



A new point mutation (D1158N) in histidine kinase Bos1 confers high-level resistance to fludioxonil in field gray mold disease

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ABSTRACT

Gray mold, caused by the fungus *Botrytis cinerea*, is one of the most important plant diseases worldwide that is prone to developing resistance to fungicides. Currently, the phenylpyrrole fungicide fludioxonil exhibits excellent efficacy in the control of gray mold in China. In this study, we detected the fludioxonil resistance of gray mold disease in Shouguang City of Shandong Province, where we first found fludioxonil-resistant isolates of *B. cinerea* in 2014. A total of 87 single spore isolates of *B. cinerea* were obtained from cucumbers in greenhouse, and 3 of which could grow on PDA plates amended with 50 µg/mL fludioxonil that was defined as high-level resistance, with a resistance frequency of 3.4%. Furthermore, the 3 fludioxonil-resistant isolates also showed high-level resistance to the dicarboximide fungicides iprodione and procymidone. Sequencing comparison revealed that all the 3 fludioxonil-resistant isolates had a point mutation at codon 1158, GAC (Asp) → AAC (Asn) in the histidine kinase Bos1, which was proved to be the reason for fludioxonil resistance. In addition, the fludioxonil-resistant isolates possessed an impaired biological fitness compared to the sensitive isolates based on the results of mycelial growth, conidiation, virulence, and osmotic stress tolerance determination. Taken together, our results indicate that the high-level resistance to fludioxonil caused by the Bos1 point mutation (D1158N) has emerged in the field gray mold disease, and the resistance risk is relatively high, and fludioxonil should be used sparingly.

1. Introduction

Gray mold, caused by the fungal pathogen *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*), is an economically important disease that attacks a wide range of plant species, including fruits, vegetables, ornamental plants, and even some agricultural crops (Williamson et al., 2007; Ren et al., 2017). Gray mold is notorious for its rapid spread and severe destructiveness, often resulting in substantial losses in both pre- and post-harvest stages (Hou et al., 2018). Currently, chemical control remains the most effective strategy for managing gray mold, primarily due to the lack of resistant varieties (Islam and Sherif, 2020). However, with the long-term and frequent application, *B. cinerea* populations have developed resistance to some types of fungicides, such as quinone outside inhibitors (QoIs), benzimidazoles, phenylpyrroles, dicarboximides, anilinepyrimidines, succinate dehydrogenase inhibitors (SDHIs) and hydroxylanilide fungicides (Elad et al., 1992; Myresiotis et al., 2007; Sang et al., 2018).

Fludioxonil [4-(2,2-difluoro-2H-1,3-benzodioxol-4-yl)-1H-pyrrole-

3-carbonitrile], developed by Syngenta, is a high-efficiency and broad-spectrum phenylpyrrole fungicide widely used for the control of various fungal diseases in crops pre- and postharvest, as well as for seed treatment (Gehmann et al., 1990; Diskin et al., 2019). The mechanism of action of fludioxonil is not fully understood at present, and the possibility is that fludioxonil, by binding to the group III histidine kinase Os1, mimics an osmotic stress through the activation of the Os-2/Hog1 mitogen-activated protein kinase (MAPK) (Kojima et al., 2004; Bersching and Jacob, 2021). This activation subsequently triggers multiple downstream reactions, such as the initiation of HC-ATPase, KC-influx and glycerol biosynthesis, and ultimately leading to increased intracellular turgor and membrane potential, which causes mycelia to swell and burst (Lew, 2010).

Fludioxonil has been used for >30 years, but only few cases of resistance in the field have been reported to date (Kilani and Fillinger, 2016), despite the fact that resistant mutants could easily be obtained for many fungal species (*B. cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus nidulans*, *Neurospora crassa*, *Ustilago maydis*) through continual exposure

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to high concentrations of fludioxonil (Vignutelli et al., 2002; Avenot et al., 2005; Taiwo et al., 2021). The field and laboratory mutants exhibit high-level resistance to fludioxonil, which is often associated with sensitivity to hyper-osmolarity and cross-resistance to dicarboximide fungicides (Leroux et al., 2002). In addition, most field and laboratory mutants, such as *B. cinerea* and *Alternaria brassicicola*, display developmental defects and reduced pathogenicity, which may be the main reason for the lack of field resistance (Ren et al., 2016; Sang et al., 2018). The majority of the reported fludioxonil-resistant mutants have point mutations in the group III histidine kinase Os1, but only few have been confirmed (Liu et al., 2023). The specific target of fludioxonil remains to be further studied.

We have been committed to the efficient control of gray mold disease and first reported the occurrence of fludioxonil resistance in China in 2016, and then in 2018 (Ren et al., 2016; Sang et al., 2018). In this study, we detected high-level fludioxonil resistance in gray mold disease in greenhouses of China in 2022, demonstrated that resistance was caused by new point mutation (D1158N) in the histidine kinase Bos1, and assessed the risk of resistance. Results of this study provide scientific guidance for the rational use of fludioxonil, and lay a foundation for elucidating the mechanism of action of fludioxonil.

2. Materials and methods

2.1. Fungal strains

The *B. cinerea* isolates were obtained by single-spore isolation from greenhouse cucumbers with typical symptom of gray mold in Shouguang City of Shandong Province in 2022. *B. cinerea* strain B05.10 and *F. graminearum* strain PH-1 were used as parental strains for transformation experiments.

2.2. Media and fungicides

Potato dextrose agar (PDA) medium (200 g potato, 20 g dextrose, and 15 g agar per

liter of tap water) was used for colony growth tests. Sterilized potato fragments were used for conidiation assays. Technical-grade fungicides fludioxonil (active ingredient: 97.9%), iprodione (active ingredient: 96.2%), and procymidone (active ingredient: 98%) were provided by the laboratory of fungicide biology in Nanjing Agricultural University.

2.3. Sensitivity test to fungicide

To determine fungicide sensitivity, mycelial plugs (5 mm in diameter) were taken from the growing edge of the 3-day-old colony and placed on the center of PDA plates amended with different concentrations of fungicides. After 3 days of incubation at 25 °C, the diameter of each colony was measured perpendicularly. The 50% effective concentration (EC₅₀) was calculated using the previously described method (Hou et al., 2018). The experiments were repeated 3 times independently.

2.4. Cloning and sequencing of Bos1 encoding gene

Genomic DNA of *B. cinerea* was extracted using the conventional cetyltrimethylammonium bromide (CTAB) method as described previously (Ren et al., 2018). Based on the genome database of *B. cinerea* (https://fungi.ensembl.org/Botrytis_cinerea/Info/Index), the open reading frame (ORF) of Bos1 encoding gene was amplified with the primers B1/B2 (Table S1). The resultant PCR products were sequenced directly and analyzed by using the BioEdit software.

2.5. Generation of point mutation mutants

To generate point mutation constructs, one portion of the Bos1

encoding gene was amplified with the primers B11/B12, and the other portion was amplified with the primers (artificially introduced point mutation) B13/B14 (Table S1), and these two portions were fused through double-joint PCR and inserted into the plasmid pNAN-OGG. The plasmid constructs were confirmed by PCR amplification and sequencing, and introduced into the protoplasts of *B. cinerea* using polyethylene glycol (PEG) mediated transformation method.

2.6. Determination of biological fitness

To determine the fitness of the *B. cinerea* isolates, 3 randomly selected sensitive isolates (Sg-3, Sg-19, and Sg-25) and 3 fludioxonil-resistant isolates (Sg-17, Sg-38, and Sg-52) were inspected for some biological characteristics, including mycelial growth, conidiation, and pathogenicity, according to the previously described methods (Ren et al., 2016).

3. Results

3.1. Monitoring of fludioxonil resistance in field gray mold disease

A total of 87 single-spore isolates of *B. cinerea* were obtained from cucumbers grown in different greenhouses, and the EC₅₀ values were mainly concentrated ranged from 0.005 to 0.03 µg/mL, while 3 isolates showed high-level resistance to fludioxonil with EC₅₀ values >20 µg/mL and minimal inhibitory concentration (MIC) > 50 µg/mL (Fig. 1), and the resistance frequency was 3.4%. In addition, the 3 fludioxonil-resistant isolates also showed high-level resistance to the dicarboximide fungicides iprodione and procymidone.

3.2. Comparison of Bos1 between fludioxonil-resistant and -sensitive isolates

Previous studies have reported that point mutations in histidine kinase os1 confers resistance to fludioxonil in fungal pathogens (Wang et al., 2021). Thus, we compared the Bos1 sequences of fludioxonil-resistant and -sensitive isolates, and the results showed that all the 3 fludioxonil-resistant isolates had a point mutation at codon 1158, GAC (Asp) → AAC (Asn) (Fig. 2A). Protein domain analysis revealed that the point mutation (D1158N) was located in the receiver domain of Bos1 (Fig. 2B).

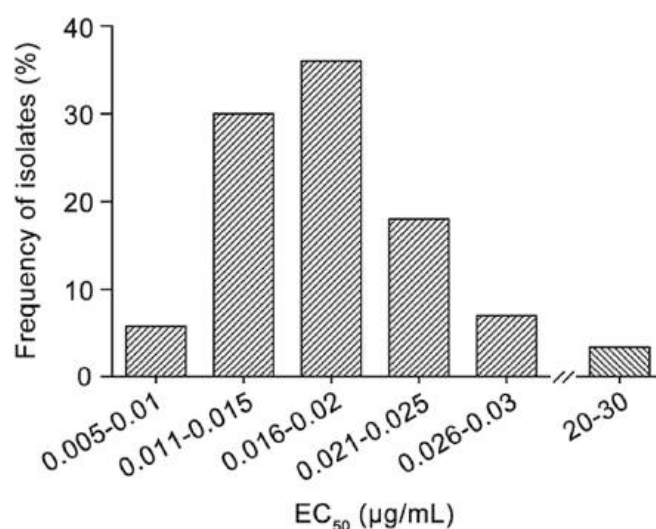


Fig. 1. Frequency distribution of fludioxonil EC₅₀ values of *Botrytis cinerea* isolates.

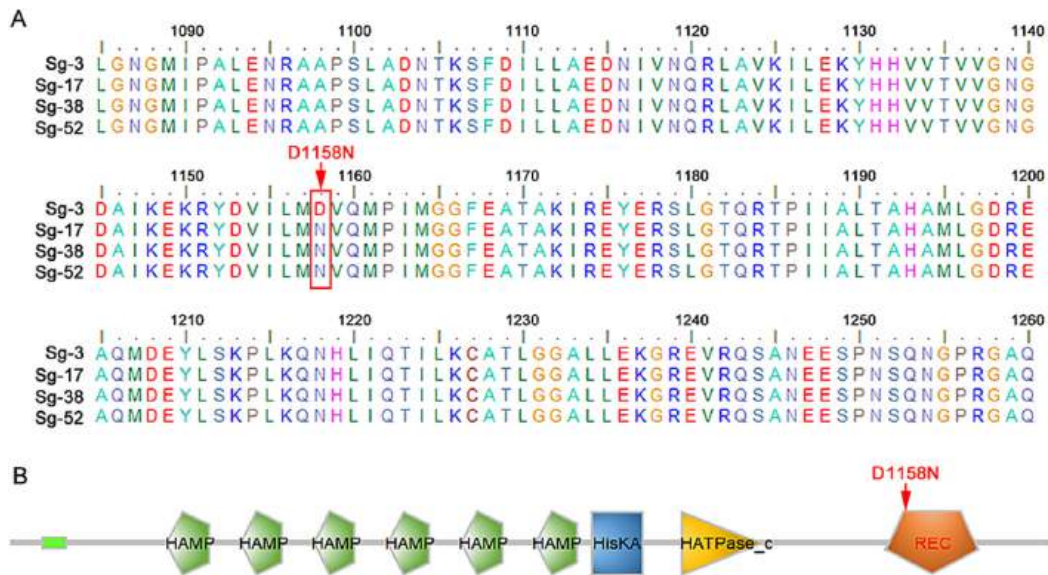


Fig. 2. Comparison and domain analysis of Bos1. (A) Alignment of partial amino acid sequences of Bos1 from fludioxonil-sensitive isolate Sg-3 and -resistant isolates Sg-17, Sg-38, and Sg-52. Amino acid changes are indicated by red box; (B) Schematic representation of Bos1 domains, including HAMP (Histidine kinase, Adenyl cyclases, Methyl binding proteins, Phosphatases), HisKA (Phosphoacceptor), HATPase_c (Histidine kinase-like ATPases), and REC (Receiver domain). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Bos1 point mutation results in high-level resistance to fludioxonil

To verify whether the high-level resistance of *B. cinerea* to fludioxonil was caused by the point mutation (D1158N) in Bos1, the wild-type (WT) or artificially constructed point-mutated Bos1 encoding gene (G3795A) was introduced into the Bos1 deletion mutant Δ Bos1, resulting in the mutants Bos1^{WT} and Bos1^{D1158N}, respectively. As shown in Fig. 3A, Δ Bos1 and Bos1^{D1158N} were able to grow on PDA plates amended with 50 μ g/mL fludioxonil, iprodione, or procymidone, while the parental strain B05.10 and Bos1^{WT} were sensitive to these fungicides. In addition, the homologous point mutation (D1105N) in Fos1 has also been shown to confer high-level resistance to fludioxonil, iprodione, and procymidone in *F. graminearum* (Fig. 3B), indicating that the universality of Bos1 (D1158N)-mediated fungal resistance to these fungicides.

3.4. Biological fitness analysis of the fludioxonil-resistant isolates

To assess the fitness of the field fludioxonil-resistant isolates, the mycelial growth, conidiation, and pathogenicity were determined. Compared with the fludioxonil-sensitive isolates, the fludioxonil-resistant isolates showed significant defects in mycelial growth rate, conidial production, and virulence (Table 1). Additionally, the fludioxonil-resistant isolates exhibited hypersensitivity to osmotic stress mediated by NaCl, KCl, or sorbitol (Fig. 4). These results suggest that the development of Bos1 (D1158N)-mediated resistance in field gray mold disease is accompanied by a fitness penalty.

4. Discussion

The gray mold fungus *Botrytis cinerea* is a high-risk pathogen for

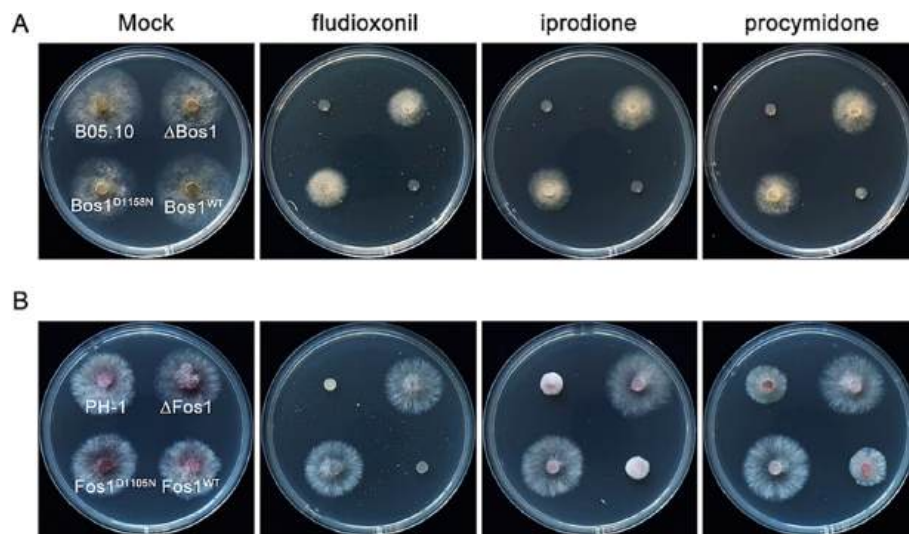


Fig. 3. Colony growth on PDA plates amended with 50 μ g/mL fludioxonil, iprodione, or procymidone. (A) *Botrytis cinerea* parental strain B05.10, Bos1 deletion mutant Δ Bos1, Δ Bos1 complemented with mutated Bos1 (D1158N) strain Bos1^{D1158N}, and Δ Bos1 complemented with wild-type Bos1 strain Bos1^{WT}; (B) *Fusarium graminearum* parental strain PH-1, Fos1 deletion mutant Δ Fos1, Δ Fos1 complemented with mutated Fos1 (D1105N) strain Fos1^{D1105N}, and Δ Fos1 complemented with wild-type Fos1 strain Fos1^{WT}.

Table 1
Biological characteristics of *Botrytis cinerea* isolates.

Isolate ^a	Growth (cm) ^b	Conidiation ($\times 10^6$)	Lesion size (cm ²)
Sg-3 (S)	5.36 \pm 0.03 a	2.84 \pm 0.14 a	1.73 \pm 0.11 a
Sg-19 (S)	5.42 \pm 0.05 a	2.87 \pm 0.12 a	1.76 \pm 0.14 a
Sg-25 (S)	5.39 \pm 0.04 a	2.91 \pm 0.16 a	1.82 \pm 0.15 a
Sg-17 (R)	5.06 \pm 0.06 b	2.52 \pm 0.15 b	1.46 \pm 0.12 b
Sg-38 (R)	4.92 \pm 0.04 b	2.48 \pm 0.14 b	1.51 \pm 0.14 b
Sg-52 (R)	4.18 \pm 0.03 c	1.76 \pm 0.12 c	1.12 \pm 0.12 c

^a S = fludioxonil-sensitive isolate, R = fludioxonil-resistant isolate.

^b Values are the means \pm standard deviations from three independent experiments. Values in each column followed by different letters are significantly different ($P < 0.05$) according to Fisher's least significant difference test.

fungicide resistance development and has developed resistance to various groups of fungicides (Sofianos et al., 2023). In 2016, we first found high-level resistant isolates of *B. cinerea* to fludioxonil in the field of Shandong province, and subsequently in Jiangsu province in 2018 (Ren et al., 2016; Sang et al., 2018). In this study, we detected high-level fludioxonil resistance of *B. cinerea* isolates in Shandong province in 2022, which also showed positive cross-resistance to dicarboximide fungicides iprodione and procymidone that is consistent with the reports in other fungal pathogens (Zhou et al., 2020a, 2020b).

Resistance to fludioxonil was previously found to be associated with mutations in histidine kinase os1 or overexpression of the drug efflux pump atrB activated by mutations in the transcription factor mrr1 in *B. cinerea* (Kretschmer et al., 2009; Grabke et al., 2014; Hu et al., 2019; Wen et al., 2022). The point mutations mainly in the HAMP domain of os1, such as F127S, G262S, G265D, G311R, R319K, I365S, Q369P, N373S, S531G, G545E, N609T have been found in the field fludioxonil-resistant *B. cinerea* mutants, but none of the mutations have been confirmed experimentally (Ochiai et al., 2001; Zhou et al., 2020a, 2020b; Wang et al., 2021; Chen et al., 2022; Oiki et al., 2022). It is worth noting that all the fludioxonil-resistant isolates of *B. cinerea* in this study had a point mutation (D1158N) in the receiver domain of Bos1, and no mutation was found in mrr1, and the point mutation (D1158N) was

proved to be the reason for fludioxonil resistance. Furthermore, the homologous point mutation (D1105N) in Fos1 has also been shown to confer high-level resistance to fludioxonil, iprodione, and procymidone in *F. graminearum*, indicating that the universality of Bos1 (D1158N)-mediated fungal resistance to these fungicides. Therefore, the new findings are of great significance for comprehensively elucidating the mechanism of action of fludioxonil.

Fludioxonil has been used for over 30 years, but there have been very few cases of field resistance, which may primarily be attributed to a significant decrease in the biological fitness of resistant strains (Kilani and Fillinger, 2016). Similarly, in this study, the fludioxonil-resistant isolates showed significantly decreased biological fitness compared with the sensitive isolates, which was consistent with our reports about fludioxonil resistance in *B. cinerea* (Ren et al., 2016; Sang et al., 2018). In addition, like in other fungal pathogens (Kilani and Fillinger, 2016), the fludioxonil-resistant isolates exhibited hypersensitivity to osmotic stress mediated by NaCl, KCl, or sorbitol. Based on these results, it is suspected that the evolution of fludioxonil resistance in fungal populations is limited to some extent unless additional mutations compensate for the penalty of fitness.

Collectively, in this study, we found for the first time that a new point mutation (D1158N) in Bos1 confers high-level resistance to fludioxonil in field gray mold disease, and the risk of resistance is relatively high. Therefore, it is necessary for farmers and agricultural advisors to stay informed about the resistance development, and fludioxonil, as well as iprodione and procymidone should be mixed with other fungicides or used at intervals, so as to extend the service life and avoid control failures.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pestbp.2023.105750>.

CRediT authorship contribution statement

Weichao Ren: Conceptualization, Funding acquisition, Investigation, Writing – original draft. **Wenjiao Han:** Data curation, Formal analysis, Investigation, Methodology. **Tinghua Huan:** Data curation,

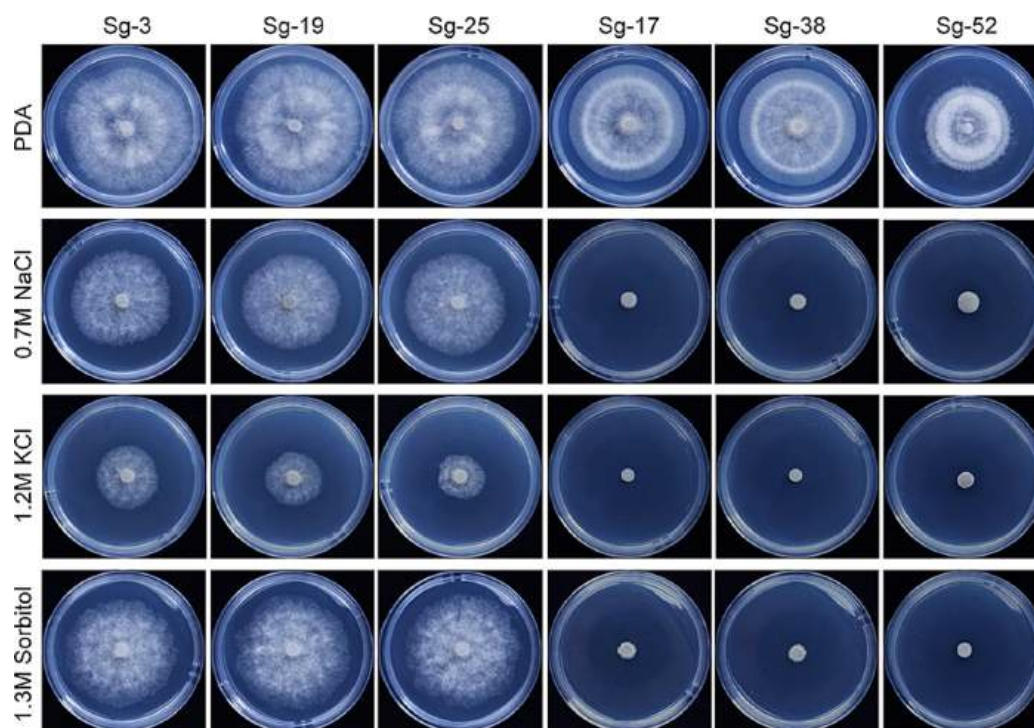


Fig. 4. Sensitivity test to osmotic stress. The fludioxonil-sensitive isolates Sg-3, Sg-19, and Sg-25 and fludioxonil-resistant isolates Sg-17, Sg-38, and Sg-52 were grown on PDA containing 0.7 M NaCl, 1.2 M KCl, or 1.3 M sorbitol, and incubated at 25 °C for 3 days.

Investigation, Software, Validation. **Meiqi Zhu**: Formal analysis, Software, Validation, Visualization. **Yihan Zhang**: Formal analysis, Investigation, Writing – original draft. **Baohua Li**: Funding acquisition, Resources, Supervision. **Na Liu**: Project administration, Supervision, Writing – review & editing.

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