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Sterol demethylation inhibitor fungicide resistance in *Colletotrichum siamense* from chili is caused by mutations in CYP51A and CYP51B

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Abstract

Anthracnose, caused by fungi of the genus *Colletotrichum*, is a serious disease of chili worldwide. Sterol 14α-demethylation inhibitors (DMIs) are a class of chemical fungicides that can effectively control anthracnose diseases. In this study, 22 *Colletotrichum* isolates collected from commercial chili fields in Zhangzhou, Fujian Province, China, were identified as *Colletotrichum siamense*. The sensitivities of the 22 *C. siamense* isolates to tebuconazole were determined based on the EC_{50} (50% effective inhibition concentration) value. The results showed that the EC_{50} values of the two isolates to tebuconazole were 0.039 ± 0.0036 and 0.042 ± 0.0012 mg/L, while the other 20 isolates showed significantly decreased sensitivities to tebuconazole, with EC_{50} values ranging from 0.61 ± 0.056 to 1.94 ± 0.11 mg/L. Sequence analysis of CYP51A and CYP51B revealed five genotype mutations (i. e., $CYP51A^{V46L, D115V, P163S, R306K, E397D}$, $CYP51A^{D115V, S164Y, R306K, E397D}$, $CYP51A^{D115V, R306K, P339T, E397D}$, $CYP51A^{D115V, R306K, E397D, S400N}$, and $CYP51A^{D115V, R306K, E397D}$CY-$P51B^{R266H}$) in the resistant isolates. The tebuconazole-resistant isolates of five genotypes suffered a fitness penalty and exhibited cross-resistance to difenoconazole, prochloraz, and propiconazole. Additionally, the five genotype mutations were validated as being responsible for tebuconazole-resistance in *C. siamense* by construction of replacement mutants. Overexpression of *CYP51A* and *CYP51B* was not detected in the replacement mutants of the five genotypes. Overall, the present study is the first to report DMI resistance in *C. siamense* and provides significant information for rational use of DMIs to control chili anthracnose.

Keywords: Anthracnose, Chili, DMI fungicides, Fitness, CYP51A and CYP51B, Resistance mechanism

Background

Chili (*Capsicum* species) is one of the most important vegetables worldwide and mainly grows in tropical and subtropical areas (Pickersgill 1997). Chili is also an important ingredient in cooking due to its high nutritional value and wide range of forms in which it can be consumed (Jiang et al. 2018). According to Food and Agriculture Organization of the United Nations (FAO, https://www.fao.org/home/en/), the total output of chili in China was 40 million tons, accounting for approximately 65% of global chili output in 2019. With the expansion of planting, fungal diseases have become a major limiting factor for chili quality and yield in both the pre- and postharvest stages (Park et al. 2012). Anthracnose, caused by *Colletotrichum* species, is a destructive disease that seriously threatens chili production (Chen et al. 2009; Wei et al. 2020). The disease can occur on chili fruit, manifested as sunken necrotic lesions accompanied with pink spore masses on surface (Lewis Ivey et al. 2004; Ashwini and Srividya 2014; Mongkolporn and Taylor 2018; Wei et al. 2021). Several *Colletotrichum* species including *C. acutatum*, *C. aenigma*, *C. gloeosporioides*,

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C. fructicola, *C. scovillei*, *C. siamense,* and *C. truncatum* have been reported as the causal agents of anthracnose on chili (Lewis Ivey et al. 2004; Than et al. 2008a; Wei et al. 2020; Shi et al. 2021), however, it's very difficult to identify a specific species based on only a few distinguishing methods (Mongkolporn and Taylor 2018; Hu et al. 2022).

Some agronomic strategies, such as crop rotation and utilization of resistant cultivars, have been developed to control chili anthracnose (Than et al. 2008b). However, current management of this disease heavily relies on chemical fungicides, including quinone outside inhibitors (QoIs), methyl benzimidazole carbamates (MBCs), and sterol demethylation inhibitors (DMIs) (Xu et al. 2014; Gama et al. 2020). These fungicides have different modes of action: QoIs (e.g., azoxystrobin) disrupt mitochondrial respiration by targeting the cytochrome b (Cyt b) at Qo sites (Bartlett et al. 2002); MBC fungicides (e.g., carbendazim) inhibit the growth of fungal pathogens by preventing microtubule assembly (Davidse 1986); DMIs (e.g., tebuconazole) inhibit fungal ergosterol synthesis by specifically binding to 14α-demethylase (CYP51), a critical enzyme for the synthesis of ergosterol (Berg et al. 1988). In plant fungal pathogens, point mutations in the *β-tubulin* gene are responsible for MBCs resistance, and mutations in Cyt b result in QoIs resistance (Ma and Michailides 2005). For DMI fungicides, there are three major resistance mechanisms: (1) point mutation in *CYP51* gene (Délye et al. 1997; Ma and Michailides 2005); (2) overexpression of *CYP51* (Luo and Schnabel 2008); and (3) enhancing drug efflux pumps by upregulation of MFS (major facilitator superfamily) or ABC (ATP-binding cassette) transporters (Sanglard et al. 1995; Nakaune et al. 1998; Hamamoto et al. 2000).

Understanding shifts in fungicide sensitivity has practical implications and is critical for disease management. So far, the intensive use of MBC and QoI fungicides has led to a rapid development of resistant *Colletotrichum* populations. A previous study showed that *C. siamense* isolates from peach and blueberry were resistant to azoxystrobin and thiophanate-methyl in the United States (Hu et al. 2015). Recently, *C. siamense* isolates from strawberry in China were shown to be sensitive to the tested DMIs but exhibited high resistance to azoxystrobin (Zhang et al. 2020). DMIs are characterized by their strong antifungal activity against a broad-spectrum of plant pathogenic fungi, and have been widely used for controlling anthracnose disease. DMIs can also enhance plant biotic and abiotic stress resistance, increase crop yield, and have a certain regulatory effect on plant growth (Liu et al. 2019). However, with the extensive use of DMIs, resistance or reduced sensitivity to this class of fungicides has been increasingly developed in many phytopathogenic fungi. In China, several DMIs are registered for controlling crop disease. Among them, tebuconazole has been widely used for controlling chili anthracnose because of its strong protective and curative activities. To date, the resistance of *C. siamense* to DMIs has not been reported. The aims of this study were to (1) determine the sensitivities of *C. siamense* isolates from chili to tebuconazole; (2) evaluate the fitness of tebuconazole-resistant *C. siamense* isolates; (3) investigate the cross-resistance between tebuconazole and other DMIs; and (4) explore the molecular mechanism of DMI resistance in *C. siamense*.

Results

C. siamense is the causal agent of chili anthracnose

A total of 22 *Colletotrichum* isolates were obtained from chili plants showing typical anthracnose symptoms in commercial chili fields of Zhangzhou, Fujian Province, China. Based on morphological characteristics and phylogenetic analysis of a combined dataset of ITS, *CAL*, *CHS1*, *GAPDH* and *ACT* sequence, all the *Colletotrichum* isolates were identified as *C. siamense* (Fig. 1).

C. siamense isolates from chili have developed resistance to tebuconazole

The sensitivities of the 22 *C. siamense* isolates to tebuconazole were determined by calculating the EC_{50} values. Among them, the EC_{50} values of two isolates, Zz14 and Zz17, to tebuconazole were 0.039 ± 0.0036 and 0.042 ± 0.0012 mg/L, respectively. In comparison, the sensitivities of the other 20 isolates to tebuconazole were significantly decreased, with EC_{50} values ranging from 0.61 ± 0.056 to 1.94 ± 0.11 mg/L and RF (resistance factor) values ranging from 15.06 to 47.90. The 20 *C. siamense* isolates exhibited high resistance to tebuconazole based on the EC_{50} and MIC values (Table 1), indicating that *C. siamense* isolates in chili fields of our sampled regions had developed resistance to tebuconazole.

Mutations in CYP51A and CYP51B of tebuconazole-resistant *C. siamense* isolates are categorized into five resistant genotypes

To investigate the resistance mechanism of *C. siamense* to tebuconazole, the full-length and promoter sequences of *CYP51A* and *CYP51B* from the tebuconazole-sensitive and tebuconazole-resistant isolates were analyzed. Sequence alignment results showed that the amino acid mutations in CYP51A and CYP51B could be categorized into five resistant genotypes. Genotype I has three mutations (D115V, R306K and E397D) in CYP51A, concomitant with a mutation R266H in CYP51B. Genotypes II, III, and IV have four mutations (D115V, S164Y, R306K, E397D), (D115V, R306K,

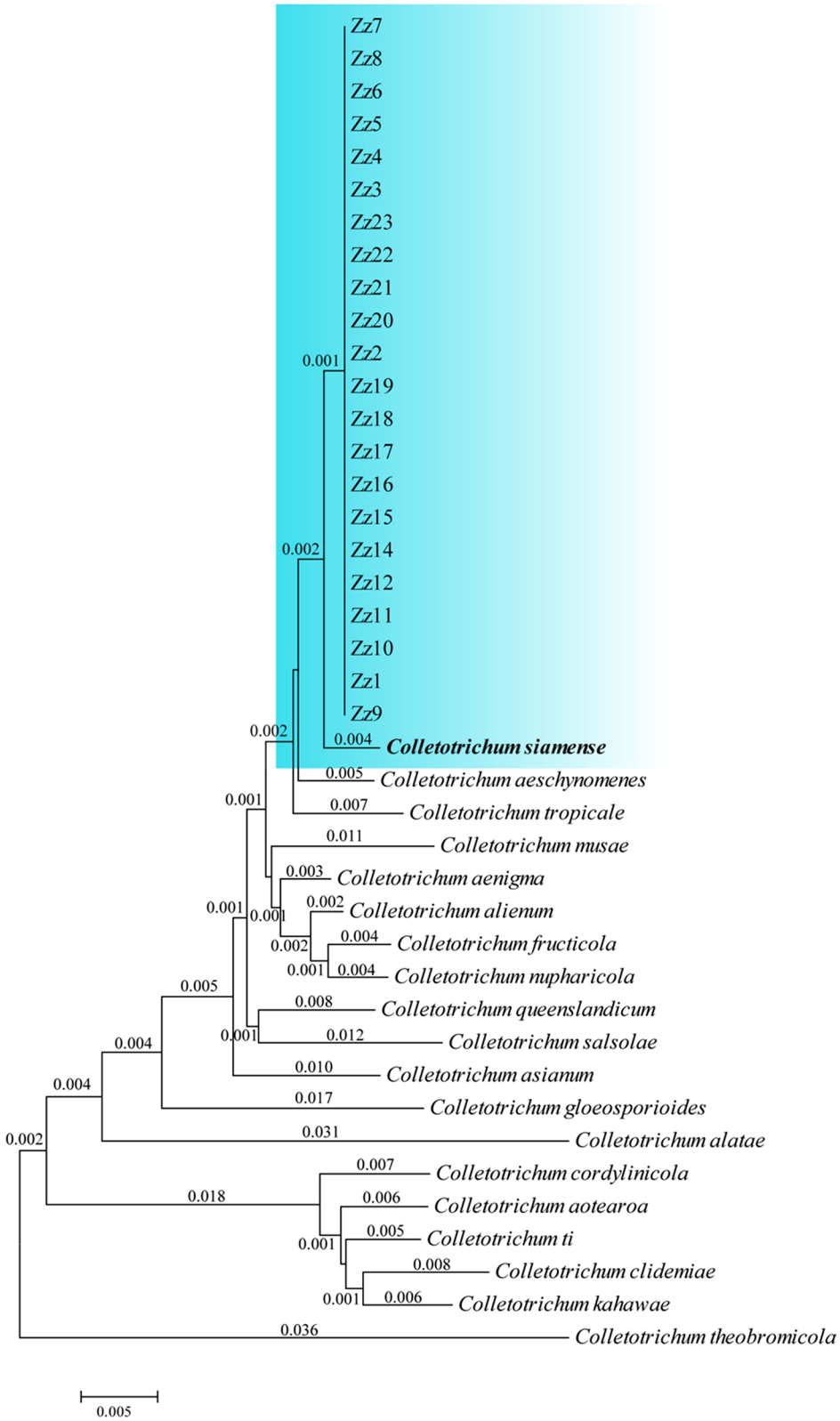

Fig. 1 Phylogenetic analysis of the *Colletotrichum* isolates from chili based on the combined sequences of ITS, *CAL*, *CHS1*, *GAPDH,* and *ACT*. Phylogenetic analysis was performed using MEGA 7 via the neighbor-joining method

Table 1 Sensitivities of *Colletotrichum* isolates from chili to tebuconazole

Isolates	Phenotype[a]	EC_{50} (mg/L)[b]	MIC (mg/L)[c]	RF for EC_{50}[d]
Zz14	Field/S	0.039 ± 0.0036e	2	–
Zz17	Field/S	0.042 ± 0.0012e	2	–
Zz1	Field/HR	1.09 ± 0.17b	60	26.91
Zz2	Field/HR	1.13 ± 0.15b	65	27.90
Zz3	Field/HR	0.68 ± 0.013 cd	55	16.79
Zz4	Field/HR	0.72 ± 0.034c	55	17.77
Zz5	Field/HR	1.73 ± 0.27a	75	42.71
Zz6	Field/HR	1.94 ± 0.11a	80	47.90
Zz7	Field/HR	1.82 ± 0.13a	80	44.93
Zz8	Field/HR	1.75 ± 0.25a	75	43.21
Zz9	Field/HR	0.61 ± 0.056d	55	15.06
Zz10	Field/HR	0.65 ± 0.023d	55	16.05
Zz11	Field/HR	1.11 ± 0.24b	65	27.41
Zz12	Field/HR	1.12 ± 0.14b	65	27.65
Zz15	Field/HR	0.71 ± 0.054 cd	55	15.06
Zz16	Field/HR	0.74 ± 0.061c	55	18.27
Zz18	Field/HR	1.14 ± 0.21b	65	28.15
Zz19	Field/HR	1.91 ± 0.17a	80	47.16
Zz20	Field/HR	1.78 ± 0.29a	75	43.95
Zz21	Field/HR	0.69 ± 0.038 cd	55	17.04
Zz22	Field/HR	1.15 ± 0.22b	65	28.39
Zz23	Field/HR	1.08 ± 0.21b	60	29.13

[a] S sensitive, HR high-resistant. [b]Values are the means ± standard deviations. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$. [c]MIC minimal inhibitory concentration. [d]RF (resistance factor) = EC_{50} value (resistant isolate)/EC_{50} value (sensitive isolate)

P339T, E397D), and (D115V, R306K, E397D, S400N) in CYP51A, respectively, but no mutations in CYP51B. Genotype V only harbored five mutations (V46L, D115V, P163S, R306K, E397D) only in CYP51A (Fig. 2, Table 2). Furthermore, no modifications were found in the promoter region of *CYP51A* and *CYP51B* from resistant isolates.

Tebuconazole-resistant *C. siamense* isolates have multiple fitness defects

Two representative tebuconazole-resistant isolates were randomly selected from each resistant genotype to determine the fitness parameters, including mycelial growth rate, conidiation capacity, and fungal virulence. These tebuconazole-resistant isolates were as follows: Genotype I: Zz9, Zz10; Genotype II: Zz5, Zz20; Genotype III: Zz11, Zz22; Genotype IV: Zz12, Zz18; Genotype V: Zz3, Zz15. The mycelial growth rates of most of the resistant isolates (Zz5, Zz20, Zz11, Zz22, Zz12, Zz18, Zz3, and Zz15) were significantly reduced compared with those of the sensitive isolates Zz14 and Zz17. However, Zz9 and Zz10 showed no significant difference from the sensitive isolates in terms of mycelial growth rates (Fig. 3a, b, Table 3). All the tebuconazole-resistant isolates exhibited no significant difference from the sensitive isolates in conidial morphology (Fig. 3c). However, conidial production of the resistant isolates was significantly decreased compared with that of the sensitive isolates (Fig. 3d, Table 3). Furthermore, we assayed the virulence of these sensitive and resistant isolates on mature chili fruit. Compared with the sensitive isolates, the lesion size caused by the resistant isolates was significantly decreased (Fig. 4a, b, Table 3). These results suggested that the tebuconazole-resistant isolates of *C. siamense* suffered a fitness penalty.

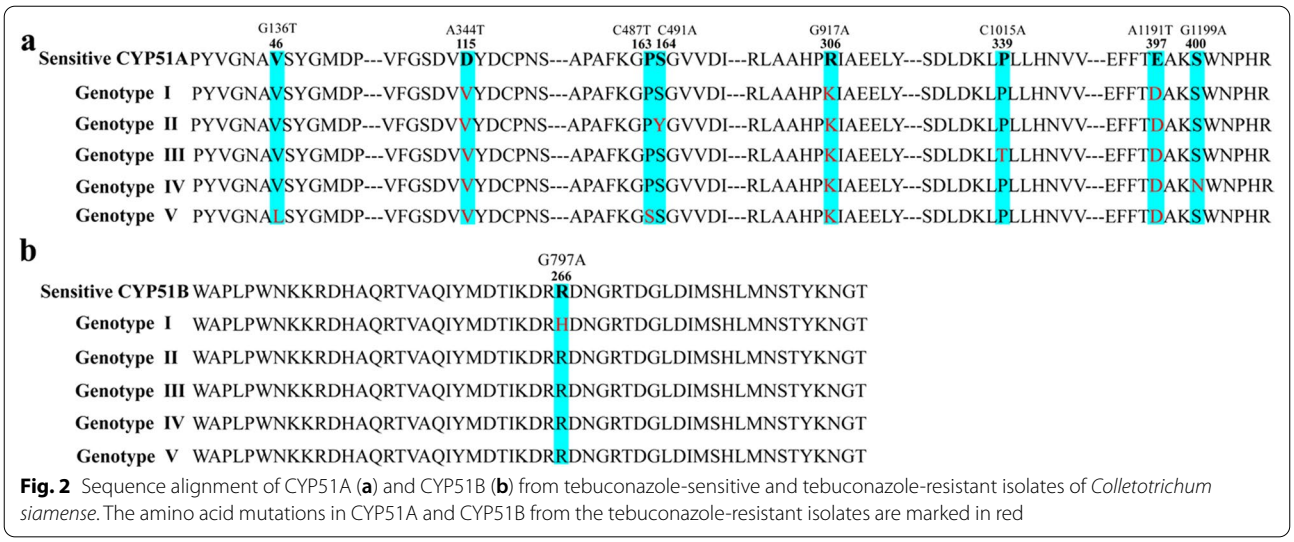

Fig. 2 Sequence alignment of CYP51A (**a**) and CYP51B (**b**) from tebuconazole-sensitive and tebuconazole-resistant isolates of *Colletotrichum siamense*. The amino acid mutations in CYP51A and CYP51B from the tebuconazole-resistant isolates are marked in red

Table 2 Mutation types of the tebuconazole-resistant isolates of *Colletotrichum siamense*

Isolates	Phenotype[a]	Genotype	Mutation sites	
			CYP51A	CYP51B
Zz14, Zz17	Field/S	–	–[b]	–
Zz9, Zz10	Field/HR	I	D115V, R306K, E397D	R266H
Zz5, Zz6, Zz7, Zz8, Zz19, Zz20	Field/HR	II	D115V, S164Y, R306K, E397D	–
Zz1, Zz2, Zz11, Zz22, Zz23	Field/HR	III	D115V, R306K, P339T, E397D	–
Zz12, Zz18	Field/HR	IV	D115V, R306K, E397D, S400N	–
Zz3, Zz4, Zz15, Zz16, Zz21	Field/HR	V	V46L, D115V, P163S, R306K, E397D	–

[a] S, sensitive; HR, high-resistant. [b]"–" indicates no mutation

Tebuconazole-resistant *C. siamense* isolates exhibit cross-resistance to other DMI fungicides

To determine the cross-resistance patterns between tebuconazole and other DMI fungicides, we assayed the sensitivities of the tebuconazole-sensitive and tebuconazole-resistant isolates to difenoconazole, prochloraz, and propiconazole. Compared with the sensitive isolates, the resistant isolates from five genotypes were less sensitive to difenoconazole, prochloraz, and propiconazole, with RF values ranging from 8.48–59.39, 1.24–3.21, and 3.43–12.18, respectively (Table 4). In addition, Spearman rank correlation analysis showed that tebuconazole has positive cross-resistance with difenoconazole ($r = 0.9515$, $P < 0.001$), prochloraz ($r = 0.9605$, $P < 0.001$) and propiconazole ($r = 0.8936$, $P < 0.001$) (Fig. 5).

Protective efficacy of tebuconazole against *C. siamense* isolates on chili fruits

Protective activity of tebuconazole against *C. siamense* isolates was determined on chili fruits. The results showed that tebuconazole possessed better protective activity on chili fruits inoculated with *C. siamense* isolates. For tebuconazole-sensitive and tebuconazole-resistant isolates, the protective activity of tebuconazole was increased with increasing dose. The protective efficacy of tebuconazole against the sensitive isolate Zz14 was $35.29 \pm 2.52\%$ and $80.55 \pm 1.12\%$ at concentrations of 50 mg/L and 100 mg/L, respectively, while that of tebuconazole was 100% at a concentration of 200 mg/L. However, the protective efficacy of tebuconazole against five representative resistant isolates (Zz9, Zz5, Zz11, Zz12, and Zz3) of five genotypes were 13.42 ± 1.65–$34.29 \pm 1.86\%$, 50.85 ± 2.63–$70.09 \pm 2.23\%$, and 67.46 ± 3.21–$81.77 \pm 2.11\%$ at concentrations of 50 mg/L, 100 mg/L and 200 mg/L, respectively (Fig. 6, Table 5).

Mutations in CYP51A and CYP51B are responsible for tebuconazole-resistance in *C. siamense*

To investigate whether mutations in CYP51A and CYP51B confer resistance to tebuconazole in *C. siamense*, replacement mutants containing each of the five-genotype mutations were constructed (Fig. 7a). All the replacement mutants were identified by PCR amplification and DNA sequencing (Fig. 7b, c). Furthermore, the sensitivities of these replacement mutants to tebuconazole and three other DMIs (difenoconazole, prochloraz, and propiconazole) were determined to validate their resistance levels. Compared with the sensitive isolates Zz14 and Zz17, the sensitivity of all the replacement mutants to tebuconazole was significantly decreased, with RF value ranging from 16.79 to 50.37 (Table 6). In addition, the mutants showed significantly reduced sensitivity to difenoconazole, prochloraz, and propiconazole. These results demonstrated that mutations in CYP51A and CYP51B confer resistance to tebuconazole in *C. siamense*.

Comparison of the expression of *CYP51A* and *CYP51B* among tebuconazole-sensitive isolates and tebuconazole-resistant mutants

Overexpression of *CYP51* is involved in DMI resistance in phytopathogenic fungi (Luo and Schnabel 2008). To further investigate the underlying fungal resistance mechanism against tebuconazole, the expression levels of *CYP51A* and *CYP51B* in tebuconazole-sensitive isolates and replacement mutants were determined with and without tebuconazole treatment (Fig. 8). Compared with the untreated groups, the expression levels of *CYP51A* and *CYP51B* were significantly increased 9.21-fold and 17.59-fold, respectively, in tebuconazole-sensitive isolate Zz14 treated with tebuconazole. In resistant mutants of the five resistance genotypes, the expression levels of *CYP51A* and *CYP51B* only increased 2.72–5.60 fold and 1.21–8.91 fold, respectively, under treatment with tebuconazole compared

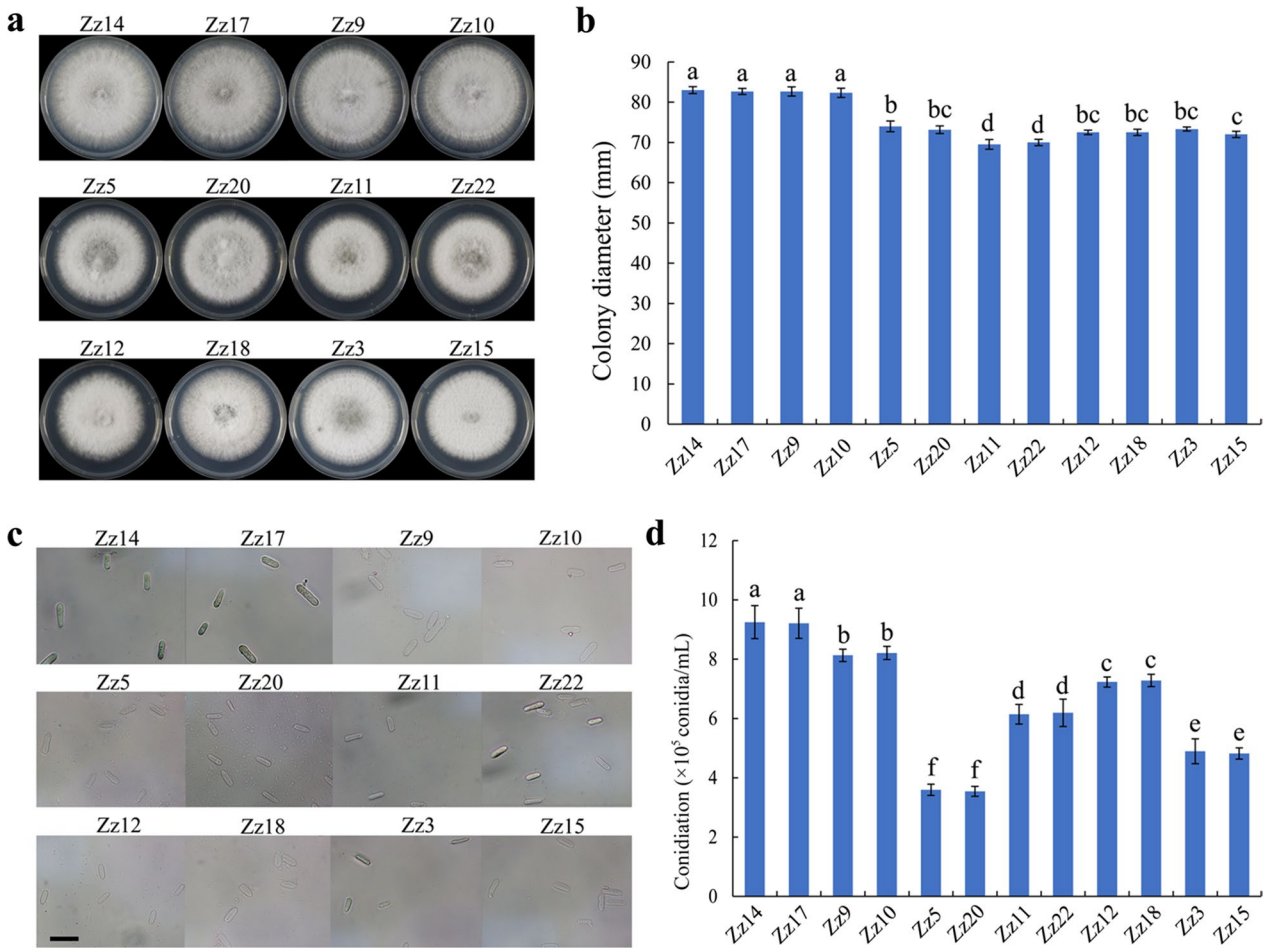

Fig. 3 Tebuconazole-resistant *Colletotrichum siamense* isolates are defective in mycelial growth and conidiation. **a** Mycelial growth of the tebuconazole-sensitive and tebuconazole-resistant isolates of *C. siamense* on PDA medium after 5 days of incubation at 25 °C in the dark. **b** Column diagram showing colony diameters of the tebuconazole-sensitive and tebuconazole-resistant isolates of *C. siamense* on PDA medium after 5 days of incubation at 25 °C. Error bars denote the standard deviations from three repeats. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$. **c** Conidial morphology of the tebuconazole-sensitive and tebuconazole-resistant isolates of *C. siamense*. Bar = 20 μm. **d** Conidiation capacity of the tebuconazole-sensitive and tebuconazole-resistant isolates of *C. siamense*. Conidial production was quantified after incubation in 30 mL PDB medium for 5 days at 25 °C. Error bars denote the standard deviations from three repeats. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$

with those in untreated groups. In addition, the expression levels of *CYP51A* and *CYP51B* in the five resistant mutants were increased 0.77–5.57 fold and 0.32–7.44 fold, respectively, compared with those in sensitive isolate Zz14 under untreated conditions, while the expression level of *CYP51A* and *CYP51B* in the five resistant mutants were increased 0.32–1.64 fold and 0.16–0.51 fold, respectively, compared with those in the sensitive isolate Zz14 under tebuconazole treatment. Taken together, these results indicated that there was no overexpression of *CYP51A* and *CYP51B* in the resistant mutants of five different resistance genotypes.

Discussion

Anthracnose caused by *Colletotrichum* spp. is an important disease of vegetables, fruits, legumes, and cereals (Than et al. 2008a; Diao et al. 2017). Tebuconazole has been widely used in controlling chili anthracnose due to its broad-spectrum antifungal activity and high efficacy (Zhang et al. 2017). In this study, the causal agent of chili anthracnose was identified as *C. siamense*, which had developed resistance to tebuconazole and other DMIs in the local fields. Based on the mutations in CYP51A and CYP51B, tebuconazole-resistant isolates were divided into five resistant genotypes. Furthermore, the fact that mutations in CYP51A and CYP51B conferred resistance

Table 3 Comparison of mycelial growth, conidiation, and virulence between the tebuconazole-sensitive and tebuconazole-resistant isolates of *Colletotrichum siamense*

Isolates	Phenotype	Genotype	Colony diameter (mm)[a]	Conidiation ($\times 10^5$ spores/mL)[b]	Lesion size (mm)[c]
Zz14	Field/S	–	83.00 ± 0.85a	9.25 ± 0.56a	11.15 ± 0.98a
Zz17	Field/S	–	82.66 ± 0.77a	9.21 ± 0.51a	10.98 ± 0.64a
Zz9	Field/HR	I	82.67 ± 1.15a	8.13 ± 0.21b	6.73 ± 0.33b
Zz10	Field/HR	I	82.33 ± 1.14a	8.21 ± 0.22b	7.21 ± 0.45b
Zz5	Field/HR	II	74.01 ± 1.34b	3.59 ± 0.19f	6.59 ± 0.28b
Zz20	Field/HR	II	73.160 ± 0.94bc	3.54 ± 0.17f	6.85 ± 0.37b
Zz11	Field/HR	III	69.50 ± 1.21d	6.14 ± 0.33d	4.84 ± 0.15c
Zz22	Field/HR	III	70.00 ± 0.79d	6.19 ± 0.46d	4.62 ± 0.26c
Zz12	Field/R	IV	72.50 ± 0.53bc	7.23 ± 0.17c	3.51 ± 0.26d
Zz18	Field/R	IV	72.51 ± 0.80bc	7.28 ± 0.21c	3.14 ± 0.24de
Zz3	Field/R	V	73.33 ± 0.49bc	4.89 ± 0.42e	2.63 ± 0.22f
Zz15	Field/R	V	72.00 ± 0.75c	4.82 ± 0.19e	2.87 ± 0.31ef

Values are the means ± standard deviations from three replicate experiments. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$. [a]The isolates were grown on PDA medium, and colony diameter of each isolate was measured after 5 days of incubation at 25 °C. [b]The sensitive and resistant isolates were incubated in PDB medium for 5 days at 25 °C, and conidia produced by each isolate were collected and determined using a hemocytometer. [c]Mycelial plugs of each isolate were inoculated on wounded chili fruits, and lesion sizes were measured after 7 days of incubation. Each isolate was inoculated on 15 chili fruits

to tebuconazole was validated by constructing replacement mutants. All the tested tebuconazole-resistant isolates exhibited low fitness according to mycelial growth, conidiation and virulence data compared with those of the sensitive isolates. In addition, the representative resistant isolates of five genotypes exhibited positive cross-resistance to the other three tested DMIs. To our knowledge, this is the first report of DMI fungicide resistance in *C. siamense*.

Mutations in CYP51A and CYP51B confer resistance to tebuconazole in *C. siamense*. Previous studies have demonstrated that mutation in CYP51 is associated with DMI resistance, whereas mutation types and resistance levels differ in *Colletotrichum* spp. (Chen et al. 2016). Three genotype mutations conferring low resistance to tebuconazole were found in CYP51A and CYP51B of *C. gloeosporioides* isolated from chili (Wei et al. 2020). Six genotype mutations conferring low resistance to difenoconazole were identified in *C. gloeosporioides* isolated from grape (Wang et al. 2020). In two other *Colletotrichum* fungi from peach orchards in the United States, *C. truncatum* isolates exhibited high resistance to flutriafol and fenbuconazole, mid resistance to tebuconazole, and low resistance to metconazole, while *C. nymphaeae* isolates were high-resistant to flutriafol and fenbuconazole (Chen et al. 2016, 2018). DMI resistance has also been reported in other phytopathogenic or clinical fungi. In *Fusarium graminearum*, the point mutation G443S in CYP51A or S169Y in CYP51B was reported to cause decreased sensitivity to tebuconazole (Chen et al. 2021a; Zhao et al. 2022). The mutation S312T in CYP51B confers high resistance to prochloraz in *Fusarium fujikuroi*, the causal agent of rice bakanae disease (Zhang et al. 2021). In the clinical fungus *Aspergillus clavatus*, mutations in CYP51A and CYP51B are related to itraconazole and posaconazole resistance (Abastabar et al. 2019). In this study, five genotype mutations conferring high tebuconazole-resistance were identified in *C. siamense* resistant isolates, and this was validated by constructing replacement mutants. The five genotype mutations in CYP51A and CYP51B of *C. siamense* have not yet been reported in other *Colletotrichum* species. Recent studies showed that *C. siamense* isolates from peach in China were sensitive to prochloraz, while those from apple in Illinois of the United States were sensitive to propiconazole (Chechi et al. 2019; Usman et al. 2021). *C. siamense* isolates from strawberry in China were sensitive to difenoconazole, tebuconazole and prochloraz (Zhang et al. 2020). Tebuconazole-resistant *C. siamense* isolates from chili in this study also showed resistance to difenoconazole, prochloraz, and propiconazole. The different sensitivities of *C. siamense* isolates to DMIs may result from the difference in hosts or the types of DMI fungicides used, and also indicated that different DMI fungicides are prone to causing selection for different mutations. Previously, it was shown that mutations or overexpression of *CYP51A* are more relevant to azole-resistance in fungi carrying two CYP51 isoenzymes (Handelman et al. 2021). Here, mutations mainly occurred in CYP51A, and three mutations (D115V, R306K and E397D) in CYP51A were common in all resistant isolates. Similar cases were also reported in *C. gloeosporioides* isolates that had

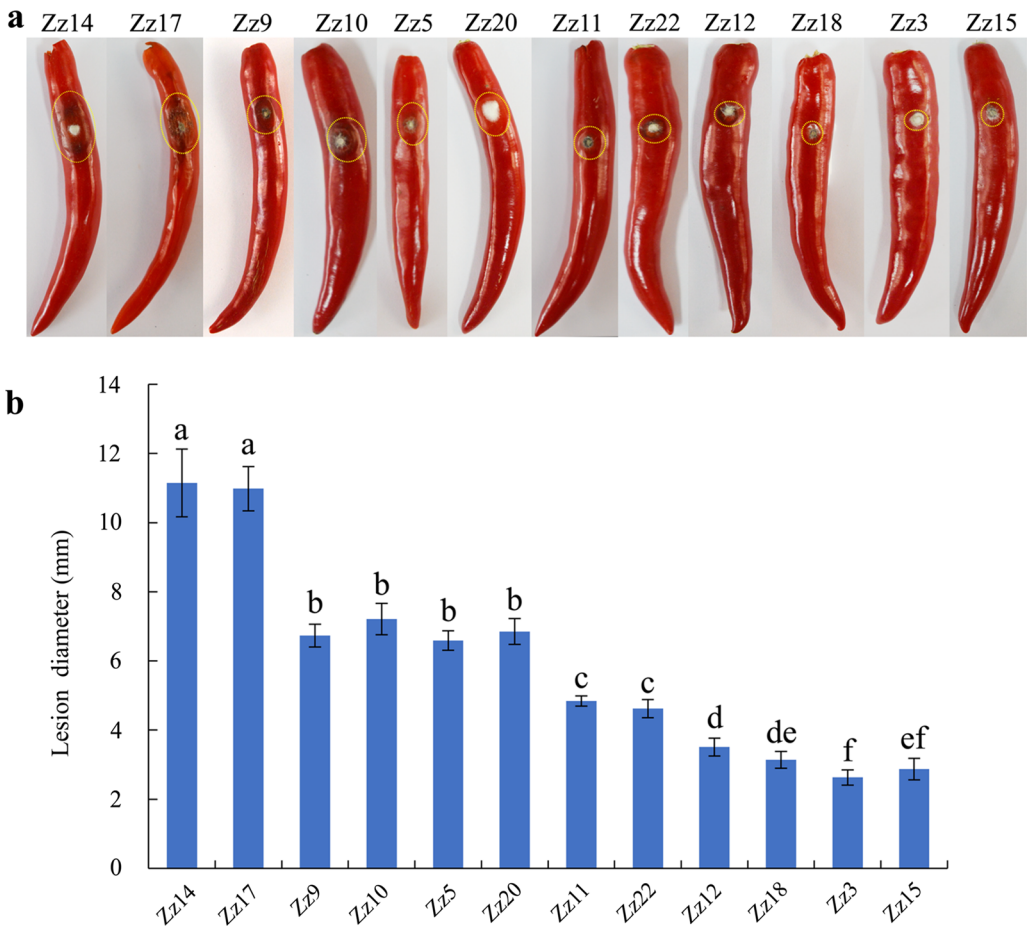

Fig. 4 Tebuconazole-resistant *Colletotrichum siamense* isolates are defective in virulence on chili fruits. **a** Fruit lesions caused by inoculation with tebuconazole-sensitive or tebuconazole-resistant isolates of *C. siamense*. Mycelial plugs (5 mm in diameter) from each isolate were used as the inocula, and disease symptom was examined after 7 days of inoculation at 25 °C. **b** Column diagram showing the lesion size in **a**. Lesion sizes were measured at 7 days post-inoculation. Error bars denote the standard deviations from three repeats. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$

developed low resistance to tebuconazole and difenoconazole (Wang et al. 2020; Wei et al. 2020).

Overexpression of *CYP51* is another mechanism conferring resistance to DMI fungicides in phytopathogenic fungi (Steffens et al. 1996). Overexpression of the gene induced by insertion of a 65-bp sequence in the promoter region of *CYP51* was shown to be correlated with DMI resistance in *Monilinia fructicola* (Chen et al. 2017). In *Ustilaginoidea virens*, overexpression is induced by the insertion of two bases (CC) at − 154 bp in the promoter of *CYP51*, which causes the resistance to propiconazole (Zhou et al. 2019). DMI fungicide resistance due to insertion-induced overexpression of the *CYP51* was also reported in *Penicillium digitatum*, *Venturia inaequalis* and *Mycosphaerella graminicola* (Schnabel and Jones 2001; Cools et al. 2012; De Ramón-Carbonell and Sánchez-Torres 2020). However, the molecular mechanism leading to the overexpression of *CYP51* has not been clarified in DMI-resistant populations in *Puccinia triticina*, *Cercospora beticola* and *Sclerotinia homoeocarp* (Stammler et al. 2009; Bolton et al. 2012; Hulvey et al. 2012). In this study, no modifications were detected in the promoter regions of *CYP51A* and *CYP51B* from the tebuconazole-resistant isolates of *C. siamense*. Under treatment with tebuconazole, the expression of *CYP51A* and *CYP51B* in tebuconazole-sensitive *C. siamense* isolates and tebuconazole-resistant mutants of five genotypes were all induced, however, the upregulation of the two genes in resistant mutants was significantly lower than that in tebuconazole-sensitive isolates. These results suggested that overexpression of *CYP51A* and *CYP51B* was not involved in tebuconazole resistance in *C. siamense*.

Table 4 Sensitivities of *Colletotrichum siamense* isolates to tebuconazole, difenoconazole, prochloraz, and propiconazole

Isolates	Phe	Gen	Tebuconazole		Difenoconazole		Prochloraz		Propiconazole	
			EC_{50} (mg/L)[a]	RF[b]	EC_{50} (mg/L)	RF	EC_{50} (mg/L)	RF	EC_{50} (mg/L)	RF
Zz14	S	–	0.039 ± 0.0036e	–	0.018 ± 0.0013e	–	0.026 ± 0.0025e	–	0.065 ± 0.0016e	–
Zz17	S	–	0.042 ± 0.0012e	–	0.015 ± 0.0024e	–	0.027 ± 0.0012e	–	0.063 ± 0.0023e	–
Zz9	HR	I	0.61 ± 0.056d	15.06	0.14 ± 0.036d	8.48	0.033 ± 0.0041d	1.24	0.22 ± 0.016d	3.43
Zz10	HR	I	0.65 ± 0.023d	16.05	0.16 ± 0.013d	9.69	0.033 ± 0.0053d	1.24	0.23 ± 0.014d	3.59
Zz5	HR	II	1.73 ± 0.27a	43.21	0.92 ± 0.031a	55.75	0.084 ± 0.0015a	3.16	0.78 ± 0.013a	12.18
Zz20	HR	II	1.91 ± 0.17a	47.16	0.98 ± 0.014a	59.39	0.085 ± 0.0016a	3.21	0.75 ± 0.027a	11.71
Zz11	HR	III	1.11 ± 0.24b	27.4	0.39 ± 0.061b	23.63	0.068 ± 0.0038b	2.56	0.45 ± 0.019b	7.03
Zz22	HR	III	1.15 ± 0.22b	28.39	0.41 ± 0.052b	24.85	0.071 ± 0.0013b	2.67	0.49 ± 0.036b	7.65
Zz12	HR	IV	1.12 ± 0.14b	27.65	0.47 ± 0.022b	28.48	0.043 ± 0.0020c	1.62	0.28 ± 0.032c	4.37
Zz18	HR	IV	1.14 ± 0.21b	28.15	0.44 ± 0.034b	26.67	0.045 ± 0.0014c	1.69	0.31 ± 0.015c	4.84
Zz3	HR	V	0.71 ± 0.054 cd	17.53	0.28 ± 0.037c	16.96	0.034 ± 0.0064d	1.28	0.29 ± 0.064c	4.53
Zz15	HR	V	0.74 ± 0.061c	18.27	0.31 ± 0.012c	18.78	0.037 ± 0.0031d	1.39	0.28 ± 0.051c	4.37

[a] Values are the means ± standard deviations from three replicate experiments. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$. [b]RF (resistance factor) = EC_{50} value (resistant isolate)/EC_{50} value (sensitive isolate)

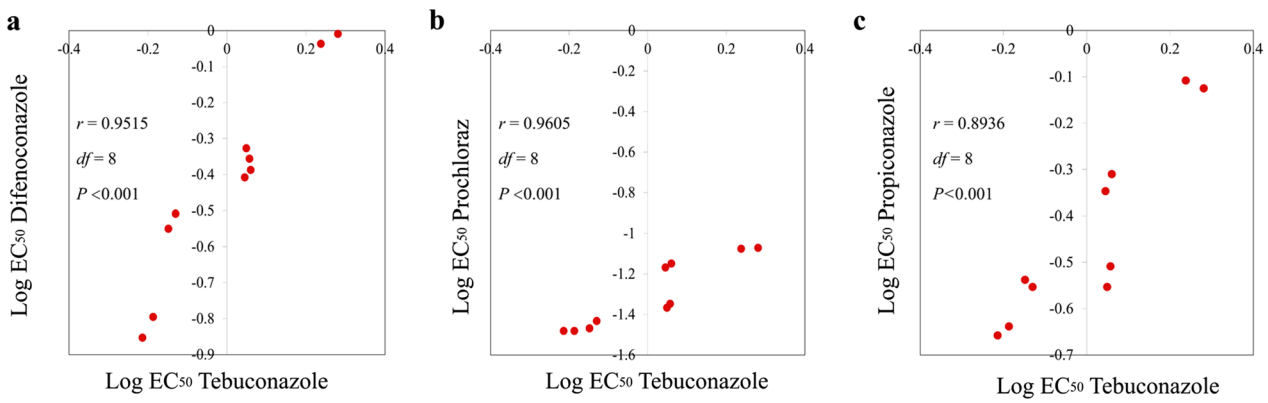

Fig. 5 Spearman rank correlation for cross resistance in *Colletotrichum siamense* between tebuconazole and difenoconazole (**a**), prochloraz (**b**), or propiconazole (**c**). The points represent the log EC_{50} values (logarithmic of EC_{50}) among the tested isolates for the indicated fungicide combinations

The assessment of the fitness of resistant isolates is important for evaluating the risk of fungicide resistance and developing efficient management strategies. In this study, the fitness of tebuconazole-resistant isolates of *C. siamense* was decreased compared with that of tebuconazole-sensitive isolates according to mycelial growth rate, conidiation capacity and virulence. Although the fitness of resistant isolates of *C. siamense* was impaired, the resistant populations still increased rapidly in some commercial chili fields. Cross resistance assays further showed that positive cross-resistance existed between tebuconazole and difenoconazole, prochloraz or propiconazole in the resistant isolates of five genotypes. Therefore, monitoring DMI fungicide resistance is necessary in chili planting regions or in a more extensive range to effectively control the disease.

Conclusions

This study represents the first report of *C. siamense* resistance to DMIs. The five genotype mutations in CYP51A and CYP51B are responsible for tebuconazole resistance in *C. siamense*. Although the isolates we tested were insufficient in number, the results provide a glimpse of DMI fungicide resistance profiles of *C. siamense* infecting chili in Fujian Province, China. The *C. siamense* isolates have developed resistance to DMIs in local commercial chili fields. Using fungicides with different modes of action should be considered for

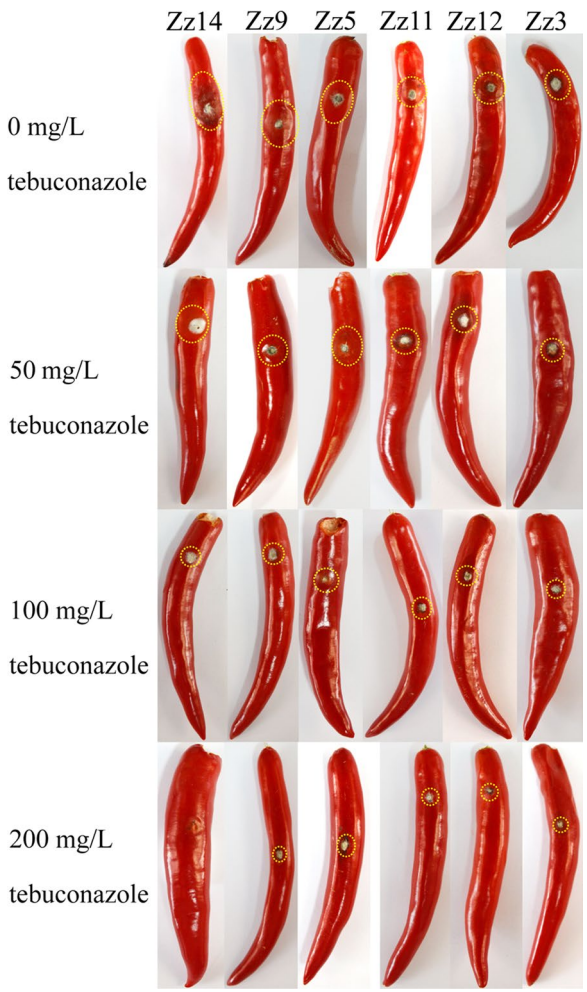

Fig. 6 Protective efficacy of tebuconazole against tebuconazole-sensitive and tebuconazole-resistant isolates of *Colletotrichum siamense* on chili fruits. The chili fruits were treated with water or different concentrations of tebuconazole for 24 h, and then were inoculated with mycelial plugs of tebuconazole-sensitive or tebuconazole-resistant isolates of *C. siamense*. Disease lesions were measured at 7 days post-inoculation

controlling chili anthracnose and delaying resistance development.

Methods

Fungicides and medium

Ninety-five percent tebuconazole, 96% difenoconazole, 96% prochloraz and 98% propiconazole were provided by Jiangsu Aijin Chemical Company. The fungicides were dissolved in methanol as stock solutions to reach a final concentration of 1×10^4 mg/L.

The *C. siamense* isolates were incubated on PDA medium (potato dextrose agar, 200 g potato, 20 g glucose, 15 g agar power and 1 L water) or PDB (potato dextrose broth, 200 g potato, 20 g glucose and 1 L water) liquid medium.

Fungal isolation and identification

Mature chili fruits with anthracnose symptoms were collected in September 2021 from commercial chili fields in Zhangzhou, Fujian Province, China. Small pieces of tissue taken from the margin between diseased and healthy tissues were soaked in 1% NaClO for 60 s and then 75% ethanol for 30 s. The samples were washed with sterilized water and then placed on PDA medium amended with 10 mg/L streptomycin sulfate. The mycelia were recovered on PDA medium after incubation at 25 °C for 5 days, and single-spore isolates were obtained according to previous methods (Chen et al. 2021b).

The internal transcribed spacer (ITS), calmodulin (*CAL*), chitin synthase 1 (*CHS1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and actin (*ACT*) sequences of 19 taxa from the *C. gloeosporioides* species complex were used as reference sequences. The ITS, *CAL*, *CHS1*, *GAPDH* and *ACT* sequences of all the isolates were sequenced and compared with those of 19 *Colletotrichum* species. Sequence alignments incorporating the above five genes were analyzed with PhyloSuite, and the phylogenetic tree was created by MEGA 7 with the neighbor-joining method based on the combined dataset of ITS, *CAL*, *CHS1*, *GAPDH*, and *ACT*. The primers used for sequencing the ITS, *CAL*, *CHS1*, *GAPDH*, and *ACT* are listed in Additional file 1: Table S1.

Sensitivity of *C. siamense* isolates to tebuconazole

To evaluate the sensitivities of *C. siamense* isolates to tebuconazole, the EC_{50} and minimal inhibitory concentration (MIC) values of each isolate to tebuconazole were assayed. In brief, fresh mycelial plugs of each isolate were transferred to PDA medium treated with different concentrations of tebuconazole, and the petri dishes were incubated for 5 days at 25 °C. The mycelial growth inhibition rate was calculated, and the EC_{50} of each isolate was obtained by using DPS software (Zhejiang University, Hangzhou, China). Each treatment had three replicate plates, and the experiment was repeated two times with similar results.

Sequencing analysis of *CYP51A* and *CYP51B* in tebuconazole-sensitive and tebuconazole-resistant *C. siamense* isolates

According to the *CYP51A* and *CYP51B* sequences of *C. siamense* strain Cs363, primers were designed for cloning the full-length sequences of *CYP51A* and *CYP51B*

Table 5 Protective activity of tebuconazole against *Colletotrichum siamense* isolates on chili fruits

Isolates	0 mg/L tebuconazole		50 mg/L tebuconazole		100 mg/L tebuconazole		200 mg/L tebuconazole	
	Lesion size (mm)[a]	Protective efficiency (%)[b]	Lesion size (mm)	Protective efficiency (%)	Lesion size (mm)	Protective efficiency (%)	Lesion size (mm)	Protective efficiency (%)
Zz14	10.85 ± 0.95a	–	7.02 ± 0.23a	35.29 ± 2.52a	2.11 ± 0.56b	80.55 ± 1.12a	ND[c]	100a
Zz9	8.56 ± 0.46b	–	6.54 ± 0.15b	23.59 ± 1.35c	2.56 ± 0.35b	70.09 ± 2.23b	1.56 ± 0.31b	81.77 ± 2.11b
Zz5	8.79 ± 0.33b	–	7.61 ± 0.45a	13.42 ± 1.65d	4.32 ± 0.12a	50.85 ± 2.63e	2.86 ± 0.45a	67.46 ± 3.21d
Zz11	6.77 ± 0.14c	–	4.78 ± 0.17c	29.39 ± 2.45b	2.14 ± 0.15b	68.39 ± 1.95c	1.76 ± 0.13b	74.00 ± 3.31c
Zz12	6.89 ± 0.24c	–	4.63 ± 0.25c	32.80 ± 2.65a	2.40 ± 0.31b	65.16 ± 2.89d	1.54 ± 0.16b	77.64 ± 1.12c
Zz3	5.98 ± 0.43d	–	3.87 ± 0.37d	34.29 ± 1.86a	2.05 ± 0.24b	65.19 ± 1.67d	1.32 ± 0.27b	77.58 ± 2.36bc

[a] Values are the means ± standard deviations from 15 replicate chili fruits. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$. [b] Protective efficacy (%) = [(lesion size of control − lesion size of treatment)/lesion size of control] × 100. [c] ND, not detected

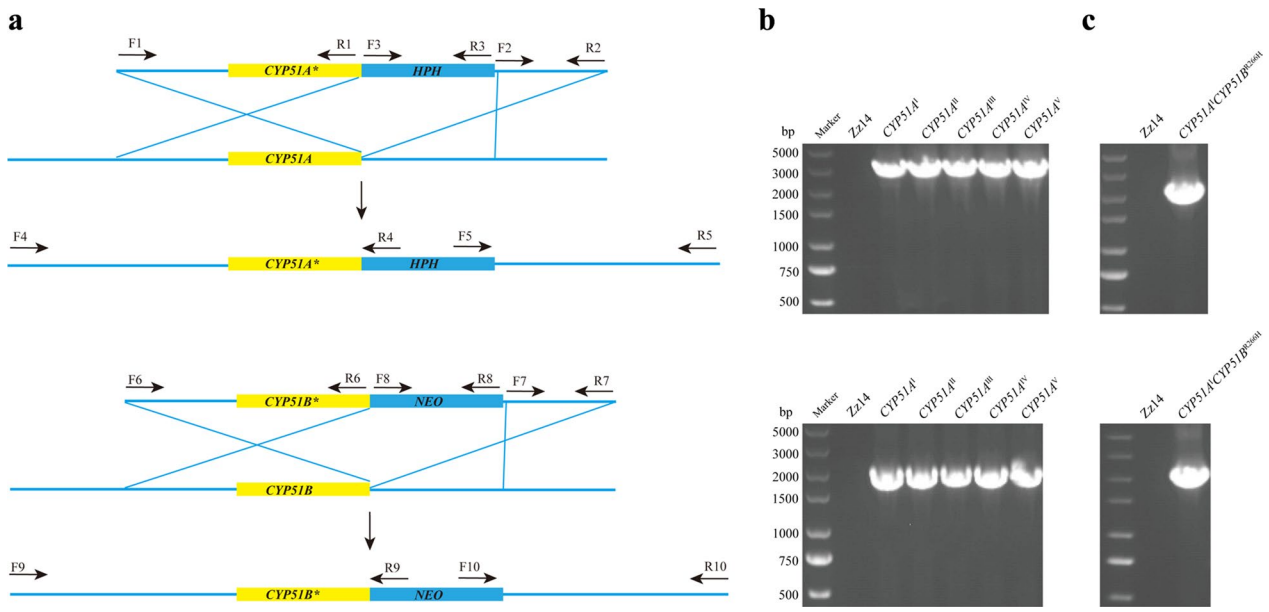

Fig. 7 Construction and identification of *CYP51A* or *CYP51B* replacement mutants of *Colletotrichum siamense*. **a** Strategies for the construction of *CYP51A* or *CYP51B* replacement mutants. The *CYP51A* and *CYP51B* replacement vectors contain the hygromycin phosphotransferase gene (*HPH*) and geneticin resistance gene (*NEO*), respectively. The primers used are indicated by arrows, and asterisks indicate the mutations in *CYP51A* and *CYP51B*. **b** PCR identification of the *CYP51A* replacement mutants. Fragments of 2.97 kb and 1.87 kb could be amplified from the *CYP51A* replacement mutants with the primer pair F4/R4 and F5/R5, respectively, but could not be amplified from the sensitive isolates. **c** PCR identification of the *CYP51B* replacement mutants. Fragments of 2.09 kb and 1.95 kb could be amplified from the *CYP51B* replacement mutants with the primer pair F10/R10 and F11/R11, respectively, but could not be amplified from the sensitive isolates

Table 6 Sensitivity of the five genotype replacement mutants to tebuconazole, difenoconazole, prochloraz, and propiconazole in *Colletotrichum siamense*

Strain	Tebuconazole		Difenoconazole		Prochloraz		Propiconazole	
	EC_{50} (mg/L)[a]	RF[b]	EC_{50} (mg/L)	RF	EC_{50} (mg/L)	RF	EC_{50} (mg/L)	RF
Zz14	0.039 ± 0.0036d	–	0.018 ± 0.0013e	–	0.026 ± 0.0025f	–	0.065 ± 0.0016d	–
Zz17	0.042 ± 0.0012d	–	0.015 ± 0.002e	–	0.027 ± 0.0012f	–	0.063 ± 0.0023d	–
Genotype I	0.68 ± 0.024c	16.79	0.14 ± 0.015d	8.48	0.030 ± 0.0043e	1.13	0.32 ± 0.045c	5.00
Genotype II	2.04 ± 0.22a	50.37	0.15 ± 0.023d	9.09	0.091 ± 0.0033a	3.43	0.83 ± 0.016a	12.96
Genotype III	1.27 ± 0.17b	31.36	0.89 ± 0.81a	53.94	0.075 ± 0.0016b	2.83	0.53 ± 0.033b	8.28
Genotype IV	1.38 ± 0.37b	34.07	0.48 ± 0.035b	29.09	0.048 ± 0.0052c	1.81	0.33 ± 0.025c	5.15
Genotype V	0.72 ± 0.023c	17.78	0.32 ± 0.014c	19.39	0.039 ± 0.0012d	1.47	0.31 ± 0.061c	4.84

[a] Values are the means ± standard deviations from three replicate experiments. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$. [b]RF (resistance factor) = EC_{50} value (resistant isolate)/EC_{50} value (sensitive isolate)

of sensitive and resistant isolates. A 50 μL PCR reaction consisted of 25 μL of PCR buffer, 2 ng/μL of DNA, 1 μL of DNA polymerase, 0.2 mM of dNTP, 0.2 μM of each forward/reverse primer and 18 μL of sterilized water (Vazyme, Nanjing, China). PCR reactions were performed with procedures: 95 °C, 5 min; 95 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min, 35 cycles; 72 °C, 10 min. PCR products were purified and sequenced by Tsingke Biotechnology (Tsingke, Beijing, China). The *CYP51A* and *CYP51B* sequences of tebuconazole-sensitive and tebuconazole-resistant isolates were compared by using BoiEdit software. The primers used for sequencing the *CYP51A* and *CYP51B* are listed in Additional file 1: Table S1.

Characterization of tebuconazole-resistant isolates

For determination of mycelial growth rates, the mycelial plugs of tebuconazole-sensitive and tebuconazole-resistant isolates of *C. siamense* were transferred to PDA

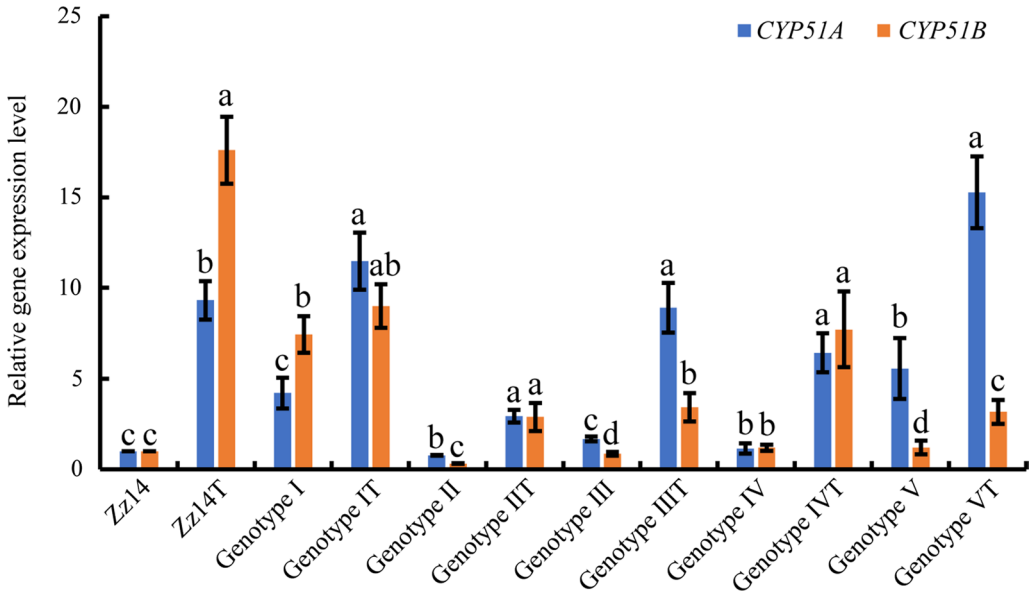

Fig. 8 Determination of constitutive and tebuconazole-induced expression levels of *CYP51A* and *CYP51B* in tebuconazole-sensitive isolates and the replacement mutants of five genotypes. The tebuconazole-sensitive isolates and replacement mutants were treated with 10 mg/L tebuconazole or left untreated. Error bars denote the standard deviations from three repeats. The significant difference was compared between the untreated and its corresponding treated groups. Different letters indicate significant differences according to the Fisher's *LSD* test at $P = 0.05$

medium and cultivated at 25 °C under darkness. Colony diameter was measured by perpendicular method after 5 days of incubation.

To determine conidial production, mycelial plugs of tebuconazole-sensitive and tebuconazole-resistant isolates were incubated in 30 mL PDB medium for 5 days at 25 °C with agitation speed of 175 rpm. The conidia produced by *C. siamense* isolates were counted with a hemocytometer, and the conidial morphology was imaged with an optical microscope (CX31, Olympus, Japan).

The virulence of tebuconazole-sensitive and tebuconazole-resistant *C. siamense* isolates was evaluated by inoculation on mature chili fruits. The mycelial plugs of each isolate were inoculated on wounded chili surface. After 5 days of incubation at 25 °C, the disease symptom of each treatment was examined, and the lesion size was measured.

Cross-resistance assay

The sensitivities of tebuconazole-sensitive and tebuconazole-resistant isolates *C. siamense* to three other DMIs including difenoconazole, prochloraz, and propiconazole were assayed. The EC_{50} values of each isolate to DMIs were determined by calculating the mycelial growth inhibition rates as described in "Sensitivity of *C. siamense* isolates to tebuconazole" section. Each treatment had three replicate plates and the experiment was repeated two times with similar results.

Protective efficacy of tebuconazole against *C. siamense* on chili fruits

The protective ability of tebuconazole against *C. siamense* was assayed on chili fruits. The fresh chili fruits were treated with water or tebuconazole to final concentrations of 50 mg/L, 100 mg/L, and 200 mg/L. After 24 h of treatment, the chili fruits were inoculated with mycelial plugs of the tebuconazole-sensitive or tebuconazole-resistant isolates of *C. siamense*. Then, the treated chili fruits were incubated in a greenhouse with a 12-h photoperiod and 80% humidity at 25 °C. The lesion size on chili fruits in each treatment was measured after 7 days of incubation. Each treatment was performed with 15 replicates. Protective efficacy was calculated according to the formula: Protective efficacy (%) = [(lesion size of control − lesion size of treatment)/lesion size of control] × 100.

Construction of *CYP51A* and *CYP51B* replacement mutants

To construct the *CYP51A* replacement mutants, the *CYP51A* and its flanking region fragments were amplified from the tebuconazole-resistant isolates and linked with the hygromycin phosphotransferase gene (*HPH*) using double-joint PCR method (Yu et al. 2004). The replacement vector of *CYP51A* was then transformed into the sensitive isolate of *C. siamense*. Similarly, the *CYP51B* and its flanking region fragments were fused with *NEO* (geneticin) gene using the same PCR strategy. The

replacement vector of *CYP51B* was transformed into the *CYP51A* replacement mutants of genotypes I. The *C. siamense* protoplast transformation was performed according to a previous study (Chung et al. 2002). The putative transformants were identified by PCR amplifications. The primers used for constructing and identifying the *CYP51A* and *CYP51B* replacement mutants are listed in Additional file 1: Table S1.

Determination of expression levels of *CYP51A* and *CYP51B*

The expression levels of *CYP51A* and *CYP51B* in tebuconazole-sensitive and tebuconazole-resistant isolates were assayed using reverse transcription-quantitative PCR (RT-qPCR). Ten microliters of conidial suspension (1×10^8 conidia/L) of each isolate were added to 100 mL of PDB liquid medium. Tebuconazole was then added to the PDB medium to a final concentration of 10 mg/L. Treatment without tebuconazole was used as a control. RNA was extracted using a total RNA extraction kit (Tiangen, Beijing, China) from fungal mycelia, and the first-strand cDNA was synthesized using an All-in-one cDNA synthesis kit (Vazyme, Nanjing, China). qPCR was conducted using a QuantStudio 6 flex system (Applied Biosystems, Foster City, USA). The *C. siamense GADPH* gene was used as internal gene, and the expression levels of *CYP51A* and *CYP51B* were determined by using the $2^{-\Delta\Delta Ct}$ method. Three biological replications were conducted for each sample. The primers used for determining the expression levels of *CYP51A* and *CYP51B* are listed in Additional file 1: Table S1.

Statistical analysis

All EC_{50} values were calculated by DPS software. The mycelial growth rate, conidial production, and virulence data were analyzed using SPSS 20 by Fisher's least significant difference test (*LSD*) with one-way ANOVA at $P = 0.05$.

Abbreviations

ACT: Actin; CAL: Calmodulin; CHS1: Chitin synthase 1; Cytb: Cytochrome b; DMIs: Sterol demethylation inhibitors; EC_{50}: 50% Effective inhibition concentration; FAO: Food and Agriculture Organization of the United Nations; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HPH: Hygromycin phosphotransferase; ITS: Internal transcribed spacer; MBCs: Methyl benzimidazole carbamates; MIC: Minimal inhibitory concentration; NEO: Geneticin; PDA: Potato dextrose agar; PDB: Potato dextrose broth; QoIs: Quinone outside inhibitors; RF: Resistance factor.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00146-w.

Additional file 1. Table S1. PCR primers used in this study.

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Author contributions

WC and FL designed the experiments and wrote the manuscript. WC, LW and RH carried out the experiments. YZ (Yangyang Zhao) and YZ (Yancun Zhao) revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Not applicable.

Author details

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