Plant Methods

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Quantification of the fungal pathogen *Didymella segeticola* in *Camellia sinensis* using a DNA-based qRT-PCR assay

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Abstract

The fungal pathogen *Didymella segeticola* causes leaf spot and leaf blight on tea plant (*Camellia sinensis*), leading to production losses and affecting tea quality and flavor. Accurate detection and quantification of *D. segeticola* growth in tea plant leaves are crucial for diagnosing disease severity or evaluating host resistance. In this study, we monitored disease progression and *D. segeticola* development in tea plant leaves inoculated with a GFP-expressing strain. By contrast, a DNA-based qRT-PCR analysis was employed for a more convenient and maneuverable detection of *D. segeticola* growth in tea leaves. This method was based on the comparison of *D. segeticola*-specific DNA encoding a Cys2His2-zinc-finger protein (NCBI accession number: OR987684) in relation to tea plant Cs18S rDNA1. Unlike ITS and *TUB2* sequences, this specific DNA was only amplified in *D. segeticola* isolates, not in other tea plant pathogens. This assay is also applicable for detecting *D. segeticola* during interactions with various tea cultivars. Among the five cultivars tested, '*Zhongcha102*' (ZC102) and '*Fuding-dabaicha*' (FDDB) were more susceptible to *D. segeticola* compared with '*Longjing43*' (LJ43), '*Zhongcha108*' (ZC108), and '*Zhongcha302*' (ZC302). Different *D. segeticola* isolates also exhibited varying levels of aggressiveness towards LJ43. In conclusion, the DNA-based qRT-PCR analysis is highly sensitive, convenient, and effective method for quantifying *D. segeticola* growth in tea plant. This technique can be used to diagnose the severity of tea leaf spot and blight or to evaluate tea plant resistance to this pathogen.

Keywords Disease progression, *Didymella segeticola*, DNA-based qRT-PCR, C2H2-ZNF, Quantification, Tea plant

Introduction

Tea plant (*Camellia sinensis*) is an economically important crop widely cultivated in tropical and temperate regions. Under high-temperature and high-humidity conditions, it often suffers from various fungal diseases, such as anthracnose, gray blight, blister blight, brown blight, leaf blight, and leaf spot [1–8]. Zhao et al. (2018) first reported that *Didymella segeticola* (Q. Chen) Q. Chen, Crous & L. Cai (syn. *Phoma segeticola*) can cause tea leaf spot in Guizhou Province, China, leading to leaf fall and a significant tea production loss [9]. Subsequently, *D. segeticola* -caused leaf spot was found in

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Sichuan and Taiwan Provinces, suggesting a preference for higher altitudes [10–12]. However, this pathogen was also isolated from diseased leaves with leaf blight symptoms in Zhejiang and Jiangsu Provinces [13]. It was thus speculated that *D. segeticola* may be spreading between different provinces along with diseased leaves. Symptomatically, *D. segeticola* infected leaves initially presents as small, pinhead-sized spots on tea leaves, which gradually expand into larger black-brown spots [9]. Infected tender leaves also show large, irregular, reddish-brown spots [11]. Tea leaf spot significantly reduces both tea production and quality, with infected shoots or leaves producing tea with a more bitter taste due to reduced amino acid derivatives [11]. Leaf blight symptoms are similar to those of large leaf spot and also severely affect tea plant growth and production [13].

Didymella segeticola was first isolated from tea plant leaves exhibiting leaf spot symptoms, identified through morphological characteristics and phylogenetic analysis using including *TUB2* (*β*-tubulin), *RPB2* (RNA polymerase II second largest subunit), ITS (internally transcribed spacer) and LSU (partial 28 S large subunit rDNA) [9]. It causes similar symptoms on leaves of *C. sinensis* cv. '*Fuding-dabaicha*' (FDDB) using needle punch and cut inoculation methods [9, 11], indicating the susceptibility of this cultivar to *D. segeticola* and its ability to invade through artificial wounds. Recently, we also isolated this pathogen from diseased leaves of *C. sinensis* cv. *Longjing43* (LJ43) and *C. sinensis* cv. *Baiye1* (BY1) exhibiting leaf blight [13]. Pathogenicity analysis showed that *D. segeticola* could invade healthy leaves of LJ43 through artificial wounds, causing necrotic lesions at inoculation sites [13]. These findings suggest that *D. segeticola* can infect various tea plant cultivars. The frequent isolation of *D. segeticola* indicates it may be a primary causal agent of foliar diseases in tea plants [11, 13]. Therefore, detecting this pathogen in tea plant tissues or diagnosing disease severity are essential for developing disease management strategies.

The simplest method to diagnose disease severity or evaluate plant resistance is conventional symptom observation and lesions measurement [14]. However, visible lesions typically appear in the late stages of infection, making it difficult to accurately determine disease severity or plant resistance in the early stage, when abundant infectious hyphae are present but not visible [15–17]. Therefore, a series of DNA-based methods such as classical PCR and quantitative real-time PCR (qRT-PCR) are used to quantify fungal growth in host cells with the advantage of high sensitivity, accuracy and reliability [18, 19]. The DNA-based qRT-PCR has been used to accurately quantify disease severity in various host-pathogen interaction systems [15, 18–22]. This quantitative method has also been developed to detect *Didymella* species in plant tissue, such as *D. pinodella* in pea and wheat roots, *D. pisi* associated with Ascochyta blight of dry pea, *D. bryoniae* in cucurbit seedlots, and *D. rabiei* in chickpea [23–26]. However, the DNA-based qRT-PCR analysis has not been applied to detect *D. segeticola* in tea plant leaves. The recent release of the whole-genome sequence of *D. segeticola* (the strain GZSQ-4) provides a valuable resource for specifically detecting this pathogen, studying its pathogenicity, and understanding the mechanisms of tea plant-pathogen interactions [27]. Additionally, high-quality transcriptome, microRNAs (miRNAs) and competing endogenous RNAs (ceRNAs) sequences from the *D. segeticola*-tea plant interaction offer important resources for studying pathogenic mechanisms and disease resistance responses [12, 28]. These bioinformatics analyses have laid the foundation for developing diagnostic methods for this commercially important phytopathogen in tea plant leaves.

In this study, we optimized qRT-PCR analysis for quantifying *D. segeticola* in infected tea plant leaves using total genomic DNA from infected leaves as templates. We designed specific primers targeting a *D. segeticola*-specific DNA sequence (NCBI accession number: OR987684), predicted to encode a Cys2His2-zinc-finger (C2H2-ZNF) protein. The specificity, sensitivity, and stability of these primers were validated by PCR or qRT-PCR analysis. This DNA-based method can be applied for detecting *D. segeticola* and evaluating tea plant resistance.

Materials and methods

Pathogen growth and infection

Three isolates of *D. segeticola* were used in this study, including YCW109 isolated from healthy tea plants in Yixing City, Jiangsu Province of China by tissue isolation, and YCW1135 and YCW2184 from diseased tea plants in Hangzhou City, Zhejiang Province of China via single-spore isolation [5]. The isolates were identified based on morphological characteristics and multi-locus (ITS, LSU, *RPB2*, and *TUB2*) phylogenetic analysis [13]. *Cladosporium angulosum* isolate YCW60, *Colletotrichum camelliae* isolate LS_19, *Colletotrichum siamense* isolate FJ1A3, *Didymella sinensis* isolate YCW1906, and *Pseudopestalotiopsis camelliae-sinensis* isolate ZJ1A1 were used as control strains in this study [5, 13, 29–31]. All strains were cultured on potato dextrose agar (PDA) plates (90 mm diameter) at 28℃ in the dark. When colonies reached approximately three-quarters of the plate diameter, 5-mm mycelial plugs from the colony edges were inoculated onto detached mature leaves (fourth leaves) of 5-year-old LJ43 tea plants with artificial wounds [13]. Leaves inoculated with 5-mm sterile PDA discs served as the control. Inoculated leaves were incubated in a growth chamber (14 h light at 25℃/10 h dark at 22℃)

with high humidity (>90%). Observations and analyses were conducted at 0, 3, 6, 12, 24, and 48 h post-inoculation (hpi), including symptom observation, lesion size measurement, and DNA extraction from collected leaves. Samples were frozen at -80℃ until DNA extraction. Each inoculation experiment included ten replicates and was independently repeated three times.

For the inoculation experiments using different tea cultivars, the healthy mature leaves (the fourth leaves) of 5-year-old FDDB, LJ43, '*Zhongcha102*' (ZC102), '*Zhongcha108*' (ZC108), and '*Zhongcha302*' (ZC302) plants were surface sterilized with 75% ethanol and washed twice with sterilized ddH_2O. Leaves were wounded with sterilized needles immediately before inoculation. *Didymella segeticola* strain YCW2184 was used. When colonies reached approximately three-quarters on PDA plates, 5-mm mycelial plugs from colony edges were inoculated onto four detached mature leaves (the fourth leaves) with the artificial wounds. Samples were collected at 72 hpi and frozen at -80℃ for DNA extraction. This experiment was independently repeated three times.

Phenotype analysis and microscopic examination

To visualize the infection process of *D. segeticola* in tea plant leaves, we generated a strain stably expressing green fluorescence protein (GFP). Plasmid pCB1532-GFP was transformed into protoplasts of the *D. segeticola* strain YCW2184, and transformants were screened by PCR and fluorescence microscopy. The phenotype and pathogenicity of a positive strain (Ds-GFP) and YCW2184 were then evaluated. For vegetable growth, 5-mm mycelial plugs from each colony were cultured at 25℃ in the dark. After 7 days, colony photographs were taken and diameters measured. For conidiation, conidia were collected from 10-day-old colonies and counted using a hemocytometer [32]. For pathogenicity, 5-mm mycelial plugs from each colony were inoculated onto detached leaves of 5-year-old LJ43 plants with the artificial wounds [13]. After 24 h, lesions on inoculated leaves were observed and measured using a vernier caliper. Tissue slices of the infected leaves were examined under a fluorescence microscopy SOPTOP-CX40RFL (SUNNY OPTICAL TECHNOLOGY (GROUP) CO., LTD, China) at 3 hpi, 6 hpi, 12 hpi, and 24 hpi. The uninfected leaves were treated as the control. Each phenotype analysis experiment was set up with three technical replicates and independently repeated three times.

Table 1 Primers designed for quantitative real-time PCR

Targeted genes	Primer names	Primer sequence (5′-3′)	Amplicon size (bp)	references
Cs18SrDNA1	S37	GACTCCGCTGGCACCTTAT	167	He et al. [18]
	S38	GCCCTTCCGTCAATTCCT		
C2H2	C2H2-F	AGCGAACGGGTAATTGATTG	204	This study
	C2H2-R	CCACGGACCAGAGGAAGAG		
ITS	ITS-F	ATTTCGCTGCGTTCTTCAT	195	This study
	ITS-R	ACCTAGAGTTGCGGGCTTT		
TUB2	TUB2-F	AATGGCACCTCGGACCTTC	163	This study
	TUB2-R	TGTACCGGGCTCCAAATCG		

Genomic DNA extraction

The genomic DNA was extracted from the entire sampled leaves using a Genomic DNA Purification Kit (Sangon Biotechnology (Shanghai) Co., Ltd., China) according to the manufacturer's instruction. DNA concentrations were determined spectrophotometrically using 260-nm absorbance with an ultra-micro ultraviolet-visible spectrophotometer ND-100 C (MIULAB, China) [33]. All DNA samples were stored at -20℃.

qRT-PCR

For qRT-PCR analysis, the extracted DNA of each sample was diluted to a concentration of 30 ng/µL. Primers were designed using Primer Premier 5 software. For the amplification of genes in *D. segeticola*, three primer pairs were used, including one pair for amplifying part of the ITS sequence (ITS-F/R), one pair for amplifying part of the *TUB2* sequence (TUB2-F/R), and one pair for a *D. segeticola*-specific DNA sequence (NCBI accession number: OR987684) which was predicted to encode a C2H2-ZNF protein (C2H2-F/R) (Table 1). The *D. segeticola*-specific DNA sequence was identified by BLAST analysis [34]. The reactions were performed using the Bio-Rad CFX real time system in a 20 µL reaction mixture consisting of 10 µL of 2 × ChamQ Universal SYBR qPCR Master Mix Green Mastermix (Vazyme Biotech (Nangjing) Co., Ltd., China), 0.4 µL of 10 mM specific primers (ITS-F/R, TUB2-F/R and C2H2-F/R primer pairs for the pathogen, while S37/S38 primer pair was used for the amplification of Cs18SrDNA1 sequence in tea plants), 2 µL of DNA template, and 7.2 µL of sterilized ddH_2O. The relative quantity of each reaction was calculated by the $2^{-\Delta\Delta CT}$ method [35]. The analysis was conducted in three replicates for each sample, and three biological replicates were maintained.

Statistical analysis

All data in this study were preprocessed with Excel 2018, and then subjected for one-way ANOVA analysis for the significance difference test using SPSS software. Each value in this study was presented as the mean ± standard

error of at least three repeats. The image processing and mapping were conducted with Photoshop CS4.

Results

Monitoring disease progression and *D. segeticola* development in tea plant leaves

To monitor the infection process of *D. segeticola* in tea plant leaves, we generated a Ds-GFP strain stably expressing GFP. We first evaluated whether there were significant differences between Ds-GFP and the wild-type (WT) strain YCW2184 in vegetative growth, conidiation, and pathogenicity. After growing on PDA plates for 7 days at 28℃ in the dark, the morphological characteristics and colony diameters of the WT and Ds-GFP strains displayed no significant differences (Fig. 1A, B). The ability of both strains to produce conidia was evaluated by washing the surface of 10-day-old cultures with 10 mL sterile ddH_2O. After filtration with three layers of lens wiping paper, approximately 8 mL conidia suspension was obtained. The WT strain produced $(1.09 \pm 0.10) \times 10^6$ spores/mL, and the Ds-GFP strain produced $(1.17 \pm 0.04) \times 10^6$ spores/mL (Fig. 1C), suggesting there was no significant difference in conidiation between the Ds-GFP strain and WT strain. Strong GFP signals were observed in the mycelia and conidia of the Ds-GFP strain, whereas no GFP signals were presented in the WT strain (Fig. 1D; Fig. S1). In addition, pathogenicity was examined by inoculating susceptible abraded LJ43 leaves with mycelial plugs from each strain (Fig. 1E). By 3 days post inoculation (dpi), necrotic lesions of consistent sizes were observed at the inoculated sites (Fig. 1E, F). These results indicated that there were no significant differences between WT and Ds-GFP strains in vegetable growth, conidiation, and pathogenicity. Therefore, the Ds-GFP strain is suitable for monitoring the infection process of *D. segeticola* in tea plant leaves.

LJ43 leaves were infected with mycelial plugs from the Ds-GFP strain using wound inoculation. At 3 hpi, 6

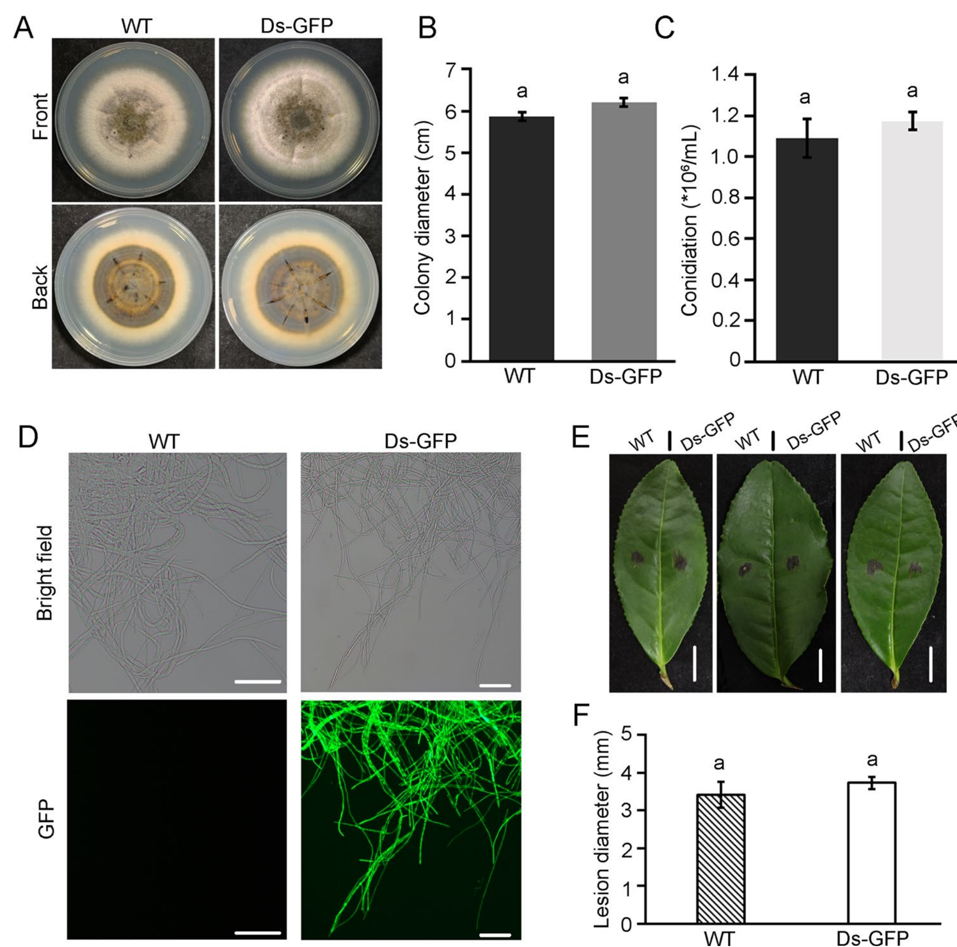

Fig. 1 No significant difference between the wild-type (WT) strain and the Ds-GFP strain of *D. segeticola* in phenotypes and pathogenicity. **A**, Colony morphology on PDA plates at 25℃ for 7 days. **B**, Radial growth on PDA plates. **C**, Bar chart showing statistical analysis of conidiation. Conidia were collected from the strain cultured on PDA plates at 25℃ for 10 days. **D**, Fluorescence microscopic observation of GFP signals in hyphae of the Ds-GFP strain. Scale bar = 50 µm. **E**, Pathogenicity test on tea leaves inoculated with mycelial plugs. Photographs were taken at 24 hpi. Scale bar = 1 cm. **F**, Statistical analysis of lesion diameters on leaves. Error bars in **B**, **C**, and **F** represent standard deviations. The same small letter indicates no significant difference ($P < 0.05$)

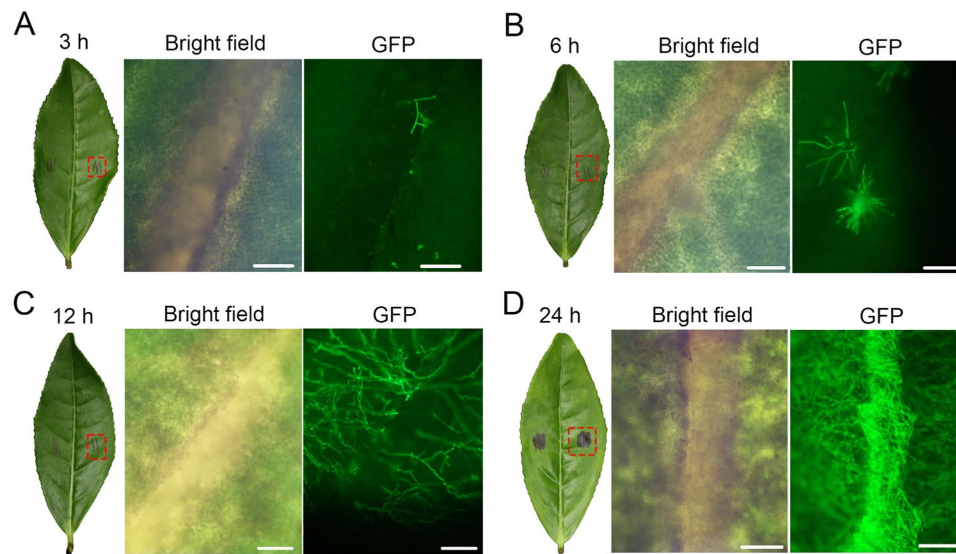

Fig. 2 The infection process of *D. segeticola* in tea cultivar LJ43 leaves. Observation of leaf symptoms on leaves inoculated with the Ds-GFP strain and the confirmation of the development of its infectious hyphae at 3 hpi (**A**), 6 hpi (**B**), 12 hpi (**C**), and 24 hpi (**D**)

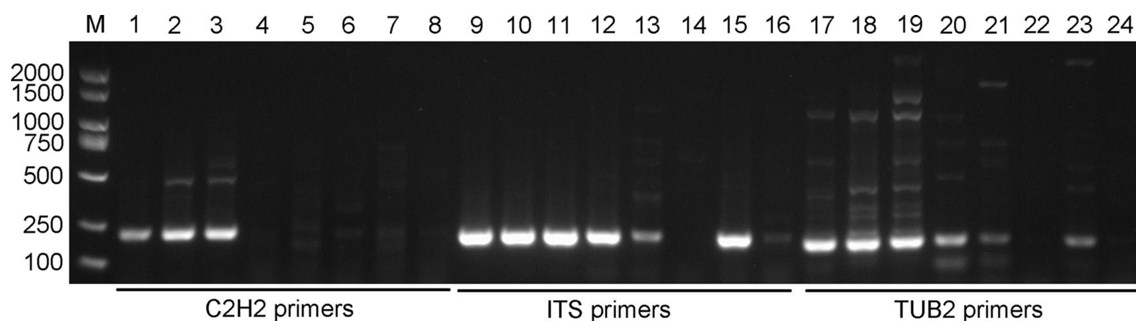

Fig. 3 Amplification results for three genes in *D. segeticola* with C2H2-F/R primers (lanes 1–8), ITS primers (Lanes 9–16), and TUB2 primers (Lanes 17–24) respectively. The DNA templates were extracted from mycelia of *D. segeticola* isolates YCW2184 (Lanes 1, 9, and 17), YCW109 (Lanes 2, 10, and 18) and YCW1135 (Lanes 3, 11, and 19), *D. sinensis* isolate YCW1906 (Lanes 4, 12, and 20), *C. camelliae* strain LS_19 (Lanes 5, 13, and 21), *Ps. sinensis* isolate ZJ1A1 (Lanes 6, 14, and 22), *Cla. angulosum* isolate YCW60 (Lanes 7, 15, and 23), and *C. siamense* isolate FJ1A3 (Lanes 8, 16, and 24). M, DL2000 DNA marker

hpi, 12 hpi, and 24 hpi, we observed the presence of diseased spots on the inoculated leaves, and checked the infectious hyphae at the inoculation sites under fluorescence microscopy. At 3 hpi, 6 hpi, and 12 hpi, no obvious diseased spots were observed, whereas more and more infectious hyphae produced at the inoculation sites with the extension of infection time (Fig. 2A-C). By 24 hpi, obvious necrotic lesions and abundant infectious hyphae were observed at the inoculation sites (Fig. 2D). The results demonstrated that *D. segeticola* successfully infected the tea plant leaves, even though no necrotic spots were not visible to the naked eye during the early stage of infection.

The specificity of *D. segeticola* quantitative primers

To quantify *D. segeticola* DNA, three qRT-PCR primer pairs targeting the ITS region, *TUB2* gene, and a gene encoding a C2H2-ZNF protein were designed (Table 1) [11]. To verify the specificity of the primers for amplifying *D. segeticola* DNA, genomic DNAs from *D. segeticola* and several control pathogens isolated from tea plants were subjected to PCR analysis. Specific amplification of the C2H2 PCR products was only detected in samples from the three *D. segeticola* strains, not in samples from other pathogens (Fig. 3). In contrast, the ITS and TUB2 PCR products were observed in the samples of other pathogens. This indicated that the C2H2-F/R primers are specific to *D. segeticola*, and suitable for further tests.

Furthermore, specific amplification of the C2H2 PCR products was detected in the samples from infected leaves with *D. segeticola* and other pathogens, as well as healthy leaves (Fig. S2), further confirming the specificity of the C2H2-F/R primers.

DNA-based qRT-PCR for the quantification of *D. segeticola* growth after infection on leaves

To conveniently quantify the biomass of *D. segeticola* on inoculated tea plant leaves, especially at the early stage of

infection, we developed a DNA-based qRT-PCR analysis. Genomic DNAs were extracted from leaves inoculated with the *D. segeticola* WT strain, with DNAs from the healthy leaves (0 hpi) serving as control. No diseased spots were observed at 3 hpi, 6 hpi, and 12 hpi, while infection lesions appeared at 24 hpi and expanded at 48 hpi (Fig. 4A, B). The Cs18S rDNA1 sequence, conserved in tea plants, was used as the standard for quantifying tea plant DNA biomass [18]. Thus, we conducted the DNA-based qRT-PCR analysis using C2H2 and Cs18S rDNA1 primers. Detection results from tea plant leaves at different infection stages showed that the amplification ratio of C2H2/ Cs18S rDNA1 increased during the infection process and reached a significant higher level at 12 hpi (Fig. 4C), and then dramatically increasing at 24 hpi and 48 hpi (Fig. 4C). These qRT-PCR data revealed a clear disease development trend during the *D. segeticola*-tea plant interaction, especially at the early stage without visible diseased spots. This contrasts with the less accurate results obtained using the lesion measurement method (Fig. 4B). In conclusion, DNA-based qRT-PCR analysis can effectively examine increases in fungal biomass over time during the pathogen-tea plant interaction.

DNA-based qRT-PCR applied for analysis of interactions between tea plants and *D. segeticola* isoates

Previous studies have identified diverse *D. segeticola* isolates from different tea cultivars in China [10, 11, 27]. Different *D. segeticola* isolates can cause distinct levels of aggressiveness on the host tea plants [11]. To evaluate whether DNA-based qRT-PCR analysis can detect *D. segeticola* during the interactions between different isolates and tea cultivars, we firstly assessed the responses of different tea cultivars to *D. segeticola* YCW2184. Five tea plant cultivars, including LJ43, ZC108, ZC102, ZC302, and FDDB, were used. Leaves of these cultivars were inoculated with mycelial plugs of the *D. segeticola* isolate YCW2184. DNA-based qRT-PCR analysis showed significantly greater fungal biomass in inoculated leaves of all five tea cultivars compared to controls, with the highest fungal growth observed on ZC102 (Fig. 5A). This indicated that ZC102 was more susceptible to *D. segeticola* compared with LJ43, ZC108, ZC302, and FDDB. Additionally, we assessed the responses of LJ43 to different *D. segeticola* isolates (YCW2184, YCW109, and YCW1135). Fungal biomass, measured by DNA-based qRT-PCR, was significantly greater in leaves infected with YCW2184, YCW109, and YCW1135 compared to controls, with

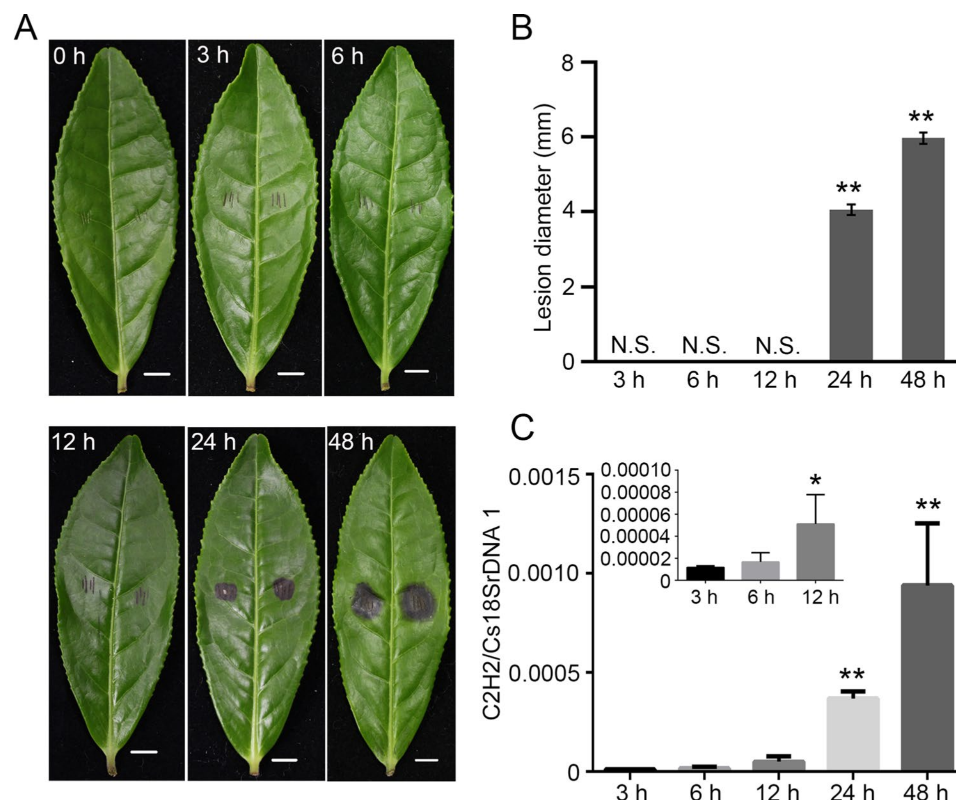

Fig. 4 Quantification of *D. segeticola* in LJ43 tea leaves using DNA-based qRT-PCR. **A**, Symptoms developed from 0 to 48 h after inoculation with the *D. segeticola* isolate YCW2184. Scale bar = 0.5 cm. **B**, Statistical analysis of lesion diameters on leaves. Error bars represent standard deviations. N. S., No statistical significance; **, $P < 0.01$. **C**, qRT-PCR-based biomass of *D. segeticola* growth in inoculated leaves. Error bars represent standard deviations. *, $P < 0.05$; **, $P < 0.01$

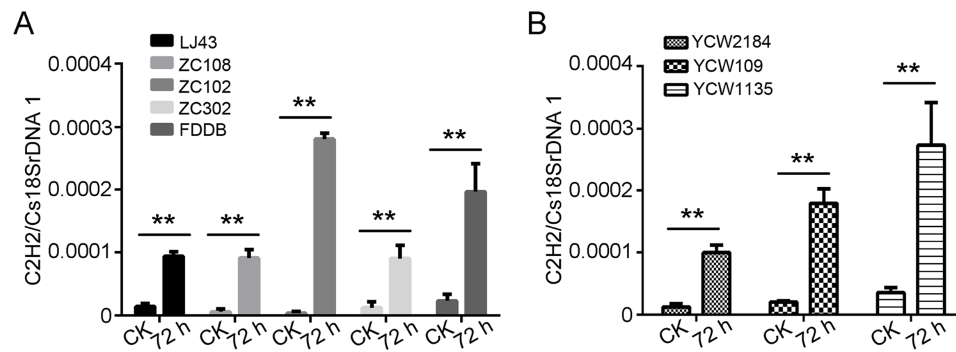

Fig. 5 Quantification of *D. segeticola* isolate YCW2184 on different cultivars of tea plants (**A**), and quantification of different isolates of *D. segeticola* on tea plant cultivar LJ43 (**B**). Error bars represent standard deviations. **, $P < 0.01$

YCW1135 showing the highest biomass (Fig. 5B). The results suggested that YCW1135 was the most aggressive isolate on the tea cultivar LJ43. Taken together, DNA-based qRT-PCR analysis revealed difference in resistance among tea cultivars to *D. segeticola* YCW2184 and in aggressiveness among *D. segeticola* isolates on LJ43. Therefore, this method is effective for analyzing of interactions between tea cultivars and *D. segeticola* isolates.

Discussion

Early detection of pathogens is critical for controlling plant diseases [36]. In this study, we observed that disease spots began to appear at 24 h after the infection of *D. segeticola* from the artificial wounds through laboratory inoculation experiments (Figs. 2 and 4A), suggesting this pathogen spread rapidly and actively killed host cells. Accordingly, to detect *D. segeticola* as early as possible is of great significance for the diagnosis and prevention of tea leaf spot. In addition, to determine how the fungal pathogenicity is established is also critical to studying the interaction between *D. segeticola* and tea plants [18].

First, we visually monitored disease progression and *D. segeticola* development in tea plant leaves inoculated with the Ds-GFP strain (Figs. 1 and 2). The results showed that *D. segeticola* could colonize the host cells from the wound at 3 hpi, with infectious hyphae developing and expanding at 6 hpi, and spreading throughout the wound and adjacent cells at 12 hpi. Despite extensive hyphal development at 12 hpi, no visible spots appeared on the inoculated leaves until 24 hpi (Fig. 2). This method allows for visualizing the infection process and observing *D. segeticola* growth in tea plant leaves with high reliability and clarity. However, this method requires constructing the Ds-GFP strain through genetic transformation of *D. segeticola*, and evaluating phenotypic differences between Ds-GFP and the WT strain to rule out any effects of the introduced GFP. This process is time-consuming, labor-intensive, and dependent on fluorescence microscopy. In consequence, we developed a DNA-based qRT-PCR method. This method is highly sensitive, reliable, simple and accurate for quantifying pathogen growth in many host-pathogen systems, especially in host-*Didymella* systems [18, 20, 22–26].

To specifically detect *D. segeticola* growth on tea plants, we selected a species-specific DNA encoding a C2H2-ZNF protein, identified through comparative genomic analysis (Data in publishing). This sequence's specificity was confirmed using C2H2-F/R primers, which specifically amplified *D. segeticola* isolates (Fig. 3). Melting curves showed single, well-defined peaks (Data not shown), and no bands of the expected size were obtained from healthy tea leaves (Fig. S2B). In contrast, ITS and *TUB2* sequences, commonly used as reference genes for fungi, were amplified in other pathogenic fungi from tea plants (Fig. 3). Therefore, the C2H2-F/R primers which amplify a 204-bp DNA fragment of *D. segeticola*, were used for the DNA-based qRT-PCR analysis. The sensitivity of the amplified fragment using C2H2-F/R primers was tested, showing brighter bands with increased infection time (Fig. S2A), and consistent results in qRT-PCR analysis (Fig. 4C). During the interaction between hosts and *Didymella* species, qRT-PCR analysis for the detection and quantification of *Didymella* species was mostly based on the conserved sequences, such as *TEF-1 alpha* gene [26]. However, high sequence similarities among pathogens' fingerprinting genes, such as ITS genes and intergenic sequences, can cause confounding results [25, 37]. For instance, Owati et al. (2019) developed a simple sequence repeat (SSR)-qPCR assay for specific detection and quantification of *D. pisi* in the presence of both host materials and other closely related species [25]. In this study, we developed a robust qRT-PCR method for quantifying *D. segeticola* growth on tea plants using a specific sequence obtained through comparative genomic analysis. This approach avoids confounding detection and diagnostic issues.

Our DNA-based qRT-PCR analysis revealed varied responses among different tea cultivars to *D. segeticola*. The tea cultivar ZC102 was more susceptible to the *D. segeticola* isolate YCW2184 compared to LJ43, ZC108,

ZC302, and FDDB (Fig. 5A). ZC102, bred from the '*Longjing*' population, is known for its strong cold resistance, but its disease resistance has not been thoroughly assessed. Zhang et al. (2021) reported that ZC102 was more susceptible to *Colletotrichum camelliae* and *C. fructicola* compared with LJ43, suggesting that ZC102 may be a susceptible variety against *Colletotrichum* infection [38]. Our study is the first to report that ZC102 also exhibits the weakest resistance to *D. segeticola* among the tested cultivars. Further field studies are necessary to evaluate the resistance of LJ43, ZC108, ZC102, ZC302, and FDDB against *D. segeticola*-induced tea leaf spot. Additionally, qRT-PCR analysis showed that LJ43 responded differently to various *D. segeticola* isolates, with YCW1135 being the most aggressive (Fig. 5B). Pathogenicity variation among isolates is well-documented in pathogens such as *C. camelliae*, *Didymella bryoniae*, *Magnaporthe oryzae*, *Fusarium graminearum*, *Pestalotiopsis*-like species, etc. [5, 39–42]. *D. segeticola* can cause distinct "large leaf spot" and "small leaf spot" symptoms on tea plants [11]. Pathogenicity of six representative *D. segeticola* isolates from infected leaves showing the small and large leaf spot symptoms indicated that different isolates caused different spot symptoms [11]. The result in this study was consistent with that in this report. In addition, the *D. segeticola* strain YCW109 was isolated from healthy leaves, and the other two strains YCW1135 and YCW2184 were isolated from the diseased leaves showing large spot symptoms, which suggests that YCW109 may be the endophytic fungi and YCW1135 and YCW2184 were possibly the pathogens. Despite this, inoculation tests showed that all three strains, including YCW109, could cause disease symptoms in LJ43 leaves. Notably, YCW109 had a greater biomass in inoculated leaves than YCW2184 (Fig. 5B), indicating higher aggressiveness. This might suggest that YCW109 was isolated from leaves in an early stage of infection, where infectious hyphae were expanding. Further research is needed to elucidate the genetic differentiation among *D. segeticola* isolates with varying aggressiveness.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-024-01284-2.

Supplementary Material 1: **Fig. S1.** GFP fluorescence in conidia produced by D. segeticola wild-type (WT) strain and the Ds-GFP strain. Scale bar = 25 μm.

Supplementary Material 2: **Fig. S2.** Validation of specific amplification results for a species-specific DNA encoding a C2H2-ZNF protein with C2H2-F/R primers. A, Amplification results using C2H2 primers (Lanes 1-6), ITS primers (Lanes 7-12), and TUB2 primers (Lanes 13-18) with the DNA templates extracted from inoculated LJ43 leaves by the D. segeticola strain YCW2184. Lanes 1-6, 7-12, and 13-18, 0 ~ 48 hpi respectively. M, DL2000 DNA marker.

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Author contributions
Zhang. Y. and Lv. W. wrote the main manuscript text. Zhang. Y.and Tu. Y. prepared Figs. 1, 2, 3, 4 and 5. Chen. Y., Fang. J., Chen, F., and Liu, L. prepared Fig. 1 and supplementary files. Wang. Y. and Lv. W. revised the manuscript. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
Not applicable.

Competing interests
The authors declare no competing interests.

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