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Evaluation of the risk of development of resistance to QoI fungicide ZJ0712 in *Podosphaera xanthii* under greenhouse conditions

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Abstract

Greenhouse-grown cucumbers were monitored over two successive growing seasons to investigate the effects of successive application of ZJ0712, a new quinone outside inhibitor (QoI) fungicide, on the development of resistance in cucumber powdery mildew (*Podosphaera xanthii*). Resistant *P. xanthii* isolates were detected after nine successive applications of ZJ0712, although the control efficacy of this fungicide against cucumber powdery mildew at that time was still higher than 80%. Seven ZJ0712-resistant *P. xanthii* isolates with resistance factor values greater than 180 were obtained, which exhibited a stable resistance to ZJ0712. These resistant *P. xanthii* isolates had similar pathogenicity to the wild-type isolate on cucumber plants. The ZJ0712 showed significant cross-resistance with azoxystrobin, enostrobilurin, or chlorothalonil, but not with the azole fungicide triadimefon. Furthermore, the most commonly reported G143A mutation in Cyt *b* associated with QoI resistance was found in five of the seven resistant isolates. These findings suggest that there is a high risk of resistance development associated with using ZJ0712 for controlling cucumber powdery mildew under greenhouse conditions, and the underlying resistance mechanisms in different *P. xanthii* isolates are not consistent and need to be further unraveled.

Keywords: *Podosphaera xanthii*, ZJ0712, Fungicide resistance, Competition, Cross-resistance, Cyt *b*

Background

Cucumber powdery mildew (CPM) caused by *Podosphaera xanthii* (also known as *Sphaerotheca xanthii*) is one of the most important foliar diseases of cucumbers in cucumber-growing areas worldwide (Braun et al. 2002; Moret et al. 2009; Pérez-García et al. 2009). White powdery colonies on leaf surfaces, petioles, and young stems are typical symptoms of CPM (Pérez-García et al. 2009). The development of cucumber cultivars resistant to powdery mildew is an efficient strategy for disease management. However, the majority of resistance genes deliberately deployed by breeders are race-specific resistance (R) genes (Collins et al. 2003), which do not always provide adequate protection when applied as a sole management practice (Urban and Lebeda 2006) because the obligate biotrophic pathogen *P. xanthii* has high evolutionary potential (McDonald and Linde 2002). Thus, the application of fungicides continues to be the principal approach for managing CPM around the world (Hollomon et al. 2002; McGrath 2015). Systemic fungicides or those with translaminar activity are particularly important in controlling CPM because they provide effective protection for abaxial leaf surfaces, where conditions are more favorable for disease development than on adaxial surfaces (McGrath 2001).

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Fungicides categorized as benzimidazole fungicides, sterol demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs) are widely used to control CPM in China (http://www.chinapesticide.org.cn). QoI fungicides, which block electron transfer between cytochrome *b* (Cyt *b*) and cytochrome *c1* in the mitochondrial respiration pathway by binding to the outer quinone-oxidizing pocket (the Qo site) of the cytochrome bc_1 complex (complex III) (Gisi et al. 2002), have been demonstrated to have a broad spectrum of activity against fungal plant pathogens (Bartlett et al. 2002). In addition to their broad-spectrum antifungal activity, QoI fungicides exhibit some other excellent properties, including low risk to human and animal health and the environment (Bartlett et al. 2002), and therefore are widely developed and applied around the world. Several novel QoI fungicides have been developed in the past few decades in China, such as ZJ0712 (Chen et al. 2006), SYP-1620 (Zhang et al. 2014), pyraoxystrobin (Li et al. 2011; Liu et al. 2011), and enestroburin (Si et al. 2003). Among these fungicides, ZJ0712, with a chemical name of methyl (*E*)-2-(2-((2,5-dimethylphenoxy) methyl) phenyl)-3-methoxyacrylate, developed by China Zhejiang Research Institute of Chemical Industry, has an excellent ability to control powdery mildew on different crops (Chen et al. 2006). It has broad-spectrum activity against various plant pathogens, including ascomycetes and oomycetes. Recently, this fungicide has been registered in China for the control of CPM and cucumber downy mildew (http://www.chinapesticide.org.cn). In our previous work (Wang et al. 2007), the sensitivities of 59 *P. xanthii* strains were determined through a leaf disc assay, and the mean EC_{50} value of ZJ0712 to these strains was 0.0105 ± 0.0028 μg/mL. No ZJ0712-resistant *P. xanthii* subpopulation was found in the field. Thus, the resistance risk evaluation was performed in laboratory by selecting resistant mutants obtained by fungicide adaption. Four ZJ0712-resistant mutants, with resistance factor (RF) values higher than 150, showed similar or greater pathogenicity and competition than those of the wild-type isolates. Thus, we speculated that ZJ0712 might be a high-risk fungicide, similar to most members of this class that have been classified as high risk for resistance development by the Fungicide Resistance Action Committee (FRAC) (https://www.frac.info).

The total risk of fungicide resistance is influenced not only by inherent factors associated with the combination of fungicide and pathogen but also by management factors including conditions of fungicide usage (Köller and Scheinpflug 1987). The conditions of fungicide usage comprise environmental factors, especially climatic and topographic conditions that affect the severity and spread of a crop disease, as well as a range of farmer-determined agronomic factors (Brent and Hollomon 1998). Due to the single-site mode of action, the resistance of CPM to QoI fungicides has been frequently reported in many countries (Ishii et al. 2001; Fernández-Ortuño et al. 2006; Lin et al. 2014). The resistance mechanisms to QoI fungicides in plant pathogens include mutations in Cyt *b* (e.g., G143A, F129L, G137R, or N261D mutations), alternative respiration, and overexpression of efflux transporters (Gisi et al. 2002; Fernández-Ortuño et al. 2008a). The resistance of *P. xanthii* to ZJ0712 in the field remains unknown, although the resistance risk has been reported in the laboratory study. The objectives of this study were to: 1) evaluate the development of resistance to ZJ0712 in *P. xanthii* under greenhouse conditions with consecutive application of this fungicide, 2) determine the biological and molecular characteristics of resistant isolates obtained from the greenhouse, and 3) clarify the resistance mechanisms of *P. xanthii* to ZJ0712.

Results

ZJ0712 showed good control efficacy against CPM with different application times and doses

Greenhouse trials on the control efficacy of ZJ0712 to CPM were performed in this study. It was shown that a control efficacy of nearly 70% was obtained when the fungicide was sprayed four times at a dose of 8 g/667 m^2 (for the first application), 4 g/667 m^2 (for the second application) and 2 g/667 m^2 (for the other two applications) in the first growing season. In the second growing season, application of ZJ0712 at a dose of 2 g/667 m^2 provided more than 80% control efficacy against CPM after the second application (Table 1).

P. xanthii isolates showed decreased sensitivity to ZJ0712 after the ninth application

The sensitivity of *P. xanthii* to ZJ0712 was determined by a leaf disc assay, and the result showed that the EC_{50} values of *P. xanthii* isolates randomly collected from ZJ0712-treated cucumber plants grown in a greenhouse were lower than 0.03 μg/mL, close to the sensitivity baseline value previously obtained by our group (Wang et al. 2007), before the ninth application of the fungicide. However, after the ninth application, the sensitivity of the randomly selected *P. xanthii* isolates to ZJ0712 was significantly reduced and the average EC_{50} value was higher than 0.9 μg/mL (Fig. 1). After the last application of ZJ0712, seven resistant *P. xanthii* isolates (NR1, NR2, NR3, NR4, NR5, NR6, and NR7) with resistance factors higher than 180 were obtained from diseased cucumber plants (Table 2). When applied at a dose of 0.1 μg/mL, ZJ0712 completely inhibited the growth of the wild-type isolate BH17, but it displayed a low inhibitory activity against the resistant isolates we obtained. Even at 1 μg/

Table 1 Control efficacy of different doses of ZJ0712 against cucumber powdery mildew in two successive growing seasons

Treatment	Date	Dose (g a.i.[a]/667 m^2)	Disease index		Control efficacy (%)
			Control	Treatment	
1st season	–	–	32.99[b]		–
1	28 April	8	26.84	19.11	28.80
2	5 May	4	21.33	8.44	60.43
3	12 May	2	23.51	12.27	47.81
4	19 May	2	30.55	10.70	67.27
5	26 May	2	35.42	8.50	76.00
2nd season	–	–	10.48[c]		–
6	4 August	2	10.83	4.38	59.57
7	11 August	2	25.88	3.46	86.63
8	18 August	2	29.07	4.16	85.69
9	25 August	2	25.78	2.98	88.44
10	1 September	2	25.14	3.53	85.96

[a] a.i. indicates active ingredients

[b] The initial disease index of cucumber powdery mildew before application of ZJ0712 in the first growing season

[c] The initial disease index of cucumber powdery mildew before application of ZJ0712 in the second growing season

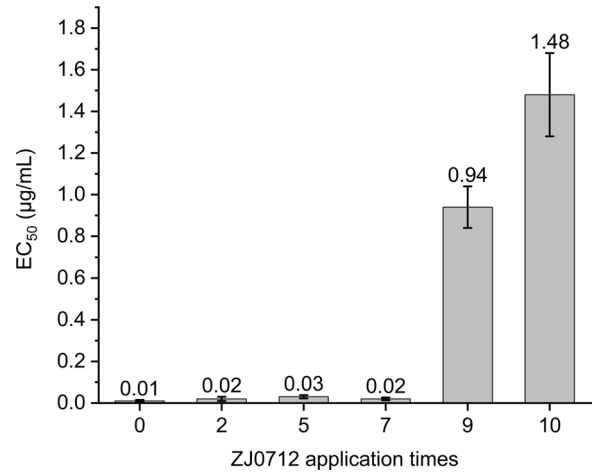

Fig. 1 Developing resistance to ZJ0712 in *Podosphaera xanthii* isolates during consecutive application of the fungicide on greenhouse-grown cucumber plants for two successive growing seasons

mL, ZJ0712 showed low inhibitory activity (less than 20%) against these resistant isolates (Fig. 2).

The ZJ0712-resistant *P. xanthii* isolates exhibited stable resistance and normal pathogenicity

The RFs of seven ZJ0712-resistant isolates were reduced after cultivation for ten asexual generations on fungicide-free cucumber leaves, but the RFs all remained above 160

Table 2 Resistance stability and pathogenicity of the ZJ0712-resistant *Podosphaera xanthii* isolates

Isolate	EC_{50} (μg/mL)/RF[a]			Lesion area (mm^2)
	1st generation	10th generation	FSC[b]	
BH17	0.01/–	0.01/–	–	97.67 ± 8.76 a[c]
NR1	2.08/208	1.64/164	0.79	85.50 ± 9.99 a
NR2	1.87/187	1.79/179	0.96	93.00 ± 11.29 a
NR3	2.93/293	2.18/218	0.74	102.00 ± 6.96 a
NR4	2.94/294	2.27/227	0.77	96.00 ± 12.43 a
NR5	2.49/249	2.09/209	0.84	93.33 ± 18.26 a
NR6	2.71/271	2.35/235	0.87	95.48 ± 7.83 a
NR7	2.24/224	1.96/196	0.88	90.67 ± 10.27 a

[a] RF, resistance factor, ratio of EC_{50} of fungicide-resistant isolates relative to 0.01 μg/mL (the sensitivity baseline of *P. xanthii* to ZJ0712, Wang et al. 2007)

[b] FSC, factor of sensitivity change; the ratio of the RF value at the tenth generation to that at the first generation

[c] Values followed by the same letter within a column do not differ significantly ($P < 0.05$)

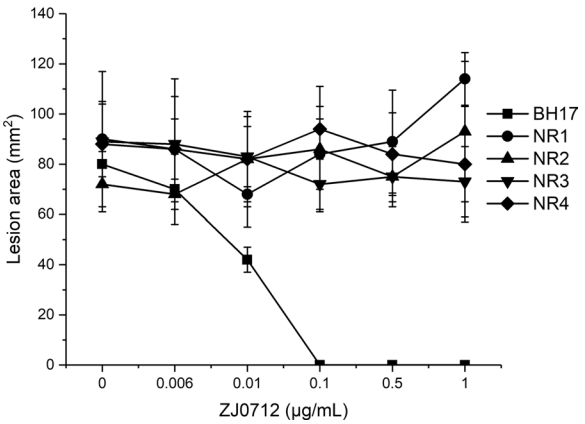

Fig. 2 Dose–response curves showing ZJ0712 sensitivity of sensitive *Podosphaera xanthii* isolate BH17 and resistant isolates (NR1, NR2, NR3, and NR4)

(Table 2). All seven ZJ0712-resistant isolates showed similar pathogenicity to that of the wild-type isolate BH17 (Table 2).

The ZJ0712-resistant *P. xanthii* isolates had a better competitive ability

In the competition experiments, no resistant progeny isolates were detected during five disease cycles of N2 and N3, two ZJ0712-sensitive *P. xanthii* isolates, on ZJ0712-free cucumber leaves, but the resistance frequency still approached 100% during five disease cycles of the ZJ0712-resistant isolates NR2 and NR3 on ZJ0712-free leaves. After consecutive cultivation of mixed population with a ratio of 1:9 for resistant to sensitive isolates for

five generations on ZJ0712-free leaves or leaves treated with 0.008 μg/mL of ZJ0712, the resistant isolates were always the dominant population despite the fact that the initial conidial ratio of these resistant isolates was lower than that of the sensitive isolates. The resistance frequencies were higher than 60% and approached 100% on the ZJ0712-free leaves and the leaves treated with 0.008 μg/mL of ZJ0712, respectively (Fig. 3).

Cross-resistance existed between ZJ0712 and azoxystrobin, enostrobilurin, or chlorothalonil in the *P. xanthii* isolates

The ZJ0712-resistant and ZJ0712-sensitive isolates were treated with four fungicides (azoxystrobin, enostrobilurin, chlorothalonil, and triadimefon) to determine whether there was any cross-resistance between these fungicides and ZJ0712. The results showed that ZJ0712 exhibited significant cross-resistance with azoxystrobin, enostrobilurin, or chlorothalonil, but not with the azole fungicide triadimefon (Table 3).

The ZJ0712-resistant *P. xanthii* isolates had mutations in Cyt *b*

A 574-bp coding fragment of the *cyt b* gene encompassing the Qo domains was sequenced from eight isolates of *P. xanthii*, seven of which were ZJ0712-resistant mutants. Amino acid G143A substitution was observed in five ZJ0712-resistant mutants, including NR2, NR3, NR4, NR5, and NR7, but not in NR1 and NR6 (Table 3). No other types of amino acid substitutions related to QoI resistance (e.g., F129L, G137R, or N261D) were detected in any of these seven ZJ0712-resistant mutants. Additionally, some other substitutions were identified in the resistant mutants, including substitution A113G, which was detected in NR2, NR3, NR4, and NR5, while I161M was found in NR1, NR3, and NR6 (Table 3).

Discussion

The development of resistance to ZJ0712 in *P. xanthii* was estimated in a greenhouse through successive applications of this fungicide during two continuous growing seasons. Although ZJ0712 showed good control efficacy against CPM (>70%) during each growing season, the sensitivity of *P. xanthii* to ZJ0712 decreased significantly after the ninth application, which indicated that the resistance to ZJ0712 was developed in *P. xanthii* at that time.

Seven ZJ0712-resistant *P. xanthii* isolates (RF>180) were obtained from the ZJ0712-treated cucumber plants. All these resistant isolates showed stable and high resistance, but were not compromised in pathogenicity. The results were similar to those of many previous studies. For example, Avila-Adame and Köller (2003) reported that azoxystrobin-resistant *Magnaporthe grisea* mutants carrying the G143A mutation had no fitness penalties. No fitness penalties were observed in QoI-resistant *Colletotrichum acutatum* isolates with the G143A mutation (Forcelini et al. 2018). A discriminatory dose was further determined, and sensitive isolates did not grow but resistant isolates grew at this dose (Moyano et al. 2004). This has been widely used to discriminate between sensitive and resistant isolates. Combined with the results from our previous study (Wang et al. 2007), 1 μg/mL was established as the discriminatory concentration to differentiate between ZJ0712-sensitive and ZJ0712-resistant *P. xanthii* isolates. This value could be

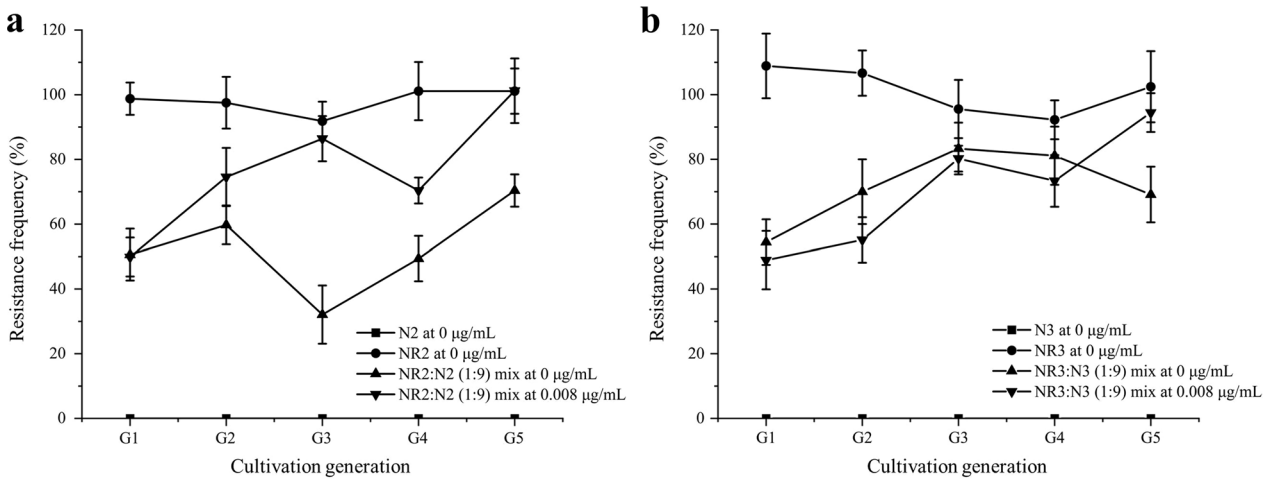

Fig. 3 Competition in vivo between ZJ0712-sensitive and ZJ0712-resistant isolates of *Podosphaera xanthii* with three different initial ratios and in two different pairs. **a** NR2/N2 pair; **b** NR3/N3 pair. Vertical lines indicate the standard error of the mean

Table 3 Amino acid substitution in Cyt *b* and sensitivity of *Podosphaera xanthii* isolates or mutants to ZJ0712 and four other fungicides

Isolate[a]	Substitution[b]	EC_{50} (μg/mL)				
		ZJ0712	Azoxystrobin	Enostrobilurin	Chlorothalonil	Triadimefon
N1	WT[c]	0.011	0.012	0.048	0.232	0.179
N2	WT	0.012	0.021	0.039	0.215	0.237
N3	WT	0.013	0.014	0.071	0.250	0.214
N4	WT	0.013	0.028	0.054	0.309	0.225
BH17	WT	0.008	0.010	0.019	0.122	0.200
SR1	G143A	1.267	1.287	1.865	1.967	0.254
BR14	G143A	1.575	1.880	1.966	1.893	0.174
NR1	I161M	1.704	1.683	2.303	2.164	0.148
NR2	A113G/G143A	1.725	1.605	1.510	2.175	0.182
NR3	A113G/G143A/I161M	3.201	5.321	1.592	2.164	0.187
NR4	A113G/G143A	3.260	3.531	1.769	1.997	0.194
NR5	A113G/G143A	2.322	2.832	1.966	3.087	0.182
NR6	I161M	2.718	2.832	1.402	1.884	0.179
NR7	G143A	2.007	5.321	1.358	2.725	0.189

[a] N1–N4 and BH17 are the wild-type isolates; SR1 and BR14 are ZJ0712-resistant mutants obtained by fungicide adaption in our previous study; NR1–NR7 are ZJ0712-resistant isolates collected from the greenhouse in this study

[b] Amino acid substitution genotype based on DNA sequencing of the *cyt b*gene

[c] WT, wild-type

used to monitor the sensitivity shifts to ZJ0712 in *P. xanthii* populations, which might simplify the monitoring procedure in the future.

In the in vitro competition test, instead of evaluating the competitive ability of a single ratio of resistant and sensitive isolates, two combinations of isolates and three different ratios of resistant and sensitive conidia were used as the inoculum. This approach was chosen because it reflected the natural conditions more closely than a single ratio of combination, for which diverse isolates with different phenotypes with respect to fungicide sensitivity would compete for dominance (Taylor et al. 2002). For two combinations of ZJ0712-resistant and ZJ0712-sensitive isolates, the resistant isolates were always dominant in the population after consecutive cultivation was accomplished for five generations on ZJ0712-free leaves or on leaves treated with 0.008 μg/mL of ZJ0712, even though the initial conidial ratio of resistant isolates to sensitive isolates was 1:9. These results indicated that the ZJ0712-resistant isolates potentially had better fitness in the field and these isolates might develop to be the dominant population in the future.

The cross-resistance experiments demonstrated that ZJ0712 showed cross-resistance with azoxystrobin and enostrobilurin, which might have been caused by their similar chemical structures and modes of action (Delp 1988; Zhou et al. 2015), but no cross-resistance with triadimefon, a sterol C14-demethylation inhibitor. Consequently, the DMI fungicides could be used as alternative fungicides with mixing or in rotation with ZJ0712 to control CPM and manage resistance to ZJ0712. This approach is currently being used in several systems, and many chemical companies in China are selling fungicides that already combine QoI and DMI fungicides to control CPM (http://www.chinapesticide.org.cn). Interestingly, the isolates with resistance to ZJ0712 exhibited resistance to chlorothalonil. Chlorothalonil is a polychlorinated aromatic with multi-site activity that is classified as a chloronitrile/phthalonitrile by FRAC (https://www.frac.info). It is an electrophile that inhibits thiol enzymes important for the spore germination of *Botrytis cinerea* (Leroux et al. 2002) and sulfhydryl groups that are important in glycolysis and fungal respiration (Tillman et al. 1973). A previous study reported that the mutation(s) for resistance to amidocarbamates iprovalicarb, benthiavalicarb, and cyanoimidazole cyazofamid also greatly reduced the sensitivity of mutant strains to the phenylamides metalaxyl, the cyanoacetamide cymoxanil, the morpholine dimethomorph, and the benzamides zoxamide and chlorothalonil (Ziogas et al. 2006). However, the relatively lower level of resistance to energy production inhibitors (QoI and QiI) and the absence of resistance to fluazinam, an inhibitor of ATP synthesis, resulted in the hypothesis that an increased energy-dependent efflux pump was the most probable mechanism for the multi-drug resistance in *Phytophthora infestans* (Ziogas et al. 2006). The fungicide SYP-14288 could

induce multi-drug resistance to several other fungicides with or without the same mode of action as SYP-14288 in *Rhizoctonia solani*, such as chlorothalonil (Cheng et al. 2020). In this study, we speculated that the efflux pump might provide an explanation for the resistance to chlorothalonil in ZJ0712-resistant isolates. It is worthwhile to determine the mechanism of cross-resistance between ZJ0712 and chlorothalonil because this is important for establishing a resistance management strategy for ZJ0712.

To elucidate whether the previously reported substitutions (e.g., G143A, F129L, G137R, or N261D) in Cyt *b* were responsible for the resistance to ZJ0712 in *P. xanthii*, the *cyt b* gene in seven ZJ0712-resistant *P. xanthii* isolates was sequenced. Similar to extensive reports for many fungal plant pathogens, including powdery mildew (*Podosphaera fusca*) (Ishii et al. 2001; Kim et al. 2003; Lesniak et al. 2011; Bolton et al. 2013), a G143A mutation was observed in five of the seven ZJ0712-resistant isolates. The results indicated that the G143A mutation was one of the mechanisms associated with the resistance to ZJ0712 in *P. xanthii*, and the resistance mechanism in NR1 and NR6 was different and not associated with the reported mutations. In the study of Ishii et al. (2001), G143A was not found in one of three resistant isolates of powdery mildew. Fernández-Ortunõ et al. (2008b) reported that no mutation or combination of mutations in the Qo domain was found to correlate with the QoI resistance in the 13 QoI-resistant *P. fusca* isolates. No amino acid change was found in any field QoI-resistant isolates of the apple scab fungus *Venturia inaequalis* (Cooke) Winter (Steinfeld et al. 2001). All these studies indicated that some other mechanisms were responsible for the QoI resistance. In this study, for the I161M mutation in Cyt *b*, as suggested by Brasseur et al. (1966), the possibility that different combinations of multiple mutations in the Qo domains of Cyt *b* responsible for the QoI-resistant phenotype could not be excluded.

Another mechanism potentially responsible for QoI resistance is the induction of alternative respiration, although this is not considered a mechanism of resistance from a practical point of view (Wood and Hollomon 2003; Fernández-Ortuño et al. 2008a;). Alternative respiration is mediated by alternative oxidase (AOX), which can be inhibited by propyl gallate or SHAM (Miguez et al. 2004). Alternative respiration has been reported to be associated with QoI resistance. For example, alternative oxidase reduces the sensitivity of *Mycosphaerella graminicola* to QoI fungicides (Miguez et al. 2004). An alternative respiratory pathway is responsible for the azoxystrobin resistance in a *Septoria tritici* mutant, which is inhibited by the addition of 2 mM SHAM (Ziogas et al. 1997). However, in this study, a high level of resistance to ZJ0712 was observed in *P. xanthii* with the addition of 100 μg/mL SHAM, ruling out the occurrence of an electron bypass via alternative oxidase as a major mechanism of QoI resistance in *P. xanthii*.

A quantitative factor involved in the resistance to fungicides is the efflux transporters located in plasma membranes that have the capacity to secrete antifungals back into the outer environment, preventing the accumulation of compounds to fungitoxic concentrations inside fungal cells (de Waard et al. 2006). The potential role of efflux transporters in the resistance to QoI fungicides in *P. xanthii* has yet to be tested. However, the high resistance factors exhibited by QoI-resistant isolates and the lack of cross-resistance between ZJ0712 and triadimefon did not seem to support a transporter-mediated resistance hypothesis in this study. The resistance mechanisms of *P. xanthii* to QoI fungicide ZJ0712 were complex and should be further investigated.

Conclusions

The evidence from this study suggested that the risk of developing resistance to ZJ0712 in *P. xanthii* was high. The absence of cross-resistance between ZJ0712 and DMI fungicides suggested that the application of ZJ0712 in mixtures with DMI fungicides might help prevent the rapid development of resistance in field populations. Future research will need to focus on the resistance mechanisms of *P. xanthii* to ZJ0712 in isolates without the reported point mutations in Cyt *b* protein.

Methods

Fungicides

Technical-grade ZJ0712 (98% active ingredient [a.i.]) and 10% ZJ0712 emulsifiable concentrate were provided by the Zhejiang Chemical Industry Research Institute, Hangzhou, China, while the other fungicides used were sourced commercially, including the strobilurin fungicides azoxystrobin (95% a.i., Syngenta Biotechnology Co. Ltd., Shanghai, China) and enostroburin (98% a.i., Shenyang Research Institute of Chemical Industry, Shenyang, China), the triazolinthione fungicide triadimefon (98% a.i., Jiangsu Runfeng Agrochemicals Co., Ltd., Jiangsu, China), and the chloronitrile fungicide chlorothalonil (98% a.i., Xinyi Agro-Chemical Co. Ltd., Jiangsu, China). ZJ0712, chlorothalonil, and triadimefon were dissolved in acetone, while the azoxystrobin, enostroburin, and salicylic hydroxamic acid (SHAM) were dissolved in methanol to prepare stock solutions and stored at 4 °C in darkness. For the sensitivity test, the stocks of fungicides were diluted with sterile distilled water containing

0.005% Tween 20. The maximum concentration of organic solvent was less than 0.1%.

Control effect test of ZJ0712 against CPM

To investigate the control efficacy of ZJ0712 against CPM, greenhouse trials were performed in two consecutive cucumber-growing seasons, with application of ZJ0712 for five times during each season at a 7-day interval. In brief, the trials were conducted in a plastic greenhouse with dimensions of 5×50 m at the Experimental Station of China Agricultural University, Beijing. Cucumber cv. Changchun mici susceptible to CPM (Wang et al. 2007) was sown in double rows in soil beds on the floor. The distance was 0.3 m apart in the beds, with 1.0 m between the bed centers. The first crop was sown on 19 March without any supplementary lighting and watered with tap water. The second crop was transplanted with a hoe when the seedlings were at the one-leaf stage on 12 June before the first crop was transferred on 20 June. The experiment consisted of ten instances of fungicide spraying in two successive seasons. The experimental plot was naturally infected with *P. xanthii* and no other fungicides had been applied previously. The first application of ZJ0712 was conducted at the appearance of white patches of powdery mildew. The fungicide application date and the dose are shown in Table 1.

Plants sprayed with tap water were used as an untreated control. The treatment group was laid out as a randomized complete block design with an area of 15 m^2 (3×5 m), containing about 80 plants per block. Each treatment had four replications. The disease severity was assessed using a 0–9 rating scale based on the percentage of foliage covered with powdery mildew. In essence, a 0 rating corresponded to no visible symptoms, 1, 3, 5, 7, and 9 corresponded to less than 5%, 6–10%, 11–20%, 21–40%, and more than 40% diseased foliage, respectively (He et al. 2017). The disease index of each treatment was calculated according to the following equation: disease index $= \sum \frac{n \times v}{VN} \times 100$, where 'n' is the number of diseased leaves in each category, 'v' is the rating value of each category, 'V' is the highest rating value, and 'N' is the total number of leaves assessed. The control efficacy was assessed based on the following equation: control efficacy (%) $= 100 \times$ (disease index of control − disease index of treatment)/disease index of control. To monitor the sensitivity of *P. xanthii* to ZJ0712, six to eight *P. xanthii* isolates were randomly collected from ZJ0712-treated cucumber plants on 28 April, 5 May, 26 May, 11 August, 25 August, and 1 September.

Sensitivity test to ZJ0712

The sensitivities of the six to eight *P. xanthii* isolates randomly obtained on the seventh day after the second, fifth, seventh, ninth, and tenth fungicide applications were determined using a leaf disc bioassay, as described previously (Zhu et al. 2007). Leaf disks (1.5 cm diameter) were collected from healthy greenhouse-grown cucumber plants (cv. Changchun mici) with four true leaves. The leaf discs were randomized and placed into containers with 50 mL of ZJ0712 solution at six concentrations (0, 0.625, 1.25, 2.5, 5, or 10 μg/mL) and 0.005% (v/v) Tween 20. To inhibit the alternative respiration pathway of the pathogen, 100 μg/mL SHAM was added to each container (Wang et al. 2007).

Leaf discs treated with distilled water containing 0.1% (v/v) methanol or acetone, 0.005% (v/v) Tween 20, and 100 μg/mL SHAM were used as a control. In total, 50 leaf discs were used for each treatment. After 30 min of soaking, the leaf discs were collected and blotted dry with sterile paper towels, then placed (adaxial surface up) on sterile filter paper in 15-cm diameter Petri dishes. A total of 15 mL of sterile water was added to each Petri dish to maintain a proper moisture level. Fresh spores of *P. xanthii* were harvested from diseased cucumber leaves by washing the lesions ten times with sterile distilled water containing 0.005% (v/v) Tween 20. The spores in suspension were counted under a microscope (UB102i) using a hemacytometer. The concentration was adjusted to 1×10^6 conidia/mL for an estimated inoculum. The leaf discs were inoculated by placing 10 μL of inoculum in the middle of each leaf disc. Un-inoculated leaf discs were used as the negative control. All of the dishes were incubated at 20℃ with a 12-h photoperiod for disease development. Ten days after inoculation, the lesion area was investigated and the mean inhibitory rate of each treatment was determined according to the equation: inhibitory rate (%) $= 100 \times$ (lesion area of control − lesion area of treatment)/lesion area of control. The median effective concentration value (EC_{50}) of ZJ0712 in reducing the diseased area caused by each isolate was further calculated using the regression of the inhibitory rate against the logarithm value of the fungicide concentration (Zhu et al. 2007). The experiment was replicated three times. The RFs were calculated for all the isolates by dividing the EC_{50} values of the isolates by the mean EC_{50} value of the previously established sensitive population (Wang et al. 2007).

Characterization of ZJ0712-resistant *P. xanthii* isolates

Dose–response to ZJ0712

To test the dose–response to ZJ0712, four resistant isolates (NR1, NR2, NR3, and NR4) and one wild-type sensitive isolate, BH17, were inoculated onto 50 leaf discs

treated with serial concentrations (0, 0.006, 0.01, 0.1, 0.5, or 1 μg/mL) of ZJ0712 and maintained at 20℃ under a 12-h photoperiod. Ten days after inoculation, the lesion area was measured. The tests were repeated three times with three replicates.

Resistance level and stability

Seven resistant isolates (NR1, NR2, NR3, NR4, NR5, NR6, and NR7) and one sensitive isolate BH17 were maintained on fungicide-free detached leaves for 10 generations at 20℃ under a 12-h photoperiod. For each generation, spores were harvested as described in the leaf disc assay and re-inoculated onto new cucumber leaves with ten drops of inoculum. After the 10th generation, the sensitivity to ZJ0712 was estimated again using the method described above.

Pathogenicity

The differences in pathogenicity between the seven resistant isolates and one sensitive isolate mentioned above were compared on full leaves from four-leaf stage healthy cucumber plants. Ten microliters of spore suspension (1×10^6 conidia/mL) of each isolate were inoculated onto 10 leaves with a 2-cm space between different spore suspension drops in Petri dishes. All of the leaves were incubated for 10 days according to the above method, and the lesion area on each leaf was measured. The tests were repeated three times with three replicates.

Competition

Two resistant isolates (NR2 and NR3) and two sensitive isolates (N2 and N3) were randomly selected to estimate the potential competition between ZJ0712-resistant and ZJ0712-sensitive isolates. The conidia of all the isolates were maintained and produced as described previously (Ishii et al. 2001). Mixed-isolate inoculations were prepared by mixing appropriate volumes of conidial suspensions (1×10^6 conidia/mL) of NR2 with N2 or of NR3 with N3 to produce suspensions containing 100% resistant (R):0% sensitive (S), 10% R:90% S, and 0% R:100% S conidia. For each pair of isolates and each conidial ratio, 50 replicate leaf discs with no fungicide treatment were inoculated as described above. For the 10% R:90% S conidial suspensions, an additional 50 replicate leaf discs treated with 0.008 μg/mL ZJ0712 were inoculated. After 10 days of incubation, the sporulating lesions were removed from each leaf disc to prepare the inoculum for the next disease cycle. The lesions from each leaf were added to 10 mL of sterile deionized water and mechanically agitated for 15 s to dislodge the conidia. The resulting suspension was used to inoculate a new set of leaf discs (10 μL/leaf disc), starting a new disease cycle. To estimate the frequency of the resistant isolates, the suspension was also used to inoculate 50 replicate leaf discs with no fungicide treatment and 50 replicate leaf discs soaked with 1 μg/mL ZJ0712 before a new disease cycle was started. The frequency of resistant isolates was calculated by dividing the lesion-area on treated leaves by the lesion-area on untreated leaves. The experiment was repeated twice and terminated after five disease cycles each time.

Cross-resistance

The sensitivities of nine ZJ0712-resistant mutants (SR1, BH14, NR1, NR2, NR3, NR4, NR5, NR6, and NR7) and five ZJ0712-sensitive isolates (N1, N2, N3, N4, and BH17) were tested using a series of concentrations of chlorothalonil-treated, triadimefon-treated, enostrobilurin-treated, or azoxystrobin-treated leaf discs in dishes incubated at 20℃ under a 12-h photoperiod. Ten days after inoculation, the lesion area on the leaf discs for each treatment was determined for the calculation of EC_{50}, as described previously. The sensitivities of the isolates to ZJ0712 and the other four fungicides were compared, and the cross-resistances were analyzed using regression analysis (Wong and Wilcox 2002).

DNA extraction and *cyt b* sequence analysis

For total DNA isolation, the biomasses of *P. xanthii* composed of conidia and hyphae were carefully harvested with a spatula from 10-day-old powdery mildew-infected cucumber leaves. The total DNA was extracted as described by Villaréal et al. (2002). Additionally, a 574-bp coding sequence of the *cyt b* gene encompassing the Qo domains was amplified using the primer pair WMF (5′-CATAGTAATACAGCTTCTGC-3′) and WMR (5′-CTGGTACTATAGCAGGTGG-3′), which was designed according to the reported QoI resistance-related mutations in *cyt b*. The polymerase chain reaction (PCR) was performed in a 50 μL reaction mixture containing 20 ng of template DNA, 1 μL of each primer (10 μM), 4 μL of dNTP mixture (2.5 μM each dNTP), 5 μL 10 × *Taq* DNA polymerase buffer, and 2.5 U of *Taq* DNA polymerase (Bodataike, Beijing, China). The PCR was performed using a MyCycler™ thermal cycler (Bio-Rad, Beijing, China) with standard program annealing at 58℃. The PCR products were sequenced by Beijing Nuosai Genome Research Center Co. Ltd., Beijing, China. The amino acid sequences of the Cyt *b* proteins from the wild-type isolates and the ZJ0712-resistant isolates were predicted and compared using DNAMAN (Version 8) software with the default parameters.

Statistical analysis

Data Processing System software version 7.05 (DPS ver. 7.05) was used to analyze the experimental data generated in the pathogenicity test. The statistical differences between the wild-type isolates and the ZJ0712-resistant isolates were analyzed using a one-way ANOVA with Tukey's multiple comparisons tests ($P < 0.05$).

Abbreviations

A113G: Substitution of alanine with glycine at codon 113 of Cyt *b*; a.i.: Active ingredients; AOX: Alternative oxidase; CPM: Cucumber powdery mildew; Cyt *b*: Cytochrome *b*; DMI: Sterol demethylation inhibitor; EC_{50}: Effective concentration at which infected area is inhibited by 50%; F129L: Substitution of phenylalanine with leucine at codon 129 of Cyt *b*; FRAC: Fungicide Resistance Action Committee; FSC: Factor of sensitivity change, the ratio of the RF value at the 10th generation to that at the 1st generation; G143A: Substitution of glycine with alanine at codon 143 of Cyt *b*; G137R: Substitution of glycine with arginine at codon 137 of Cyt *b*; I161M: Substitution of isoleucine with methionine at codon 161 of Cyt *b*; N261D: Substitution of asparaine with aspartic acid at codon 261 of Cyt *b*; PCR: Polymerase chain reaction; QoI: Quinone outside inhibitor; RF: Resistance factor, the ratio of the EC_{50} value of a resistant isolate to the mean value of baseline sensitivity; SHAM: Salicylic hydroxamic acid.

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Author contributions

XL and QP designed the research. QP, MW, JF, MC, and ZH performed the research. QP, MW, ZL, and JM analyzed the data. QP, JM, and XL wrote the manuscript. All authors read and approved the final manuscript.

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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.

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