Phytopathology Research

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Detection and characterization of difenoconazole resistance in *Stagonosporopsis citrulli* from watermelon and muskmelon in Zhejiang Province of China

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Abstract

The watermelon and muskmelon productions are important agriculture pillar industries of Zhejiang Province in China. Difenoconazole is an imidazole-class sterol, 14-demethylase inhibitor (DMI), which has been in use for several years to control gummy stem blight (GSB) caused by *Stagonosporopsis* species. However, the detection and characterization of difenoconazole resistance in GSB have not been clarified. In this study, a total of 112 isolates were collected from samples of GSB on watermelon and muskmelon in five locations of Zhejiang Province, China. All of the isolates were identified as *Stagonosporopsis citrulli* through morphology and multiplex PCR analysis. The determination of their resistance to difenoconazole via the discriminatory dosage method showed that the total resistance frequency was 89.3%. Among the resistant sub-population, 36.6% had high-level resistance to difenoconazole (Dif^{HR}), while 46.4% and 6.3% had low- (Dif^{LR}) and moderate-level resistance (Dif^{MR}), respectively. Additionally, the difenoconazole showed a positive cross-resistance with four DMIs, i.e., tebuconazole, prochloraz, metconazole, and mefentrifluconazole, but not hexaconazole. The phenotypic analysis found that the difenoconazole resistant (Dif^{R}) isolates demonstrated attenuated ability in both the mycelial growth and sporulation compared with the difenoconazole sensitive (Dif^{S}) isolates, while there was no significant difference in pathogenicity on watermelon leaves between the Dif^{R} and Dif^{S} isolates. Further exploration of the mechanism related to difenoconazole resistance of *S. citrulli* isolates revealed that the resistance to difenoconazole involved four types of mutations in CYP51, i.e., G463S for Dif^{LR}, I444M, Y446H, and A464G for Dif^{HR}. No over-expression of the *cyp51* gene was found in the tested Dif^{R} isolates. Furthermore, it was found that 5% of the Dif^{R} isolates were significantly more sensitive to difenoconazole after being treated with 20 μg/mL chlorpromazine hydrochloride, indicating that the efflux mechanism may be involved in these difenoconazole-resistant isolates. Together, our study results suggested that *S. citrulli* had a strong resistance to difenoconazole on watermelon and muskmelon, and the mutations in *cyp51*and changes in fungicide efflux were responsible for the emergence of difenoconazole resistance in *S. citrulli*.

Keywords Watermelon, Muskmelon, Gummy stem blight, Fungicide resistance, Difenoconazole, *cyp51*

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Background

Watermelon (*Citrullus lanatus*) and muskmelon (*Cucumis melo*) are annual trailing herbs of Cucurbitaceae (Zhang et al. 2019). Globally, nearly three-quarters of the world's watermelon and muskmelon are produced in Asia, with China being the leading producer. Watermelon and muskmelon are widely cultivated in China, among which the dominant areas are the following 12 provinces: Tibet, Sichuan, Yunnan, Chongqing, Hubei, Hunan, Jiangxi, Anhui, Jiangsu, Zhejiang, Guizhou, and Shanghai. In 2019, the cultivation area and yield of watermelon and muskmelon in Zhejiang Province were 82800 hectares and 2.467 million tons, respectively, accounting for 83.81% harvest area and 88.12% yield of fruit melons in Zhejiang Province, and 4.28% harvest area and 3.26% yield in China (Zhejiang Provincial Bureau of Statistics 2019). As an important infectious disease, gummy stem blight (GSB) occurs throughout all stages of these melons' growth and development, severely impacting their production and quality. The incidence of GSB in fields is generally between 20% and 40%, while in greenhouse planting and continuous cropping, its incidence rate can increase to 80%. In GSB epidemic regions, the yield reduction can reach up to 15% (Babadoost and Zitter 2009). GSB is caused by three morphologically similar yet genetically distinct *Stagonosporopsis* species: *S. cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae* (Brewer et al. 2015; Stewart et al. 2015). A rapid PCR-based method has been developed to distinguish these three morphologically similar species. To date, the pathogen of GSB in China has not been clearly identified and analyzed.

At present, chemical control is the main approach against GSB, in which demethylation inhibitor (DMI) fungicides, such as difenoconazole, are widely employed (Shamshad et al. 2009). Due to their single action site and extensive applications, many plant pathogens have evolved various degrees of resistance (Cools et al. 2013; Price et al. 2015). Although the *S. cucurbitacearum* isolates from South Carolina State of the USA remain sensitive to the DMI fungicides (Keinath and Hansen 2013), low- and moderate-level resistance of GSB to difenoconazole has been reported in Chongqing and Guizhou provinces of China. However, the mechanism of difenoconazole resistance in *Stagonosporopsis* spp. has not been reported (Liu 2016).

DMI fungicides inhibit ergosterol biosynthesis in fungi by targeting lanosterol 14a-demethylase, which is encoded by the *cyp51* gene (Price et al. 2015). The resistance mechanisms of DMI fungicides include the following: (1) mutations in the coding region of *cyp51*, leading to changes in the amino acid sequence of CYP51, so that it cannot be recognized by DMIs (Cools et al. 2011; Frenkel et al. 2014; Schmitz et al. 2014; Zhang et al. 2021); (2) over-expression of *cyp51* (Schnabel and Jones 2001; Bolton et al. 2012; Cools et al. 2012; Ma and Tredway 2013; Zhang et al. 2021); and (3) over-expression of transporters (Kretschmer et al. 2009; Marin et al. 2016). The latter mechanism is mainly caused by one of the two efflux systems: ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (Cools and Fraaije 2013).

Compounds regulating the activity of ABC or MFS transporters can reverse resistance in some cases due to their inhibitory effects on toxicants efflux, and these compounds have been described in medical publications as 'regulators', 'reversers', 'inhibitors', or 'chemosensitizers' to treat resistance (Robert and Jarry 2003) caused by transporters in cells. These compounds include antipsychotic drugs (such as amitriptyline, chlorpromazine, and thioridazine), immunosuppressants (such as cyclosporine A and tacrolimus), steroid or hormone analogues (such as progesterone and diethylstilbestrol), and various plant metabolites (such as curcumin, flavone, and quercetin) (Roohparvar et al. 2007). Several of these regulators have been shown to increase the toxicity of DMI against fungi, especially in the strains overexpressing ABC transporters (Schuetzer-Muehlbauer et al. 2003; Hayashi et al. 2010). However, whether the emerging DMI resistance is due to transporters-related drug efflux is a matter that has not been addressed. In this study, we isolated the GSB-causing agents in the five locations of Zhejiang Province, China, and characterized their resistance to difenoconazole. Additionally, we assessed the potential mechanisms involved in difenoconazole resistance in the *S. citrulli* isolates. The results of this study will provide a scientific basis for resistance management in *S. citrulli* to the DMI fungicides in future production.

Results

Identification of GSB causing agents

A total of 112 single-spored isolates were isolated in 2019 from five cities in Zhejiang Province. Among them, 67 were isolated from watermelon, and 45 from muskmelon. According to the morphology of the colony and characteristics of conidia, the pathogens were first identified as *Stagonosporopsis* sp. Then, further identification via multiplex PCR showed that all of the isolates were *S. citrulli* (Additional file 1: Figure S1).

Resistance of *S. citrulli* to difenoconazole

The sensitivity of 112 isolates of *S. citrulli* to difenoconazole was determined by the method of discriminatory dosage in vitro. The results indicated that 12 isolates were sensitive to difenoconazole, which was then named Dif^S. The remaining 100 isolates were resistant to

Table 1 Difenoconazole resistance in the *Stagonosporopsis citrulli* isolates collected from different regions

City	No. of isolates	No. of resistance isolates*			Resistance frequency (%)		
		LR	MR	HR	LR	MR	HR
Taizhou	3	2	0	1	66.7	0	33.3
Wenzhou	24	12	3	6	50	16.7	20.8
Jinhua	60	31	2	23	51.7	3.3	38.3
Huzhou	12	1	0	7	8.3	0	58.3
Ningbo	13	6	2	4	46.2	15.3	30.8

*LR, MR, and HR mean low-, moderate-, and high-level resistance to difenoconazole, respectively

difenoconazole to varying degrees, and the frequency of resistance was 89.3%. Of all the resistant isolates (Dif^{R}), 52 demonstrated low resistance (Dif^{LR}), with a frequency of 46.4%; seven isolates were moderately resistant (Dif^{MR}), with a frequency of 6.3%; and 41 isolates were highly resistant (Dif^{HR}), with a frequency of 36.6% (Table 1). In view of the two hosts, the resistance frequency of the *S. citrulli* isolates from watermelon was 88.1%, including 40.3% Dif^{LR}, 4.5% Dif^{MR}, and 43.3% Dif^{HR} isolates; meanwhile, the respective frequencies of the Dif^{R}, Dif^{LR}, Dif^{MR}, and Dif^{HR} isolates from muskmelon were 91.1, 55.5, 8.9, and 26.7%. Taken together, these results showed that the *S. citrulli* isolate from both plant hosts had developed strong resistance to difenoconazole.

Growth, sporulation, and pathogenicity of Dif^{S} and Dif^{R}

No significant differences in mycelial growth, sporulation, and pathogenicity were observed between the Dif^{S} and Dif^{LR} or Dif^{MR} isolates (Table 2). The pathogenicity of the four types of isolates on watermelon leaves also had no significant difference. However, a fitness penalty in mycelial growth and sporulation was found for the Dif^{HR} isolates. The mycelial growth rates of Dif^{S} and Dif^{HR} isolates on PDA were 18.75 ± 0.25 (mean $\pm$ SD) and 17.36 ± 0.60 mm/d, respectively. There was a significant difference in mycelial growth rate among the two types of isolates ($F = 5.082$, $P < 0.05$) and the mycelial growth rates of the Dif^{HR} isolates were significantly reduced compared with those of the Dif^{S} isolates. The respective sporulation abilities of the Dif^{S} and Dif^{HR} isolates were $(10.13 \pm 2.89) \times 10^{6}$ and

Table 2 Biological characteristics of difenoconazole-sensitive and -resistant isolates

Isolate	Phenotypes*	Growth (mm/d)	Sporulation ($\times 10^{6}$ conidia/mL)	Pathogenicity (Lesion diameter/mm)
HZXGJ-3	Dif^{S}	18.33 ± 0.22ab	29.38 ± 0.16a	25.3 ± 1.5ab**
JHTGY-7		17.67 ± 0.08c	5.56 ± 0.28b	24.0 ± 1.8ab
WZTGJ-4		19.79 ± 0.08a	4.05 ± 0.06c	27.8 ± 2.6a
WZTGJ-3		19.21 ± 0.18a	1.53 ± 0.07d	25.3 ± 1.8ab
JHXGJ-14	Dif^{HR}	18.71 ± 0.15ab	1.25 ± 0.17d	18.7 ± 1.0b
WZXGJ-11		14.29 ± 0.04d	1.24 ± 0.27d	24.3 ± 2.5ab
JHTGY-5		17.58 ± 0.21c	0.23 ± 0.09e	23.7 ± 1.4ab
JHXGJ-35		18.88 ± 0.19ab	0.15 ± 0.02e	26.2 ± 1.4ab
WZXGJ-21	Dif^{MR}	17.23 ± 0.08c	6.17 ± 0.28b	28.3 ± 2.1a
WZXGJ-17		19.85 ± 0.08a	28.46 ± 0.12a	25.1 ± 1.2ab
JHTGY-7		19.13 ± 0.11a	7.33 ± 0.24b	23.3 ± 1.1ab
NBXGY-6		18.25 ± 0.18ab	4.26 ± 0.05c	24.9 ± 1.4ab
NBCGY-7	Dif^{LR}	17.76 ± 0.07c	4.37 ± 0.05c	28.4 ± 1.8a
JHXGJ-8		19.46 ± 0.10a	6.34 ± 0.11b	25.0 ± 1.0ab
JHXGJ-3		19.95 ± 0.08a	30.35 ± 0.23a	27.1 ± 1.6a
TZXGJ-4		18.27 ± 0.13ab	6.96 ± 0.29b	27.1 ± 1.9a

*Dif^{S}, Dif^{LR}, Dif^{MR}, and Dif^{HR} mean difenoconazole sensitive, low-level resistant, moderate-level resistant, and high-level resistant isolates, respectively. **Mean values in the same column with the same letters were not statistically different ($P > 0.05$) according to the least significant difference (LSD) test

$(0.72 \pm 0.62) \times 10^6$ conidia/mL. According to one-way ANOVA ($F = 10.546$, $P < 0.01$), it can be inferred that the sporulation ability of the Dif^{HR} isolates was significantly lower than that of the Dif^{S} isolates (Table 2).

Cross-resistance between difenoconazole and other DMIs in *S. citrulli*

The sensitivities of *S. citrulli* to difenoconazole, tebuconazole, prochloraz, hexaconazole, metconazole, and mefentrifluconazole are shown in Additional file 2: Table S1. Positive cross-resistance was observed between difenoconazole and tebuconazole ($\rho = 0.947$, $P = 0.000$), prochloraz ($\rho = 0.938$, $P = 0.000$), metconazole ($\rho = 0.881$, $P = 0.004$), and mefentrifluconazole ($\rho = 0.842$, $P = 0.049$). However, there was no cross-resistance observed between difenoconazole and hexaconazole ($\rho = 0.389$, $P = 0.512$) (Fig. 1).

cyp51 mutations in the difenoconazole-resistant isolates

The *cyp51* gene in the Dif^{S} isolates (n = 3), Dif^{LR} isolates (n = 5), Dif^{MR} (n = 4), and Dif^{HR} isolates (n = 7) were amplified by PCR with primers CQ82 and CQ83, and

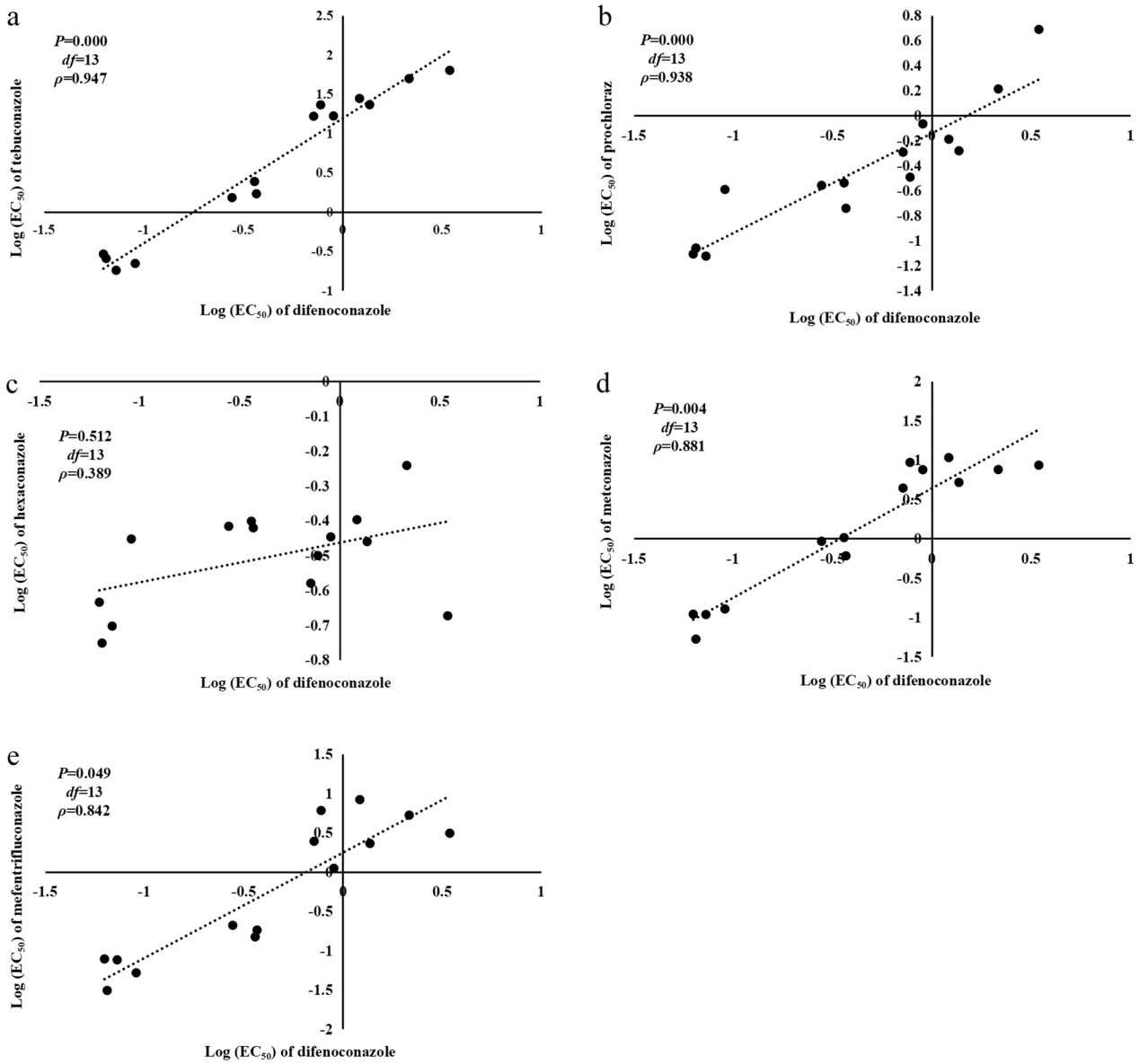

Fig. 1 Cross-resistance among difenoconazole and tebuconazole (**a**), prochloraz (**b**), hexaconazole (**c**), metconazole (**d**), and mefentrifluconazole (**e**)

the target DNA fragment with a length of 1516 bp was obtained and submitted to NCBI (NCBI accession No. OL677535–OL677549). Four types of nucleotide mutations, including C to G, T to C, G to A, and C to G, were found at the positions of 2551, 2555, 2603, and 2607 of the *cyp51* gene coding sequence, respectively. Accordingly, the 444th amino acid was mutated from isoleucine (I) to methionine (M), the 446th from tyrosine (T) to histidine (H), the 463rd from glycine (G) to serine (S), and the 464th from alanine (A) to glycine (G). Among them, three types of mutations, namely I444M, Y446H, and A464G, were associated with the Dif^{HR} isolates, G463S was found in the Dif^{LR} isolates, and no mutation was observed in the Dif^{MR} isolates (Table 3).

cyp51 expression between the Dif^{S} and Dif^{R} isolates

The expression levels of the *cyp51* gene in Dif^{S} (JHTGY-7 and WZTGJ-3), Dif^{LR} (JHXGJ-3 and JHXGJ-8), Dif^{MR} (WZXGJ-17 and NBXGY-6), and Dif^{HR} (WZXGJ-11 and JHXGJ-35) were determined using real-time quantitative PCR. Based on the one-way ANOVA analysis, there was no over-expression of *cyp51* in the resistant strains. Aside from the expression of *cyp51* in Dif^{LR} (JHXGJ-3) having decreased significantly, there was no significant difference among the tested isolates (Table 4).

Effect of chlorpromazine hydrochloride on sensitivity to difenoconazole

The intrinsic effect of chlorpromazine hydrochloride (CH) on mycelial growth was investigated, and the results showed that CH had no obvious effect on the growth of *S. citrulli* at a concentration of 20 μg/mL without having amended difenoconazole. When the isolates were treated with difenoconazole + CH, most isolates did not change their sensitivity phenotype to difenoconazole, indicating that Dif^{S} cannot grow on 5 μg/mL difenoconazole or

Table 3 Comparison of the *cyp51* gene and amino acid sequences between Dif^{S} and Dif^{R}

Isolates	Phenotypes*	Codon bases and coding amino acids			
		2549–2551 (444th)	2555–2557 (446th)	2603–2605 (463th)	2606–2608 (464th)
NBCGY-5	Dif^{S}	AT**C** (Ile)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
WZXGJ-1		AT**C** (Ile)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
WZTGJ-3		AT**C** (Ile)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
NBCGY-7	Dif^{LR}	AT**C** (Ile)	**T**AC (Tyr)	**A**GC (Ser)	G**C**C (Ala)
JHXGJ-3		AT**C** (Ile)	**T**AC (Tyr)	**A**GC (Ser)	G**C**C (Ala)
JHXGJ-8		AT**C** (Ile)	**T**AC (Tyr)	**A**GC (Ser)	G**C**C (Ala)
JHXGJ-10		AT**C** (Ile)	**T**AC (Tyr)	**A**GC (Ser)	G**C**C (Ala)
JHXGJ-16		AT**C** (Ile)	**T**AC (Tyr)	**A**GC (Ser)	G**C**C (Ala)
WZXGJ-21	Dif^{MR}	ATC (Ile)	TAC (Tyr)	GGC (Gly)	GCC (Ala)
WZXGJ-17		ATC (Ile)	TAC (Tyr)	GGC (Gly)	GCC (Ala)
JHTGY-7		ATC (Ile)	TAC (Tyr)	GGC (Gly)	GCC (Ala)
NBXGY-6		ATC (Ile)	TAC (Tyr)	GGC (Gly)	GCC (Ala)
HZXGJ-4	Dif^{HR}	AT**G** (Met)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
JHXGJ-32		AT**G** (Met)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
JHXGJ-35		AT**G** (Met)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
WZXGJ-5		AT**G** (Met)	**T**AC (Tyr)	**G**GC (Gly)	G**C**C (Ala)
JHXGJ-14		AT**C** (Ile)	**C**AC (His)	**G**GC (Gly)	G**C**C (Ala)
JHXGJ-26		AT**C** (Ile)	**C**AC (His)	**G**GC (Gly)	G**C**C (Ala)
NBCGY-11		AT**C** (Ile)	**T**AC (Tyr)	**G**GC (Gly)	C**G**C (Gly)

*Dif^{S}, difenoconazole-sensitive isolate; Dif^{LR}, difenoconazole low-level resistant isolate; Dif^{MR}, difenoconazole moderate-level resistant isolate; and Dif^{HR}, difenoconazole high-level resistant isolate

Bold and red color mean the wild-sensitive and mutated base of the codon, respectively

Table 4 Relative expression of the *cyp51* gene in the *Stagonosporopsis citrulli* isolates with varying levels of sensitivities to difenoconazole

Isolate	Phenotypes*	Relative expression
JHTGY-7	Dif^{S}	1.00 ± 0.07a**
WZTGJ-3		1.06 ± 0.09a
JHXGJ-8	Dif^{LR}	0.84 ± 0.12a
JHXGJ-3		0.40 ± 0.04b
WZXGJ-17	Dif^{MR}	0.96 ± 0.10a
NBXGY-6		1.03 ± 0.09a
WZXGJ-11	Dif^{HR}	1.01 ± 010a
JHXGJ-35		0.93 ± 0.11a

*Dif^{S}, Dif^{LR}, Dif^{MR}, and Dif^{HR} mean difenoconazole sensitive, low-level resistant, moderate-level resistant, and high-level resistant isolates, respectively. **Mean values in the same column with the same letters were not statistically different ($P > 0.05$) according to the least significant difference (LSD) test

5 μg/mL difenoconazole + 20 μg/mL CH, while Dif^{R} can grow on 5 μg/mL difenoconazole or 5 μg/mL difenoconazole + 20 μg/mL CH. Only five resistant isolates (Dif^{R}) out of all 100 Dif^{R} became more sensitive to difenoconazole and changed their respective sensitivity phenotypes (Table 5). Two isolates (JHXGJ-10 and NZCGY-8) changed from Dif^{LR} isolates to Dif^{S}, and three (HZXGJ-12, JHXGJ-24, and JHXGJ-7) changed from Dif^{HR} isolates to Dif^{LR}.

Discussion

Difenoconazole, a sterol demethylation inhibitor, is an important fungicide for managing watermelon and muskmelon diseases. Currently, several hundred products containing difenoconazole have been registered to control watermelon and muskmelon diseases in China. The Fungicide Resistance Action Committee (FRAC) has listed DMIs as moderate- and high-risk fungicides, and many other plant pathogens have been reported to possess varying degrees of resistance to them, including *Puccinia triticina* (brown rust in wheat crops) (Stammler et al. 2009) and *Pyrenopeziza brassicae* (light leaf spot in oilseed rape) (Helen et al. 2014).

GSB is a serious disease of watermelon and muskmelon in eastern China, especially in Zhejiang Province, where difenoconazole is one of the commonly used fungicides to control the disease. It has been reported that *D. bryoniae* has developed resistance to difenoconazole in Chongqing and Guizhou provinces. The resistance frequency of difenoconazole reached 0.67% in Chongqing and 2.03% in Guiyang (Liu 2016). In the present study, 112 isolates of *S. citrulli* were sampled and isolated, and their resistance to difenoconazole was determined. The results indicated that the resistance frequency was 89.3%, significantly higher than in other regions in China and other countries (Keinath and Hansen 2013; Liu 2016). Many previous studies have shown that the adaptability of resistant isolates will decrease after most pathogens have become resistant. The cost of fungicide resistance may be reflected in the ability of the fungus to complete its life cycle and may specifically affect conidial production and dispersal, infection efficiency, mycelial growth, overwintering capabilities, and other life cycle components. It may also affect its ability to compete with fungicide-sensitive isolates in a field environment (Standish et al. 2019). Comparing the fitness of Dif^{S} and Dif^{R} revealed a significant decrease in both the growth rate and sporulation ability of Dif^{HR}. The attenuated fitness has also been consistently reported in the DMI-resistant isolates of *Fusarium oxysporum* f. sp. *fragariae* and *Lasiodiplodia theobromae*. In *F. oxysporum* f. sp. *fragariae*, it was reported that the tebuconazole-resistant isolates had lower growth rates, sporulations, and pathogenicity than the sensitive isolates (Gu et al. 2010). Moreover, when *L. theobromae* from papaya in Brazil developed resistance to difenoconazole, its fitness also decreased (Li et al. 2020). Cross-resistance is defined as resistance to two or more fungicides conferred by the same resistance mechanism (Dutra et al. 2020). Generally, cross-resistance is common between fungicides belonging to

Table 5 Effect of chlorpromazine hydrochloride on the sensitivity of the *Stagonosporopsis citrulli* isolates to difenoconazole

Isolate	Phenotypes	Dif (μg/mL) + CH (μg/mL)*					
		0 + 0	5 + 0	0 + 20	5 + 20	50 + 0	50 + 20
JHXGJ-10	Dif^{LR}	26.3 ± 0.7a**	18.5 ± 0.9b	25.9 ± 0.7a	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
NZCGY-8	Dif^{LR}	27.2 ± 0.3a	18.7 ± 0.7b	26.1 ± 0.3a	0. ± 0.0c	00 ± 0.0c	00 ± 0.0c
HZXGJ-12	Dif^{HR}	36.1 ± 0.4a	34.5 ± 0.3a	35.9 ± 0.4a	17.5 ± 0.3b	15.0 ± 0.6b	0.0 ± 0.0b
JHXGJ-24	Dif^{HR}	24.3 ± 0.5a	21.5 ± 0.3a	23.8 ± 0.4a	21 ± 0.0a	13.5 ± 0.3b	0.0 ± 0.0c
JHXGJ-7	Dif^{HR}	16.7 ± 0.2a	15.5 ± 0.3a	16.5 ± 0.2a	15.5 ± 0.3a	13.0 ± 0.6a	1.0 ± 0.6b

*Chlorpromazine hydrochloride (CH), difenoconazole (Dif). Dif^{LR}, difenoconazole low-level resistant isolate; Dif^{HR}, difenoconazole high-level resistant isolate. **Data were colony diameter (mm) provided as mean ± SD, mean values in the same line with the same letters were not statistically different ($P > 0.05$) according to the least significant difference (LSD) test

the same chemical class, sharing a similar mode of action. However, this is not true in all cases (Xu et al. 2014). For the DMI fungicides, due to a range of various resistance mechanisms, such as mutations in the 14a-demethylase (*cyp51*) gene, overexpression of *cyp51* gene, and overexpression of ABC transporters, the lack of or the variety of cross-resistance among DMIs has been reported in many phytopathogenic fungi (Xu et al. 2014). For instance, in *F. graminearum*, there was weak cross-resistance between difenoconazole and metconazole, yet no cross-resistance with tebuconazole, prothioconazole, and metconazole (Fu et al. 2021). Additionally, no cross-sensitivity was observed in *Monilinia fructicola* to prothioconazole and the other DMIs tested (Dutra et al. 2020). In *F. fujikuroi*, our previous reports showed that prochloraz-resistant isolates only showed cross-resistance to prothioconazole, with no cross-resistance with tebuconazole, difenoconazole, propiconazole, metconazole, hexaconazole, triadimefon, or mefentrifluconazole (Mao et al. 2020; Zhang et al. 2021; Liu et al. 2022). In this study, we found that there was no cross-resistance between difenoconazole and hexaconazole, while there was positive cross-resistance between difenoconazole and other four DMIs: tebuconazole, prochloraz, metconazole, and mefentrifluconazole, indicating variability in the cross-resistance among DMIs and same DMI may even have distinct cross-resistance with other DMIs in the different fungi species. Thus, monitoring the resistance development and analyzing the specific cross patterns of pathogen populations to DMIs were important for guiding DMIs application and disease management. The most widely reported mechanism of DMI resistance in field isolates is the *cyp51* mutations. These mutations lead to changes in the affinity of fungicides to this enzyme, resulting in tolerance to azoles. *cyp51* mutations have been reported in some fungal plant pathogens (Cools et al. 2013; Helen et al. 2014; Wang et al. 2014). In this study, to analyze the resistance mechanisms in *S. citrulli* to difenoconazole, we first scan the *cyp51* sequence among the Dif^{S}, Dif^{LR}, Dif^{MR}, and Dif^{HR} stains. We found four mutations within *cyp51* in the isolates with different resistant levels, i.e., G463S in the Dif^{LR} isolates, I444M, Y446H, and A464G in the Dif^{HR} isolates. G463S and A464G had been previously detected in *Stagonosporopsis* species for tebuconazole resistance (Li et al. 2016), whereas I444M and Y446H were reported for the first time. Surprisingly, no mutation was observed in *cyp51* of Dif^{MR}, indicating other mechanisms except mutations in *cyp51* were responsible for the emergence of Dif^{MR}. The overexpression of the *cyp51* gene is another reported mechanism involved in the reduction of sensitivity to the DMI fungicides. This mechanism has been reported in some pathogens, such as *Blumeriella jaapi* (cherry leaf spot) (Ma et al. 2006; Proffer et al. 2006), *Venturia inaequalis* (apple scab) (Schnabel and Jones 2001), and *P. brassica* (Helen et al. 2014). However, the over-expression of *cyp51* was not observed in the resistant isolates of *S. citrulli*. To investigate the involvement of efflux protein in the resistance of *S. citrulli* to difenoconazole, chlorpromazine hydrochloride was used. Chlorpromazine hydrochloride is a compound that can regulate the activity of ABC or MFS transporters by inhibiting the efflux of toxicants from cells (Leroux and Walker 2013). It has been previously shown that chlorpromazine can enhance the sensitivity of *Botrytis cinerea* to DMIs (Hayashi et al. 2010), and Leroux and Walker (2013) confirmed this finding. Although the expression level of *cyp51* was not determined in all the resistant isolates, we assessed the involvement of efflux protein in difenoconazole resistance in all the *S. citrulli* isolates with combined treatment of difenoconazole and chlorpromazine hydrochloride. Among all the 112 isolates, 5% of Dif^{R} isolates became more sensitive to difenoconazole after the treatment of chlorpromazine hydrochloride. Some highly resistant isolates became moderately resistant, and some low-resistant isolates became sensitive when chlorpromazine hydrochloride was added (Table 5), indicating that the efflux of fungicide was also involved in the emergence of DMIs resistance in certain *S. citrulli* isolates. Taken together, our results prove that the difenoconazole resistance mechanisms were complicated, and different strains, even from the same species, may have distinct mechanisms in resistance development. There are four types of mutations in the *cyp51* gene as well as the changed fungicide efflux activity responsible for the emergence of difenoconazole resistance in *S. citrulli*.

Conclusions

In summary, GSB on watermelon and muskmelon caused by *S. citrulli* in Zhejiang Province, eastern China, had developed serious resistance to difenoconazole. Four types of mutations in the *cyp51* gene and the changed fungicide efflux activity are responsible for the emergence of difenoconazole resistance in *S. citrulli*.

Methods

Origin, collection, and identification of isolates

In 2019, 16 sampling sites were selected from five main producing areas of watermelon and muskmelon in Zhejiang Province (Wenzhou, Taizhou, Ningbo, Jinhua, and Huzhou), and three to eight greenhouses were selected for each sampling site. The GSB samples were collected in each greenhouse by the random sampling method. Each sample was packed separately in a sampling bag to avoid cross-contamination, and the time and location of the sample collection were recorded. The watermelon variety

was Zaojia (8424). The muskmelon varieties were netted melon, Baishami No. 1, Lübaoshi, and Cucumis Melon. The tissue separation method (Fang 1998) was used to isolate the pathogen from the samples. The samples were cut, and the infected tissue (5 × 5 mm) was incubated on PDA (200 g potato, 20 g glucose, 20 g agar, and 1 L distilled water) at 25 °C for 3 days. The mycelium at the edge of the colonies was transferred to fresh PDA to obtain a pure culture. A single-spore culture was prepared for each isolate and maintained on PDA slants at 4 °C. First, the isolates were identified by morphology (Wei 1979). Then, DNA for each isolate was extracted by the CTAB method (Guo et al. 2010) and further analyzed by multiple PCR (Brewer et al. 2015). Information on the multiplex PCR reaction, primer sequence (CQ176–CQ181), and the PCR reaction system of *Stagonosporopsis* sp. is provided in Table 6 (Stewart et al. 2015). The primers (CQ176–CQ181) were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Fungicides

The tested technical fungicides were 95.4% difenoconazole from Zhejiang Tianyi Agricultural Chemical Co., Ltd.; 97% prochloraz from Zhejiang Tianfeng Biological Science Co., Ltd.; 97% tebuconazole from Jiangsu Yang Agrochemical Co., Ltd.; and 95% metconazole from Jiangsu Huifeng Biological Agriculture Co., Ltd. In addition, hexaconazole (97%) was obtained from Hubei Sanonda Tianmen Agrochemical Co., Ltd.; and mefentrifluconazole (98%) was provided by Target Molecule Corp. These technical fungicides were dissolved in acetone or methanol as stock solutions of 5×10^4 μg/mL.

Determination of resistance to difenoconazole

Mycelium plugs with a diameter of 5 mm were obtained after 3-day-old culture plates. The mycelium plugs were placed onto PDA plates supplemented with difenoconazole at 5, 20, and 50 μg/mL (Li 2007). The control PDA plates (CK) were amended with only acetone or methanol, and the experiment was repeated three times. All plates were incubated in the dark at 25 °C for 3 days to observe the growth of the isolates. The different growth phenotypes of the measured isolates were defined as follows: The sensitive isolate (Dif^S) could not grow on PDA medium containing 5 μg/mL difenoconazole. The low-level resistant isolate (Dif^{LR}) grew on PDA medium with 5 μg/mL difenoconazole, yet could not grow at 20 μg/mL difenoconazole. The moderate-level resistant isolate (Dif^{MR}) grew at 20 μg/mL, but not at 50 μg/mL difenoconazole. The isolate with a high level of resistance (Dif^{HR}) was able to grow on 50 μg/mL difenoconazole (Li 2007). The resistance frequency was calculated as follows:

$$F_R(\%) = (N_R/N_T) \times 100,$$

where F_R is the resistance frequency, N_R is the number of resistant isolates, and N_T is the total number of isolates.

Comparison of growth, conidial production, and pathogenicity between sensitive and resistant isolates

A total of 16 isolates from four sensitive types were chosen at random. The mycelial plugs (5 mm in diameter) were taken from the edge of 3-day-old PDA plates and inversely placed onto the center of the PDA media. They were placed in an incubator at 25 °C for 4 days, and the diameter of each colony was measured. The experiment was repeated three times for each isolate. The mean colony diameter (measured diameter: 5 mm) was adopted to represent mycelial growth ability (Zhang et al. 2009; Xu et al. 2014). Conidia production was determined according to the previously described sporulation method (Li 2007; Wang et al. 2017). The mycelial plugs (5 mm in diameter) were taken from the edge of a 3-day-old PDA medium and placed onto the center of potato ammonium dihydrogen phosphate agar medium (PDA 200 mL,

Table 6 Primers used in the study

Primer	Sequence	Description	Reference
CQ176	F:5′-CGGTCCGGTCAACCTACTAC-3′	Amplify ~ 360 bp amplicons from the *Stagonosporopsis citrulli citrulli* isolate	Stewart et al. (2015)
CQ177	R:5′-CACGCCAGCAAATCACACTA-′		
CQ178	F:5′-GGTGACATCTTGCGTGAATG-3′	Amplify ~ 270 bp amplicons from the *S. citrulli* and *S. cucurbitacearum* isolates	
CQ179	R:5′-TGGTTGTTTGGTTGTTTGGA-3′		
CQ180	F:5′-TATGACGTTGGGCAAGTGAG-′	Amplify ~ 220 bp amplicons from the *S. caricae*, *S. citrulli* and *S. cucurbitacearum* isolates	
CQ181	R:5′-TTTGCTGGGATGGTGTTGTA-3′		
CQ82	F:5′-TCCGCCGTTCCTCATTG-3′	Sequencing of the *cyp51* coding region	This study
CQ83	R:5′-GCTGCCGTTCACATTCC-3′		
CQ146	F:5′-CAGAACCCTGGTCTTATTG-3′	Determination of the *cyp51* gene expression	This study
CQ147	R:5′-GAGGCTGCTTGACTTTAC-3′		

2 g ammonium dihydrogen phosphate). Each experiment was repeated three times for each isolate. Isolates were incubated in the dark at 25 °C for 7 days. All aerial hyphae were scraped off, and the petri dish was sealed with a sealing film and placed under black light tubes (36 W) with a 12-h light and 12-h dark illumination cycle. Another 4 days later, the conidia were washed with 20 mL ddH_2O, and the number of conidia was counted by hemocytometer (Shi et al. 2012; Xu et al. 2014). For pathogenicity, a mycelial plug (5 mm in diameter) was taken from the edge of the 3-day-old PDA medium and placed on a 50-day-old watermelon leaf (Zaojia variety). The experiment was repeated three times with three replicate plates for each tested isolate. They were then incubated in a 25 °C incubator for 48 h. The size of the lesion (mm) was measured, and the mean size (mm) was calculated for each isolate to represent the pathogenicity (Li 2007).

Cross-resistance assay

The sensitivity assay was conducted using a mycelium growth inhibition assay. Three replicates were used for each treatment, and the tests were repeated three times. For each fungicide, the stock solutions were diluted to the desired concentrations (Additional file 2: Table S2). Colony diameters were measured after 5 days of incubation at 25 °C, and medium effective inhibitory concentration (EC_{50}) values were calculated for each isolate–fungicide combination by linear regression of the percent inhibition of mycelial growth relative to the control versus the log10 transformation for each of the fungicide concentrations. The $logEC_{50}$ of difenoconazole was used as the x-coordinate, while the $logEC_{50}$ of other DMI fungicides was individually plotted as the y-coordinate to establish the linear regression equations. Spearman rank correlation calculations were then used to assess the cross-resistance between two fungicides, wherein $P<0.05$ and $\rho>0.6$ indicated a strong positive cross-resistance between the two fungicides (Sun et al. 2021).

Analysis of *cyp51* mutations in the difenoconazole-resistant isolates

Four types of isolates were adopted at random: Dif^{S} (n = 3), Dif^{LR} (n = 5), Dif^{MR} (n = 4), and Dif^{HR} (n = 7). The complete *cyp51* gene sequence (KX246903) of *S. cucurbitacearum* was obtained from NCBI, and the primers for the *cyp51* gene of *S. citrulli* were designed by Primer Premier 5. The primers (CQ82 and CQ83) were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. (Table 6). The gene sequence of *cyp51* was amplified by PCR using the DNA of *S. citrulli* as a template. PCR was conducted using a 25 μL volume reaction as follows: 12.5 μL of 2 × Taq PCR Master Mix; 1 μL of template DNA (50 ng/μL); 1.5 μL of each pair of primers (10 μM); and 2.5 μL of ddH_2O. The thermal cycling conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. PCR products of the expected size were excised from 1% agarose gel, and the amplicons were sequenced at Sangon (Shanghai, China). Finally, the DNA sequences were analyzed using DNAMAN6.0 to analyze mutations in *cyp51*.

RNA extraction and reverse transcription

RNA extraction was conducted according to the instructions of the UNIQ-10 column Trizol total RNA extraction kit. All materials used in the experiment were sterilized and de-enzymatic. The M-MuLV first-strand cDNA synthesis kit was used for RNA reverse transcription. A volume mixture of 12 μL was added to the test tube of the ice bath, including 0.5 μg RNA extract (100 ng/μL) and 1 μL Oligo (dT) (0.5 μg/mL), and the volume was fixed to 12 μL with RNase-free ddH_2O. The mixture was incubated at 65 °C for 5 min, followed by an ice bath for 30 s. Reverse transcription was conducted in a volume of 20 μL, including a 12 μL reaction solution in the previous step, 4 μL 5 × reaction buffer, 2 μL dNTP mix (10 mmol/L), 1 μL M-MuLV RT (200 U/μL), and 1 μL RNase inhibitor (40 U/μL). This was mixed gently and centrifuged for 3–5 s. Then, reverse transcription was performed on the PCR instrument under the following conditions: 42 °C for 45 min, and 70 °C for 10 min. Finally, the complementary DNA was stored at −20 °C.

Real-time quantitative PCR

The SGExcel UltraSYBR Mixture kit was used for gene expression analysis in real-time quantitative PCR. Each reaction was conducted in a 20 mL volume containing 12.5 μL 2 × SGExcel Ultra SYBR mixture, 0.5 μL of each primer (0.2 μM), 1 μL cDNA, and 5.5 μL ddH_2O. According to the instructions, the reaction process was set as follows: initial heat at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 20 s, then final heating at 72 °C for 25 s. The relative expression of the target gene was calculated by the $2^{-\Delta\Delta CT}$ method. The relative expression of the *cyp51* (target gene) was calculated with *Actin* as the reference gene and the sensitive isolate as the control group (CK). ΔC_T (test sample) = C_T (target gene, test sample) – C_T (reference gene, test sample). ΔC_T (control sample) = C_T (target gene, control sample) – C_T (reference gene, control sample). $\Delta\Delta C_T = C_T$ (test sample) – C_T (control sample) (Kenneth and Thomas 2002).

Analysis of the effect of chlorpromazine hydrochloride on resistance to difenoconazole

Chlorpromazine hydrochloride, from Shanghai Sangon Bioengineering Co., Ltd., was dissolved in anhydrous ethanol as a stock solution of 4×10^4 μg/mL. For all 112 isolates, mycelial plugs (5 mm diameter) were respectively taken from the edge of a 3-day-old colony and placed onto the plates supplemented with 20 μg/mL chlorpromazine hydrochloride, 5 μg/mL difenoconazole, 50 μg/mL difenoconazole, 5 μg/mL difenoconazole + 20 μg/mL chlorpromazine hydrochloride, 50 μg/mL difenoconazole + 20 μg/mL chlorpromazine hydrochloride, and control medium (containing only acetone) (Leroux and Walker 2013). All plates were cultured in the dark at 25 °C for 4 days, and the colony diameter was recorded. Three replicates were used for each treatment, and the tests were repeated twice.

Statistical analysis

SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis to determine significant differences. One-way analysis of variance (ANOVA) with the least significance difference (LSD) test was used to evaluate the variance between the sensitive and resistance groups. Cross-resistance between fungicides was determined using Spearman's rank-order correlation analysis.

Abbreviations

A	Alanine
ABC	ATP-binding cassette
CH	Chlorpromazine hydrochloride
Dif^{HR}	High-level resistance to difenoconazole
Dif^{LR}	Low-level resistance to difenoconazole
Dif^{MR}	Moderate-level resistance to difenoconazole
DMI	14α-Demethylase inhibitor
FRAC	The Fungicide Resistance Action Committee
G	Glycine
G	Glycine
GSB	Gummy stem blight
H	Histidine
I	Isoleucine
M	Methionine
MFS	Major facilitator superfamily
S	Serine
T	Tyrosine

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00156-8.

Additional file 1: Figure S1. Representative identification of 112 single-spored isolates of *Stagonosporopsis* species causing watermelon and melon gummy stem blight.

Additional file 2: Table S1. EC_{50} of difenoconazole, tebuconazole, prochloraz, hexaconazole, metconazole, and mefentrifluconazole. **Table S2.** Series concentration for determine EC_{50} of difenoconazole, tebuconazole, prochloraz, hexaconazole, metconazole, and mefentrifluconazole.

Acknowledgements

Not applicable.

Authors' contributions

CZ (Chuanqing Zhang) designed the research. ZK, YZ, CZ (Chaojie Zhuang), and CM performed the experiments, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by the 'Sannongliufang' Research Joint Project of Zhejiang Province (2021SNLF019).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 9 July 2022 Accepted: 26 December 2022
Published online: 13 January 2023

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