

Identification of the causal agent of stem basal rot in *Cyclocodon lancifolius* and screening of potential biocontrol bacteria

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Abstract

Background: Stem base rot disease poses a serious threat to the *Cyclocodon lancifolius* industry, yet its causal agent remains unidentified.

Aims: In this study, we aimed to identify the pathogenic fungi responsible for this disease and to screen for effective biocontrol strains against the causal agent.

Methods: We isolated and identified the fungal pathogen from the stem base of diseased plants and confirmed its pathogenicity by fulfilling Koch's postulates. Multilocus sequence analysis identified the pathogen as *Stemphylium lycopersici*.

Results: An antagonistic strain, *Bacillus subtilis* YHYS1, exhibited significant inhibition (51.8%) of *S. lycopersici* growth in a plate confrontation assay. Volatile compound analysis further confirmed the fungistatic potential of YHYS1. These findings highlight the promise of YHYS1 as an eco-friendly alternative to chemical fungicides for controlling stem base rot disease in *C. lancifolius* caused by *S. lycopersici*.

Conclusions: Our characterization of this novel disease in *C. lancifolius* has elucidated the pathogenic features of *Stemphylium* species, an essential yet understudied group of plant pathogens. These findings provide valuable references for the diagnosis and control of diseases affecting *C. lancifolius*.

Keywords: *Bacillus subtilis*, *Cyclocodon lancifolius*, stem base rot disease, *Stemphylium lycopersici*.

Introduction

C. lancifolius is a perennial, erect or trailing herbaceous plant of the Campanulaceae family, primarily distributed in the Guizhou and Yunnan provinces of China. As a unique

medicinal and edible resource in Guizhou, it is valued for its rhizome, which is believed to reinforce Qi (vital energy in traditional Chinese medicine), tonify deficiency, dispel stasis, and relieve pain. Due to *C. lancifolius* being a perennial plant, with the extension of planting years,

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Abbreviations: BCAs - effective biocontrol agents; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; ISR - induced systemic resistance; ITS - internal transcribed spacer; LB - Luria-Bertani; ML - maximum likelihood; NJ - Neighbor-joining; NNI - Nearest Neighbor Interchange; PDA - potato dextrose agar; TEF-1 α - translation elongation factor 1-alpha; VOC - volatile organic compound.

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Conflict of interest: The authors declare that they have no conflict of interest.

stem base rot disease has become a recurring issue. The consequences of this phenomenon include vascular tissue necrosis, plant wilting and lodging, which collectively result in a substantial reduction in crop yield (Wang et al., 2024). The initial presentation of stem basal rot disease is typically characterized by the emergence of water-soaked lesions that expand radially, undergo a transition from brown to black over time, and propagate longitudinally along the stem. This progression ultimately culminates in plant wilting and death (Agrios, 2005). The primary causative agents of stem basal rot are soil-borne fungal pathogens (Feng et al., 2021), including *Fusarium* spp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Phytophthora* spp. (Boro et al., 2022; Poveda and Eugui, 2022; Adkar-Purushothama et al., 2025). Therefore, it is both valuable and urgent to find effective and environmentally friendly methods to reduce the damage caused by stem base rot disease to *C. lancifolius* production.

It is estimated that plant diseases are responsible for 10 - 40% of annual yield loss in major food crops worldwide, resulting in economic losses exceeding \$220 billion globally (Savary et al., 2019). For decades, chemical control measures have played a pivotal role in safeguarding crop security (Qiao et al., 2010; 2011; Yang et al., 2022). However, the indiscriminate application of chemical agents has precipitated a cascade of ecological and agronomic concerns, including environmental degradation, fungicide residues, and the emergence of pathogen resistance (Chowdhury et al., 2025). Biological control has been widely recognized as a viable and sustainable alternative to chemical pesticides in plant disease management (Lahlali et al., 2022). Distinguished by its efficacy, non-toxicity, and environmental compatibility, this approach not only addresses the pressing issues of pesticide residues and pathogen resistance but also aligns with the principles of ecological sustainability. Consequently, biological control has emerged as an indispensable strategy in modern agricultural practices for mitigating plant diseases.

The utilization of beneficial microorganisms for the biological control of fungal plant diseases has been demonstrated to be a sustainable alternative to chemical fungicides (Sarrocco, 2023). Effective biocontrol agents (BCAs) include bacterial genera such as *Bacillus*, *Pseudomonas*, and *Streptomyces*; fungal genera such as *Trichoderma*, *Gliocladium*, and *Coniothyrium*; and yeasts such as *Candida* and *Pichia*. These BCAs suppress pathogens through mechanisms including antibiosis (e.g., antibiotics, lytic enzymes, volatiles), nutrient competition, mycoparasitism, and induced systemic resistance (Dehbi et al., 2023).

Trichoderma and *Bacillus* are the most extensively studied and commercialized BCAs. *Trichoderma*-based products constitute over 60% of registered fungal biocontrol products worldwide, targeting soil-borne pathogens such as *Rhizoctonia*, *Fusarium*, *Pythium*, and *Sclerotinia* (Andrade-Hoyos et al., 2023). It has been demonstrated that *Bacillus* species, most notably

B. subtilis and *B. amyloliquefaciens*, are capable of producing resistant endospores, thereby facilitating formulation and storage (Kumar et al., 2025). However, field efficacy remains inconsistent due to environmental variability (temperature, humidity, soil type, indigenous microbes) (Schoenborn et al., 2023). The current research priorities are as follows: firstly, the screening of locally adapted strains; secondly, the improvement of formulation technologies; and thirdly, the development of microbial consortia within integrated pest management (IPM) frameworks.

Despite the severity of stem basal rot in *C. lancifolius*, the causal agent of this disease remains unidentified, and effective control strategies are lacking. The present study concentrated on necrotic basal stem tissues of *C. lancifolius*, with three principal objectives: the isolation and polyphasic characterization of fungal pathogens, the identification and functional assessment of antagonistic microbiota, and the development of molecular and phenotypic diagnostic protocols for integrated disease management strategies.

Materials and methods

Experimental materials: Diseased tissue from *C. lancifolius* exhibiting stem base rot was collected during the epidemic phase from medicinal plant germplasm fields at Guizhou University of Traditional Chinese Medicine. A randomized sampling strategy was employed, with samples collected from three individual plants per plot. The samples were subsequently transported in sterile bags to the laboratory for pathogen isolation. Field soil was independently collected using a five-point sampling protocol (Zhou et al., 2022). In parallel, five healthy *C. lancifolius* seedlings were randomly selected, and their rhizosphere soil was aseptically collected from a depth of 20 cm. All samples were immediately placed in sterile bags, transported to the laboratory on ice, and stored at 4°C until further processing.

Isolation and purification of pathogenic fungi: Pathogens from stem base rot samples were isolated and purified using tissue isolation and single-spore purification techniques. Prior to this, the diseased tissues were thoroughly rinsed under running tap water. Following surface sterilization in a clean bench through a sequence of 75% ethanol (30 s), three sterile water rinses, 0.1% HgCl₂ (3 min), three sterile water rinses, the second 75% ethanol (30 s), and a final three sterile water rinses, the tissues were blotted dry on sterile filter paper. Subsequently, segments (0.5 × 0.5 cm) from the lesion borders were excised and plated on potato dextrose agar (PDA). Pure cultures were obtained by subculturing mycelial tips from the advancing colony margins onto fresh PDA, followed by incubation at 28°C in darkness. The purified isolates were stored at -80°C in 30% sterile glycerol (Fei et al., 2019; Prasgi et al., 2025; Solomon et al., 2016).

Pathogenic fungi pathogenicity assay: To fulfill Koch's postulates, the pathogenicity of the isolates was

tested on aseptically grown 3-month-old *C. lancifolius* seedlings. Prior to inoculation, standardized puncture wounds with dimensions of 1 mm in diameter and 2 mm in depth were created in the basal stem regions using a sterile needle. The scars were inoculated by placing a 5-mm-diameter mycelial plug (from a 5-day-old culture grown on PDA at 28°C in the dark) directly into each site. The inoculated area was then wrapped with sterile moist cotton wool for 24 h to maintain high humidity. Control plants were treated identically but inoculated with sterile PDA plugs only ($n = 3$ plants per group). All plants were maintained in controlled-environment growth chambers at 28°C with 85% relative humidity under a 12 h light/12 h dark photoperiod. Disease progression was assessed daily for disease symptom development. The pathogen was re-isolated from the diseased tissues both to confirm Koch's postulates and to enable morphological characterization.

Morphological and molecular biological identification of pathogenic fungi:

We aseptically transferred mycelial plugs (5 mm in diameter) from purified fungi to fresh PDA plates, with three replicate plates per isolate. Morphological characteristics, including mycelial color, aerial hyphae, and conidial production, were observed and recorded after 7 days of incubation. After the PDA plates were fully colonized by the pathogen, mycelia were harvested from the culture surface using a sterile spatula and ground into a fine powder. Genomic DNA was then extracted from the resulting powder with a commercial DNA extraction kit (*Ezup Column Fungal Genomic DNA Extraction Kit*, Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Three genomic regions – the internal transcribed spacer (ITS) of ribosomal DNA (Sun *et al.*, 2014), translation elongation factor 1- α (TEF-1 α) (Dong, 2022), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Qiao, 2015) – were amplified by polymerase chain reaction (PCR) (Table 1). PCR reactions were performed in a total volume of 25 μ L containing 10.5 μ L dd H₂O, 0.5 μ L template DNA (final concentration: 1 ng/ μ L), 1 μ L of each forward and reverse primer (final concentration: 0.4 μ M each), and 12.5 μ L of 2 $\times$ *FidCycle Evo High-Fidelity PCR Master Mix* (Sangon Biotech Co., Ltd., Shanghai, China). This *Master Mix* contains *Super-Fidelity Evo DNA Polymerase*, MgCl₂, dNTPs, PCR additives, and a blue dye, allowing direct electrophoresis of PCR products. No additional

dNTPs were added. The thermal cycling protocol was performed in a *Longji A200 Gene Amplifier* (Hangzhou Longji Scientific Instruments Co., Ltd., Hangzhou, China). The thermal cycling protocol consisted of an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gels and visualized using an *EPK APK-Mate G510 Gel Imaging System* (Anhui EPK Instruments Co., Ltd., Hefei, China). The target amplicons were sent to *Sangon Biotech* (Shanghai, China) for sequencing. The obtained sequences were analyzed using *BLAST* on the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Sequences with high homology were downloaded and aligned with those of our isolates using *MAFFT* developed by Kazutaka Katoh, CBRC, Tokyo, Japan. A phylogenetic tree was constructed using the maximum likelihood (ML) method in *MEGA* software (version 11.0) with 1 000 bootstrap replications.

Isolation and purification of biocontrol bacteria:

Rhizosphere soil samples were sieved (20-mesh) to homogenize them, and a 1.00 g aliquot was transferred into a flask containing 10 mL of sterile distilled water. The resulting soil suspension was shaken at 200 rpm for 1 h at 25°C. A 1 mL aliquot of the supernatant was serially diluted in sterile distilled water to yield final concentrations of 10⁻⁴, 10⁻⁵, and 10⁻⁶. Subsequently, 200 μ L of each dilution was spread onto Luria-Bertani (LB) agar plates (*Shanghai Bo Microbial Technology Co., Ltd.*, Shanghai, China). After incubation at 30°C for 48 h, we selected morphologically distinct colonies based on differences in size, shape, and color. Representative colonies were purified through repeated streaking on fresh LB agar plates. All pure isolates were assigned unique strain designations, subcultured in LB broth at 28°C for 24 h, and stored at 4°C for subsequent molecular identification.

Screening of biocontrol bacteria: Antagonistic bacteria were screened using the plate confrontation method. A mycelial plug (5 mm in diameter) of the pathogen was placed in the center of a PDA plate. Each bacterial isolate was then inoculated symmetrically at four points located 2.5 cm from the central plug. Sterile distilled water applied in the same manner served as the negative control. Each treatment was replicated three times, and all plates were incubated at 28°C for 5 days.

Table 1. DNA amplification primer sequences.

Gene name	Primer	Primer sequence (5'-3')	Annealing temperature (°C)
ITS	ITS1	TCCGTAGGTGAACCTGCGG	56
	ITS4	TCCTCCGCTTATTGATATGC	
TEF-1 α	TEF1-728F	CATCGAGAAGTTCGAGAAGG	56
	TEF1-986R	TACTTGAAGGAACCCCTTACC	
GAPDH	gpd1	CAACGGCTTCGGTCGCATTG	56
	gpd2	GCCAAGCAGTTGGTTGTGC	

After incubation, the fungal colony radius toward each bacterial inoculation point (R_i) was measured, and the inhibition zone radius was calculated as $2.5 - R_i$. The percentage inhibition of mycelial growth was calculated using the formula:

$$\text{inhibition rate (\%)} = (R_c - R_i) / R_c \times 100,$$

where R_c is the fungal colony radius in the control group. The experiment was performed with three independent biological replicates. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using *Student's t*-test, with $P < 0.05$ considered statistically significant.

Morphological and physiological-biochemical characterization of biocontrol bacteria: As outlined in the section Isolation and purification of biocontrol bacteria, the antagonistic strains were cultivated on LB agar plates at 25°C for a period of 72 h. A comprehensive set of observations and measurements was conducted to document colony characteristics, encompassing surface texture, opacity, and colour. The Gram staining procedure was executed in accordance with the standard protocol delineated by Cappuccino and Sherman (2014), involving sequential application of crystal violet (primary stain), iodine (mordant), ethanol (decolorizer), and safranin (counterstain).

Molecular identification of biocontrol bacterial strains: The selected antagonistic bacterial strains were cultivated in LB broth at $30 \pm 1^\circ\text{C}$ with orbital shaking at 180 rpm until the mid-logarithmic growth phase. Genomic DNA was extracted using a standard protocol. The 16S ribosomal DNA (rDNA) region was then amplified by PCR using the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR amplification protocol consisted of an initial denaturation at 94°C for 4.5 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 53°C for 45 s, and extension at 72°C for 80 s, and a final extension at 72°C for 10 min. The purified PCR products were then submitted to bidirectional sequencing by Sangon Biotech (Shanghai, China). The obtained sequences were aligned against the NCBI database using BLAST. Following the implementation of manual corrections, the alignment results were imported into MEGA 11.0 software (developed by the Molecular Evolutionary Genetics Analysis team, Temple University, Philadelphia, PA, USA), and a phylogenetic tree was constructed using the ML method. The optimal nucleotide substitution model was evaluated and selected in MEGA 11.0 based on the Tamura-Nei model. During the process of constructing the tree, instances of missing sites and gaps were addressed through the implementation of a partial deletion strategy. The initial tree was automatically generated using the Neighbor-joining (NJ) algorithm, and the tree-building heuristic search employed the Nearest Neighbor Interchange (NNI) algorithm. The assessment of node

support was conducted through the implementation of 1 000 bootstrap repetitions.

Assessment of antimicrobial activity of volatile compounds from antagonistic strains: To assess the antifungal activity of bacterial fermentation broth, a double-chamber culture plate assay was conducted. A 5 μL aliquot of the bacterial fermentation broth (prepared as described in the section Molecular identification of biocontrol bacterial strains) was applied to the center of the PDA medium on one side of a double-chamber culture plate, while the opposing compartment received a 5-mm mycelial plug of the target pathogen. For the control treatments, 5 μL of sterile medium was used instead of the fermentation broth. After incubation at 28°C for 72 h, antifungal activity was evaluated based on the inhibition of mycelial growth. The experiment was performed with three independent biological replicates. Data are presented as mean $\pm$ SD. Statistical significance was determined using *Student's t*-test, with $P < 0.05$ considered statistically significant.

Determination of antimicrobial spectrum of antagonistic strain YHYS1: The biocontrol potential of strain YHYS1 was tested against three pathogenic fungi previously isolated from *C. lancifolius*: *Alternaria alternata* (DHY1), *Fusarium oxysporum* (DHJ1), and *Fusarium solani* (DHJ4). These fungal pathogens were originally isolated in our laboratory. The experimental procedure followed the method detailed in section Screening of biocontrol bacteria.

Results

Isolation and identification of pathogens causing *Cyclocodon lancifolius* stem rot disease: A total of 34 fungal strains were isolated from the stems and basal stems of symptomatic *C. lancifolius*. Preliminary classification based on morphological characteristics grouped these isolates into three groups. The first group, comprising 16 strains (47% of the total), was tentatively identified as *S. lycopersici*. The 16 strains in this group were further subdivided into 4 morphotypes based on morphological variations and isolation time, with one representative strain selected from each. These representative strains were designated DHJ8, DHJ9, DHJ10, and DHJ11. The second group consisted of 12 strains (35.2% of the total) and was morphologically identified as *F. solani*. This group was subdivided into 4 morphotypes, with the representative strains from each being DHJ2, DHJ3, DHJ4, and DHJ7. The third group consisted of 6 strains (17.6% of the total) and was morphologically identified as *F. oxysporum*. This group was differentiated into 2 morphotypes, represented by strains DHJ1 and DHJ5 (Fig. 1).

Pathogenicity assay of stem rot pathogens: Pathogenicity tests revealed that only the fungal isolates from Group I could induce stem rot symptoms in

aseptically grown *C. lancifolius* seedlings. Representative strains from this group were selected to fulfill Koch's postulates. The results showed that all inoculated stems developed stem rot symptoms identical to the natural infections. Initially, water-soaked brown lesions appeared at the stem base and then expanded, causing stem girdling. The lesions progressively spread upward and downward along the stem and darkened from brown to black.

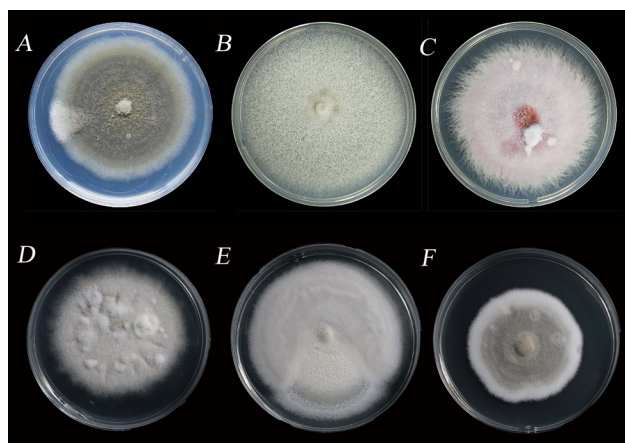


Fig. 1. Morphology of fungal isolates from *Cyclocodon lancifolius* stem basal rot. (A) Class I fungi (representative strain DHJ9), (B) Class II fungi, (C) Class III fungi, (D) Strain DHJ8, (E) Strain DHJ10, (F) Strain DHJ11.

In contrast, no symptoms developed on the control plants (Fig. 2). The pathogen was re-isolated from the diseased stem tissue of inoculated plants and was shown to be morphologically identical to the original isolates used for inoculation. Among the 4 representative strains (DHJ8, DHJ9, DHJ10, and DHJ11) isolated from Group I, strain DHJ9 was selected for multilocus sequence analysis and pathogenicity tests as the representative isolate, based on its morphological characteristics consistent with *Stemphylium lycopersici*. Strain DHJ9 was selected based on its typical morphological characteristics and consistent recovery from diseased tissues. All inoculated seedlings (9/9) developed typical stem rot symptoms, fulfilling Koch's postulates and confirming that *S. lycopersici* is the causal agent of stem base rot in *C. lancifolius*. Given that strains DHJ8, DHJ10, and DHJ11 were morphologically and molecularly identified as the same species and originated from the same disease sample, they are presumed to be pathogenic based on their taxonomic identity and association with the disease.

Morphological and molecular re-identification of pathogenic strains: The representative strain DHJ9, isolated from *C. lancifolius* with stem base rot, produced tan to pale pink mycelium on PDA and exhibited a radial growth pattern with distinct, often white, advancing margins. The conidia were clavate-conical to fusiform, brown, and characterized by 1 to 8 transverse septa and

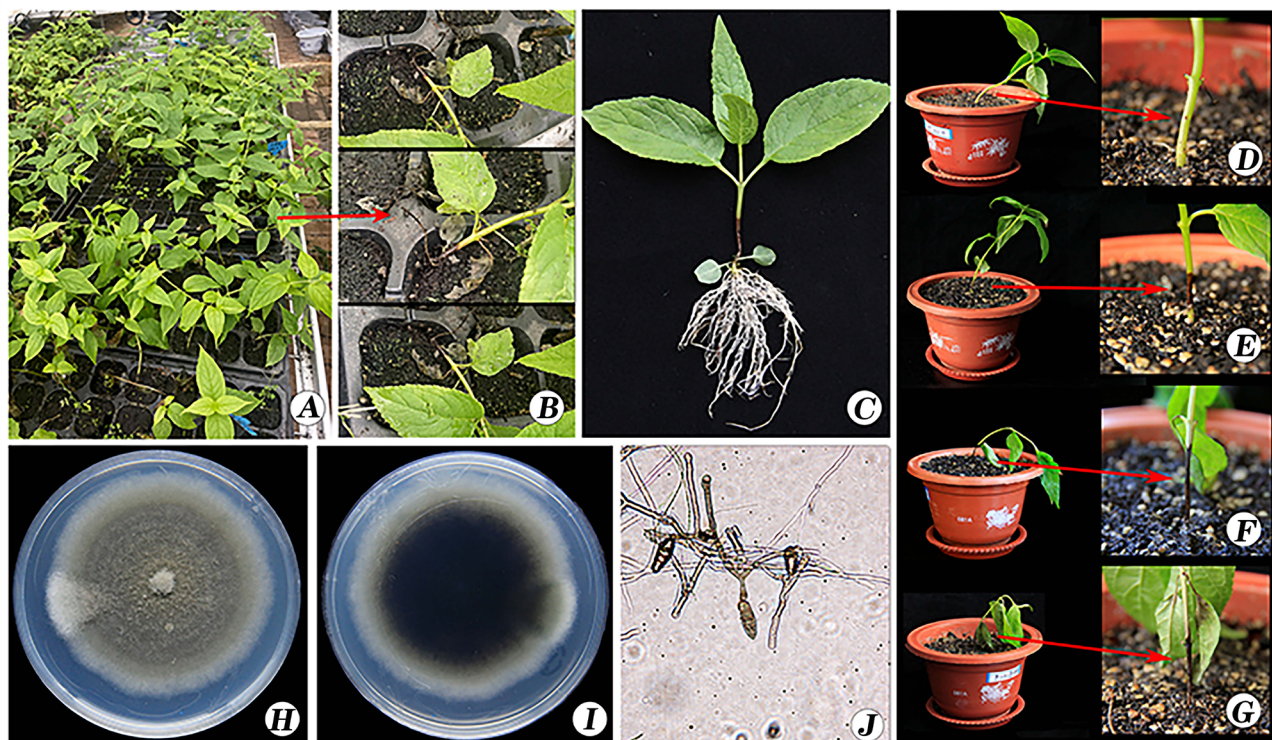


Fig. 2. Signs and morphological features of stem rot of *C. lancifolius*. The experiment was performed with 3 independent biological replicates (total $n = 9$ inoculated plants; $n = 9$ control plants). (A, B, C) Field symptoms of stem basal rot, (D) Control, (E, F, G) Symptoms on inoculated stem at 15 days post-inoculation (dpi). Water-soaked brown lesions appeared at the stem base, expanded to girdle the stem, and progressively extended upward and downward, with colour deepening from brown to dark brown/black, (H, I) Photographs of DHJ9 colonies (H - surface, I - reverse), (J) Conidia.

1 to 2 longitudinal septa. Conidia measured $14.82 - 31.56 \times 7.26 - 14.28 \mu\text{m}$ ($n = 50$) (Fig. 2). These morphological characteristics were consistent with previous descriptions of *S. lycopersici* by Nasehi et al. (2014), thereby supporting the preliminary identification of strain DHJ9 as *S. lycopersici*. Three genomic DNA regions of strain DHJ9 were amplified and sequenced. BLAST analysis revealed 99 - 100% sequence identity with *S. lycopersici* reference sequences. For phylogenetic analysis, a ML tree was constructed in MEGA 11.0 using concatenated sequences of the internal transcribed spacer (ITS) (GenBank accession PV276963), translation elongation factor 1-alpha (TEF1- α) (GenBank accession PV805043), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession PV805042). As shown in the phylogenetic tree (Fig. 3), isolate DHJ9 clustered robustly with *S. lycopersici* type strains, while exhibiting a clear distinction from other *Stemphylium* species. Based on the combined morphological and molecular data, the pathogenic strain DHJ9 was conclusively identified as *S. lycopersici*.

Isolation, purification, and screening of biocontrol bacteria: A total of 15 bacterial strains were isolated from the rhizosphere soil of healthy plants (Fig. 1 Suppl.). Following an initial antagonistic assay against *S. lycopersici*, 4 strains showing inhibitory activity were selected. After purification on LB medium, strain YHYS1 exhibited the most potent inhibition (Fig. 4). Based on 3 independent biological replicates, the fungal colony radii were 4.29 ± 0.09 cm for the control group and 2.07 ± 0.01 cm for the treatment group. The inhibition zone radius, calculated as $2.5 - R_t$, was 0.43 ± 0.01 cm. The inhibition rate was calculated to be $51.8 \pm 2.2\%$ (mean $\pm$ SD, $n = 3$). Statistical analysis using Student's *t*-test revealed a significant difference between the treatment and control groups ($P < 0.01$).

Identification of biocontrol bacteria: When cultured on LB medium, strain YHYS1 initially formed circular to semi-circular colonies that were transparent, moist, glistening, and adherent. Upon extended incubation, the colonies dried, became wrinkled, and turned

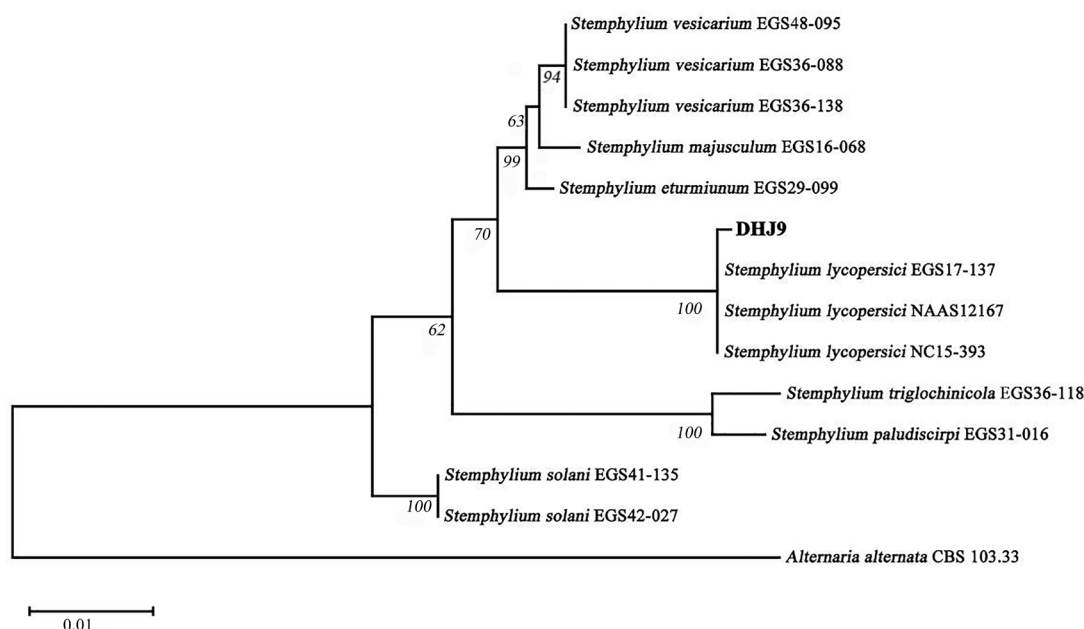


Fig. 3. Phylogenetic tree of strain DHJ9. A maximum likelihood phylogenetic tree was constructed based on concatenated nucleotide sequences of the ITS region and the TEF-1 α and GAPDH genes.

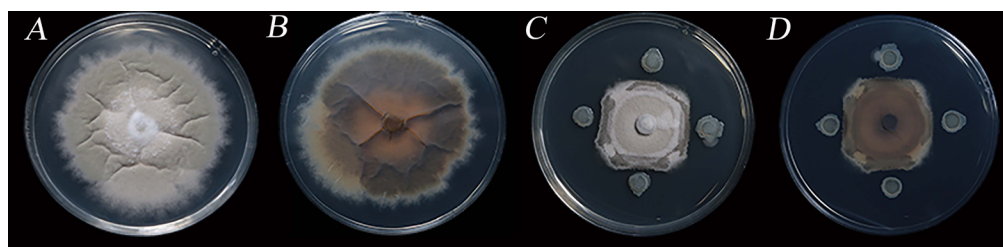


Fig. 4. Antagonistic activity of *Bacillus subtilis* YHYS1 against *Stemphylium lycopersici* DHJ9. The experiment was performed with 3 independent biological replicates. The inhibition rate was $51.8 \pm 2.15\%$ (mean $\pm$ SD, $n = 3$). Student's *t*-test revealed a significant difference between the treatment and control groups ($P < 0.1$). (A) *S. lycopersici* colonies (front), (B) *S. lycopersici* colonies (reverse), (C, D) Inhibitory effect of strain YHYS1 on *S. lycopersici* (C - front side; D - back side).

a creamy white color. The cells were rod-shaped and were determined to be Gram-positive (Fig. 2 Suppl.). Strain YHYS1 was tentatively identified as a *Bacillus subtilis*-like bacterium based on its morphological, physiological, and biochemical characteristics. To confirm this identification, a phylogenetic tree was constructed using the 16S rDNA sequence of strain YHYS1 and those of 12 other *Bacillus* species. The results showed that strain YHYS1 clustered closely with *B. subtilis* and exhibited 100% sequence identity with *B. subtilis* strain OQ874691. On the basis of these results, strain YHYS1 (PQ237068) was identified as *B. subtilis* (Fig. 5).

Determination of antimicrobial activity of volatile compounds from an antagonistic strain: The inhibitory activity of volatile organic compounds (VOCs) produced by strain YHYS1 against strain DHJ9 was assessed using

a double-chamber culture plate assay (Fig. 6). Based on 3 independent biological replicates, the fungal colony radius in the control group was 0.22 ± 0.008 cm, while that in the treatment group was 0.16 ± 0.01 cm. The inhibition rate was calculated to be $28.6 \pm 4.2\%$ (mean $\pm$ SD, $n = 3$). Statistical analysis using *Student's t*-test revealed a significant difference between the treatment and control groups ($P < 0.01$).

Antimicrobial spectrum of the antagonistic strain: Strain YHYS1 demonstrated antagonistic activity against all 3 tested fungal pathogens (Fig. 7). The greatest inhibition rate was observed against *A. alternata* (DHY1) (GenBank accession OQ128111) at 49.4%, followed by *F. oxysporum* (DHJ1) (GenBank accession OQ443098) at 49.1%, and *F. solani* (DHJ4) (GenBank accession OQ443101) at 33.7%. (Table 1 Suppl.)

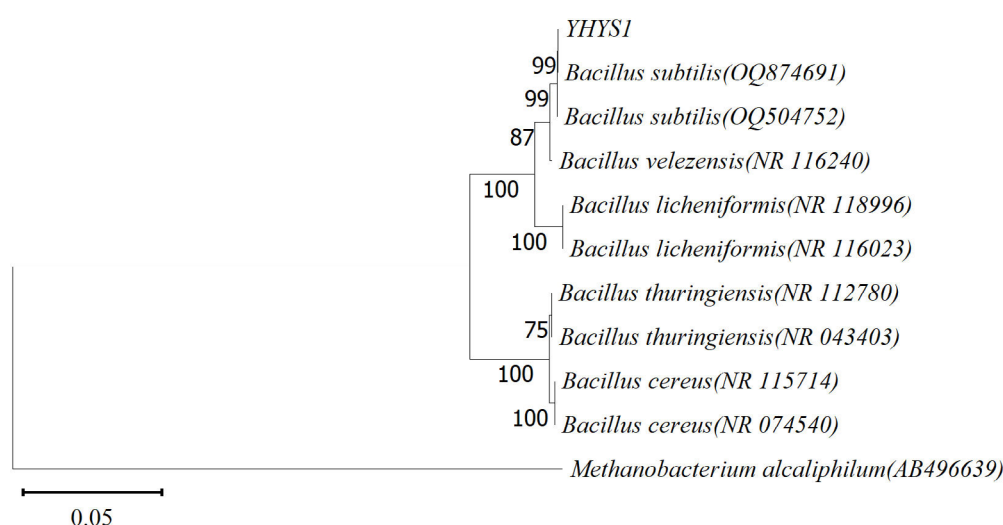


Fig. 5. A maximum likelihood phylogenetic tree of strain YHYS1 constructed based on 16S rDNA sequences.

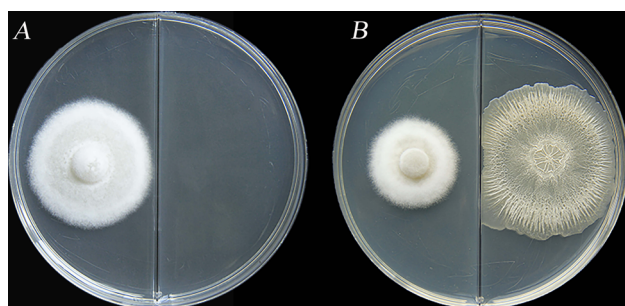


Fig. 6. The inhibitory activity of volatile organic compounds produced by strain YHYS1 against *S. lycopersici* strain DHJ9. The experiment was performed with 3 independent biological replicates. The inhibition rate was $28.6 \pm 4.2\%$ (mean $\pm$ SD, $n = 3$). *Student's t*-test revealed a significant difference between the treatment and control groups ($P < 0.01$). (A) *S. lycopersici* colonies (left compartment contains mycelium block of *S. lycopersici*; right compartment contains 5 μ L sterile culture medium), (B) The inhibitory effect of volatile organic compounds produced by strain YHYS1 against *S. lycopersici* strain (left compartment contains mycelium block of *S. lycopersici*; right compartment contains 5 μ L YHYS1 fermentation broth).

Discussion

Stem base rot is a destructive plant disease primarily caused by fungal pathogens, leading to tissue decay, vascular obstruction, and ultimately plant death (Bharudin *et al.*, 2022; Song *et al.*, 2024). Fungal pathogens have been identified as a critical factor in the triggering of this disease, with species from genera such as *Fusarium*, *Rhizoctonia*, and *Sclerotinia* frequently reported as causal agents across various crops (Michielse and Rep, 2009; Wang *et al.*, 2024). In the present study, we identified *S. lycopersici* as the pathogen responsible for stem base rot in *C. lancifolius*, thereby clarifying the etiology of this disease.

S. lycopersici is a globally distributed phytopathogen with a broad host range (Yang *et al.*, 2017; Tagele *et al.*, 2018; Zhang *et al.*, 2022). It was initially isolated from tomato plants, where it causes gray leaf spot. This pathogen employs phytotoxic secondary metabolites to disrupt plant cells during infection. *S. lycopersici* was first documented in 2001 in Tai'an City, Shandong Province, China, and its taxonomy has been well

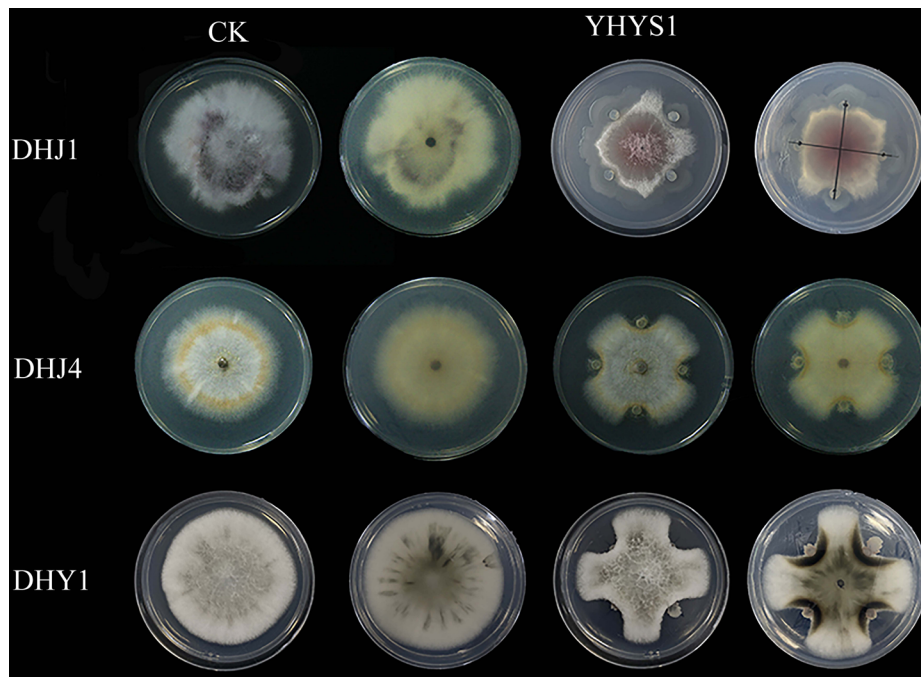


Fig. 7. Inhibitory effect of strain YHYS1 on three phytopathogenic fungi: *F. oxysporum* DHJ1, *A. alternata* DHY1, and *F. solani* DHJ4. For each pathogen, the two images on the left (front and bottom views) represent the control group (CK = control). The two images on the right (front and bottom views) represent the treatment group (YHYS1 = treated). For each group, the same plate is shown from the front (left column) and from the bottom (right column).

established (Xue et al., 2005). This fungal pathogen can cause severe leaf spot diseases on economically important crops, including tomato (Zhang et al., 2022) and *Polygonatum kingianum* (Chen et al., 2022), and leads to significant economic losses. Additionally, *S. lycopersici*-induced stem rot in Wilford's swallowwort leads to complete plant wilting and mortality (Hong et al., 2012). An important finding of this study is the relationship between the stem basal rot caused by *S. lycopersici* in *C. lancifolius* and the leaf blight caused by the same pathogen species reported by Yang et al. (2024) on the same host. Comparative analysis reveals that the *S. lycopersici* strain DHJ9 from this study (causing stem rot) is genetically and morphologically highly similar to the leaf blight-causing isolate described by Yang et al. The ITS sequences of both strains show 100% identity, and multi-locus phylogenetic analysis confirms they cluster within the same clade with high bootstrap support.

This raises the intriguing possibility that leaf spot and stem rot may represent different manifestations of the same disease syndrome caused by *S. lycopersici* in *C. lancifolius*. This phenomenon is not unprecedented in *S. lycopersici* pathology. Hong et al. (2012) reported that *S. lycopersici* causes both leaf spot and stem rot on Wilford's swallowwort leading to complete plant wilting. The pathogen's ability to infect multiple tissue types may be attributed to its repertoire of cell wall-degrading enzymes and effectors that enable adaptation to different host microenvironments (Zhang et al., 2022).

We hypothesize that leaf spot and stem rot in *C. lancifolius* caused by *S. lycopersici* are different

manifestations of the same disease, with symptom expression potentially influenced by infection site, environmental conditions (humidity, temperature), and infection routes (aerial dispersal for leaves vs. soil contact or wounding for stems). Future research should include cross-inoculation studies to determine if the same isolate can reproduce both symptom types and investigate tissue-specific gene expression patterns during infection. Understanding the relationship between these symptom manifestations will be crucial for developing integrated disease management strategies that target the pathogen regardless of infection site.

B. subtilis exhibits multifaceted biocontrol mechanisms: (1) competitive colonization of plant surfaces, tissues, and rhizosphere soils; (2) direct parasitism via lytic enzyme production that degrades pathogen hyphae; (3) antimicrobial secondary metabolites that disrupt spore cell walls. In a related study, Shao et al. investigated the effects of the endophytic biocontrol strain *Bacillus subtilis* R31 on the quality, transcriptome, and metabolome of sweet corn. Through field experiments and multi-omics analysis, they elucidated the molecular mechanisms through which *B. subtilis* R31 enhances disease resistance and nutritional value (Shao et al., 2023). Ratanaprom et al. demonstrated that the combined application of *B. subtilis* supernatant and sodium bicarbonate not only directly inhibits the growth of *Neoscytalidium dimidiatum*, the pathogen causing stem brown spot disease in dragon fruit, but also activates the plant's natural defense system (e.g., defense-related enzyme activity and lignin deposition), achieving dual protection (Ratanaprom et al.,

2021). This biocontrol strategy exhibited outstanding preventive and therapeutic effects in field trials, offering a safe and sustainable solution. In field trials in Siberia, the application of this bacterium significantly reduced damage to potato plants, decreasing the disease development index from 40.9% to 12.0% (Maslennikova *et al.*, 2023). *B. subtilis* enhances stress tolerance in its plant hosts by inducing the expression of stress-response genes, as well as genes involved in the biosynthesis of phytohormones and stress-related metabolites (Hashem *et al.*, 2019). Induced systemic resistance (ISR) elicited by *Bacillus* spp. has been reported to protect plants against a wide range of pathogens, including leaf-spotting fungi and bacteria, systemic viruses, crown- and stem-rotting fungi, root-knot nematodes, as well as diseases such as damping-off, blue mold, and late blight.

Our results demonstrate that *B. subtilis* YHYS1 inhibits *S. lycopersici* mycelial growth *in vitro*, suggesting its potential as a biocontrol agent for further investigation against *C. lancifolius* stem rot.

B. subtilis strains that demonstrate comparable *in vitro* inhibitory profiles have been employed with success for the purpose of plant disease control through a variety of application methods, including seed coating, root drenching, and foliar spraying (Xie *et al.*, 2021; Hansel *et al.*, 2024). For *C. lancifolius*, a perennial medicinal plant primarily cultivated in Guizhou Province, China, root drenching with YHYS1 suspension during the seedling stage or at the onset of disease symptoms could be a practical approach to establish protective colonization in the rhizosphere and stem base tissues. It is important to note that the transition from *in vitro* antagonism to effective disease suppression in the field depends on multiple factors, including formulation stability, rhizosphere competence, and environmental conditions (Negi *et al.*, 2024). Consequently, subsequent studies should concentrate on formulating appropriate YHYS1 preparations and evaluating its biocontrol efficacy in greenhouse and field trials. In addition, research should be conducted into its compatibility with existing agricultural practices for *C. lancifolius* cultivation.

Conclusions

In this study, the causative agent of stem base rot in *C. lancifolius* was isolated and identified by fulfilling Koch's postulates. Subsequently, potential biocontrol strains were screened using antagonistic assays. This work makes two key contributions to plant pathology: first, it provides the first evidence that *Stemphylium lycopersici* is a pathogen of *C. lancifolius*, a novel finding in disease etiology that necessitates updates to diagnostic protocols; secondly, the potential of *B. subtilis* YHYS1 as a promising candidate for biocontrol is revealed, suggesting that it may offer a sustainable alternative to chemical fungicides such as azoxystrobin. Further validation is required through plant-based and field experiments to confirm this hypothesis. Future research should investigate the molecular basis of this pathosystem, particularly

the role of fungal effector proteins in stem tissue colonization and the genetic regulation of antimicrobial compound production in *B. subtilis* under field conditions. This study has expanded the existing body of knowledge surrounding fungal diseases affecting medicinal plants. Furthermore, it has highlighted the potential of microbial antagonists as a sustainable tool for crop protection.

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