Characterization of the Resistance Mechanism and Risk of *Fusarium verticillioides* to the Myosin Inhibitor Phenamacril

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ABSTRACT

Fusarium verticillioides is a major pathogen of maize that causes ear rot and produces mycotoxins. Phenamacril is a novel cyanoacrylate fungicide that exhibits favorable activity against *Fusarium* species. In this study, the phenamacril-resistant mutants of *F. verticillioides* were obtained by ultraviolet mutagenesis. Single point mutations of S73L or E276K in the myosin-1 FvMyo1 were proven to be responsible for the high-level resistance of *F. verticillioides* to phenamacril. Phenamacril had a significant impact on the localization of the wild-type FvMyo1 (FvMyo1^{WT}-green fluorescent protein [GFP]), but not on the mutated

FvMyo1 (FvMyo1^{S73L}-GFP and FvMyo1^{E276K}-GFP) at the hyphal tips. Molecular docking analysis suggested that mutation (S73L or E276K) in FvMyo1 altered the binding mode and decreased the binding affinity between phenamacril and myosin-1. There was no significant fitness penalty in mycelial growth, conidiation, and virulence of *F. verticillioides* associated with resistance to phenamacril. The results will enhance our understanding of the resistance mechanism of *F. verticillioides* to phenamacril and provide new reference data for the management of maize ear rot.

Maize (*Zea mays* L.), also known as corn, is one of the most important crops in the world and has a wide variety of uses, including food, feed, and industrial material (Ranum et al. 2014). *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) is one of the most common pathogens of maize that causes ear and stalk rot of maize worldwide (Munkvold 2003). In addition to high yield losses, *F. verticillioides* can also synthesize mycotoxins (most notably, fumonisins) in infested corn (Rheeder et al. 2002; Seo et al. 2001). The structure of fumonisins is similar to that of sphinganine and they disrupt sphingolipid metabolism by inhibiting ceramide synthase (Nelson et al. 1993). Elevated levels of fumonisins in naturally contaminated corn have been associated with high incidences of several animal diseases as well as cancer in humans (Fink-Grenmels 1999; Norred and Voss 1994). Despite its enormous negative impact on corn production, *F. verticillioides* is not adequately controlled with currently available commercial fungicides, resistant maize cultivars, or cultural practices. Therefore, the development of research-supported effective control measures is urgently needed.

The cyanoacrylate fungicide phenamacril (experimental code JS399-19; active ingredient: 2-cyano-3-amino-3-phenylacrylic acetate), developed by the Jiangsu Branch of the National Pesticide Research and Development Center of China, is a *Fusarium*-specific fungicide (Li et al. 2008). In China, phenamacril has been registered for controlling wheat head blight (WHB) and rice bakanae caused by *F. graminearum* and *F. fujikuroi*, respectively, since 2012 (Hou et al. 2018). Phenamacril exhibits satisfactory control efficiency and

deoxynivalenol (DON) suppression for WHB in the field (Tang et al. 2018). For many years, phenamacril-resistant (PR) strains of *F. fujikuroi* and *F. graminearum* were not found in the field. However, resistant strains of *F. fujikuroi* were isolated from a rice field in Zhejiang Province in 2016 (Hou et al. 2018). Previous studies showed that phenamacril showed moderate to high resistance risk for controlling *F. graminearum*, *F. fujikuroi*, and *F. oxysporum* f. sp. *melonis* (Hou et al. 2018; Li et al. 2016; Zhang et al. 2018). Previous studies showed that the class I myosin-5 was the target of phenamacril and mutations in myosin-5 conferred resistance to the fungicide in *F. graminearum*, *F. fujikuroi*, and *F. oxysporum* f. sp. *melonis* (Hou et al. 2018; Zhang et al. 2015, 2018; Zheng et al. 2015). In addition, myosin-5 interacts with Tril and actin and participates in DON-toxisome formation in *F. graminearum* (Tang et al. 2018).

Given its good efficacy on *Fusarium* species, phenamacril is considered as a potential alternative to control *F. verticillioides*. Yet the biology of phenamacril resistance in *F. verticillioides* is still unknown. In this study, the molecular basis of *F. verticillioides* to phenamacril was characterized, and the resistance risk was preliminarily assessed.

MATERIALS AND METHODS

Strains, media, and fungicides. *F. verticillioides* strain 7600 (FGSC7600) was used as the parental strain for phenamacril-resistance mutagenesis. Potato dextrose agar (PDA; 200 g of potato, 20 g of glucose, 15 g of agar, and 1 liter of water) was used for mycelial growth and colony maintenance. Carboxymethyl cellulose (CMC; 1 g of NH₄NO₃, 1 g of KH₂PO₃, 0.5 g of MgSO₄·7H₂O, 1 g of yeast extract, 15 g of carboxymethylcellulose, and 1 liter of water) was used for conidiation. Phenamacril (95% active ingredient) was provided by Jiangsu Pesticide Research Institute Co., Ltd. and dissolved in dimethylsulfoxide to make a stock solution (10 mg ml⁻¹). Tebuconazole (97%) was provided by Jiangsu Pesticide Research Institute Co., Ltd. and dissolved in methanol to make a stock solution (10 mg ml⁻¹). Carbendazim (98%) was provided by Jiangsu Lanfeng Biochemical Limited Co., Ltd. and dissolved in 0.1 M of hydrochloric acid to make a stock solution (10 mg ml⁻¹).

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Generation of PR mutants. To obtain PR mutants, 1×10^6 conidia of strain 7600 were spread onto PDA plates supplemented with $100 \mu\text{g ml}^{-1}$ of phenamacril and exposed to ultraviolet (UV) light for 90 s. After UV radiation, the plates were incubated at 25°C for 7 days, and the PR colonies growing on the plates were obtained. A total of 12 independent single-spore PR colonies were isolated for *FvMYO1* sequence analysis.

Cloning and sequence analysis of *FvMYO1*. Genomic DNA of *F. verticillioides* was extracted using the conventional cetyltrimethylammonium bromide method (Möller et al. 1992). Based on the fungal genome database (<https://fungidb.org/fungidb/>), the open reading frames (ORFs) of myosin-1 gene *FvMYO1* were amplified from the sensitive and resistant strains with the primer pair P1/P2 (Supplementary Table S1). The resultant PCR products were sequenced directly, and the sequences were aligned with BioEdit software.

Construction of the *FvMyo1*-GFP fusion cassette and microscopy. To observe the localization of *FvMyo1* in *F. verticillioides*, the *FvMYO1* gene ORF including the native promoter region was fused with carboxy-terminal green fluorescent protein (GFP) and transformed into wild-type (WT) strain 7600 by the polyethylene glycol-mediated method as described previously (Shim and Woloshuk 2001). *FvMyo1*-GFP fluorescence signals in fresh hyphae of each strain harvested from yeast extract peptone dextrose medium amended with or without $2 \mu\text{g ml}^{-1}$ of phenamacril were examined with the Zeiss LSM780 confocal microscope (Carl Zeiss AG, Jena, Germany).

Sensitivity test to different fungicides. For the mycelial sensitivity assays, mycelial plugs were placed on PDA plates amended with phenamacril at 0, 0.5, 1, 2, 4, and $8 \mu\text{g ml}^{-1}$ or at 0, 25, 50, 100, 200, and $400 \mu\text{g ml}^{-1}$, with carbendazim at 0, 0.1, 0.2, 0.4, 0.8, and $1.6 \mu\text{g ml}^{-1}$, and with tebuconazole at 0, 0.01, 0.02, 0.04, 0.08, and $0.16 \mu\text{g ml}^{-1}$. After cultures were kept at 25°C for 4 days, the radial growth on each plate was measured perpendicularly and averaged to calculate the percentage of growth inhibition. For the conidial sensitivity assays, $5\text{-}\mu\text{l}$ conidia suspensions ($1 \times 10^5 \text{ ml}^{-1}$) of each strain were incubated on 12-well plates containing PDA with phenamacril at 0, 1, 5, 10, 50, 100, 200, and $400 \mu\text{g ml}^{-1}$. Photographs were taken after incubation at 25°C for 2 days. The effective concentration for 50% inhibition of mycelial growth (EC_{50}) was calculated by the regression of the probit of the percentage of inhibition of radial growth against the logarithmic value of phenamacril concentration. The experiments were performed three times independently.

Determination of biological fitness. To determine the mycelial growth rate, the PR mutants (transformed mutants and UV mutants) and corresponding parental strain were placed on PDA plates. After incubation at 25°C for 4 days, the colony diameters were measured perpendicularly and averaged. There were three replicates in each test and the experiment was repeated three times independently.

To determine conidiation, an equal amount of mycelia from each strain was cultured in an equal volume of CMC medium. After incubation at 25°C for 4 days in a shaker (180 rpm), conidia were quantified with a hemocytometer. The experiment was repeated three times independently.

To determine virulence, conidia of each strain were collected and adjusted to a concentration of 1×10^6 conidia ml^{-1} . Ten microliters of conidial suspension was used to inoculate maize stalks via a hole punched in the stem. The control maize stalks were inoculated with $10 \mu\text{l}$ of sterilized water. Each strain was represented by 10 replicates. After inoculation, the maize stalks were maintained at $25 \pm 2^\circ\text{C}$ under 80% relative humidity and a 12-h/12-h light/dark cycle. After 15 days, the disease lesion sizes on maize stalks were measured and averaged. The experiment was repeated four times independently. Data analysis was carried out with SPSS 20 software (IBM). Significance tests adopted least significant difference and Duncan methods. Error bars in the figures represent the standard deviations from three independent

experiments and values on the bars followed by the same letter are not significantly different at $P = 0.05$.

Molecular docking analysis. The protein sequence of *FvMyo1* (XP_018756374.1) was obtained from the NCBI database. The three-dimensional structure of myosin-1 was built by the crystal structure of the human *Mus musculus* Myosin-V (Protein Data Bank identifier 2DFS) by using the SWISS-MODEL web server (Liu et al. 2006; Waterhouse et al. 2018). The binding mode of phenamacril in the binding site of myosin-1 was predicted by AutoDock (version 4.2.6) (Morris et al. 2009). The proteins and ligand were prepared by AutoDockTools software (version 1.5.6). A grid box size of $60 \times 60 \times 60$ dimensions, with a spacing of $0.375 \text{ \AA}$ between the grid points, was implemented and covered almost the entire actin binding cleft of myosin-1. The Lamarckian genetic algorithm (LGA) was utilized for global conformational sampling. In the LGA runs, trials of 200 dockings and the number of energy evaluations were set to 2,500,000, and other settings were set as default. The lowest binding energy conformation was considered for further analysis.

RESULTS

Generation of *F. verticillioides* mutants resistant to phenamacril. A total of 12 PR mutants were obtained by UV mutagenesis, selecting for strains that grew well on PDA plates amended with $100 \mu\text{g ml}^{-1}$ of phenamacril after 3 days of incubation. In contrast, WT strain 7600 failed to grow even at $15 \mu\text{g ml}^{-1}$.

Myosin-1 gene sequence analysis in PR mutants. The myosin-1 gene *FvMYO1* (FVEG_09486) (Fig. 1A) from all PR mutants was sequenced and aligned with *FvMYO1* of WT strain 7600. The PR mutants contained two types of single point mutation in *FvMYO1*, designated as PR-I and PR-II. For PR-I mutants, codon175 (TCA) for serine was changed to a codon (TTA) for leucine. For PR-II mutants, codon276 (GAG) for glutamic acid was changed to a codon (AAG) for lysine (Fig. 1B). Both of the mutation sites were located in the motor domain of myosin-1.

Mutation in myosin-1 confers resistance of *F. verticillioides* to phenamacril. To verify whether the resistance of *F. verticillioides* to phenamacril is caused by a point mutation (S73L or E276K) in *FvMyo1*, the constructs encoding the WT (without mutations) or individual-point mutated (C218T or G826A) *FvMYO1* were introduced into WT strain 7600, resulting in strains *FvMyo1*^{WT}, *FvMyo1*^{S73L}, and *FvMyo1*^{E276K}, respectively. As shown in Figure 2, both *FvMyo1*^{S73L} and *FvMyo1*^{E276K} strains exhibited lower sensitivity to phenamacril and were able to grow on PDA plates amended with $400 \mu\text{g ml}^{-1}$ of phenamacril, which was consistent with the PR mutants (PR-I and PR-II), whereas *FvMyo1*^{WT} remained sensitive to phenamacril and failed to grow even at $30 \mu\text{g ml}^{-1}$. In addition, similar results were observed in the conidial sensitivity assays (Fig. 3). Notably, mutations in myosin-1 did not cause significant changes in

Fig. 1. Structure and sites of the mutation in myosin-1 associated with phenamacril resistance in *Fusarium verticillioides*. **A**, Structure of the *FvMyo1* protein. The conserved motor domain, myosin tail homology 1 (TH1), and Src homology domain 3 (SH3) are highlighted. The mutation sites Ser73 and Glu276 are indicated with triangles. Bar represents 100 amino acids (aa). **B**, Alignment of partial amino acid sequences of *FvMyo1* from the parental strain 7600 and the phenamacril-resistant (PR) mutants PR-I and PR-II. The amino acid changes in phenamacril-resistant mutants are indicated with boxes.

sensitivity toward other fungicides with different modes of action, such as carbendazim and tebuconazole (Table 1). Collectively, these results suggested that point mutations S73L or E276K in FvMyo1 conferred specific resistance to phenamacril in *F. verticillioides*.

Fig. 2. Mycelial growth of *Fusarium verticillioides* strains on potato dextrose agar (PDA) plates amended with phenamacril. The parental strain 7600, the laboratory-induced phenamacril-resistant (PR) mutants PR-I and PR-II, the artificially constructed strains FvMyo1^{S73L} (7600 transformed with the mutated *FvMYO1* containing the point mutation S73L), FvMyo1^{E276K} (7600 transformed with the mutated *FvMYO1* containing the point mutation E276K), and FvMyo1^{WT} (7600 transformed with the wild-type *FvMYO1*) were grown on PDA plates amended with serial concentrations of phenamacril for 2 days at 25°C. DMSO = dimethylsulfoxide.

Fig. 3. Conidial germination of *Fusarium verticillioides* strains on potato dextrose agar (PDA) plates amended with phenamacril. Conidia of each strain were cultured on PDA plates amended with serial concentrations of phenamacril for 2 days at 25°C. DMSO = dimethylsulfoxide. PR = phenamacril resistant and WT = wild type.

Subcellular localization analysis of FvMyo1-GFP. To explore the effect of phenamacril on FvMyo1 location in mycelium, the WT and mutated *FvMYO1* fused with GFP were transformed into WT strain 7600, respectively. Microscopic examination showed that strong fluorescent signals of FvMyo1^{WT}-GFP were observed at the hyphal tips and diminished dramatically after the treatment with 2 µg ml⁻¹ of phenamacril for 4 h (Fig. 4). However, the fluorescent signals at the hyphal tips of mutated proteins FvMyo1^{S73L}-GFP and FvMyo1^{E276K}-GFP were not affected dramatically by phenamacril treatment (Fig. 4). These results indicated that mutation (S73L or E276K) in FvMyo1 can overcome the dislocation caused by phenamacril.

Analysis of binding affinity between myosin-1 and phenamacril. The interactions between myosin (WT/S73L/E276K) and phenamacril were evaluated by molecular docking analysis. The docking results indicated that phenamacril formed four hydrogen bonds with the residues of Leu-70, Ser-73, and Lys-393 in the WT myosin (Fig. 5A). However, phenamacril in S73L myosin exhibited a significant difference from the WT pattern that forms three hydrogen bonds with the residues of Gly-77, Ile-79, and Glu-276 (Fig. 5B). In addition, phenamacril in S73L myosin showed obvious movement out of the binding pocket, which may be attributable to the loss of the two important hydrogen bonds between phenamacril and Ser-73 that resulted in the instability of phenamacril in the binding site. As for the mutation of E276K, the increase in the amino acid length of Glu-276 to Lys-276 may hinder the binding of phenamacril to the active site (Fig. 5C).

Biological fitness analysis. To assess the fitness of PR mutants, mycelial growth rate, conidiation, and virulence were determined. Compared with WT strain 7600, the UV-induced resistant mutants showed a significant decrease in mycelial growth, conidiation, and virulence (Fig. 6; Table 2). However, the artificially constructed point mutation strains exhibited similar phenotypes to WT strain 7600 (Fig. 6; Table 2). These results indicated that there was no significant fitness penalty of *F. verticillioides* associated with resistance to phenamacril.

DISCUSSION

Phenamacril exhibits high antifungal activity against *Fusarium* species, and it has great potential for registration for controlling corn ear rot mainly caused by *F. verticillioides*. To date, there is no report on the biology of phenamacril against *F. verticillioides*. In this study, we investigated the resistance mechanism and assessed the resistance risk of *F. verticillioides* to phenamacril.

Like other *Fusarium* species, phenamacril displayed strongly inhibitory activity against mycelial growth and conidial germination of *F. verticillioides*. Considering that *F. verticillioides* can be seed transmitted and cause systemic maize infection (Wilke et al. 2007), phenamacril is likely to be developed as a seed coating formulation.

Previous studies showed that phenamacril resistance in *F. graminearum* was caused by a mutation in myosin-5 (Zhang et al. 2015; Zheng et al. 2015). For the laboratory-induced resistant mutants of *F. graminearum*, mutations at different sites resulted in different resistance levels, and the single point mutations A135T,

TABLE 1. Sensitivity (EC₅₀/MIC) of *Fusarium verticillioides* strains to phenamacril, carbendazim, and tebuconazole^z

Strain	EC ₅₀ (µg ml ⁻¹)			
	Phenamacril	Carbendazim	Tebuconazole	Phenamacril MIC (µg ml ⁻¹)
7600	2.18 ± 0.17 a	0.42 ± 0.057 a	0.027 ± 0.0023 a	<15
PR-I	138.54 ± 1.37 d	0.42 ± 0.059 a	0.029 ± 0.0027 a	>800
PR-II	140.73 ± 1.43 d	0.43 ± 0.071 a	0.030 ± 0.0038 a	>800
FvMyo1 ^{S73L}	115.69 ± 1.29 c	0.41 ± 0.048 a	0.029 ± 0.0034 a	>800
FvMyo1 ^{E276K}	117.35 ± 1.33 c	0.42 ± 0.062 a	0.028 ± 0.0036 a	>800
FvMyo1 ^{WT}	13.41 ± 0.86 b	0.43 ± 0.065 a	0.029 ± 0.0041 a	<30

^z Values are the means ± standard deviations of three repeats. Values in a column followed by the same letters are not significantly different at *P* = 0.05. EC₅₀ = concentration causing 50% growth inhibition, MIC = minimal inhibitory concentration, PR = phenamacril resistant, and WT = wild type.

V151M, P204S, I434M, A577T, R580G/H, or I581F caused low resistance; the single point mutations S418R, I424R, and A577G caused moderate resistance; and the single point mutations K216R/E, S217P/L, and E420K/G/D caused high resistance (Li et al. 2016). In *F. fujikuroi*, only two types of point mutation, S219P/L, in myosin-5 were found in the field-resistant mutants (Hou et al. 2018). For the laboratory-induced resistant mutants of *F. oxysporum* f. sp. *melonis*, S175L may mediate resistance toward phenamacril (Zhang et al. 2018). Amino acid substitutions (Val₁₅₁ to Ala and Ser₄₁₈ to Thr) in the FvMyo5 motor domain led to natural resistance to phenamacril in *F. oxysporum* (Zheng et al. 2018). In this study, we obtained 12 PR strains by UV mutagenesis, which showed two types of mutations: S73L (83%) and E276K (17%). Both types of mutation conferred high-level resistance to phenamacril, which is consistent with the homologous loci mutation for phenamacril resistance in *F. graminearum* (Zhang et al. 2015; Zheng et al. 2015).

Proteins can only function properly if they are in the right location (Čmielová and Řezáčová 2011). In this study, the subcellular localization of the WT FvMyo1 protein was affected dramatically by phenamacril, whereas the effects of phenamacril on the mutated FvMyo1 protein (S73L or E276K) were not significant. Thus, the inhibitory activity of phenamacril against *F. verticillioides* may be achieved by interfering myosin-1 distribution. In most cases, resistance is related to point mutations in the target gene, which resulted in changes of amino acid sequences at the fungicide binding site (Ma and Michailides 2005). In this study, the binding affinity between phenamacril and the WT FvMyo1 protein was significantly higher than that between phenamacril and the mutated FvMyo1 protein (S73L or E276K), which may be the main reason for the resistance of *F. verticillioides* to phenamacril.

The biological fitness of resistant mutants is an extremely important parameter regarding the risk for development of resistance (Hawkins and Fraaije 2018). For the laboratory-

induced PR mutants of *F. graminearum* and *F. oxysporum* f. sp. *melonis*, there is no significant relationship between phenamacril resistance and fitness (Li et al. 2016; Zhang et al. 2018). In field populations of PR mutants of *F. fujikuroi*, point mutation S219P in myosin-5 did not result in a significant fitness penalty compared with sensitive strains. However, the fitness of resistant strains conferred by the S219L mutation was less compared with sensitive strains (Hou et al. 2018). In this study, both types of UV-induced PR strains showed a decrease in biological fitness, including mycelial growth, conidiation, and virulence. However, the artificially constructed strains exhibited similar phenotypes to the WT strain. In addition to having mutations that increased resistance, PR-I and PR-II may have had other mutations that affected fungal growth and development, and point mutations (S73L and E276K) in myosin-1

Fig. 4. Subcellular localization of FvMyo1 in *Fusarium verticillioides*. Mycelia expressing the wild-type FvMyo1 (FvMyo1^{WT}-GFP) and mutated FvMyo1 (FvMyo1^{S73L}-GFP and FvMyo1^{E276K}-GFP) were incubated in liquid yeast extract peptone dextrose for 24 h and then treated with or without phenamacril (2 µg ml⁻¹) for 4 h. The boxes indicate the hyphal tip. Scale bar = 10 µm. DMSO = dimethylsulfoxide and GFP = green fluorescent protein.

Fig. 5. Molecular docking analysis of phenamacril in the activity cavity of FvMyo1. The three-dimensional binding poses between phenamacril and **A**, the wild-type (WT) FvMyo1, **B**, mutated FvMyo1^{S73L}, and **C**, FvMyo1^{E276K}. The right black box indicates the zoom-in view of the predicted binding site of FvMyo1. The key residues are shown in the stick representation and labeled by residue name and position.

are not at the expense of fitness in *F. verticillioides*. We therefore suggest that the resistance risk of *F. verticillioides* to phenamacril may be moderate to high. To avoid the rapid development of resistance to phenamacril in the field, growers should reduce the number of phenamacril applications and should apply phenamacril in mixtures with multisite fungicides or with fungicides that lack cross-resistance with phenamacril.

In conclusion, single mutation S73L or E276K in myosin-1 conferred high resistance to phenamacril in *F. verticillioides*, which was not at the expense of biological fitness. The results of this study

will provide a scientific basis for the registration of phenamacril for the control of maize ear rot and the subsequently rational application.

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Fig. 6. Mycelial growth of *Fusarium verticillioides* strains. **A**, Colony morphology of each strain that was grown on potato dextrose agar plates for 4 days at 25°C. **B**, Mycelial growth rate of each strain. Bars denote standard deviations from three experiments. Values on the bars followed by different letters are significantly different at $P = 0.05$. PR = phenamacril resistant and WT = wild type.

TABLE 2. Comparison of mycelial growth, conidiation and virulence in *Fusarium verticillioides* strains^a

Strain	Growth (cm)	Conidiation (×10 ⁶)	Lesion area (cm ²)
7600	7.51 ± 0.19 a	8.52 ± 0.42 a	21.34 ± 1.46 a
PR-I	7.12 ± 0.16 b	7.57 ± 0.38 b	18.63 ± 1.53 b
PR-II	7.06 ± 0.16 b	7.24 ± 0.33 b	17.18 ± 1.48 b
FvMyo1 ^{S73L}	7.48 ± 0.18 a	8.43 ± 0.47 a	21.29 ± 1.44 a
FvMyo1 ^{E276K}	7.41 ± 0.21 a	8.32 ± 0.39 a	20.95 ± 1.39 a
FvMyo1 ^{WT}	7.56 ± 0.24 a	8.65 ± 0.41 a	22.03 ± 1.47 a

^a Values are the means ± standard deviations of three repeats. Values in a column followed by the same letters are not significantly different at $P = 0.05$. PR = phenamacril resistant and WT = wild type.