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Biological and molecular characterizations of fluxapyroxad-resistant isolates of *Botrytis cinerea*

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Abstract

Gray mold caused by *Botrytis cinerea* leads to serious losses in various crops. Fluxapyroxad, a succinate dehydrogenase inhibitor (SDHI) fungicide, has been used to control gray mold for several years in China. In this study, we identified six fluxapyroxad-resistant (FluR) isolates from 96 isolates of *B. cinerea* collected in fields. Phenotypic assays showed that the FluR isolates exhibited defects in mycelial growth, conidiation, sclerotium formation, stress tolerance and virulence. Analysis of nucleotide sequences of succinate dehydrogenase subunit-coding genes revealed that various point mutations, including P225F, N230I, K283N and H272R in BcSdhB, G37S and P80H in BcSdhC, and V9A in BcSdhD, were associated with the resistance to fluxapyroxad in *B. cinerea*. Sensitivity assays showed that the FluR isolates also exhibited resistance to another SDHI boscalid, but there was no cross-resistance between fluxapyroxad and other fungicides with different modes of action. These results indicate that the resistance of *B. cinerea* to fluxapyroxad has occurred in fields, and further monitoring and management of the resistance to fluxapyroxad in *B. cinerea* should be carried out.

Keywords: *Botrytis cinerea*, Gray mold, Fluxapyroxad, Resistance

Background

Gray mold caused by *Botrytis cinerea* is an important plant disease that seriously threatens the yield and quality of economically important crops, including vegetables, fruits and ornamental plants (Williamson et al. 2007). To date, the common method for controlling gray mold worldwide is the application of chemical fungicides (Hahn 2014; Fan et al. 2017). Unfortunately, *B. cinerea* has been considered a plant pathogen with a high resistance-risk against fungicides due to its wide host range, fast growth rate, strong reproductive capacity and high genetic variability (Leroux et al. 2010). To date, *B. cinerea* populations collected from different crop fields have exhibited resistance to various fungicides with different modes of action, including benzimidazole carbamates, dicarboximides, anilinopyrimidines, quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs) and phenylpyrroles (Leroux et al. 2002; Williamson et al. 2007; Fernández-Ortuño et al. 2013; Yin et al. 2018; Shao et al. 2021). The resistance has led to a decrease or a failure in control efficiency in the management of gray mold when using the aforementioned fungicides.

Fluxapyroxad is an SDHI fungicide produced by the BASF Corporation. It binds to the succinate dehydrogenase complex (Sdh, complex II) of the respiratory chain and disrupts cellular respiration (Lin et al. 2021). Fluxapyroxad has been reported to inhibit mycelial growth, germ tube development, spore germination and appressorium formation (Yang et al. 2019). Owing to its broad-spectrum and excellent antifungal activity,

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fluxapyroxad has been used to control various crop diseases caused by fungi (Li et al. 2020). The Sdh complex constitutes four subunits, SdhA, SdhB, SdhC and SdhD (Hägerhäll 1997), and amino acid mutations in SdhB, SdhC and SdhD lead to resistance to SDHIs in *B. cinerea* (Sierotzki and Scalliet 2013; Veloukas et al. 2013). Moreover, in *B. cinerea*, P225L/F/T, N230I or H272L/R/Y mutations in SdhB endows different resistance levels to boscalid (a commonly used SDHI), and the H132R mutation in SdhD is also responsible for the resistance to SDHIs (Leroux et al. 2010; Shao et al. 2021). Additionally, different amino acid mutations in SdhC were identified in field isolates of *B. cinerea*; these mutations are responsible for the decreased sensitivity of *B. cinerea* to SDHIs (Shao et al. 2020). Furthermore, compared with sensitive isolates, boscalid-resistant isolates containing single amino-acid mutations (H272L/R/Y, P225F or N230) in SdhB have decreased virulence on host plants (Veloukas et al. 2013). Yin et al. (2018) reported that boscalid-resistant isolates containing the H272R mutation in SdhB did not exhibit significant differences in mycelial growth, osmotic sensitivity and virulence compared with boscalid-sensitive isolates. To date, various SDHIs have been registered to control gray mold in fields, and therefore, investigation into the development of resistance of *B. cinerea* to SDHIs is essential for the management of gray mold.

In China, fluxapyroxad was registered to control fungal diseases including gray mold in 2014, and the mixture of fungicide products containing fluxapyroxad was subsequently used to spray twice to control gray mold during growth of greenhouse tomato plants. The application of mixed fungicide products containing fluxapyroxad for years has placed selective pressure on *B. cinerea* to generate resistance to this fungicide. To investigate the occurrence of fluxapyroxad resistance in *B. cinerea*, we collected 96 *B. cinerea* isolates from tomato fields in four districts of Zhejiang Province in this study. The sensitivity of all these isolates to fluxapyroxad was analyzed via the discriminative method (Yin et al. 2018), and six FluR isolates were identified. Moreover, the fitness of the six FluR isolates was evaluated by determining the mycelial growth rate, conidiation capability and spore germination rate, sclerotium formation, stress tolerance and virulence. Additionally, we investigated the molecular characteristics of subunits within the succinate dehydrogenase (Sdh) complex in FluR isolates. The results lay the basis for management of resistance of *B. cinerea* to fluxapyroxad, and also for further understanding of the underlying resistance mechanism of plant pathogens to SDHI fungicides.

Results

Frequency and level of resistance to fluxapyroxad in *B. cinerea* isolates

To determine the sensitivity of *B. cinerea* to fluxapyroxad, we collected 96 *B. cinerea* isolates from four districts in Zhejiang Province, China in 2020. Among them, six isolates exhibited resistance to fluxapyroxad. The resistance frequency of *B. cinerea* to fluxapyroxad was 6.25% (Table 1). These six FluR isolates (JS-16R, NB-13R, NB-20R, XS-15R, XS-17R and YH-18R) and six randomly selected fluxapyroxad-sensitive (FluS) isolates (JS-5S, NB-10S, NB-19S, XS-12S, XS-23S and YH-27S) were used for further studies. The EC_{50} values of the six FluS isolates ranged from 0.2014 to 0.2505 μg/mL; however, the EC_{50} values of the six FluR isolates ranged from 5.2472 to 38.5692 μg/mL (Table 2). Moreover, the six FluR isolates also exhibited resistance to boscalid, whereas the FluR isolates and FluS isolates showed similar sensitivity to tebuconazole, fluazinam and fludioxonil (Table 2), indicating that positive cross-resistance occurred between fluxapyroxad and boscalid but not between fluxapyroxad and tebuconazole, fluazinam or fludioxonil.

FluR isolates exhibit defects in mycelial growth, conidiation, spore germination and sclerotium formation

To detect if there are differences in growth and reproductive characteristics between FluR and FluS isolates of *B. cinerea*, we carried out investigations on mycelial growth rate, conidiation, spore germination and sclerotium formation. The results of mycelial growth assay showed that there was no difference in mycelial growth rate between FluR and FluS isolates on PDA; however, compared with that of FluS isolates, the mycelial growth rate of FluR isolates was obviously decreased on MM (Fig. 1a–d). For conidiation assay, all the tested isolates generated vast amounts of conidia on both PDA and MM; however, the conidiation capability of FluR isolates was reduced compared with that of FluS isolates (Fig. 1e, f). For spore germination assay, the spore germination rate of FluR

Table 1 Frequency and distribution of FluR isolates from different districts in Zhejiang Province, China

Location	The number of isolates tested	The number of resistant isolates	Frequency of resistant isolates (%)
Jiashan	19	1	5.26
Ningbo	26	2	7.69
Xiasha	23	2	8.69
Yuhang	28	1	3.57
Total	96	6	6.25

Table 2 Sensitivity analysis of FluR and FluS isolates to fluxapyroxad, boscalid, fluazinam, fludioxonil and tebuconazole

Isolates	Sensitivity	Origin	Fluxapyroxad		Boscalid		Fluazinam		Fludioxonil		Tebuconazole	
			EC_{50} (μg/mL)	RF	EC_{50} (μg/mL)	RF	EC_{50} (μg/mL)	RF	EC_{50} (μg/mL)	RF	EC_{50} (μg/mL)	RF
JS-5S	S	Field	0.2014 C	–	0.7291 C	–	0.0164 A	–	0.0071 A	–	0.2245 A	–
NB-10S	S	Field	0.2204 C	–	0.9238 C	–	0.0183 A	–	0.0078 A	–	0.2236 A	–
NB-19S	S	Field	0.2505 C	–	0.7234 C	–	0.0156 A	–	0.0068 A	–	0.2644 A	–
XS-12S	S	Field	0.2239 C	–	0.6919 C	–	0.0193 A	–	0.0066 A	–	0.2273 A	–
XS-23S	S	Field	0.2438 C	–	0.7620 C	–	0.0188 A	–	0.0081 A	–	0.2266 A	–
YH-27S	S	Field	0.2127 C	–	0.7486 C	–	0.0121 A	–	0.0069 A	–	0.2587 A	–
JS-16R	R	Field	38.5692 A	171.08	45.3007 A	59.36	0.0112 A	0.67	0.0079 A	1.09	0.2623 A	1.10
NB-13R	R	Field	5.5389 B	24.57	9.5309 B	12.49	0.0178 A	1.06	0.0073 A	1.01	0.2526 A	1.06
NB-20R	R	Field	5.2472 B	23.27	9.3567 B	12.26	0.0176 A	1.05	0.0076 A	1.05	0.2268 A	0.95
XS-15R	R	Field	5.3613 B	23.78	9.5774 B	12.55	0.0137 A	0.82	0.0067 A	0.93	0.2552 A	1.07
XS-17R	R	Field	5.6009 B	24.84	9.2296 B	12.09	0.0172 A	1.03	0.0078 A	1.08	0.2537 A	1.07
YH-18R	R	Field	5.6381 B	25.01	9.5973 B	12.58	0.0163 A	0.97	0.0082 A	1.14	0.2276 A	0.96

RF, resistance factor, the ratio of EC_{50} of a FluR isolate relative to the mean value of EC_{50} of FluS isolates

S indicates an isolate is sensitive to fluxapyroxad; R indicates an isolate is resistant to fluxapyroxad

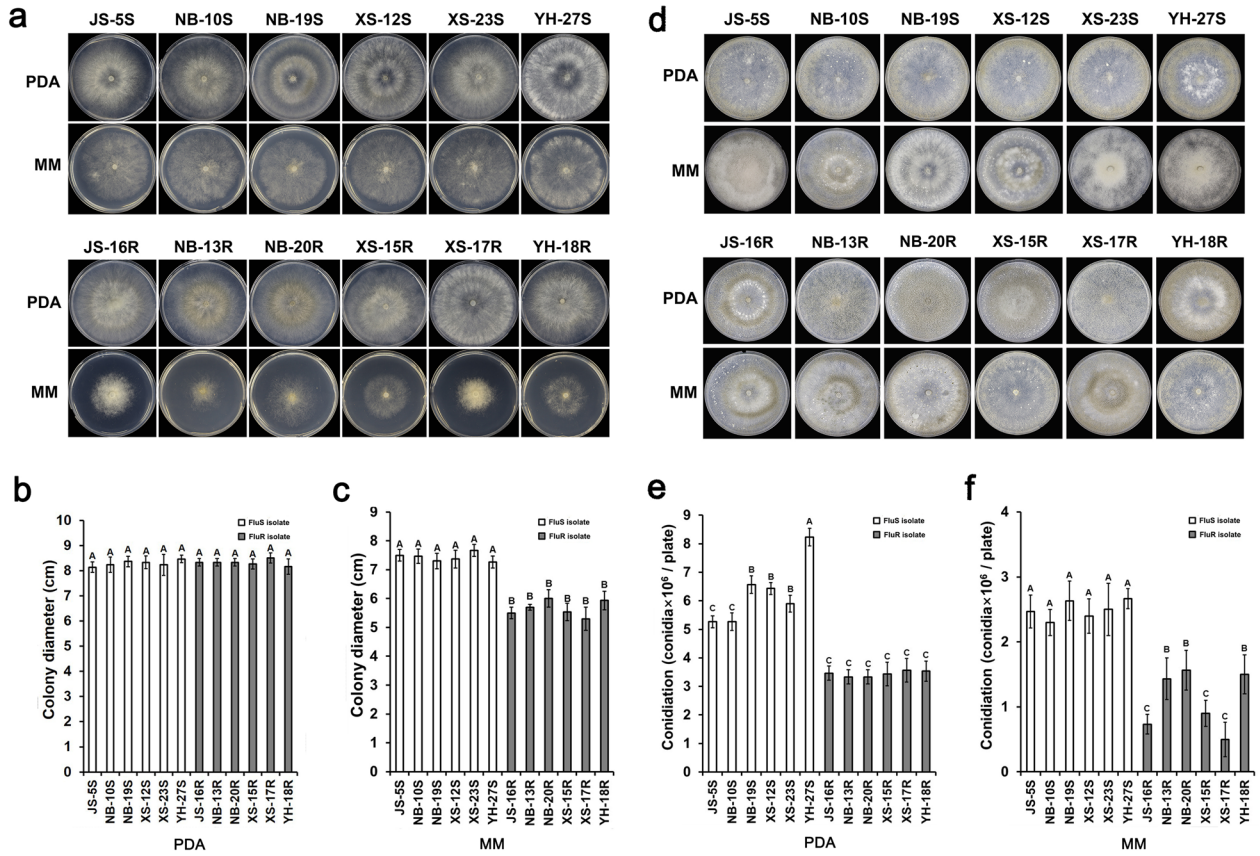


Fig. 1 Mycelial growth rate and conidiation capacity of FluS and FluR isolates of *B. cinerea*. **a** Colony morphology of FluS and FluR isolates grown on PDA and MM medium at 25 °C for 3 days. Column diagram showing colony diameter of FluS and FluR isolates grown for 3 days on PDA (**b**) and MM (**c**). **d** Conidiation of FluS and FluR isolates grown on PDA and MM at 16-h photoperiod for 14 days. Column diagram showing conidiation capacity of FluS and FluR isolates grown on PDA (**e**) and MM (**f**). For statistical analysis, bars denote standard errors of three repeated experiments. Values on bars followed by the same letter are not significantly different from each other at $P = 0.05$

isolates was significantly lower than that of FluS isolates on WA; however, the spore germination rate of FluR isolates was obviously higher than that of FluS isolates on WA supplemented with 5 μg/mL fluxapyroxad (Fig. 2a, b). In sclerotium formation assay, we found that the number of sclerotia produced by FluR isolates was lower than that produced by FluS isolates (Fig. 2c, d). These results indicate that compared with FluS isolates, FluR isolates exhibit defects in mycelial growth, conidiation, spore germination and sclerotium formation, and as expected, FluR isolates also exhibit resistance to fluxapyroxad during spore germination.

FluR isolates have increased sensitivity to multiple stresses and decreased virulence on host plants

To determine if the stress tolerance of FluR isolates is changed compared with FluS isolates, the stress tolerance assay was performed on PDA medium supplemented with Congo red, KCl, NaCl or H_2O_2, and the results showed that the mycelial growth inhibition rate of FluR isolates was significantly higher than that of FluS isolates under all these stress conditions (Fig. 3). For the virulence assay, each isolate caused obvious lesions on apple and tomato; however, FluR isolates caused obviously smaller disease spots than FluS isolates (Fig. 4). These results indicate that FluR isolates exhibit increased sensitivity to various stresses and decreased virulence on host plants.

FluR isolates have multiple types of mutation in subunits of succinate dehydrogenase complex

To investigate the molecular mechanism underlying the resistance of *B. cinerea* to fluxapyroxad, we compared nucleotide sequences coding for the subunits of succinate dehydrogenase, the target of SDHI fungicides, between FluR and FluS isolates, and seven types of mutations were identified in FluR isolates. The different types of mutations in FluR isolates were as follows: BcSdhB^{P225F} in JS-16R; BcSdhB^{N230I} in NB-13R; BcSdhB^{N230I} and BcSdhD^{V9A} in NB-20R; BcSdhB^{N230I}, BcSdhB^{K283N} and BcSdhD^{V9A} in XS-15R; BcSdhC^{G37S} in XS-17R; and BcSdhB^{H272R} and BcSdhC^{P80H} in YH-18R (Table 3).

Discussion

Fluxapyroxad, an important SDHI fungicide, has been used to control gray mold for several years. In general, continuous application of a fungicide on a certain plant pathogenic fungus will promote the generation of resistant fungal isolates against this fungicide, and as a result, the resistant isolates may gradually develop into dominant populations that can lead to serious crop losses (Yin et al. 2018). To investigate whether FluR isolates of *B. cinerea* have emerged in the tomato fields where fluxapyroxad were used to control gray mold, our study focused on monitoring and characterizing FluR isolates of *B. cinerea*. Our data indicated that a total of six FluR isolates were identified among 96 isolates collected from fields in four districts in Zhejiang Province, China. As is well known, fitness is an extremely essential parameter for evaluating the potential risk of fungicide-resistant populations (Wang et al. 2021). In current study, the FluR isolates exhibited defects in mycelial growth, conidiation, spore germination, sclerotium formation, stress tolerance and virulence compared with FluS isolates, indicating that the fitness of FluR isolates is weaker than that of FluS isolates, and therefore the potential risk for FluR isolates to develop into dominant populations is low currently. However, the 6.25% distribution frequency for FluR isolates is still relatively high; furthermore, FluR isolates were found in all the sampled regions, indicating that fluxapyroxad-resistant *B. cinerea* strains had been widely distributed in tomato fields. Therefore, it is necessary to seek some resistance management measures to help avoid or delay the development of FluR *B. cinerea* populations, including enhancing resistance monitoring, mixing or alternating application of fluxapyroxad and other fungicides with different modes of action (MoA). In previous studies, many *B. cinerea* isolates were found to exhibit multiple drug resistance (Shao et al. 2021), and it looks like alternation of fungicides with different MoA could speed up selection for multi-resistance in *B. cinerea*, thus, for management of fluxapyroxad resistance, the frequency and dosage of fungicides with different MoA should be scientifically evaluated and applied.

Previous studies have shown that the amino acid mutations SdhB$^{P225L/F/T,\ N230I\ or\ H272L/R/Y}$ and SdhD^{H132R} are associated with the resistance of *B. cinerea* to SDHIs (Sierotzki and Scalliet 2013; Veloukas et al. 2013; Shao et al. 2021), and amino acid substitutions in SdhC are also responsible for the decreased sensitivity of *B. cinerea* to SDHIs (Shao et al. 2020). Moreover, it was reported that various mutations are involved in different resistance level of *B. cinerea* to SDHIs, including SdhB^{N230I} and SdhB$^{H272R/Y}$, which were found in moderately boscalid-resistant isolates, and SdhB^{H272L} and SdhB$^{P225F/L}$,

(See figure on next page.)

Fig. 2 Conidia germination rate and sclerotium production of FluS and FluR isolates of *B. cinerea*. The conidia germination rate of FluS and FluR isolates incubated at 25 °C for 10 h on WA (**a**) and WA supplemented with 5 μg/mL fluxapyroxad (+ Flu) (**b**). **c** Observation of sclerotium production of FluS and FluR isolates grown on PDA for 3 days at 25 °C and then incubated for 4 weeks at 10 °C. **d** Column diagram showing the amount of sclerotia of FluS and FluR isolates after incubation on PDA for 3 days at 25 °C and then for 4 weeks at 10 °C. For statistical analysis, bars denote standard errors of three repeated experiments. Values on bars followed by the same letter are not significantly different from each other at $P = 0.05$

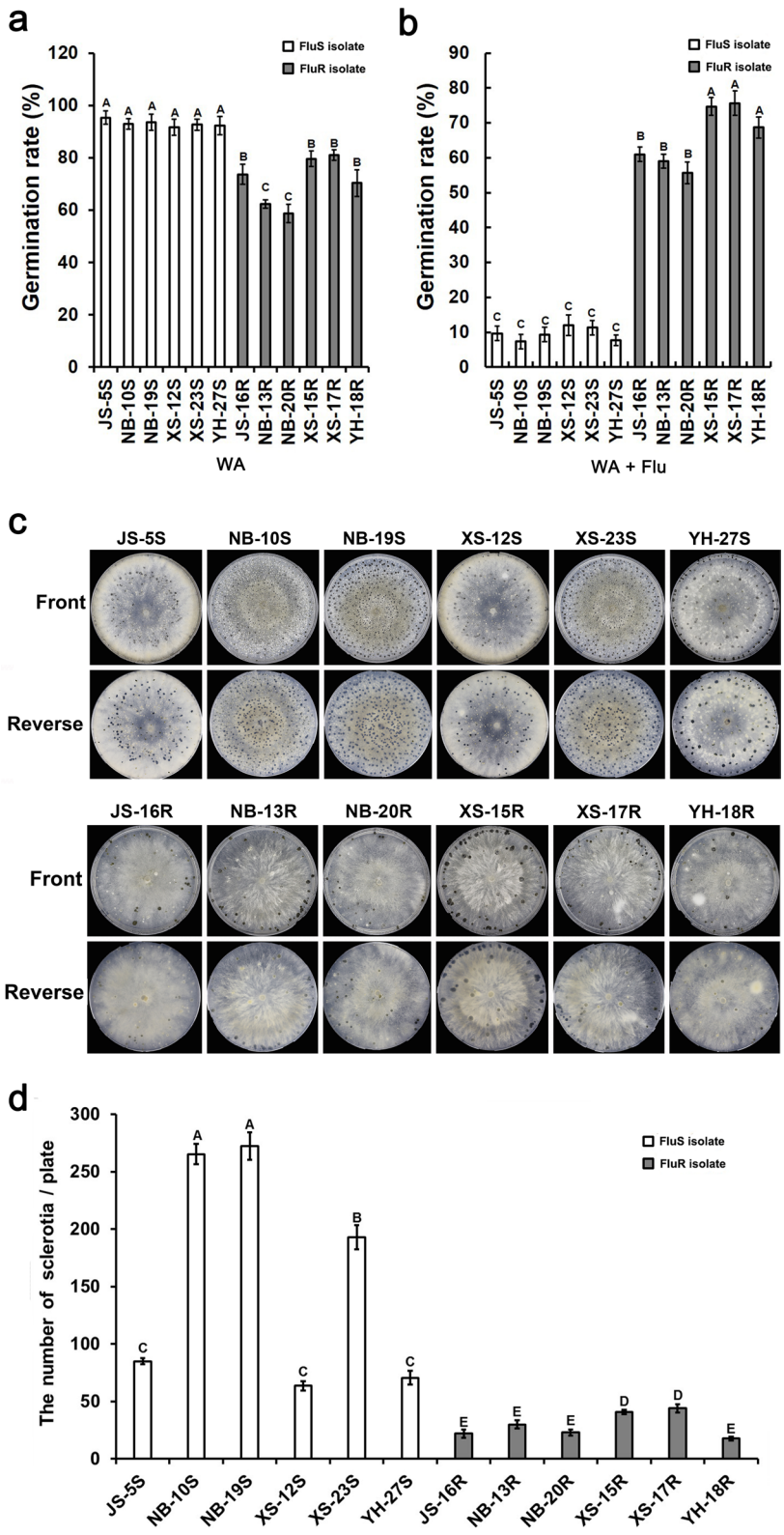


Fig. 2 (See legend on previous page.)

which were identified in highly boscalid-resistant isolates (Veloukas et al. 2013). In this study, seven different mutations, including BcSdhB^{P225F}, BcSdhB^{H272R}, BcSdhB^{K283N}, BcSdhB^{N230I}, BcSdhC^{G37S}, BcSdhC^{P80H} and BcSdhD^{V9A} were identified in FluR isolates. Among them, BcSdhB^{K283N}, BcSdhC^{P80H}, BcSdhC^{G37S} and BcSdhD^{V9A} are new types of mutation that were identified for the first time in SDHI-resistant *B. cinerea* isolates. Before the application of fluxapyroxad, another SDHI fungicide boscalid was ever used to control gray mold in these sampled tomato fields where FluR isolates were obtained. Therefore, we infer that the mutations BcSdhB^{P225F}, BcSdhB^{H272R} and BcSdhB^{N230I} might have existed in *B. cinerea* before the application of fluxapyroxad and are induced by boscalid, whereas the selective pressure exerted by fluxapyroxad leads to the generation of new types of mutation including BcSdhB^{K283N}, BcSdhC^{P80H}, BcSdhC^{G37S} and BcSdhD^{V9A}. In sensitivity assays of *B. cinerea* isolates to fluxapyroxad and boscalid, we found that the resistance factor (RF) of JS-16R containing the BcSdhB^{P225F} mutation was higher than that of other FluR isolates, and there was no difference in RF among NB-13R, NB-20R, XS-15R, XS-17R and YH-18R (Tables 1, 2). Lalève et al. (2014) also reported that the BcSdhB^{P225F} mutation in *B. cinerea* confers a high-level resistance to boscalid and fluopyram (2014). These results indicate that the BcSdhB^{P225F} mutation in *B. cinerea* is responsible for a high-level resistance to fluxapyroxad, boscalid and fluopyram. It was found that mutations in BcSdhB could decrease Sdh inhibition, leading to the resistance to SDHIs, and different BcSdhB modifications exhibit differences in interfering with SDHI binding (Lalève et al. 2014). Accordingly, we consider that the mutation BcSdhB^{P225F} confers a higher resistance to fluxapyroxad in *B. cinerea* than other types of mutation due to that it causes a more serious defect in the binding activity of fluxapyroxad to Sdh. Moreover, mutations in BcSdhB or BcSdhC were demonstrated to affect Sdh activity, respiration and carbon source utilization (Lalève et al. 2014; Shao et al. 2020); thus, we infer that those mutations in FluR isolates may also affect Sdh activity, respiration and carbon source utilization, leading to defects in mycelial growth, conidiation, spore germination, sclerotium formation, stress tolerance and virulence. Additionally, inconsistent with this study, previous studies found much difference in fitness of the SDHIs-resistant isolates (Yin et al. 2018; Petrasch et al. 2019; Shao et al. 2021), and the variation in fitness cost among SDHIs-resistant isolates might be due to the differences in some specific mutations.

In this study, several FluR isolates were found to include different types of mutation: BcSdhB^{N230I} and BcSdhD^{V9A} were found in NB-20R; BcSdhB^{N230I}, BcSdhB^{K283N} and BcSdhD^{V9A} were identified in XS-15R; BcSdhB^{H272R} together with BcSdhC^{P80H} were identified in YH-18R. However, there is no difference in resistance level to fluxapyroxad among these three FluR isolates. From the perspective of resistance evolution, we conclude that one or two mutations in these FluR isolates are single nucleotide polymorphisms that are not associated with the resistance of *B. cinerea* to SDHIs. This is the first report of multiple different mutations existing in a single SDHI-resistant *B. cinerea* isolate. The biological mechanism behind a single resistant isolate generating multiple different mutations is worth exploring.

Conclusions

We collected 96 *B. cinerea* field isolates, of which six exhibited resistance to fluxapyroxad. Moreover, all FluR isolates exhibited defect in fitness. There was no cross-resistance between fluxapyroxad and other fungicides with different modes of action, including tebuconazole, fluazinam or fludioxonil. Moreover, new types of mutations, BcSdhB^{K283N}, BcSdhC^{P80H}, BcSdhC^{G37S} and BcSdhD^{V9A}, were identified in FluR isolates. These results provide directions for the management of fluxapyroxad resistance in *B. cinerea* and further improve our understanding of the underlying resistance mechanism of plant pathogens to SDHIs.

Methods

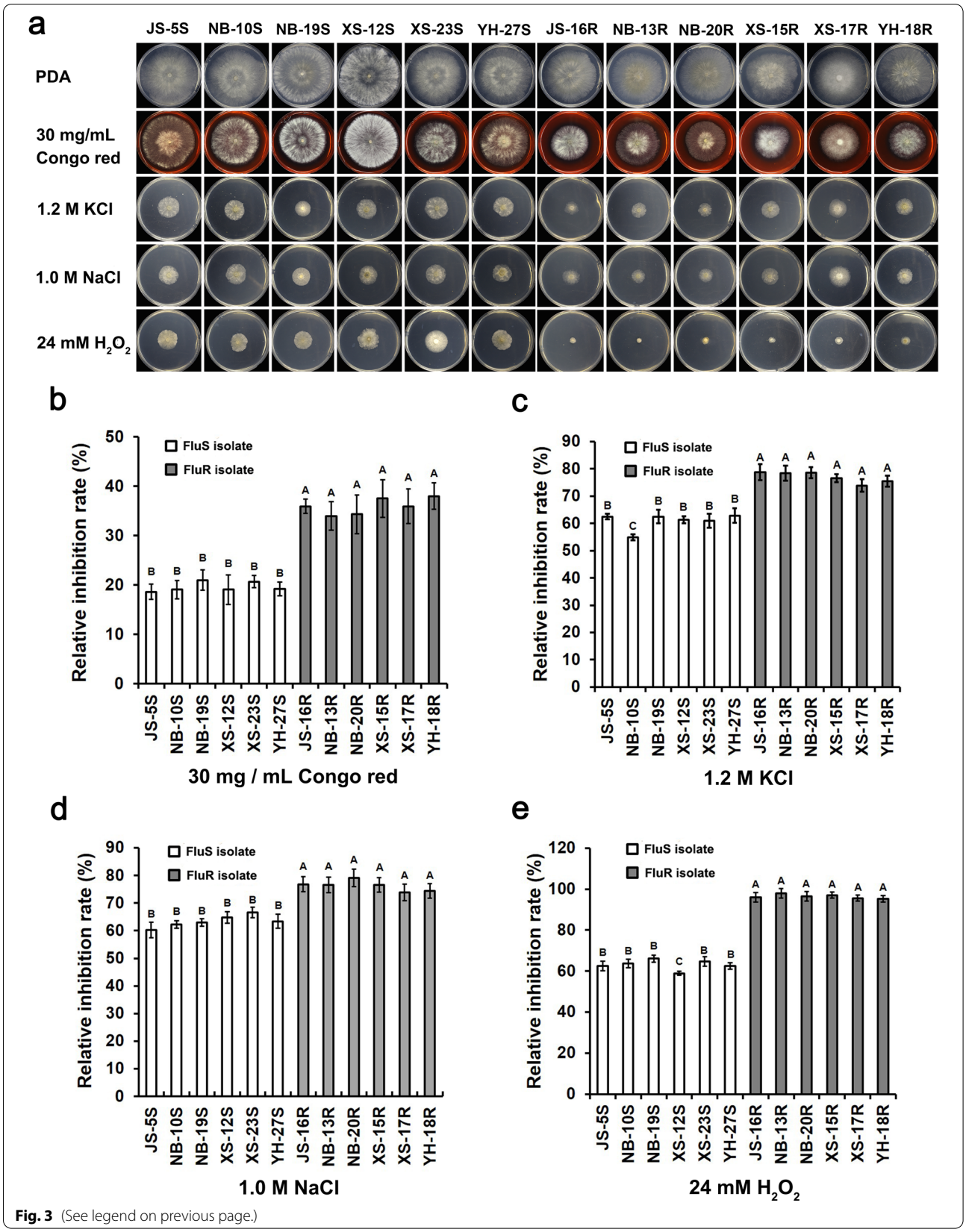
Culture media and fungicides

Culture media used in this study include potato dextrose agar (PDA, 200 g of potato, 20 g of dextrose and 18 g agar per liter of distilled water), potato dextrose broth (PDB, 200 g of potato and 20 g of dextrose per liter of distilled water), alkylester agar medium (AEA, 5 g of yeast extract, 6 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, 0.25 g $MgSO_4$, 20 mL of glycerol and 20 g of agar per liter of distilled water).

Technical grades of fluxapyroxad (96.5%), boscalid (98%) fluazinam (98%), fludioxonil (95%) and tebuconazole (95%) were provided by Badische Anilin Soda Fabrik Ga (Germany), Jingbo Agrochemical Technology Co. LTD, Zhejiang Hetian Chemical Co., LTD, Qingdao Runnong Chemical Co., LTD and Jiangsu Changfeng

(See figure on next page.)

Fig. 3 Sensitivity of FluS and FluR isolates of *B. cinerea* to various stresses. **a** Colony morphology of FluS and FluR isolates grown on PDA modified with or without stress agents for 3 days. The relative growth rate of FluS and FluR isolates grown for 3 days on PDA supplemented with 30 mg/mL Congo red (**b**), 1.2 M KCl (**c**), 1.0 M NaCl (**d**) or 24 mM H_2O_2 (**e**). For statistical analysis, bars denote standard errors of three repeated experiments. Values on bars followed by the same letter are not significantly different from each other at $P = 0.05$



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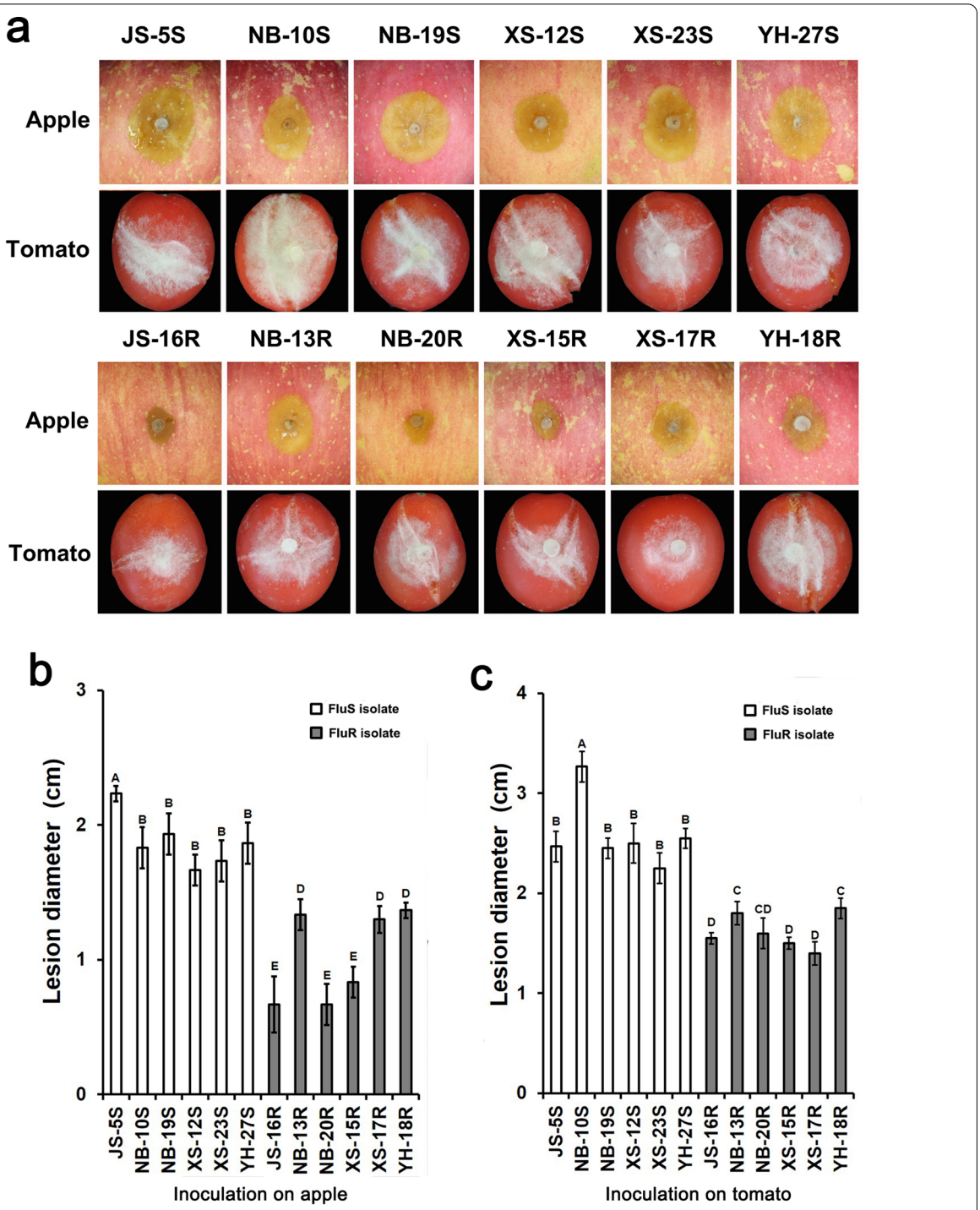

Fig. 4 Virulence assay of FluS and FluR isolates of *B. cinerea* on apple and tomato fruits. **a** Symptoms on wounded apple and tomato fruits after inoculated with *B. cinerea* isolates for 72 h. Lesion diameter measured at 72 h post-inoculation (hpi) on apple fruits (**b**) and tomato fruits (**c**). For statistical analysis, bars denote standard errors of three repeated experiments. Values on bars followed by the same letter are not significantly different from each other at $P = 0.05$

Table 3 Mutations in BcSdhB, BcSdhC and BcSdhD of FluR isolates

Isolates	Origin	Sensitivity	SDHB sequence	SDHC sequence	SDHD sequence
JS-5S, NB-10S, XS-12S	Field	S	WT	WT	WT
JS-16R	Field	R	CCC (P) 225 TTC (F)		
NB-13R	Field	R	AAC (N) 230 ATC (I)		
NB-20R	Field	R	AAC (N) 230 ATC (I)		GTC (V) 9 GCC (A)
XS-15R	Field	R	AAC (N) 230 ATC (I), AAG (K) 283 AAT(N)		GTC (V) 9 GCC (A)
XS-17R	Field	R		GGC (G) 37 AGC (S)	
YH-18R	Field	R	CAC (H) 272 CGC (R)	CCT (P) 80 CAT (H)	

S indicates an isolate is sensitive to fluxapyroxad; R indicates an isolate is resistant to fluxapyroxad

Agrochemical Co. LTD. respectively. All fungicides were dissolved in methanol to obtain a stock solution containing 10 mg active ingredient per mL.

Collection of *B. cinerea* isolates

A total of 96 *B. cinerea* isolates were isolated from tomato plants at two locations in Jiashan (19), three locations in Ningbo (26), two locations in Xiasha (23) and three locations in Yuhang (28) in Zhejiang Province, China in 2020, with a distance of at least 0.5 km between different sampling sites within a location. In these sampling locations, Orkestra, a fluxapyroxad-containing fungicide, was used to control gray mold. To obtain *B. cinerea* isolates, small tissue fragments cut from lesion margins were disinfected in 1% NaClO for 2 min, washed three times with sterile water, and then put onto PDA medium supplemented with streptomycin sulfate at a final concentration of 100 μg/mL. After incubation at 25 °C for 3 days, mycelial plugs cut from the margins of colony were transferred onto fresh PDA medium and incubated at 25 °C under a 16 h-photoperiod for 12 days to obtain conidia. All isolates were purified by single-spore isolation and then maintained on PDA slant at 4 °C. In total, 96 single-spore isolates of *B. cinerea* were obtained from field-grown tomato plants.

Assessment of sensitivity of *B. cinerea* isolates to fluxapyroxad and other fungicides

To determine the sensitivity of *B. cinerea* isolates to fluxapyroxad, a mycelial plug cut from the edge of a 3-day-old colony was transferred onto AEA medium modified with fluxapyroxad at a discriminatory concentration of 5 μg/mL, with three replicates for each isolate. After incubated for 3 days at 25 °C, the isolates that were able to grow were identified as potential FluR isolates, while those unable to grow were designated as FluS isolates. The resistance frequency was calculated via the following formula: Resistance frequency (%) = [(Number of FluR isolates /Total number of isolates) × 100]. To further measure sensitivity of FluR isolates to fluxapyroxad, the EC_{50} (effective concentration for 50% inhibition of mycelial growth) of six FluR isolates and six randomly selected FluS isolates were calculated. For each isolate, a 5 mm mycelial plug was cut from the edge of a 3 day-old-colony and put onto AEA medium modified with pydiflumetofen at a final concentration of 0.03125, 0.0625, 0.15625, 1.25, 2.5, 5, 10, 20 or 40 μg/mL. After 3 days of incubation at 25 °C in the dark, the mycelial growth inhibition rate was measured and the EC_{50} value of each isolate was analyzed with DPS software as previously reported (Zhou et al. 1994; Chen et al. 2021; Zhang et al. 2021). EC_{50} of these isolates to other fungicide (boscalid, fluazinam, fludioxonil and tebuconazole) were also measured via the same method. The experiment was repeated independently for two times, with three replicates for each treatment.

Measurement of mycelial growth rate, conidiation capacity, spore germination rate and sclerotia production of *B. cinerea* isolates

For mycelial growth assay, a 5 mm mycelial plug was cut from the edge of an actively growing colony and put onto PDA or MM medium plates, with three replicates for each isolate on each culture medium. After 3 days of incubation at 25 °C in the dark, the diameter of colony was measured, and the original mycelial plug diameter (5 mm) was subtracted from measured data. The experiment was repeated independently for two times.

For conidiation assay, a mycelial plug was cut from the edge of a 3-day-old colony of each isolate and placed onto PDA or MM medium plates, with three replicates for each isolate on each culture medium. After incubated at 25 °C for 12 days under a 16-h photoperiod, conidia were washed down with 20 mL ddH_2O per plate. The collected conidia suspension was filtrated with lens-wiping paper and centrifuged at 1,300 × *g* for 5 min, after which the conidia were resuspended in 5 mL ddH_2O and counted on a hemacytometer. The experiment was repeated independently for two times.

For spore germination assay, spore suspension of each isolate was prepared as described above, and adjusted to a concentration of 10^5/mL. A volume of 200 μL spore suspension was added and smeared evenly onto WA medium supplemented with or without 5 μg/mL of fluxapyroxad, with three replicates for each isolate. After incubated at 25 °C for 10 h in darkness, the spore germination rate of each isolate was calculated by the following formula: Spore germination rate (%) = [(Number of germinated spores/Total number of spores) × 100]. The experiment was repeated independently for two times.

To measure production of sclerotia, a mycelial plug cut from the edge of a 3-day-old colony of each isolate was transferred onto PDA medium, incubated at 25 °C for 3 days in darkness and then incubated for two weeks at 10 °C. Finally, the amount of sclerotia produced by each isolate was measured. Three replicates for each treatment. The experiment was repeated two times.

Determination of stress sensitivity and virulence of *B. cinerea* isolates

To analyze stress sensitivity, a 5 mm mycelial plug cut from the edge of an actively growing colony and transferred onto PDA medium modified with 30 mg/mL of Congo red, 1.2 M KCl, 1.0 M NaCl or 24 mM H_2O_2, with three replicates for each treatment. PDA medium without stress agents was served as the control treatment. The diameter of colony was measured after incubated at 25 °C for 3 days in darkness. The percentage of inhibition of mycelial radial growth (PIMG) was calculated using the following formula: PIMG (%) = [(C − N)/(C − 5)] × 100, in which C is the colony diameter of the untreated control and N is that of a treatment. The experiment was repeated independently for three times.

To measure virulence, all apple and tomato fruits were surface-sterilized with 75% alcohol, wounded with a sterilized needle and then inoculated with mycelial plugs cut from the edge of 3-day-old colony of each isolate, with five replicates for each isolate. The inoculated fruits were incubated at 25 °C and 90% relative humidity under a 16-h photoperiod. The lesion diameter was measured at 72 hpi. The experiment was repeated independently for two times.

Cloning and sequencing of target genes

Genomic DNA of FluR and FluS isolates was extracted using the method described previously (Yin et al. 2011). The Sdh complex in *B. cinerea* contains four subunits including BcSdhA (BCIN_15g01180), BcSdhB (BCIN_01g04980), BcSdhC (BCIN_02g03080) and BcSdhD (BCIN_05g04430). The nucleotide sequences coding for these Sdh subunits were amplified in six FluR isolates and six FluS isolates using the corresponding specific primer pairs (Additional file 1: Table S1) and Phanta Max PCR kit (Vazyme, Nanjing, China). The PCR reactions were conducted using the following procedure: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1–3 min, and a final extension at 72 °C for 10 min. The PCR products were then purified and sequenced. DNA sequences coding for the Sdh subunits were analyzed using Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Abbreviations

AEA: Alkylester agar medium; EC_{50}: Effective concentration for 50% inhibition of mycelial growth; FluR: Fluxapyroxad-resistant; FluS: Fluxapyroxad-sensitive; MM: Minimal medium; PCR: Polymerase chain reaction; PDA: Potato dextrose agar; PDB: Potato dextrose broth; PIMG: Percentage of inhibition of mycelial radial growth; QoIs: Quinone outside inhibitors; RF: Resistance factor; Sdh: Succinate dehydrogenase; SDHI: Succinate dehydrogenase inhibitor; WA: Water agar medium.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00107-3.

Additional file 1: Table S1. Primers used in this study.

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Authors' contributions

WS and ZM conceived and designed the experiments. KL, ZW and WS carried out the experiments. All authors analyzed the data. KL, ZM and WS wrote the manuscript. All authors read and approved the final manuscript.

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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.

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