Phytopathology Research

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Treatment with succinate dehydrogenase inhibitor Y12196 protects strawberries from boscalid-resistant *Botrytis cinerea* with the H272R mutation in SDH B

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Abstract

Gray mold, caused by *Botrytis cinerea,* poses a significant threat to the strawberry industry worldwide. *B. cinerea* is a high-risk pathogen in the sense of fungicide resistance. The sensitivities of *B. cinerea* isolates collected from Zhejiang Province, China, to the succinate dehydrogenase inhibitors (SDHIs) boscalid and Y12196 were determined based on discriminatory dose or 50% effective concentration (EC_{50}). Of the 42 isolates collected in 2018, 15 were resistant to boscalid (35.7%), and 3 were resistant to Y12196 (7.1%). Among the 84 isolates collected in 2019, the EC_{50} values for boscalid ranged from 0.097 to 54.162 mg/L, while the EC_{50} values for Y12196 ranged from 0.284 to 20.147 mg/L. Sequence analysis showed that the *B. cinerea* isolates carrying P225F (proline → phenylalanine) and N230I (asparagine → isoleucine) mutations in SDH subunit B exhibited cross-resistance between boscalid and Y12196. However, boscalid-resistant isolates with a point mutation at position 272 of SDH B (H272R, histidine → arginine) were more sensitive to Y12196. Consistent with this, Y12196, but not boscalid, could successfully inhibit the growth of *B. cinerea* carrying the H272R mutation ($BcSDH^{B-H272R}$) on detached strawberries and leaves. Molecular docking simulations further revealed that the hydrogen bonds and π-π interactions were formed between Y12196 and $BcSDH^{B-H272R}$, but not between boscalid and $BcSDH^{B-H272R}$, explaining why the boscalid-resistant *B. cinerea* H272R mutant isolates were sensitive to Y12196. Together, our results suggested that Y12196 could effectively control boscalid resistance associated with the H272R mutation.

Keywords *Botrytis cinerea*, Y12196, Boscalid resistance, H272R mutant, Binding mode

Background

Strawberries are among the most popular fruit crops worldwide due to their high nutritional content and sweet taste. China is the largest strawberry producer in the world (Wang et al. 2021). In Zhejiang Province alone, about 5333 ha are dedicated to the strawberry crop, with an annual yield of 100,000 tons and a total output value of more than RMB 1.5 billion (Liao and Hu 2018). However, the profitability of the strawberry industry worldwide is seriously threatened by gray mold disease, which is caused by the fungus *Botrytis cinerea* Pers (Boff et al. 2001).

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In modern production, fungicide application is the most effective and economical strategy for controlling *B. cinerea* in the fields (Liu et al. 2016; Petrasch et al. 2019). Fungicides with a single-site mode of action, such as carbendazim (benzimidazoles, MBCs), procymidone (dicarboximides, DCFs), fludioxonil (phenypyrroles), difenoconazole (demethylation inhibitors, DMIs), azoxystrobin (quinone outside inhibitors, QoIs), and boscalid (succinate dehydrogenase inhibitors, SDHIs) have been used to manage gray mold throughout the world (Sun et al. 2010; Fan et al. 2017; Petrasch et al. 2019; Weber et al. 2019; Shao et al. 2021). However, with the continuous use of fungicides in the field, *B. cinerea* isolates have developed resistance, causing the fungicides to be ineffective (Mosbach et al. 2017; Rupp et al. 2017; Amiri et al. 2020; Toffolatti et al. 2020). Fungicide resistance in *B. cinerea* was severe in China; for example, carbendazim, pyrimethanil, and procymidone are no longer effective in controlling gray mold on tomato and grape in Shandong and Jiangsu provinces (Sun et al. 2010; Zheng et al. 2019). In addition, multiple resistance to fungicides has been commonly detected in *B. cinerea* from various hosts such as strawberry, cherry, nectarine, tomato, grape, and vegetable crops (Sun et al. 2010; Zhang et al. 2010; Fan et al. 2017; Adnan et al. 2018; Yin et al. 2018; Zheng et al. 2019).

SDHIs are a class of compounds that can target the mitochondrial respiratory Complex II or succinate dehydrogenase (SDH), a key enzyme in the tricarboxylic acid cycle and the mitochondrial respiratory chain (Sierotzki and Scalliet 2013). Boscalid, an SDHI fungicide, was the first SDHI to control a broad range of fungal pathogens in many host plants (Stammler et al. 2015). Because boscalid inhibits SDH function in *B. cinerea,* this fungicide is extremely effective in controlling gray mold (Sierotzki and Scalliet 2013; Hua 2018). However, *B. cinerea* has been shown to develop strong resistance to boscalid in the field following continuous fungicide applications (Veloukas et al. 2011; Liu et al. 2016). Previous studies have shown that specific amino acid point mutations at different positions in three *B. cinerea* SDH subunits (B, C, and D) were attributed to resistance against SDHIs, including boscalid (Stammler et al. 2015; Rupp et al. 2017). In recent years, the boscalid-resistant *B. cinerea* isolates have been detected frequently in China and are mainly caused by point mutations in SDH subunit B. In Shandong Province, the field boscalid-resistant *B. cinerea* isolates were confirmed to carry point mutations of P225F, N230I, H272Y, and H272R in SDH subunit B (He et al. 2020; Cui et al. 2021). In Shanghai, Hubei, and Zhejiang provinces, the H272R mutation in *B. cinerea* was the primary cause of boscalid resistance in greenhouse strawberries (Fan et al. 2017; Lin et al. 2018; Liu et al. 2018). Amino acid mutations in the *sdh* gene also contribute to SDHI resistance in other fungal plant pathogens (Avenot et al. 2012, 2014; Wang et al. 2015). To date, fluopyram, pydiflumetofen, and penthiopyrad also have been reported to have inhibitory activities on *B. cinerea* growth (Amiri et al. 2014; Vitale et al. 2016; He et al. 2020). Positive cross-resistance was always detected amongst the fungicides with the same mechanisms of action; however, there were exceptions. For instance, no positive cross-resistance was observed between boscalid and fluopyram, even though they both belong to SDHI fungicides (Laleve et al. 2014).

Y12196 (*N*-(2-(2,4-dichlorophenoxy) phenyl)-3-(difluoromethyl) -1-methyl-1*H*-pyrazole-4-carboxamide) (Additional file 1: Figure S1) is a new SDHI that was developed using pharmacophore-linked fragment virtual screening (PFVS) by Central China Normal University, and it has bioactivity against *Rhizoctonia solani* and *Sphaerotheca fuliginea* (Xiong et al. 2017; Shi et al. 2018). Like boscalid, Y12196 has an amide moiety, an aromatic ring, and a nitrogen-containing heterocycle (Additional file 1: Figure S1). These chemical structures are essential for fungicidal activity because they facilitate hydrogen bonds and π-π interactions between SDHI and pathogen SDH (Horsefield et al. 2006; Ruprecht et al. 2009). The structure–activity relationship between Y12196 and SDH is affected by the structure of the pyrazole-carboxamide-benzoxazol hybrid of Y12196 (-CF_2H on the pyrazole ring, 2,4-dichloro on the aromatic ring; Additional file 1: Figure S1). Indeed, Y12196 decreased the SDH enzyme activity, which was assessed by evaluating succinate-DCIP reductase activity (Xiong et al. 2015, 2017). Previously, Y12196 was shown to control *Fusarium* head blight caused by an isolate of *Fusarium asiaticum* carrying the A64V mutation in the SDH subunit C, which is associated with resistance to the SDHI fungicide pydiflumetofen (Chen et al. 2021). However, to our knowledge, the sensitivity of strawberry-infecting *B. cinerea* to Y12196 has not yet been investigated. In this study, we aimed to address this knowledge gap by evaluating the ability of Y12196 to control gray mold and to assess the cross-resistance of *B. cinerea* to Y12196 and boscalid. Specifically, we aimed to (i) investigate the sensitivity of the *B. cinerea* isolates from strawberry to Y12196; (ii) explore the differences between the fungicidal activities of Y12196 and boscalid in *B. cinerea*; and (iii) reveal the mechanisms underlying the differences in fungicidal mode of action between Y12196 and boscalid.

Results

Resistance of the *B. cinerea* isolates to Y12196 and boscalid

The *B. cinerea* isolates ($n=42$) collected in 2018 were assessed for their sensitivity to Y12196 based on the

discriminatory dose of 5 mg/L. The result showed that the spore germination of 39 isolates was significantly inhibited when they grew on YBA plates amended with 5 mg/L Y12196, and the spore germination rate varied from 4.8 to 9.5% (Fig. 1a); 15 isolates showed the spore germination rates above 90% on YBA plates supplemented with 5 mg/L boscalid (Fig. 1a). In addition, of the 42 isolates, 3 isolates (representative isolate TXB-4) showed spore germination rates above 90% on YBA plates amended with 5 mg/L Y12196 or 5 mg/L boscalid (Fig. 1a). Thus, 35.7% of the *B. cinerea* isolates were resistant to boscalid, while only 7.1% of the isolates were resistant to Y12196. Similar results were also observed for the mycelial growth of the isolates at 96 h (Fig. 1b).

EC_{50} values of the *B. cinerea* isolates for Y12196 and boscalid

To precisely determine the sensitivity of the *B. cinerea* isolates to Y12196 and boscalid, EC_{50} values of 84 *B. cinerea* isolates collected in 2019 were assessed based on 50% mycelial growth inhibition. The EC_{50} values varied from 0.284 to 20.147 mg/L for Y12196, with an average EC_{50} value (± standard error, SE) of 5.582 ± 0.478 mg/L. For most of the 84 isolates tested (84.5%), the EC_{50} values were between 0.284 to 9.885 mg/L, with about 53.6% of the assayed isolates having an EC_{50} of less than 5 mg/L. A single *B. cinerea* isolate had an EC_{50} value greater than 20 mg/L for Y12196 (Fig. 2a).

The EC_{50} values ranged from 0.097 to 54.162 mg/L for boscalid, with an average EC_{50} of 13.452 ± 1.297 mg/L (Fig. 2b). About a quarter of the 84 isolates tested (24 isolates; 28.6%) had the EC_{50} values less than 5 mg/L, and another quarter (20 isolates; 23.8%) had the EC_{50} values greater than 20 mg/L. These data suggested that the *B. cinerea* populations were more sensitive to Y12196 than boscalid. However, for the 84 isolates collected in 2019, there was a positive correlation between sensitivity to Y12196 and boscalid ($\rho = 0.575$, $P < 0.05$; Fig. 2c).

Mutations of SDH linked with boscalid resistance

The PCR-amplified SDH subunits B, C, and D were 1114, 712, and 784 bp long, respectively. Compared to the reference *sdh* B, *sdh* C, and *sdh* D genes, several mutations in the SDH subunits B and C were detected in the field isolates collected in 2018 and 2019 (Fig. 3 and Additional file 2: Table S1). In particular, we identified three types of point mutations in SDH subunit B, associated with boscalid resistance: P225F (CCC → TTC at codon 225); N230I (AAC → ATC at codon 230); and H272R (CAC → CGC at codon 272) (Fig. 3a). Among the 42 isolates collected in 2018, the H272R mutation was the most common (twelve isolates, 28.6%), followed by N230I (two isolates, 4.8%), and P225F (one isolate, 2.4%) (Fig. 3b). Importantly, the isolates carrying the H272R mutation were sensitive to Y12196, whereas the isolates carrying the P225F mutation or N230I mutation were resistant to Y12196 (Y^RBOS^R, Fig. 1a).

The *B. cinerea* isolates carrying the P225F, N230I, and H272R mutations were also detected among the 84 isolates collected in 2019 (Fig. 3b): 3 (3.6%), 19 (22.6%), and 42 (50%) isolates carried, respectively. Consistent with 2018, the *B. cinerea* isolates carrying the H272R mutation were the dominant mutants in field isolates collected in 2019. Of the 42 H272R mutants collected in 2019, 38 isolates had an EC_{50} lower value for Y12196 than for boscalid (Additional file 2: Table S1). The average EC_{50} values of these 38 H272R mutant isolates were 4.889 ± 0.518 mg/L for Y12196 and 20.089 ± 1.913 mg/L for boscalid, respectively, indicating that the majority of H272R mutants were somewhat more sensitive to Y12196 than to boscalid.

Among the 126 isolates collected in 2018 and 2019, 27 the *sdh C*-mutant isolates were detected, including 25 isolates carrying four concurrent mutations (G85A, I93V, M158V, and V168I) and 2 isolates carrying double mutations (I93V and V168I or G85A and V168I). Of the *sdh C*-mutant isolates collected in 2019, 86.7% (13/15) were the isolates with the H272R mutation, and 13.3% (2/15) were the isolates without the H272R mutation (Additional file 2: Table S1).

Efficacy of Y12196 and boscalid against the H272R isolates on detached fruits and leaves

Four isolates, including two isolates without the H272R mutation (B05.10 and PB-4) and two H272R mutant isolates (ZB-4 and JDGZ-9), were used to investigate the control efficacy of Y12196 and boscalid on detected fruits and leaves. Both Y12196 and boscalid significantly inhibited the growth of isolate B05.10 and PB-4 on strawberry fruits. Treatment with up to 500 mg/L boscalid did not, however, inhibit the growth of the ZB-4 and JDGZ-8 isolates, which carried the H272R mutation, on strawberry fruits (Fig. 4a). In contrast, treatment with 240 mg/L Y12196 noticeably inhibited the growth of the ZB-4 and JDGZ-8 isolates on strawberry fruits, and strawberries treated with 480 mg/L Y12196 showed no evidence of mold growth within 3 days of inoculation (Fig. 4a).

Similarly, both Y12196 and boscalid inhibited the development of the B05.10 and PB-4 isolates on leaves. However, only Y12196 effectively inhibited the development of the ZB-4 and JDGZ-8 isolates (Fig. 4b). Lesion development was significantly decreased on the leaves sprayed with 80 mg/L boscalid or 80 mg/L Y12196, compared with the water treatment, for all the four isolates (Fig. 4b). For the *B. cinerea* isolates without the H272R mutation, application of Y12196 or boscalid resulted in a

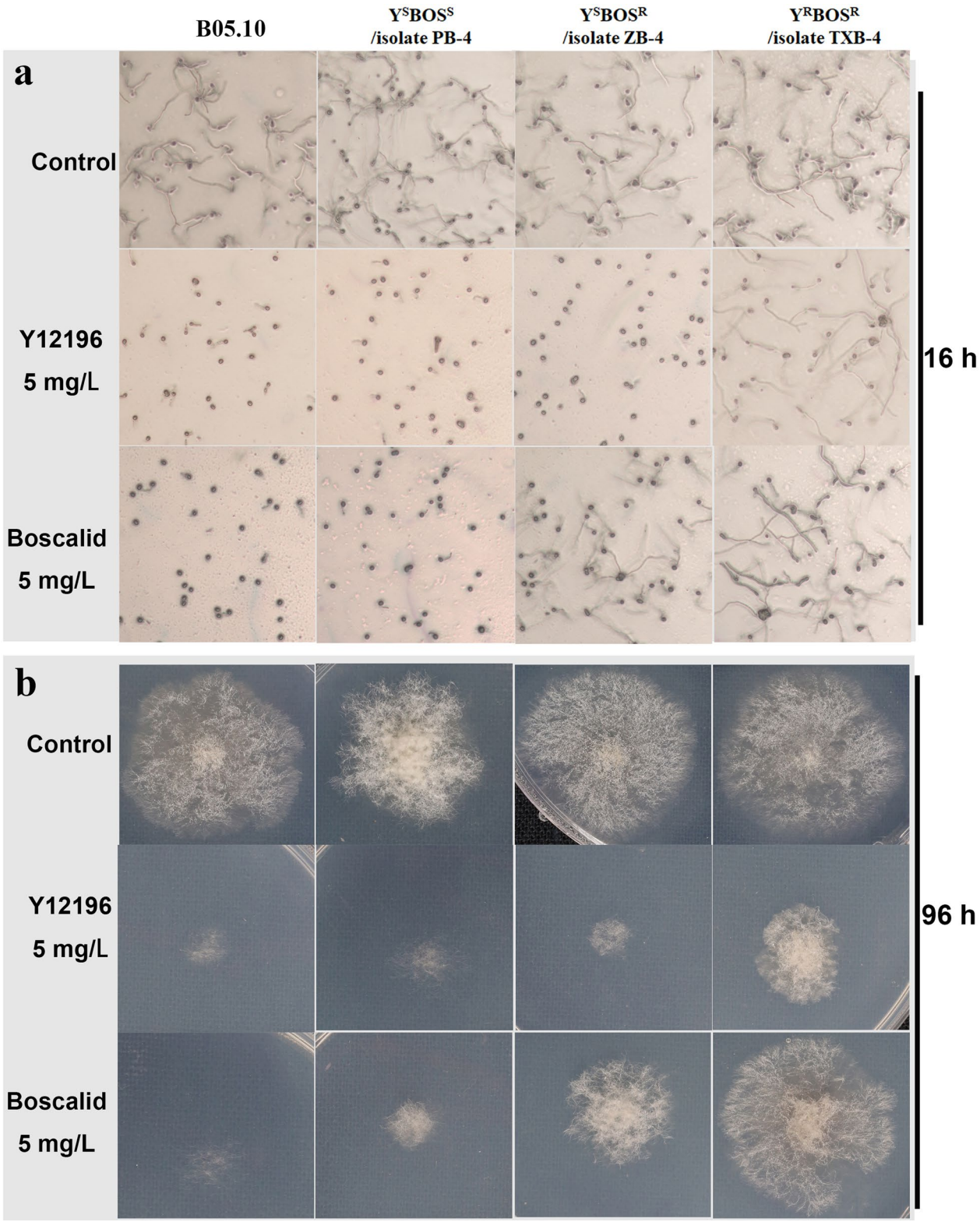


Fig. 1 Response of *Botrytis cinerea* isolates collected in 2018 to Y12196 and boscalid. Spore germination (**a**) and mycelial growth (**b**) of isolates were observed at 16 h and 96 h after isolates grew on the fungicide-YBA plate, and the isolates grown on methanol-YBA were used as control (Control). The *B. cinerea* isolate B05.10, was sensitive to both Y12196 and boscalid. The $Y^{S}BOS^{S}$ isolate PB-4 was sensitive to both Y12196 and boscalid. The $Y^{S}BOS^{R}$ isolate ZB-4 was sensitive to Y12196 but resistant to boscalid. The $Y^{R}BOS^{R}$ isolate TXB-4 was resistant to both Y12196 and boscalid

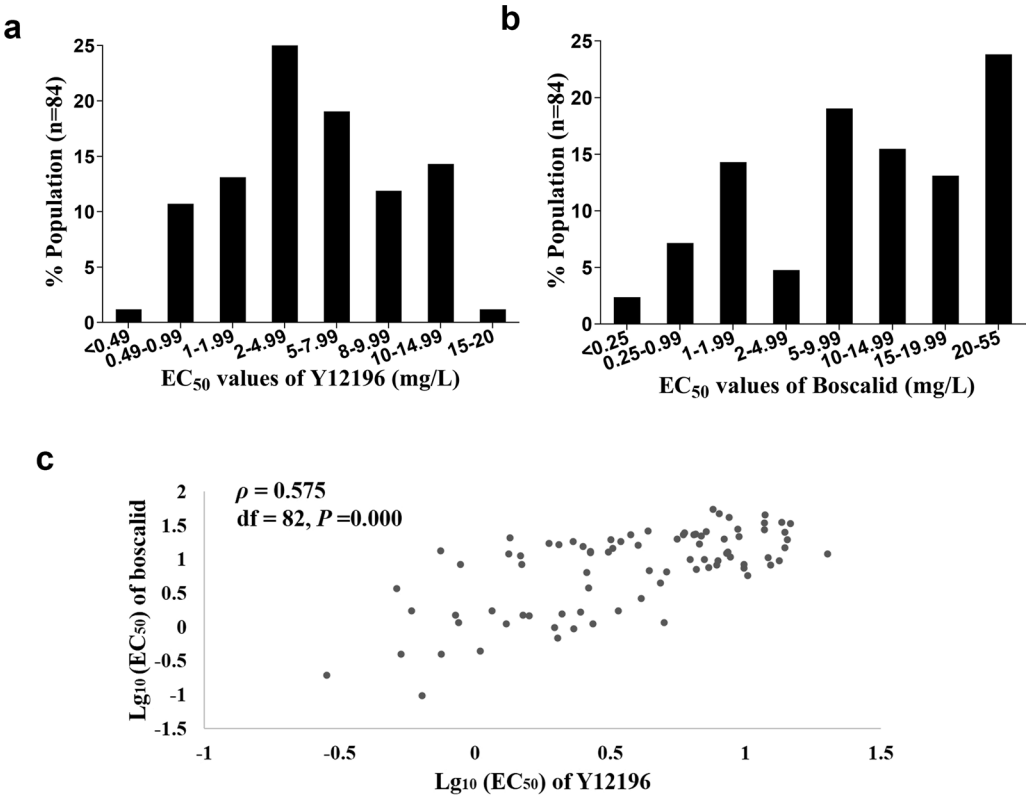


Fig. 2 Y12196 and boscalid sensitivity of the *Botrytis cinerea* isolates collected from Zhejiang Province in 2019. Frequency distributions of EC_{50} values for Y12196 (**a**) and boscalid (**b**) against 84 *B. cinerea* isolates. **c** Correlation between Y12196 and boscalid. EC_{50}, 50% effective concentration value inhibiting mycelial growth

similar control efficacy. For the B05.10 isolate, the control efficacies of Y12196 and boscalid at 80 mg/L were 69.81% and 75.47%, respectively, and for the PB-4 isolate were 59.68% and 64.52%, respectively (Fig. 4c). However, the control efficacy of the Y12196 application for the H272R mutants was significantly higher than that obtained for boscalid application. For example, spray application of 80 mg/L Y12196 provided a control efficacy of 65.78% for the H272R mutant isolate ZB-4, significantly higher ($P=0.004$) than that observed for boscalid application (Fig. 4c).

Molecular docking of Y12196 to *B. cinerea* SDH (BcSDH)

Using the crystal structure of SDH from the porcine heart as a template, we predicted the structure of BcSDH with high credibility (Additional file 1: Figure S2b, c). The binding mode of Y12196 to BcSDH was similar to that of boscalid to BcSDH (Fig. 5a, c): one hydrogen bond was present between the B_W229 residue of SDH and the amide oxygen atom of SDHI, while the other hydrogen bond was formed between the C_S84 residue of SDH and the pyrazole nitrogen of Y12196 (Fig. 5a) or the pyridine nitrogen of boscalid (Fig. 5c). The dichlorobenzene rings of both SDHIs also exhibited π-π interactions with the C_W228 residue of SDH (Fig. 5a, c). In contrast, no hydrogen bonds or π-π interactions were observed between boscalid and BcSDH carrying the H272R mutation ($BcSDH^{B-H272R}$) (Fig. 5d). However, the binding mode of Y12196 with $BcSDH^{B-H272R}$ showed that a hydrogen bond was formed between $BcSDH^{B-H272R}$ residue C_H71 and the amide oxygen atom of Y12196, while π-π interactions were observed between the Y12196 aromatic ring and the B_W229, C_81, C_Y75, and C_H71 residues of $BcSDH^{B-H272R}$ (Fig. 5b). These results indicated that the binding affinity of boscalid for $BcSDH^{B-H272R}$ was broken, and that of Y12196 for $BcSDH^{B-H272R}$ was kept. This may theoretically explain why the *B. cinerea* H272R mutants were resistant to boscalid and sensitive to Y12196.

Discussion

Strawberry gray mold, caused by the fungus *B. cinerea*, is a major threat to strawberry production. Several SDHI fungicides have recently been developed and released for field use, including boscalid (BASF), fluopyram (Bayer), and pydiflumetofen (Syngenta). These fungicides have quickly become widely used in many countries (Hua

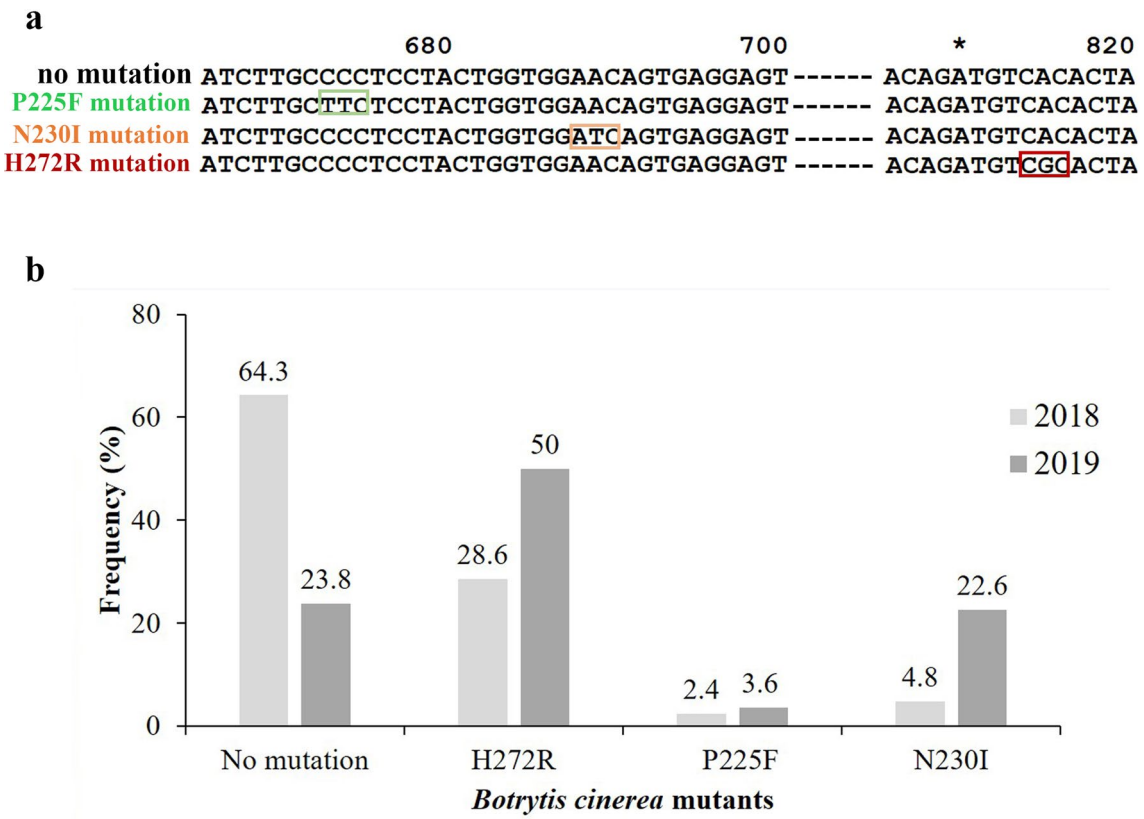


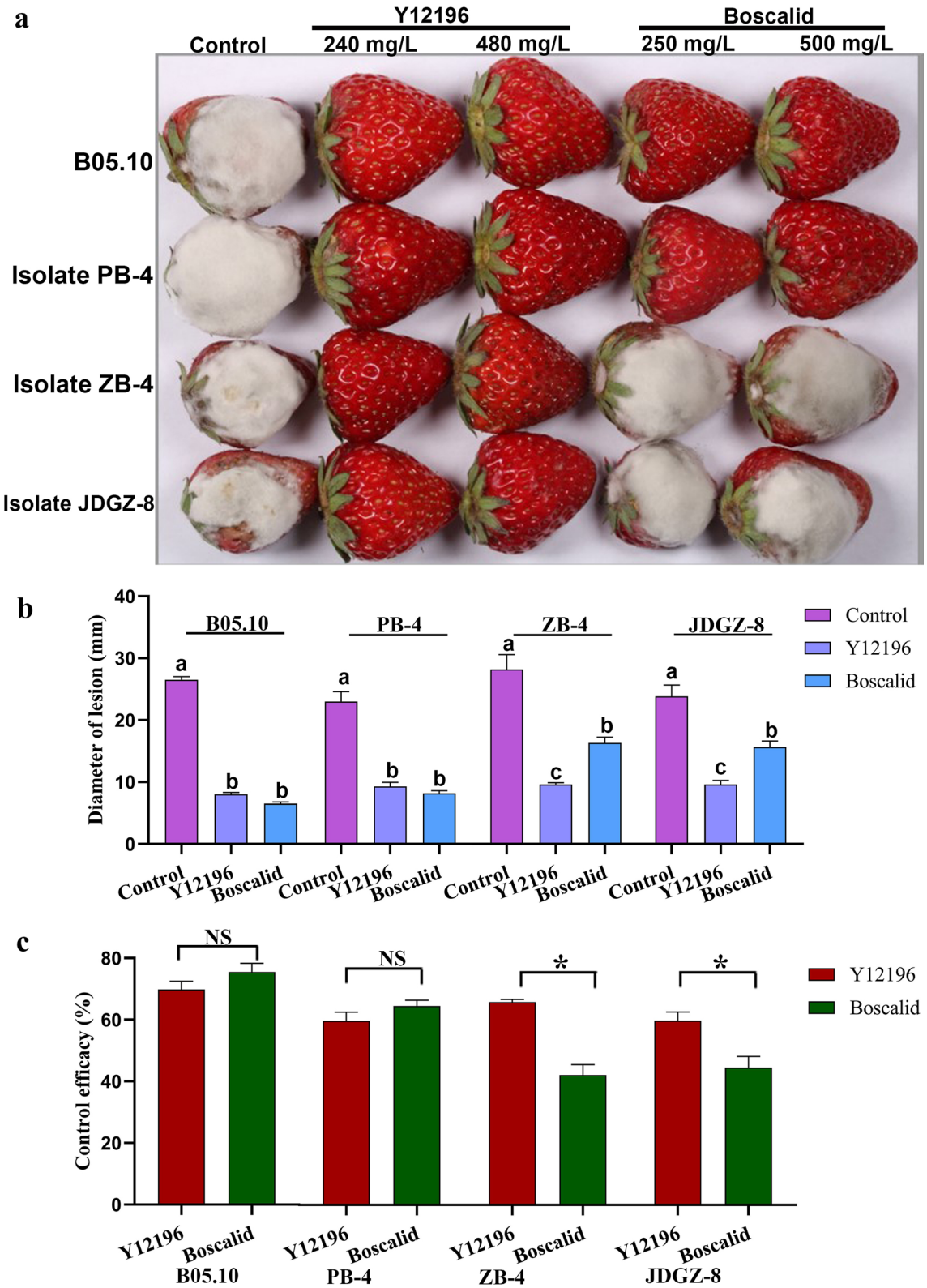
Fig. 3 Characterization of mutations in the succinate dehydrogenase subunit B in *Botrytis cinerea* isolates. **a** Four genotypes of the SDH B subunit were found in this study. Sequence alignment was done using partial SDH B subunit nucleotide (nt) sequences: P225F, mutation changed the codon 225 from CCC (P) to TTC (F); N230I, mutation changed the codon 230 from AAC (N) to ATC (I); H272R, mutation changed the codon 272 from CAC (H) to CGC (R); 680, 700 and 820 indicated that bases position at 680, 700, 820 in the *sdh* B gene, respectively; green box, orange box and red box indicated that the codon 225, 230 and 272 of SDH B, respectively. **b** Frequency of the four genotypes found in field isolates in 2018 and 2019. The numbers on the bar were the frequency of each mutant

2018; Sang et al. 2018; Duan et al. 2019; He et al. 2020; Neves and Bradley 2020). However, continuous application over several years has led to increasing levels of SDHI fungicide resistance. For example, the emergence of boscalid-resistant *B. cinerea* has sharply reduced the control efficacy of boscalid against gray mold (Veloukas et al. 2011; Toffolatti et al. 2020; Cui et al. 2021). Here, we showed that Y12196, an SDHI newly developed using PFVS, has a different mode of bioactivity against *B. cinerea* compared with boscalid. Although we detected some positive cross-resistance between Y12196 and boscalid in a few *B. cinerea* isolates tested, fungicide sensitivity monitoring and control efficacy assays showed that the *B. cinerea* H272R mutants were resistant to boscalid and were sensitive to Y12196.

In the present study, we collected 126 *B. cinerea* isolates from strawberry greenhouses in Zhejiang Province. Sensitivity testing showed that boscalid-resistant *B. cinerea* isolates were found in 2018 and 2019. Mutations in *B. cinerea* SDH subunit B have previously been shown to be associated with SDHI fungicide resistance (Fernández-Ortuño et al. 2012; Veloukas et al.

(See figure on next page.)

Fig. 4 Control efficacy of Y12196 for *Botrytis cinerea* infections on strawberry fruits and leaves. **a** Compared in vivo performances of Y12196 and boscalid in controlling gray mold on strawberries. Fruits were treated with Y12196 (240 mg/L and 480 mg/L) or boscalid (250 mg/L and 500 mg/L) before inoculating with a conidial suspension of *B. cinerea* isolates. Lesion diameter (**b**) and control efficacy (**c**) on leaves treated with Y12196 and boscalid. Leaves sprayed with 80 mg/L Y12196 or 80 mg/L boscalid before inoculating with isolates. Values are shown as the means ± SE of ten lesions measured in two independent experiments. Bars with different lowercase letters are significantly different ($P < 0.05$) according to Fisher's least significant difference test (lesion diameter); asterisks denote significant difference ($P < 0.05$) and NS denotes no statistical significance according to Student's *t*-test (control efficacy). B05.10 and PB-4, the isolates lacking the H272R mutation; ZB-4 and JDGZ-8, the isolates with the H272R mutation in SDH B



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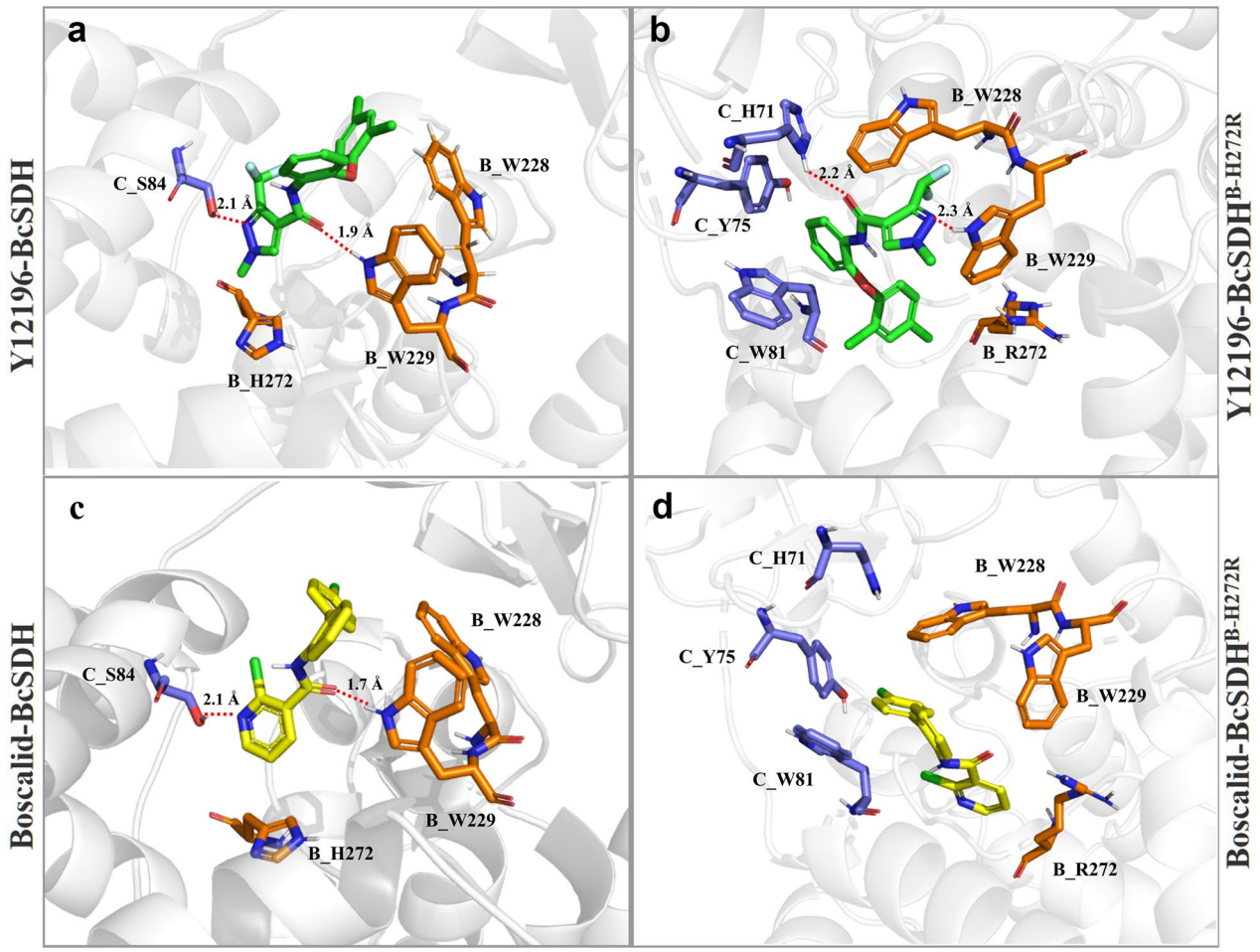

Fig. 5 Docking prediction for Y12196 (green sticks) or boscalid (yellow sticks) with BcSDH or BcSDHB^{H272R}. Binding of Y12196 to BcSDH (**a**), Y12196 to BcSDHB^{H272R} (**b**), boscalid to BcSDH (**c**), and boscalid to BcSDHB^{H272R} (**d**). The dotted line indicated the distance between the H atom and O/N atoms in the protein–ligand complex; white sticks indicated that H atoms; BcSDH: *Botrytis cinerea* without the H272R mutation; BcSDHB^{H272R}: *Botrytis cinerea* carrying the H272R mutation

2013; Amiri et al. 2014; Stammler et al. 2015; Rupp et al. 2017): mutations at amino acids 225 (P225F/L/T), 230(N230I), and H272L of the SDH B subunit were responsible for the resistance to boscalid, fluopyram isopyrazam, and penthiopyrad; H272R and H272Y mutants showed resistance to boscalid and isopyrazam. Among these, SDH B-H272R and SDH B-H272Y mutations in *B. cinerea* were the most frequently detected in the field boscalid-resistant isolates. Similarly, in other plant pathogenic fungi, amino acid substitutions at the conserved histidine residue in the SDH B subunit (H260R in *Blumeriella jaapii*, H277R/Y in *Alternaria alternata*, and H267Y in *Sclerotinia homoeocarpa*) were the main mutation types for boscalid resistance (Avenot et al. 2012, 2014; Popko et al. 2018; Outwater et al. 2019). Furthermore, in *B. cinerea*, the H132R mutation within SDH subunit D was also detected in the boscalid-resistant isolates in the field, albeit at a lower frequency (Leroux et al. 2010). There were seventy-nine *sdhB*-mutant isolates detected, including 54 *B. cinerea* isolates carrying the H272R mutation, 21 isolates carrying the N230I mutation, and 4 isolates carrying the P225F mutation, which were responsible for boscalid resistance. In recent years, the H272Y and P225F mutations in the SDH B subunit were the main mutation types for boscalid-resistant isolates in Shandong Province (He et al. 2020; Cui et al. 2021). However, in Zhejiang Province, the H272R mutants were the dominant boscalid-resistant populations, which was in agreement with a previous study (Lin et al. 2018). Meanwhile, SDH B-H272 L/Y, SDH B-P225L/T, and SDH D-H132R mutations were not detected in the field isolates collected in Zhejiang Province.

Twelve *B. cinerea* H272R mutant isolates were collected in 2018, and the spore germination of each strain was inhibited by Y12196, a new SDHI, while not

significantly inhibited by boscalid. Furthermore, of the 38 H272R mutant isolates collected in 2019, each strain had a lower EC_{50} value for Y12196 than for boscalid. Meanwhile, Y12196 was also confirmed to effectively inhibit the development of boscalid-resistant *B. cinerea* H272R mutants on detached strawberry fruits and leaves. These results showed that SDH B-H272R boscalid-resistant isolates were sensitive to Y12196, which is in agreement with several previous studies that revealed the H272R mutation in *B. cinerea* SDH does not confer resistance to fluopyram and pydiflumetofen (Laleve et al. 2014; He et al. 2020). Moreover, the absence or lack of cross-resistance between boscalid and structurally different SDHI fungicides in boscalid-resistant mutants carrying SDH B mutations also has been observed in other fungal species, such as *A. alternata*, *Corynespora cassiicola*, and *D. bryoniae* (Ishii et al. 2011; Avenot et al. 2012, 2014).

Structural differences between SDHIs may suggest differences in binding properties to the target SDH of pathogenic fungi. As shown previously (Horsefield et al. 2006; Ruprecht et al. 2009), the binding modes of SDHIs with SDH are crucial for fungicidal activity. The affinity of a given SDHI for SDH is determined by hydrogen bonds and π-π interactions formed between SDH and the aromatic ring and amide moiety of the SDHI (Stammler et al. 2015). Mutations at various positions in three SDH subunits (B, C, and D) may alter the binding mode of the SDHI with fungal SDH, reducing binding affinity and fungicidal activity (Horsefield et al. 2006; Ruprecht et al. 2009). Several studies suggest that boscalid-resistant mutants carrying SDH B mutations do not affect their binding to fluopyram may be linked to the molecular structure of this benzamide derivative and its interaction with SDH B (Leroux et al. 2010; Ishii et al. 2011). Y12196 was designed and synthesized as an SDHI, and it was a diphenyl ether-containing pyrazole-carboxamide derivative: 2,4-dichloro on the aromatic ring in Y12196 is favorable to its binding to SDH (Xiong et al. 2015, 2017). Molecular docking simulations showed that the binding affinity between Y12196 and the H272R mutant was maintained by a hydrogen bond and π-π interactions involving the Y12196 amide oxygen atom and aromatic ring. In contrast, neither hydrogen bonds nor π-π interactions were formed between boscalid and $BcSDH^{B-H272R}$. This suggested that the sensitivity of the *B. cinerea* H272R mutant to Y12196 resulted from the maintenance of binding affinity due to the formation of new bonds and interactions between Y12196 and $BcSDH^{B-H272R}$.

In the current study, we also detected mutations in *B. cinerea* SDH subunit C in 12 of the 42 isolates collected in 2018 and 15 of the 84 isolates collected in 2019. Previously, it was shown that the amino acids at positions 85, 93, 158, and 168 in *B. cinerea* SDH C were located in the binding pocket, and that the simultaneous mutation of these amino acids reduced the sensitivity of *B. cinerea* to fluopyram (Amiri et al. 2020). Interestingly, other studies found that simultaneous mutations of the same amino acids did not affect boscalid resistance (Cui et al. 2021; Liu et al. 2021). Consistent with these studies, we detected simultaneous mutations of these four amino acids (G85A, I93V, M158V, and V168I) in subunit C of wild-type SDH and SDH carrying the H272R mutation in subunit B. Thus, our results also suggested that these mutations in SDH C subunit do not affect the sensitivity of *B. cinerea* to boscalid and Y12196.

Conclusions

In this study, H272R was the most common mutation conferring boscalid resistance among the *B. cinerea* isolates collected in 2018 and 2019 in Zhejiang Province, and Y12196 has bioactivity against the H272R mutants. Therefore, we suggest that Y12196 might have potential utility for controlling boscalid-resistant gray mold infections in strawberries. We also propose that the protein of mutants found in the field and created in the lab can be utilized as a direct target when developing a new fungicide.

Methods

Fungicides and media

Potato dextrose agar (PDA) medium (200 g potato, 20 g glucose, and 20 g agar in 1 L sterile deionized water) was prepared and used for routine growth and sporulation assays. In addition, yeast peptone acetate (YBA) medium (10 g yeast extract, 10 g Bacto peptone, 20 g sodium acetate, and 15 g agar in 1 L sterile deionized water) was prepared and used to determine fungal sensitivity to Y12196 and boscalid.

Boscalid (98% active ingredient) and Y12196 (90% active ingredient) were each dissolved in 100% methanol to prepare stock solutions of 40,000 mg/L. Each stock solution was stored in the dark at 4°C. Commercial formulations of Y12196 (24% SC; Zhengbang Group, Jiangxi, China) and boscalid (Cantus 50% WG; BASF SE, Ludwigshafen, Germany) were used in the detached strawberry fruit and leaf assays.

Evaluation of the sensitivity of *B. cinerea* to Y12196 and boscalid

In 2018, diseased strawberries were collected from five strawberry greenhouses located 500 m apart in Jiande County in Zhejiang Province. All sampled greenhouses were in conventional production without the Y12196 application. Spores collected from diseased fruits were directly transferred onto 2% water agar plates supplemented with 50 mg/L streptomycin sulfate, and the plates

were incubated at 22°C for 12 h. From these plates, 42 single-conidial *B. cinerea* isolates were obtained.

Resistance of the 42 isolates to boscalid and Y12196 was assessed using a conidial germination assay on YBA medium amended with 5 mg/L of each fungicide, as described previously (Vitale et al. 2016). Conidial suspensions of each strain (2×10^5 spores/mL) were prepared, and 5 µL drops of each conidial suspension were added to separate YBA medium plates amended with 5 mg/L of Y12196 or boscalid. Spores were added to YBA plates with methanol as a control. The spore germination rate of each isolate was calculated after 16 h incubation at 22°C: spore germination was quantified at three sites by counting 100 conidia per site, and the germination percentage was calculated. We distinguished between the SDHI-resistant isolates (i.e., germ tube growth was clearly visible at an SDHI concentration of 5 mg/L, and > 90% of the conidia were able to germinate well) and the SDHI-sensitive isolates (i.e., < 10% of the conidia were able to germinate at 5 mg/L) (Kim and Xiao 2010; Fernández-Ortuño et al. 2012; Amiri et al. 2014). Each treatment was replicated three times, and the entire experiment was performed three times. The mycelial growth of each isolate was observed at 96 h.

Determination of the EC_{50} of Y12196 and boscalid for *B. cinerea* mycelial growth

To determine the sensitivity of *B. cinerea* isolates to boscalid and Y12196, the field *B. cinerea* isolates collected in 2019, their EC_{50} values for boscalid and Y12196 were calculated based on mycelial growth as described previously (Wu et al. 2020). We collected 84 single-conidial *B. cinerea* isolates from strawberry greenhouses in four counties of Zhejiang Province in 2019: Jiande, Xiaoshan, Yuhang, and Shaoxing. There was no Y12196 application in these greenhouses. A fresh mycelium plug (5 mm in diameter) was taken from the edge of each colony and placed on YBA plates amended with 0, 1, 2, 4, 8, 16, and 32 mg/L of Y12196 or boscalid. If the EC_{50} value was below this dose range, the mycelium plug was transferred to the center of a YBA plate amended with 0, 0.0625, 0.125, 0.25, 0.5, 1 mg/L Y12196 or boscalid. Conversely, if the EC_{50} was above this dose range, the mycelium plug was transferred to the center of a YBA plate amended with Y12196 or boscalid at 0, 2, 4, 8, 16, 32, 64 mg/L. Each treatment was replicated three times, and the entire experiment was performed three times. We investigated the cross-resistance between Y12196 and boscalid by regressing the individual $\log_{10}$ EC_{50} values for Y12196 against the $\log_{10}$ EC_{50} values for boscalid and determining the Spearman rank correlation (ρ) (Lu et al. 2010).

Sequencing of the *sdh* B, *sdh* C, and *sdh* D genes

Genomic DNA from the 126 field-collected *B. cinerea* isolates (42 collected in 2018 and 84 collected in 2019) was extracted using Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). The primers used to amplify subunits B, C, and D are listed in Table 1. Each PCR reaction (50 µL) contained 25 µL of 2×PrimeSTAR Max Premix (TaKaRa, Dalian, China), 2 µL genomic DNA (50 ng/µL), 1 µL of each primer (10 µM), and 21 µL of sterile water. The amplicons were purified from 1% agarose gels after electrophoresis using the SanPrep Column PCR Product Purification Kit (Shanghai, China), and the purified products were sequenced by Sangon Biotech (Shanghai, China). The complete sequences of the *sdh* B, *sdh* C, and *sdh* D genes were compared with reference sequences (Gene ID 5,428,850, *sdh* B; 5,427,541, *sdh* C; and 5,440,404, *sdh* D) retrieved from the National Center for Biotechnology Information (NCBI) database using DNAstar v.6.0, to identity the mutations related to boscalid and Y12196 sensitivity.

Control efficacy of Y12196 against the *B. cinerea* H272R mutant

Preliminary results indicated that a mutation in the *sdh B* gene, H272R, conferred resistance to boscalid but not to Y12196. To more precisely determine how well the two SDHIs inhibited the *B. cinerea* H272R mutant on strawberry fruits and leaves, B05.10 (wild sensitive isolate lacking the H272R mutation), the isolate PB-4 (lacking the H272R mutation, a field isolate collected in 2018) and isolate ZB-4, isolate JDGZ-8 (possessing the H272R mutation, two field isolates collected in 2018 and 2019, respectively) were selected for efficacy assays,

Table 1 List of primers used for amplification and sequencing in this study

Primer		Primer sequence (5′ → 3′)	Gene ID in NCBI	Purpose
CQZ3	F	CTATCTTACCATTGATACATAC	5,428,850	To amplify and sequence the SDH B subunit
CQZ4	R	GAAATGCTATCTCATCAAG		
CQZ5	F	ATGTTTTCACAGAGAGCAAC	5,427,541	To amplify and sequence the SDH C subunit
CQZ6	R	CTACAAGAAAGCAACCAAC		
CQZ7	F	GACTCTTTTACACTCAATAATTG	5,440,404	To amplify and sequence the SDH D subunit
CQZ8	R	CCTTGATATTGCACTGCGCGTAC		

as described previously (Vitale et al. 2016). The isolates B05.10, PB-4, ZB-4, and JDGZ-8, had the EC_{50} values of 1.907, 2.512, 4.796, and 3.187 mg/L for Y12196 and had the EC_{50} values of 0.633, 0.661, 11.443, 19.383 mg/L for boscalid, respectively. Spore suspensions of each isolate were prepared (2×10^5 spores/mL). We tested the following concentrations of fungicides on fruits: 240 and 480 mg/L Y12196 or 250 and 500 mg/L boscalid. Before spray application, fruits were surface disinfected with 1% NaClO for 30 s, followed by three rinses in sterile water. Next, the disinfected fruits were sprayed with fungicides or sterile water (control). After air-drying, each fruit was inoculated with a 20 µL drop of the spore suspensions (2×10^5 spores/mL). The inoculated fruits were incubated in a growth chamber at 22 ± 3 °C with a photoperiod of 12 h and 90% relative humidity. Control assays were performed twice, with 15 fruits per treatment per assay. After 3 days, strawberries displaying symptoms of gray mold were counted as an indicator of control efficacy.

For assessing the control efficacy of Y12196 against the *B. cinerea* H272R mutant at a lower concentration, we tested the following concentrations of fungicides on leaves: 80 mg/L Y12196 or boscalid. After disinfection of the surface and air-drying, the leaves were wounded with a sterile needle and then were sprayed with fungicides or sterile water, followed by incubation of the spore suspensions (20 µL, with a density of 2×10^5 spores/mL). These control assays were also performed twice, using 10 leaves per treatment per assay. After cultivation for 3 days as described in the previous section, the lesions on the leaves were measured, and the average diameters of the lesions associated with each treatment were used to determine control efficacy as follows:

Control efficacy (%) = [(average lesion diameter on the control leaves—average lesion diameter on the fungicide-treated leaves)/average lesion diameter on the control leaves] × 100%.

Molecular docking simulations of Y12196 with SDH

To investigate the molecular interactions between Y12196 and the *B. cinerea* H272R mutant, we used homology models and model docking analyses to construct binding models for Y12196 with different SDH genotypes. Although the three-dimensional (3D) structure of *B. cinerea* SDH is currently unavailable, the crystal structures of SDH from *Escherichia coli*, porcine heart, chicken heart, and several parasites have previously been reported (Yankovskaya et al. 2003; Huang et al. 2005; Inaoka et al. 2015). Previous studies have suggested that the residues within the B, C, and D subunits might form binding pockets for SDHIs (Horsefield et al. 2006; Xiong et al. 2017). Here, we predicted the 3D structures of the SDH B, C, and D subunits based on their protein sequences (GenBank accession numbers XP_001548350.1, XP_001547075.1, and XP_001559774.1, respectively; Additional file 1: Figure S2a) using Discover Studio 3.5 (Accelrys Inc., 2013). The crystal structure of porcine heart SDH (PDB-code: 3ABV) was chosen as the modeling template using Pymol 2.5 (Sun et al. 2005). Y12196 and boscalid were added individually into the docking site in the homology-based model using the Maestro 10.2 Glide matrix (Zhu et al. 2014; Morris et al. 2015).

Statistical analysis

The data analyses were performed using SPSS v. 19.0 software. The EC_{50} values of each *B. cinerea* strain for Y12196 and boscalid were calculated by regressing the percent relative growth against the $\log_{10}$ values of the fungicide concentrations. The cross-resistance relationships between Y12196 and boscalid were evaluated based on the correlations between EC_{50} values transformed into $\log_{10}$ values. All the data were represented as the mean ± standard error (SE). To assess control efficacy, the diameters of the lesions on the leaves were measured for each isolate, and analyses of variance (ANOVA) followed by Fisher's least significant difference test were performed to identify significant differences in mean diameter. We considered $P<0.05$ statistically significant.

Abbreviations

ANOVA	Analyses of variance
$BcSDH^{B-H272R}$	*Botrytis cinerea* carrying the H272R mutation
EC_{50}	50% effective concentration
G85A	Amino acid at position 85 change from glycine to alanine
H272L/R/Y	Amino acid at position 272 change from histidine to leucine/arginine/tyrosine
I93V	Amino acid at position 93 change from isoleucine to valine
M158V	Amino acid at position 158 change methionine to valine
N230I	Amino acid at position 230 change from asparagine to isoleucine
P225F/L/T	Amino acid at position 225 change from proline to phenylalanine/leucine/threonine
PDA	Potato dextrose agar
SC	Suspension concentrate
SDH B	Succinate dehydrogenase subunit B
SDHIs	Succinate dehydrogenase inhibitors
SE	Standard error
V168I	Amino acid at position 168 change valine to isoleucine
WG	Water dispersible granule
YBA	Yeast peptone acetate

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00173-1.

Additional file 1. Figure. S1. Chemical structures of the succinate dehydrogenase inhibitorsboscalid and Y12196. Figure. S2. Homology-based three-dimensional modeling of *Botrytis cinerea* SDH.
Additional file 2. Table S1. Sensitivity of the 42 *Botrytis cinerea* H272R mutants collected in 2019 to Y12196 and boscalid.

Acknowledgements
Not applicable.

Authors' contributions
JW, FW, and CZ designed the experiments; JW, HW, and CZ wrote the manuscript; JW, HM, and YS carried out the experiments. All authors read and approved the final manuscript.

Funding
This research was funded by the Key Important Program of China National Tobacco Corporation (110202101048(LS-08)), the National Natural Science Foundation of China (31801779), the Postdoctoral Science Foundation of Zhejiang Province, China (ZJ2021121), and the Research Training Project for College Student (2021KX0006).

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: 29 November 2022 Accepted: 10 April 2023
Published online: 23 May 2023

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