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FUNGAL DISEASES

Variations in mycelial growth and virulence below 26 °C among five *Colletotrichum* strains from strawberry

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Abstract

Anthracnose caused by *Colletotrichum* spp. is a serious threat to many crops, and *C. siamense* has become increasingly prevalent worldwide. However, the mycelial growth and virulence of many of these species, especially below 26 °C, has largely remained unknown. Here, mycelial growth of five representative *Colletotrichum* strains (three *C. siamense* [Cs], one *C. fructicola* [Cf] and one *C. gloeosporioides* s.s. [Cg]) from diseased strawberry was measured at 10, 14, 18, 22 and 26 °C. Their virulence on *Fragaria vesca* and highly susceptible *F.* × *ananassa* cv. Benihoppe was tested at 14 °C and 22 °C. The strains did not differ much in mycelial growth between 10 and 26 °C, although growth of the Cs and Cg strains was somewhat faster than that of Cf. However, the strains varied greatly in their virulence on the two hosts. Strain Cs GQHZJ19, which grew fastest at 22 °C, was also the most virulent on both hosts at 14 °C and 22 °C. But growth rate of Cs was only positively correlated with virulence on susceptible cv. Benihoppe after 3 days at 22 °C. Based on the virulence analysis of the five *Colletotrichum* strains on hosts that vary in susceptibility at distinct temperatures, host genotype might play a decisive role in disease severity at an early stage, while temperature might influence the host–*Colletotrichum* spp. interaction at a later stage. More *Colletotrichum* strains (especially *C. siamense*) need to be tested to determine the mycelial growth and virulence in a wider temperature range and thus we can effectively manage disease.

Keywords *Colletotrichum* spp. · Temperature · Mycelial growth · Strawberry · Virulence

Introduction

Cultivated strawberry, popular all over the world, belongs to the genus *Fragaria* (*Rosaceae*). The octoploid strawberry *F.* × *ananassa* Duchesne ($2n = 8x = 56$) is the major cultivated group (Davis et al. 2007), and the diploid *F. vesca* L. ($2n = 2x = 14$) is the predominant subgenome provider for octoploid strawberry (Edger et al. 2019; Oosumi et al. 2006). Strawberry is susceptible to a wide range of phytopathogenic organisms and is considered a model system for the *Rosaceae* (Amil-Ruiz et al. 2011). Among plant families, *Rosaceae* has the most records associated with the filamentous ascomycete *Colletotrichum* fungi, with as many as 41 pathogenic *Colletotrichum* species (Talhinhas and Baroncelli 2021).

The genus *Colletotrichum* comprises more than 280 species and consists of 16 species complexes and 15 singletons (Liu et al. 2022). Many *Colletotrichum* species are widespread plant pathogens, causing anthracnose leaf blight or fruit/stem rot on more than 3,000 species of

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plants (O'Connell et al. 2012). Anthracnose, caused by *Colletotrichum* spp., is a serious threat to the strawberry industry because the fungi can infect and colonize a wide range of strawberry tissues, causing black spots on leaves, petioles, stolons, and fruits that are sunken or necrotic, and a crown rot that can lead to wilting and death of the entire plant (Howard et al. 1992). *C. siamense* and *C. fructicola* of the *C. gloeosporioides* sensu lato complex are currently the most prevalent species endangering strawberry in Asia (Ji et al. 2022) due to their broad host range, environmental adaptability and genetic variability (da Silva et al. 2020; Wilson et al. 2019).

Epidemics caused by *Colletotrichum* spp. generally occur in wet, warm weather between 20 and 30 °C (Ngugi et al. 2000; Sharma and Kulshrestha 2015; Soares-Colletti and Lourenço 2014). The temperature requirements of *Colletotrichum* fungi vary depending on the biological process, fungal genotype and ecotype (Chung et al. 2020; Moreira et al. 2021; Soares-Colletti and Lourenço 2014). The optimal temperatures for mycelial growth of *Colletotrichum* spp. largely range from 24 to 28 °C (Salotti et al. 2022; Ji et al. 2023). *C. siamense*, one of the most predominant species threatening strawberry worldwide, can tolerate higher temperatures, especially for mycelial growth (Ji et al. 2022, 2023). Although the morphology and virulence of different *Colletotrichum* species under high temperatures have been described (Damm et al. 2014; Dubrulle et al. 2020; He et al. 2019; Ji et al. 2023; Munir et al. 2016; Weir et al. 2012), they have not been described for *Colletotrichum* species, including *C. siamense*, at low temperatures conditions. Thermal phenotyping of the prevalent *Colletotrichum* species at low temperatures is thus necessary to assess risk and manage *Colletotrichum* spp. over a wide range of temperatures.

The mission of our laboratory is to provide useful information to manage anthracnose. Our previous work (Ji et al. 2023) stimulated several questions. Do the strains of *C. siamense* with growth superiority above 30 °C pose relatively less risk for crops below 26°C? Is the growth rate of *Colletotrichum* spp. correlated with their virulence on strawberry at lower temperatures? Is there a potential trade-off between the tolerance to high temperature among *C. siamense* strains and their virulence to susceptible hosts as previously suggested (Chen et al. 2017; Ji et al. 2023)? Our specific objectives in this study of five representative *Colletotrichum* strains was (1) to evaluate the potential risk of *Colletotrichum* spp. below 26 °C by modeling mycelial growth and disease severity, (2) to examine the possible trade-off between temperature adaptation and virulence, and (3) to compare the relative influence of temperature and host genotype on strawberry disease severity.

Materials and methods

Plant materials and growth conditions

The woodland strawberry 'Ruegen' (*F. vesca*) was grown from seed, and the highly susceptible commercial octoploid strawberry 'Benihoppe' (*F.* × *ananassa* Duch.) was propagated from stolons (Jin et al. 2014) in a strawberry greenhouse at the Institute of Forestry and Fruit Tree Research, Shanghai Academy of Agricultural Sciences. All plants were grown at 24 ± 2 °C with 14 h light/10 h dark and watered twice a week. Fertilizer (0.1%) containing N-P-K 15-15-15 (YaraMila, Norway) was applied once a week. Old leaves were removed, then the plants were sprayed with fungicides. Fungicides and fertilizer were no longer applied in 2 weeks before inoculation. The third and fourth leaves were freshly detached and used for site-specific fungal inoculation or mock treatment.

Fungal strains

Five *Colletotrichum* strains belonging to the *C. gloeosporioides* complex and isolated from strawberry fields in eastern China were chosen for this study. *C. siamense* strains GQHAH2, Nj-2, and GQHZJ19, which vary in virulence on 'Benihoppe', were isolated, respectively, from Anhui, Jiangsu and Zhejiang Provinces (Han et al. 2016; Zhang et al. 2020). *C. gloeosporioides* sensu stricto (s.s). strain JSH-7-1 was isolated in Hubei Province (Han et al. 2016). Highly virulent *C. fructicola* accession CGMCC3.17371 (depository) was isolated in Shanghai Municipality and maintained in our laboratory (Ren et al. 2008; Zhang et al. 2016).

The strains were rejuvenated in 2022 by single-spore isolation from infected leaf tissues of strawberry 'Benihoppe'. Before the temperature tests, each strain was propagated on PDA (Difco Potato Dextrose Agar; Becton, Dickinson Company, USA) plates for 7 days at 28 °C in the dark.

Characterizing in vitro mycelial growth

Three pieces (1–2 mm^2) from the margin of the 7-day-old cultures (described in the previous section) were transferred to a plate (Φ = 90 mm) of PDA, four replicate plates were used for each temperature × strain combination. The 20 plates (4 replicates × 5 strains) per temperature were placed in a completely randomized block design in a low-temperature incubator (model KT053, BINDER, Tuttlingen, Germany) and incubated in the dark at 10, 14, 18, 22 or 26 °C.

After 3 d, both sides of the colonies were photographed; colony texture, density, color, and shape were recorded.

Colony diameter was measured using ImageJ software (Rueden et al. 2017), and average growth rate over 3 d (mm/day) was calculated. The diameter data for each strain at each temperature were used for modeling and statistical analysis. The experiment was done twice (2022 and 2023).

Preparation of conidial inoculum

About 10 mycelial squares (ca 1 × 1 cm) cut from the margin of 7-d-old cultures on PDA incubated at 14 or 22 °C were transferred to a 250 ml conical flask with 100 ml PDB (Difco Potato Dextrose Broth, Becton, Dickinson Co., Franklin, Lakes, NJ, USA), and incubated on a rotary shaker at 200 rpm for 4–7 days at the same incubation temperature as the original culture. Then, the culture was filtered through two layers of lens tissue, and the conidia were quantified with a hemocytometer. The concentration was adjusted to 2×10^6 conidia per ml either by dilution with distilled water for all cultures grown at 22 °C, except for *C. siamense* strain Nj2, or by centrifugation enrichment for all *Colletotrichum* strains grown at 14 °C. All the harvested conidia except for those of Nj2 at 22 °C were used for inoculation in PDB that was diluted nearly 50% with distilled water. The potential effect of PDB concentrations on pathogenesis was also evaluated using conidia from strain Cs GQHZJ19 (grown at 22 °C) that were suspended in 0, 25%, 50%, 75% and 100% (v/v) PDB.

Virulence assay

The virulence of each of the five *Colletotrichum* strains was assessed by inoculating 15 wounded, detached leaves on filter paper that was saturated with sterile water in petri plates, largely as previously reported (Ji et al. 2023). On the adaxial surface of each leaflet from at least eight plants of each strawberry variety for each temperature × strawberry variety combination, each half of the leaflet was wounded at two sites (toward the tip [apical] and the base [basal] with the needle of a 1-ml syringe. On each leaf, the apical wound on the right was treated with 10 µl of sterile water containing 0.01% (v/v) Tween-20 as the mock treatment, and the other three sites were inoculated with a 10-µl drop (2×10^6 per ml) of a conidial suspension of the respective strains (left two for one strain, right basal for another strain). In all for each combination of temperature × variety, 42 sites were inoculated with conidia and 15 sites with sterile water. In total for the experiment, 75 leaflets were used. All plates were sealed with parafilm to maintain the humidity above 90%, then incubated in the dark at 14 or 22 °C (the same temperature for mycelial culture and conidia production) for 48 h, then with 14 h light/10 h dark. After 3 and 5 days, the leaves were photographed to calculate lesion area using ImageJ software and to score symptoms and calculate a disease index (%) as described previously (Jin et al. 2014; Zhang et al. 2020). Two independent experiments were done in 2023.

In 2024, two additional virulence assays at 22 °C were done using 'Benihoppe'. To evaluate the potential influence of PDB on disease development, conidia of Cs GQHZJ19 were suspended in 0, 25%, 50%, 75% or 100% PDB, which were each used to inoculate two to three wounded sites on each of six to eight leaflets. Each leaflet had two to three wounded sites on the left side that were inoculated with conidia and one toward the apex on the right side that was mock-inoculated with PDB. In addition, to compare virulence on wounded and nonwounded sites, we inoculated an apical wound on the left side and an intact basal site on the right side of 10 leaflets with conidia of one fungal strain. Five other leaflets were mock-treated with PDB on wounded and nonwounded sites on the opposite sides of the leaf.

Model fitting and statistical analyses

To assess mycelial growth of each *Colletotrichum* strain in response to temperatures (10–26 °C), we used a two-way ANOVA (Tukey test, $P \leq 0.001$). Mycelial growth rate was compared between fungal strains using Duncan's two factor test ($P \leq 0.05$). A polynomial model was used to establish a response curve for the growth rate of *Colletotrichum* mycelium at different temperatures. The mycelial growth rate of each strain at different temperatures was analyzed using linear regression analysis in SPSS Statistics version 25 (IBM, Armonk, NY, USA), modelled with a quadratic function, and written as:

$$y(T) = b_0 + b_1 T + b_2 T^2, \quad (1)$$

where T is the independent variable temperature, y is the dependent variable mycelial growth rate, and b_0 is the intercept and b_1 and b_2 are coefficients of T and T^2.

In addition, we used a linear regression to analyze the relative influence of temperature and host genotype on disease severity caused by the *Colletotrichum* strain in strawberry, which was modelled with a linear function as:

$$y = ax + b, \quad (2)$$

where y is the disease index as the dependent variable, x is temperature or host genotype as the independent variable, a is the coefficient for a certain independent variable (slope) and b is a constant (intercept).

We used Pearson's correlation analysis to test for a correlation between PDB concentration and disease index on wounded leaflets of 'Benihoppe' inoculated with Cs GQHZJ19 at 22 °C. Parametric correlation analysis between mycelial growth rate (at 14/22 °C) and disease index (in *F. vesca*/*F.* × *ananassa*), and nonparametric correlation (Spearman's rank correlation coefficient) for temperature, host

genotype and fungal ecotype with disease severity were performed using the CORREL analysis (Wang and Chu 2014) in SPSS 25. Spearman's coefficient > 0 indicates a positive correlation, < 0 a negative correlation; the closer the correlation is to 1 or –1 the stronger the correlation, and 0 indicates no correlation. SPSS Statistics 25.0 was used for all regression and statistical analysis.

Results

Colony morphology of the *Colletotrichum* strains from strawberry at 10–26 °C

After 3 days in the dark on PDA at 10–26 °C (Fig. 1), the colony morphology of the five strains from eastern China differed at all temperatures tested except for 10 °C, but their growth rate did not differ significantly. At 14 °C, the colony of *C. siamense* (Cs) GQHZJ19 was relatively denser and *C. fructicola* (Cf) CGMCC3.17371 was the least dense among the five strains. At 18–26 °C, Cs and *C. gloeosporioides* s.s. (Cg) were mostly greyish white with abundant mycelium, intact margin, and a pale-orange dorsal surface. At 18 °C, five strains were greyish white with markedly elevated centers, although their reverse sides had a uniformly orange center except for Cf. At 22 °C, Cs GQHZJ19 produced white, dense mycelium that gradually thinned toward the edge, and brown conidial mounds formed in the center, which was much clear than that at 18 °C and 26 °C. Generally, Cs Nj2 looked like Cf CGMCC3.17371 at 22–26 °C, but grew relatively faster. The colonies of Cf at 18–26 °C were greyish white, with relatively sparse and fluffy edges, and the mycelium was visible on the back of all plates as clockwise circular growth. There was no discernible difference among the colonies at 22 °C and 26 °C.

Morphologically, the mycelium of Cg JSH-7-1 was basically the same as Cs GQHAH2 at 18–26 °C, but was more abundant and compact.

The above observations largely coincided with those of Ji et al. (2023), although clear differences were observed for Cs GQHZJ19 and Cf CGMCC3.17371. They reported that the Cs GQHZJ19 colony had a markedly raised center at 22 °C and 26 °C, which we did not observe. For *C. fructicola*, we did not observe the olivaceous gray flocculent in the center that was previously reported. These minor differences are likely due to differences in cultural conditions such as duration, starting inoculum, or unknown factors.

Growth rates of *Colletotrichum* strains at different temperatures

Significant differences among *Colletotrichum* strains were detected for all temperatures tested (Fig. 2). At 10 °C, Cf CGMCC3.17371 grew slower than Cs Nj2 (Fig. 2a), and it grew the slowest at all temperatures. Generally, growth of the other strains did not differ significantly; however, the

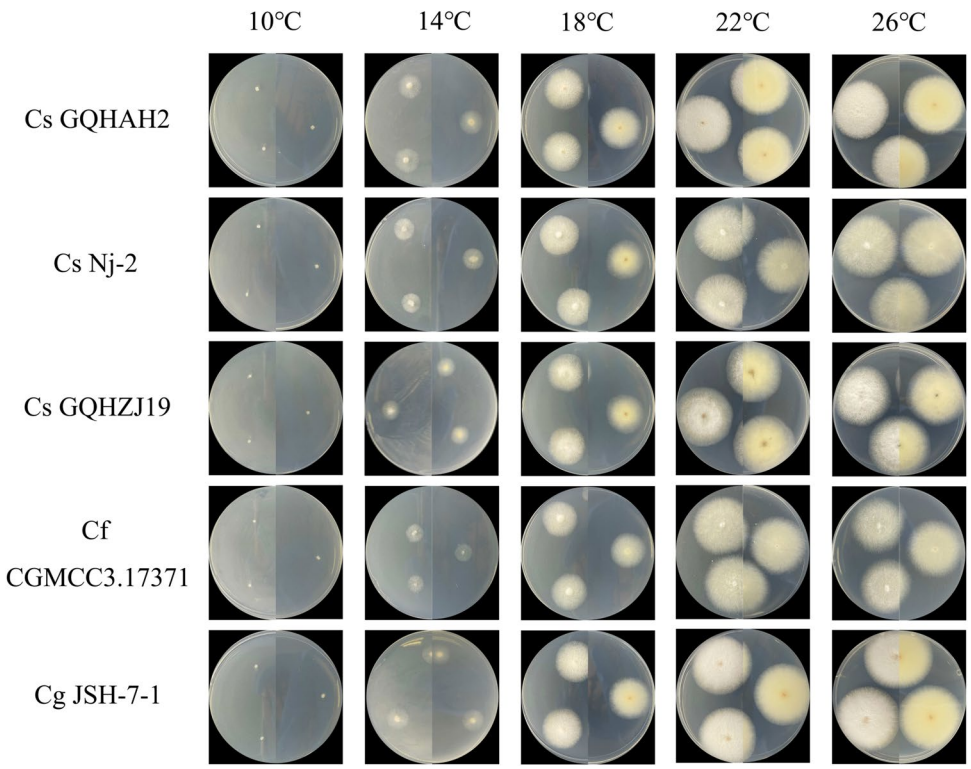

Fig. 1 Typical colony morphology of five *Colletotrichum* strains isolated from strawberry in eastern China: *C. siamense* (GQHAH2, Nj-2, GQHZJ19), *C. fructicola* (CGMCC3.17371) and *C. gloeosporioides* sensu stricto (JSH-7-1). Mycelial disks (ca 1–2 mm^2) were transferred to a PDA plate and incubated at 10 °C, 14 °C, 18 °C, 22 °C and 26 °C in the dark for 3 days. Each image is a composite: the top of plate is on the left; the back of the plate is on the right

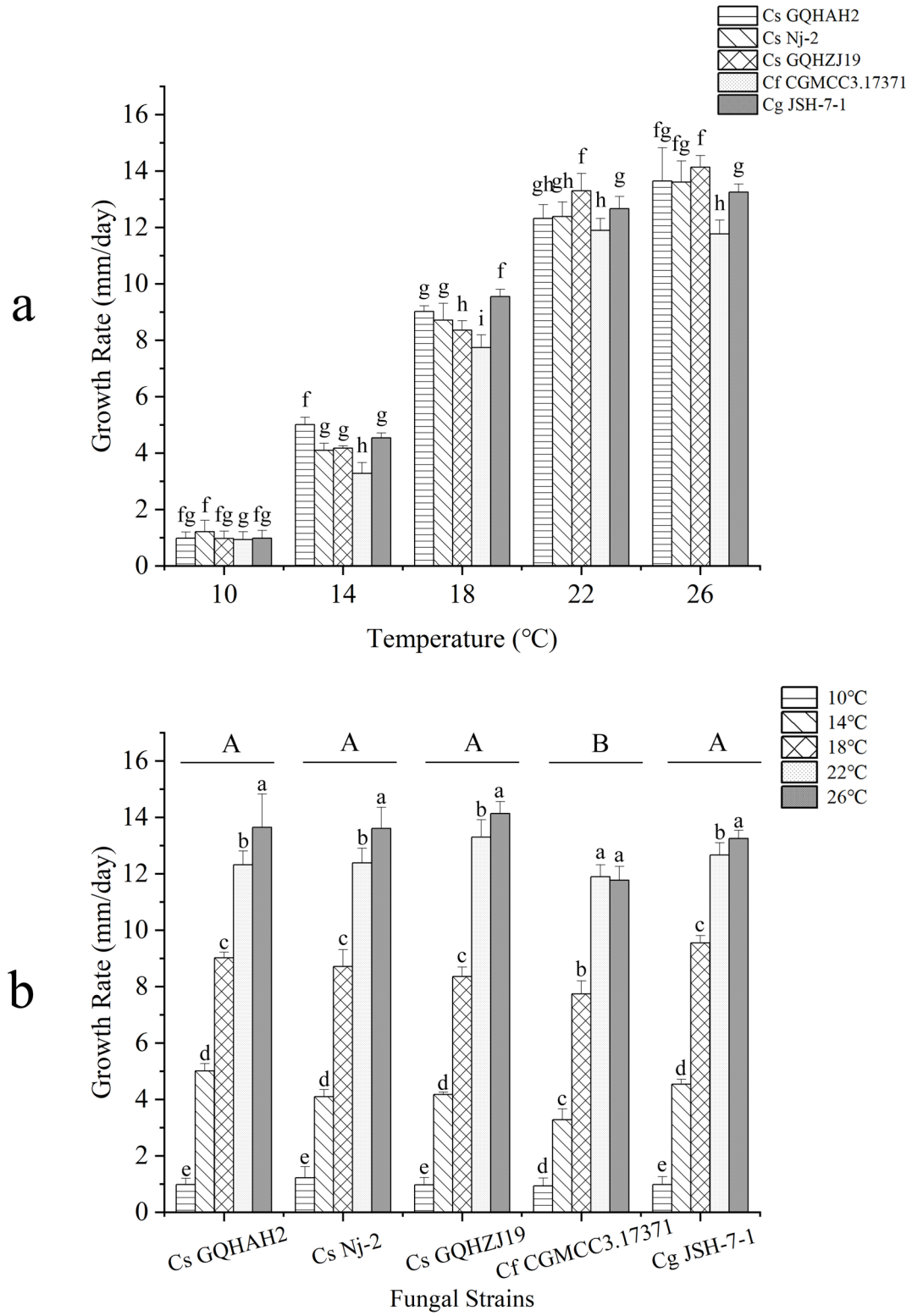

Fig. 2 Mean (±SD) colony growth rates of the five *Colletotrichum* strains isolated from strawberry and grown at 10–26 °C for 3 days in the dark. Twelve replicate plates were measured for each temperature × strain combination. **a** Difference among the growth rates of five fungal strains at the same temperature. Different lowercase letters indicate a significant difference among strains at that temperature (Duncan's test, $P < 0.05$). **b** Growth rate of *Colletotrichum* strains at different temperatures. Different lowercase letters indicate a significant difference among growth rates at the different temperatures for the strain (Duncan's test, $P < 0.05$). Different uppercase letters indicate a significant difference in growth rates among the five strains in a two-way ANOVA (followed by Tukey's test, $^{**}P < 0.001$)

growth rate of Cs GQHAH2, Cg JSH-7-1 and Cs GQHZJ19 peaked at 14 °C, 18 °C and 22 °C, respectively. Morphologically similar Cg JSH-7-1 and Cs GQHAH2 grew at similar rates at 22–26 °C, but their growth rates differed significantly at 14 °C and 18 °C. Cf CGMCC3.17371 and Cs Nj2 grew at significantly distinct rates at all temperatures except 22 °C (11.89 and 12.38 mm/d, respectively). The growth rate of Cs GQHZJ19 was highest at 22 °C (13.30 mm/d) and 26 °C (14.13 mm/d), but the difference in growth rates between it and the other four strains was significant only at 22°.

As temperatures increased from 10 to 22 °C, the growth rate of all five strains sharply increased (Fig. 2b). *C. siamense* and *C. gloeosporioides* strains grew faster from 22 to 26 °C, but Cf CGMCC3.17371 did not, and the increase in rate was less than that observed below 22 °C. Two-way ANOVA (followed by a Tukey test) statistical results suggested that all four *C. siamense* and *C. gloeosporioides* strains had similar thermal responses at 10–26 °C, but the growth rate of Cf CGMCC3.17371 at the different temperatures differed significantly from the other four strains.

These observations did not completely agree with those of our previous study (Ji et al. 2023), in which the average mycelial growth among these five strains did not differ significantly during 5 days at 22 °C; Cf CGMCC3.17371 and Cg JSH-7–1 grew significantly faster than Cs GQHAH2 during 4 days at 26 °C. However, our present study might provide a more accurate comparison of the strains and thus differentiate mycelial growth of these *Colletotrichum* strains in an identical duration (3 d).

Polynomial curve fitting for *Colletotrichum* mycelial growth at different temperatures.

The generalized beta-function of the nonlinear regression model (Hau et al. 1985) fit *Colletotrichum* mycelial growth rates from 22 to 36 °C in our previous study (Ji et al. 2023), but in the present study, based on a series of derivations in SPSS, the generalized beta-function was not applicable from 10 to 26 °C (data omitted). Because fungal growth rate against temperature might follow a quadratic parabola (Hau and Kranz 1990), a polynomial function was used to fit the mycelial growth rates in this study (Eq. 1; Table 1; Fig. S1). The coefficient of determination (R^2) was used as an index of model fitting to quantify the strength of the relationship between the response variable and the dependent variable. As shown in Table 1, all R^2 and adjusted R^2 values for the current modelling were greater than 0.95.

Mycelial growth was fastest at 26 °C, when the model curve still showed an increasing trend for all *Colletotrichum* strains (Fig. S1), which was consistent with their optimum temperature above 26 °C proposed based on the generalized beta-function (Ji et al. 2023). The growth rate of the *C. fructicola* strain was obviously the lowest among five strains at all tested temperatures except 10 °C. *C. gloeosporioides* s.s. growth was significantly faster than Cf from 14 to 22 °C, which was not easily distinguished from the patterns of *C. siamense*. From 26 °C and above, radial growth of the three *C. siamense* strains was consistently slightly faster than that of *C. gloeosporioides* s.s. In brief, the growth rate of the *C. siamense* strains conformed to a quadratic function curve, showing that these strains grew relatively faster at all temperature ranges.

Virulence of *Colletotrichum* strains on two strawberry hosts at 14 °C and 22 °C

The virulence test at 14 °C and 22 °C of the five strains on leaves of the two strawberry hosts that varied in susceptibility showed that all strains were pathogenic on the wounded leaves of *F. vesca* and *F.* × *ananassa*, but disease severity varied with temperature, host type, fungal strain and time after inoculation. No necrotic lesions occurred on mock-treated leaves of two strawberries at any phase after inoculation. Representative leaves with typical symptoms are in Fig. 3.

At 14 °C, necrosis was rarely observed on *F. vesca* at 3 dpi, and minor necrotic points was discernible only at 5 dpi with Cs GQHZJ19 and Cf CGMCC3.17371. By contrast, at the same temperature, on the leaves of the highly

Table 1 Response surfaces for mycelial growth rate of different *Colletotrichum* strains as a function of temperature described by the equation: $y(T) = b_0 + b_1T + b_2T^2$

Statistic	Cs GQHAH2	Cs Nj-2	Cs GQH ZJ19	Cf CGMCC3.17371	Cg JSH-7–1
b_0	–14.48051 ± 0.99366	–12.42774 ± 1.20755	–12.94563 ± 1.2914	–13.30115 ± 1.49075	–16.71172 ± 0.98774
b_1	1.80004 ± 0.11816	1.51054 ± 0.14359	1.52446 ± 0.15356	1.61068 ± 0.17859	2.07545 ± 0.11745
b_2	–0.02732 ± 0.00325	–0.019 ± 0.00395	–0.01773 ± 0.00423	–0.02393 ± 0.00495	–0.03498 ± 0.00323
Sum of squared residuals	25.95995	38.3392	43.84781	51.98874	25.65142
R^2	0.98059	0.97192	0.97199	0.95251	0.9811
Adjusted R^2	0.97991	0.97094	0.97101	0.95069	0.98044

Cs, *C. siamense*; Cf, *C. fructicola*; Cg, *C. gloeosporioides*

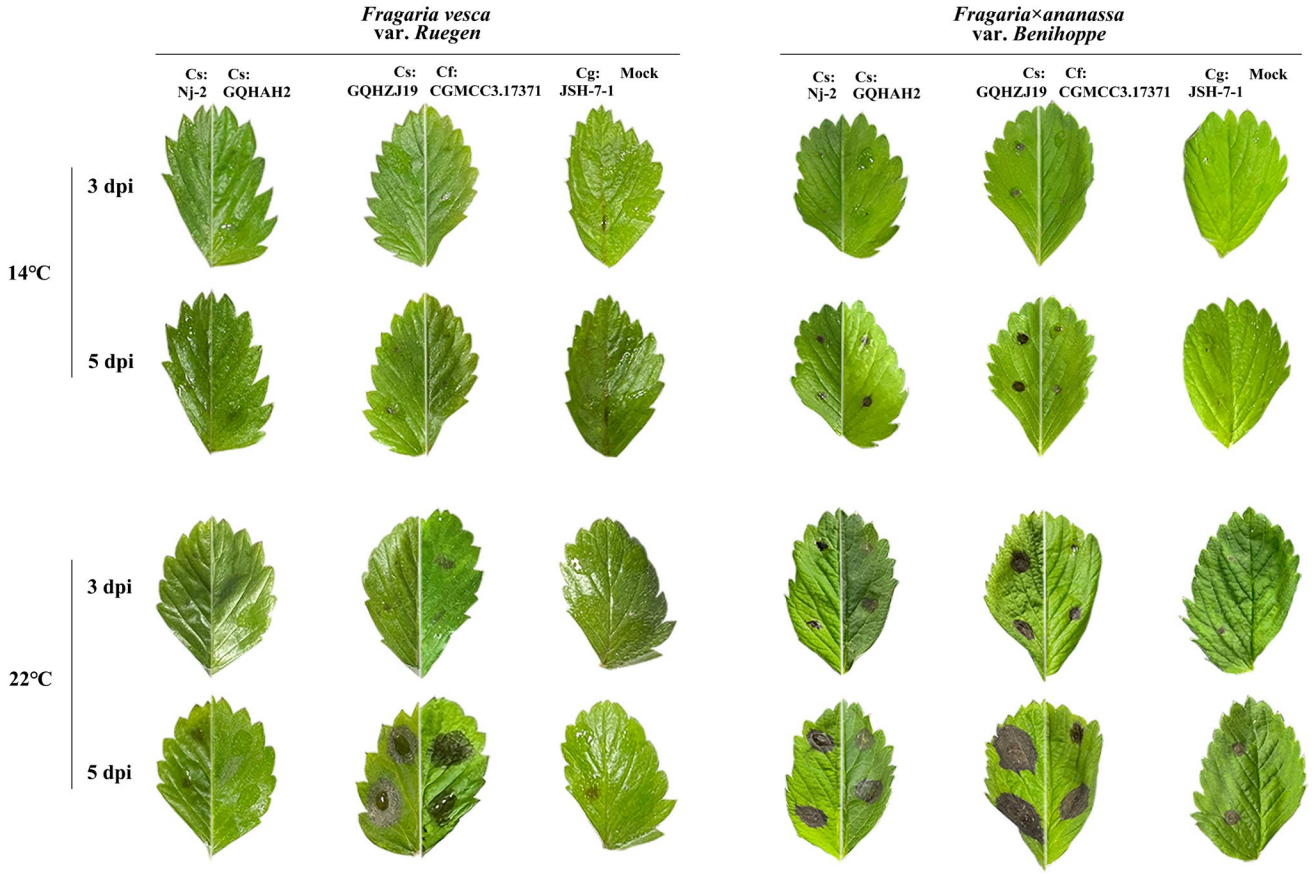

Fig. 3 Anthracnose symptoms on wounded strawberry leaves inoculated with *Colletotrichum* spp. and incubated at 14 °C or 22 °C for 3 or 5 days post inoculation (dpi). Two sites on the left side of each leaf were inoculated with 10 µl of a conidial suspension (2×10^6 per ml) of the strain. The right image shows a different leaflet that was similarly inoculated with the strain shown, except for Mock, which shows representative leaves from the actual experiment; the right side of each leaf was inoculated with 10 µl of sterile water (apical right) and 10 µl of the conidial suspension of the test strain (basal right) for three triplicate tests of each strain

susceptible 'Benihoppe', necrosis was obvious at 3 dpi with Cs GQHZJ19. Indeed, necrotic points were also discernible on 'Benihoppe' at this stage after inoculation with the other two *C. siamense* strains. By 5 dpi, all five *Colletotrichum* strains had caused necrosis on 'Benihoppe' leaves at 14 °C, although the lesions caused by the three *C. siamense* strains were significantly larger than those caused by *C. fructicola* and *C. gloeosporioides* s.s.

At 22 °C, at 3 dpi with Cs Nj2, Cs GQHZJ19 and Cf CGMCC3.17371, leaves of *F. vesca* had necrotic points. By 5 dpi, necrotic lesions were present on *F. vesca* inoculated with all strains except for Cs GQHAH2. Comparatively, the relative lesion areas on *F. vesca* at 5 dpi were somewhat equivalent to those observed at 3 dpi on 'Benihoppe'. Actually, the virulence of each fungal strain was remarkably pronounced on the wounded leaves of 'Benihoppe' at 5 dpi under 22 °C. Both *C. fructicola* and Cs GQHZJ19 caused necrotic symptoms at all 42 inoculation sites per strain by 3 dpi at 22 °C on 'Benihoppe'. By 5 dpi at 22 °C, the number of necrotic spots increased, the area of the necrosis expanded, and every strain caused necrotic lesions on all 42 inoculated sites.

The above observations are based on several typical infected leaves and, thus preliminary. In the present study, results from two independent replications of the virulence tests were generally consistent for the strains on each host at each time and temperature. With the disease index based on necrosis at the 42 inoculated sites, the relative lesion areas were summarized to show the overall virulence per temperature × host type × fungal strain at each time after inoculation for one independent experiment (Fig. 4). Clearly, low temperature such as 14 °C limited the virulence in both hosts, while the 22 °C temperature optimum for host and pathogen fostered the development of anthracnose. The virulence of the strains varied with host type, infection duration and temperature.

At 3 dpi and 14 °C on the less susceptible *F. vesca*, the virulence of the five strains was too weak to be distinguishable from each other, but at 22 °C, their relative virulence could be ranked as Cs GQHZJ19 > Cf

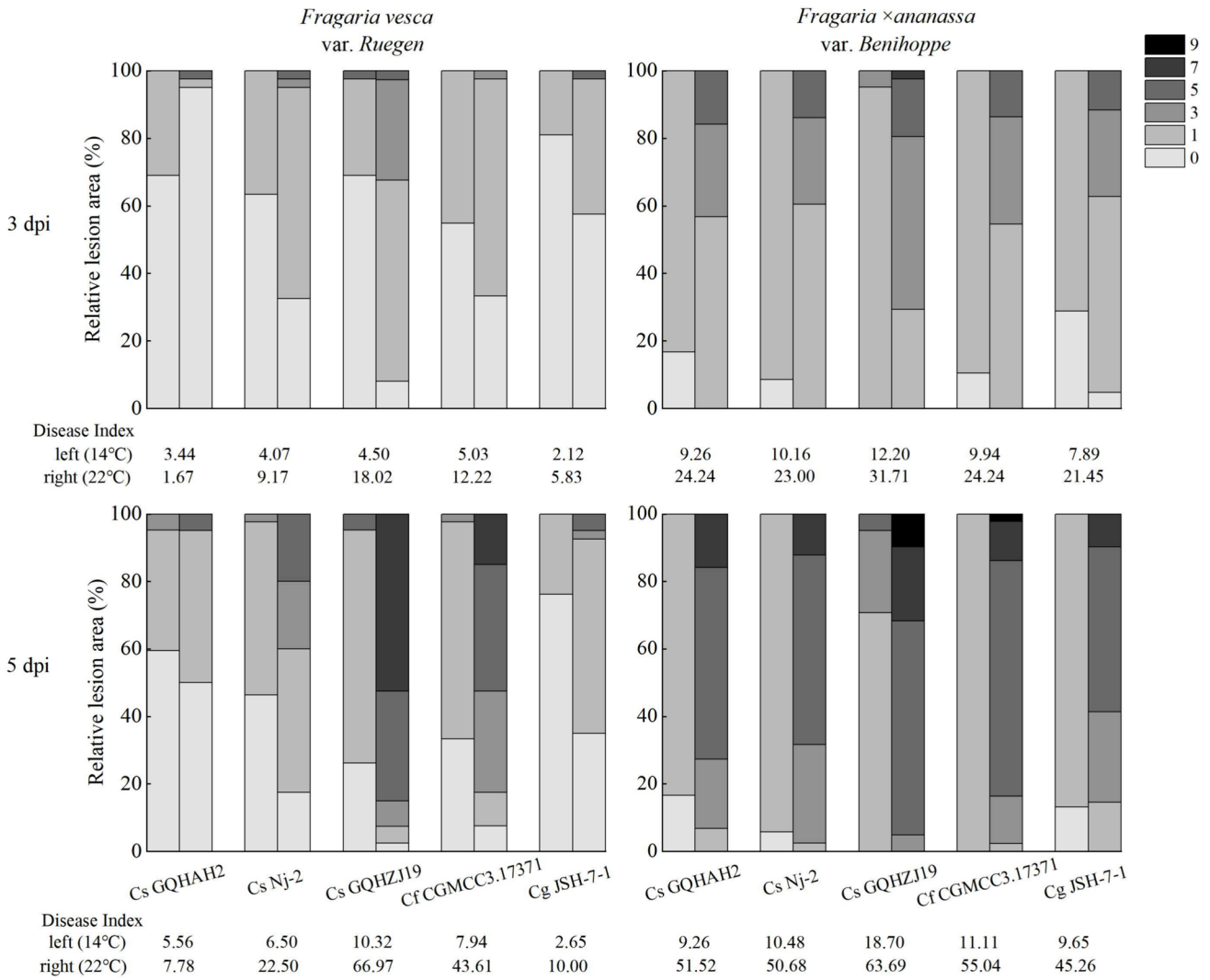

Fig. 4 Anthracnose severity on wounded, detached strawberry leaves at 14 °C and 22 °C. Relative lesion area (%) is based on the severity at all 42 inoculated sites per strain × temperature combination scored as described by Jin et al. (2014) with minor modification. The disease index (%), calculated using the formula of Zhang et al. (2020), is provided below each strain name

CGMCC3.17371 > Cs Nj2 > Cg JSH-7–1 > Cs GQHAH2. By 5 dpi, the relative virulence of five strains largely ranked as Cs GQHZJ19 > Cf CGMCC3.17371 > Cs Nj2 and Cs GQHAH2 > Cg JSH-7–1, regardless of temperature. The virulence of *C. siamense* strains NJ2 and GQHAH2 on *F. vesca* was difficult to differentiate.

For the highly susceptible 'Benihoppe', Cs GQHZJ19 still was the most virulent (Fig. 4). At 3 dpi at 14 °C and at 22 °C with CGMCC3.17371, Cs Nj2 and Cs GQHAH2, disease severity on 'Benihoppe' was similar, and a little more severe than with Cg JSH-7-1 but less severe than with Cs GQHZJ19. By 5 dpi at 14 °C, the virulence of all but Cs GQHZJ19 on 'Benihoppe' was largely similar. At 22 °C at 5 dpi, the relative virulence of five strains on 'Benihoppe' was the same as on *F. vesca*.

For the most virulent strain, Cs GQHZJ19, when wounded leaves of 'Benihoppe' were inoculated with conidia in different concentrations of PDB, the PDB concentration was not correlated with the disease index at 3 dpi and 5 dpi (Tables S1–S3). Although conidia in 50% and 100% PDB caused relatively higher disease severity than those in 0, 25% and 75% PDB (Fig. S2), the difference was not significant ($P < 0.05$) at either 3 dpi or 5 dpi.

Compared with inoculation of wounds on 'Benihoppe', inoculation of intact sites resulted in a significant delay in the first appearance of necrosis but did not alter the relative virulence of five fungal strains (Figs. S3, S4). Cg JSH-7–1 did not cause any visible necrosis until 10 dpi (data not shown), although Han et al. (2016) reported necrosis appeared at 30 dpi at 25–35 °C for this strain. Inoculating

wounds is thus better for distinguishing differences in the virulence of fungal strains and is convenient for quantifying and comparing distinct host–pathogen interactions. We thus inoculated wounds for subsequent assays.

Correlation of mycelial growth with *C. siamense* virulence under 14 °C and 22 °C

The above work indicated that the mycelial growth rate of the *C. fructicola* strain was always lowest of the five strains, but its virulence was significantly higher than that of the *C. gloeosporioides* s.s. strain. Clearly, mycelial growth rate was not correlated with the virulence of the various *Colletotrichum* strains and species. Since slower mycelial growth might contribute to a reduction in the virulence of *C. gloeosporioides* (Zhang et al. 2023), the potential correlation of mycelial growth rate with fungal virulence (disease index, that is, the severity of symptoms) was evaluated among strains of *C. siamense* using a parametric correlation analysis in SPSS (Table 2).

Interestingly, the mycelial growth rate of *C. siamense* strains was always negatively correlated at 14 °C and positively correlated at 22 °C with their virulence on strawberry at the same temperature, independent of host susceptibility and time after inoculation, although not at a significant level in most cases. The only exception was that, on the highly susceptible 'Benihoppe' at 3 dpi the growth rate of *C. siamense* strains at 22 °C was significantly and positively correlated with the disease index.

Influences of temperature, host genotype and fungal ecotype on *Colletotrichum* virulence

In the analysis of temperature, host genotype, fungal strain and disease index using a non-parametric correlation analysis in SPSS, similar results were obtained when data for all five *Colletotrichum* strains or only the three *C. siamense* strains were analyzed. The results for all five strains are shown in Table 3. When the disease index was treated as the dependent variable reflecting fungal virulence, fungal strain was not correlated with severity (disease index) at an early (3 dpi) or late (5 dpi) stage. Notably, host genotype was significantly correlated with the disease index at both stages. In contrast, temperature was only significantly correlated with virulence at 5 dpi. This analysis suggested that at an early stage, host genotype had crucial, extremely significant effect on pathogenesis near 14–22 °C.

At 5 dpi, host genotype and temperature were significantly correlated with the disease index. To analyze the influence of temperature and host genotype on disease severity, we used a linear regression (Eq. 2). From Table 4, the *t*-value for the analyzed temperature was 4.670, the *t*-value for host genotype was 2.306, and the *P*-value for the two factors was less than 0.05, suggesting that temperature and host genotype had a significant effect on disease index. The VIF value was less than 5.0, indicating that the two regression lines were not covariant. Beta, the standardized coefficient for current regression modelling was higher, indicating that the dependent variable was more responsive to the independent variable, that is, the greater the degree of influence. Obviously, later in pathogenesis, temperature had a greater

Table 2 Parametric correlation analysis of mycelial growth and the virulence of *Colletotrichum siamense* at different times after inoculation of two strawberry species

Statistic	*F. vesca* var. Ruegen				*F.* × *ananassa* cv. Benihoppe			
	14 °C		22 °C		14 °C		22 °C	
	3 dpi	5 dpi	3 dpi	5 dpi	3dpi	5dpi	3dpi	5dpi
Correlation coefficient[a]	−0.984	−0.651	0.855	0.958	−0.695	−0.644	0.997*	0.959
P	0.113	0.549	0.347	0.186	0.511	0.554	0.045	0.183

[a]Correlation coefficient for mycelial growth rate and disease index

*Significant at $P < 0.05$ (2-tailed)

Table 3 Nonparametric correlation of temperature (T), host genotype and fungal ecotype with *Colletotrichum* virulence (disease severity)

Method	Dependent variable	Parameter	3 dpi			5 dpi		
			T	Host genotype	Fungal ecotype	T	Host genotype	Fungal ecotype
Spearman's rho	Disease index	Correlation coefficient	0.305	0.638**	0.000	0.538*	0.486*	0.018
		Sig. (2-tailed)	0.112	0.001	1.000	0.014	0.030	0.939
		N	20	20	20	20	20	20

*Significant at 0.05 level (2-tailed)

**Significant at 0.01 level (2-tailed)

Table 4 Influence of temperature and host genotype on disease index at 5 dpi described as $y = ax + b$

Parameters	Nonstandardized coefficients[a]		Standardized coefficient[b]	Values for influence		
	b	*a*	Beta	*t*	*P*	VIF
Constant	–57.690 ± 16.087			–3.586	0002	
Temperature		3.415 ± 0.731	0.703	4.670	0.000	1.000
Host genotype		13.490 ± 5.850	0.347	2.306	0.034	1.000
R^2	0.615					
Adjusted R^2	0.569					

[a]*b*: constant of the equation. *a*: coefficient for certain independent variables. Dependent variable: disease index (*y*)

[b]Beta is the standardized coefficient of regression modelling. R^2 value is the coefficient of determination, an indicator of model fit, the closer the value is to 1 the better the fit

influence ($\beta = 0.703$) on severity (disease index) than host genotype ($\beta = 0.347$). The adjusted R^2 value suggested that temperature and host genotype explained at least 56.9% variation in *Colletotrichum* virulence to strawberry at a later stage.

Discussion

In this work, modelling of mycelial growth based on a polynomial linear regression analysis revealed that the *C. siamense* strains and the *C. gloeosporioides* strain had similar growth trends at 10–26 °C, but the rates differed significantly from that of the *C. fructicola* strain. Indeed, our current and previous work (Ji et al. 2023) together suggested that growth of *C. siamense* is vigorous at 10–40 °C, and the thermotolerant strain Cs GQHZJ19 also grew fast at 22/26 °C.

In the present study, Cs GQHZJ19 was highly virulent on both *F. vesca* and the highly susceptible *F.* × *ananassa* cv. Benihoppe at 14 °C and 22 °C. The relative virulence of three *C. siamense* strains was largely consistent with previous work on 'Benihoppe' at 25 °C (i.e., virulence rank: GQHAH2, weak; Nj2, moderate; GQHZJ19, strong; Ji et al. 2023; Zhang et al. 2020). However, Cs GQHAH2 was significantly more virulent than Cs Nj2 on 'Benihoppe' at 5 dpi at 30 °C (Ji et al. 2023). Since Cs GQHZJ19 grew fastest at a wide range of temperatures over 22 °C, these observations are counter to the suggestion of a potential trade-off between tolerance to high temperature and virulence of a fungal strain on susceptible host (Chen et al. 2017). Accordingly, we propose that *C. siamense*, which is favored by global warming, can harm host crops at a wide range of temperatures, and its potential threat to agricultural production is not reduced below 26 °C.

Plants and pathogens coevolve in an arms race of host resistance against the mode of pathogen attack (Zhan et al. 2014, 2015). Plants have evolved qualitative and quantitative types of resistance to improve their survival when confronted with pathogens (Lo Iacono et al. 2012; Poland et al. 2009). Host resistance is one of the most important biological factors shaping not only the populations but also the evolutionary structure of pathogens (McDonald and Linde 2002). Our statistical analysis evaluated the effects of host genotype, fungal strain and temperature on the risk of strawberry to disease caused by *Colletotrichum*. Host genotype always significantly affected *Colletotrichum* virulence, and strawberry genotype was especially important in preventing disease early in the disease process. Thus, breeding novel strawberry varieties with improved resistance to *Colletotrichum* fungi is the best option for managing anthracnose.

Temperature is well known to affect almost all aspects of biological and biochemical processes (Nadeem et al. 2014; Park et al. 2011). Our present study suggested that temperature might not be a significant influence at an early stage of pathogenesis at 14/22 °C; however, during the late stage, temperature surpassed host genotype in determining the final outcome of the host–pathogen interaction. In addition, fungal ecotype was not correlated with anthracnose severity.

This preliminary work addressed questions that arose from previous work on five representative *Colletotrichum* strains from strawberry. To better evaluate the growth rate and virulence of *Colletotrichum* strains, especially *C. siamense*, more fungal strains need to be characterized over a wider temperature range. Determining the temperature requirements of *C. siamense* for sporulation, conidial germination, and the formation of appressoria should provide a more complete analysis of their thermal requirements and help in designing the best management for these pathogens.

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Author contributions XL, CG, KD and QG conceived the study and designed the experiments. XL and KD wrote the manuscript. XL, XH, SC, JY and FL did the experiments. XL, CG, YH and KD analyzed and interpreted the data. XL, KD and QG revised the manuscript. All authors approved the submitted version.

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Declarations

Conflict of interest The authors declare that they had no commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval The manuscript does not contain experiments using animals and human studies.

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