust in China. *Cladosporium* exhibit a range of ecological adaptations, including endophytic behavior, human pathogenicity, phytopathogenicity, saprotrophic nutrition, and the ability to thrive in extreme environments. The majority of known *Cladosporium* species are associated with plants, frequently causing damage and manifesting as spots on plant leaves[13]. The management of plant diseases through the utilization of hyperparasitic fungi, which specifically reduce damage to host plants via specialized parasitism, represents an innovative approach for biological control[14]. Recent studies have demonstrated that secondary metabolites produced by *Cladosporium* play a protective role in enhancing the capacity of plants to adapt to novel habitats and to defend against biotic and abiotic stresses. Consequently, these findings support the potential application of *Cladosporium*-derived metabolites as plant biostimulants in agricultural practices. This survey represents the inaugural identification of three strains of *Cladosporium* hyperparasitic fungi associated with small grain coffee leaf rust in China. This finding holds considerable significance for the utilization of indigenous hyperparasitic fungal resources in the management of coffee leaf rust. Nevertheless, the parasitism efficiency and the mechanisms underlying hyperparasitism of these three strains on coffee leaf rust remain poorly understood, necessitating further investigation.

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