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Myosin as a Selective Target for the Fungicide Phenamacril

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ABSTRACT

Phenamacril (development code number: JS399-19), i.e. 2-cyano-3-amino-3-phenylancryic acetate, is a *Fusarium*-specific fungicide that is especially effective against *Fusarium graminearum* and *F. moniliforme*. Its molecular target in *F. graminearum* is the protein myosin-5, and amino acid point mutations in myosin-5 confer resistance to the fungicide. This report describes recent progress in understanding the toxicology and mechanism of action of phenamacril.

INTRODUCTION

Fusarium head blight (FHB) or scab caused by *Fusarium graminearum* or other *Fusarium* species is one of the most common fungal diseases of cereal crops worldwide. Only a few cultivars with effective resistance to FHB are available, and fungicides for controlling the disease are limited. In China, carbendazim (MBC) and its mixtures with other fungicides are the main compounds used to control the disease. MBC-resistant field populations, however, have developed and are increasingly common.

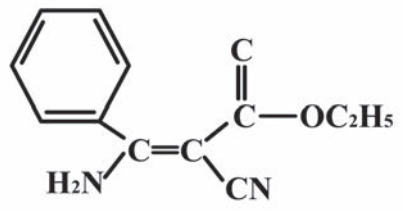

Figure 1 The chemical structure of phenamacril

Phenamacril (development code number: JS399-19), i.e. 2-cyano-3-amino-3-phenylancryic acetate (Figure 1), is a cyanoacrylate fungicide synthesized by the Pesticide Institute of Jiangsu Province, collaborating with Nanjing Agricultural University in studing the biology of the chemical. In China, phenamacril was temporarily registered in 2007 and formally registered for controlling Fusarium head blight (FHB) of wheat by spraying and rice bakanae disease by seed dressing in 2012.

ACTIVITIES AND RESISTANCES

Activity against Fusarium graminearum and Fusarium moniliforme *in vitro*

Phenamacril is a *Fusarium*-specific fungicide and is especially effective against *Fusarium graminearum, F. asiaticum* and *F. moniliforme* (Wang et *al.* 2004 and Zhang CQ et *al*. 2015), which are the main pathogens of FHB of wheat and of bakanae disease of rice, respectively. The 50% effective concentration (EC_{50}) of phenamacril against *F. graminearum* isolates averaged 0.126±0.027 (± SD) μg/ml and ranged from 0.108 to 0.141 μg/ml (Li et *al.* 2008); the mean EC_{50} was 4 times lower than that of MBC against *F. graminearumin in vitro*. Phenamacril was also active against *F. moniliforme* (EC_{50}= 0.459 μg/ml) but phenamacril was inactive against other fungi and oomycetes. For example, the EC_{50} values of phenamacril against *Phytophthora capsici*, *Alternaria solani*, and *Blumeria graminis* were >100 μg/ml (Table 1 and Figure 2) (Li et *al*. 2008). Treatment of a sensitive isolate of *F. graminearum* with this fungicide decreased the rate of conidial germination, strongly inhibited conidial germ tube growth, and increased the ratio of germ tubes that emerged from the basal parts of conidia to those emerging from the middle parts of conidia. Phenamacril also caused swelling and contorting of germ tubes of a sensitive isolate of *F. graminearum* (Chen et *al.* 2007).

Activity against Fusarium graminearum *in vivo*

Phenamacril failed to translocate basipetally in wheat but showed local systemic activity in leaves (Li and Zhou, 2006). In the greenhouse, phenamacril provided excellent protective and curative activity against *F. graminearum* when applied at various intervals (Li and Zhou, 2006). In the field, FHB control was better with phenamacril at 562.5 g ai ha^{-1} than with MBC at 750 g ai ha^{-1} (Li et *al.* 2008). The excellent FHB control provided by phenamacril makes it especially useful in areas of China where MBC-resistant populations of *F. graminearum* have developed. Compared to MBC, phenamacril significantly reduced the incidence of FHB-infected spikelets, reduced the amount fungal DNA in the grain, reduced the total DON content in the grain, and increased the 1000 grain weight (Zhang et *al.* 2009). Importantly, phenamacril, like azoxystrobin and tebuconazole delayed wheat senescence and increased wheat grain yield, but phenamacril was generally the most effective of the three fungicides (Zhang et *al.* 2010).

Resistance risk

Although phenamacril provided excellent control of FHB in the field, phenamacril-resistant mutants can be easily obtained in the laboratory. Through UV irradiation and through selection for resistance to the fungicide, 76 resistant mutants derived from five wild-type isolates of *F. graminearum* were obtained with an average frequency of 1.71×10^{-7}% with UV radiation and 3.5% with selection following fungicide exposure (Chen et *al.* 2008). These mutants could be divided into those with low resistance (LR), moderate resistance (MR), and high resistance (HR) based on EC_{50} values of 1.5–15.0 μg/ml, 15.1–75.0 μg/ml, and > 75.0 μg/ml, respectively.

Table 1 Toxicity of phenamacril (JS399-19) against 12 fungal plant pathogens that are economically important in agriculture (Li et *al.* 2008). Toxicity was based on *in vitro* growth inhibition.

Pathogen	EC_{50} (µg/ml)
F. asiaticum	0.141
F. moniliforme	0.459
F. oxysporum	3.565
Colletotrichum capsici	28.160
Dothiorella gregaria	39.690
Sclerotinia selerotiorum	72.070
Botrytis cinerea	72.188
Magnaporthe grisea	77.080
Phytophthora capsici	111.410
Alternaria solani	133.290
Pseudoperonospora cubensis	12.740
Blumeria graminis	>1000

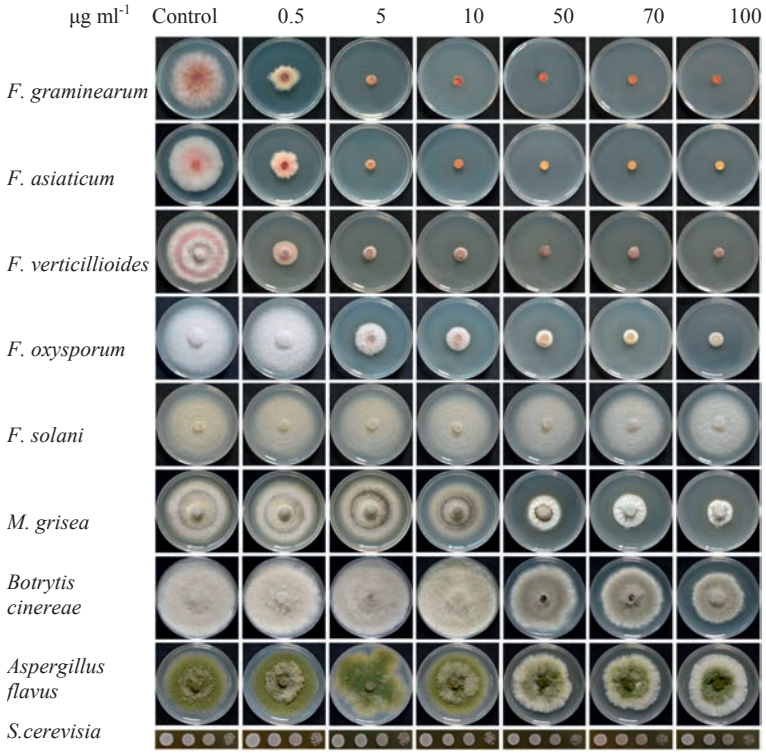

Figure 2 Antifungal activity of phenamacril (JS399-19) against *Fusarium graminearum, F. asiaticum, F. verticillioides, F. oxysporum, F. solani, Magnaporthe oryzae, Botrytis cinerea, Aspergillus flavus* and *Saccharomyces cerevisiae* (Zhang et *al.* 2015). The concentrations of phenamacril are indicated at the top of each column.

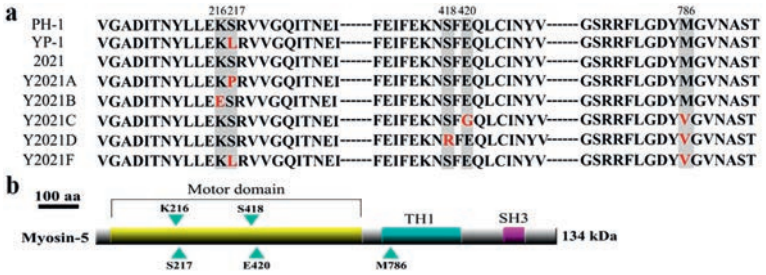

Figure 3 Phenamacril-resistant mutants of *Fusarium graminearum* contain point mutations at codon 216, 217, 418, 420, or 786 of myosin-5.(a): Alignment of the partial deduced amino acid sequences of myosin-5 of reference strain PH-1, resistant strain YP-1 deduced from PH-1, wild-type strain 2021, and its deduced resistant strains Y2021A, B, C, D, and F. The vertical boxes indicate the amino acid changes at codons 216, 217, 418, 420, and 786 that are responsible for phenamacril resistance. (b): Schematic presentation of *Fusarium graminearum* myosin-5. Sites of lysine, serine, glutamic acid, and methionine mutations are indicated by blue arrowheads. The conserved motor domain, the myosin tail (TH1), and the src homology domain 3 (SH3) are highlighted by yellow, blue, magenta coloured bars (Zheng et *al.* 2015).

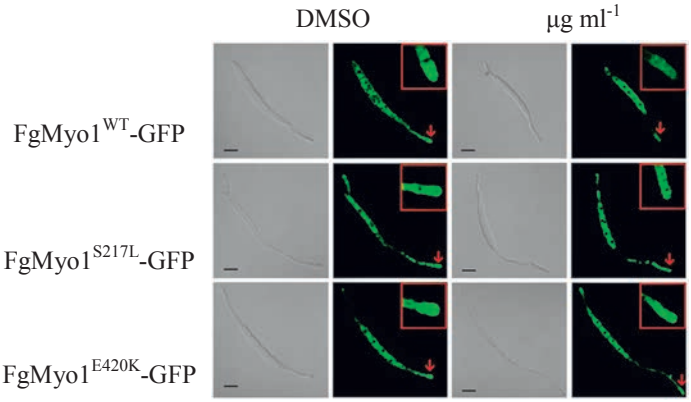

Figure 4 Localization and intensity of FgMyo1-GFP in germinating conidia of *Fusarium graminearum*. Conidia were incubated in 2% sucrose supplemented with DMAS (control) or DMSO containing 0.5 μg/ml phenamacril (JS399-19) at 25°C and were imaged after 3 h. The tips of germlings are shown at increased magnification in the insets. Phenamacril greatly affected the intensity of GFP-fluoresnece in the wild-type FgMyo1 (FgMyo1^{WT}-GFP) but not of the mutated FgMyo1 (FgMyo1^{S217L}-GFP and FgMyo1^{E420K}-GFP). Scale bars = 10 μm (Zhang et *al.* 2015).

There was no positive cross resistance between phenamacril and fungicides belonging to other chemical classes, such as benzimidazoles, ergosterol biosynthesis inhibitors, and strobilurins, suggesting that phenamacril has a new biochemical mode of action. Most of the resistant mutants had fitness levels comparable to their parental strains and had MR or HR levels of resistance (Chen et *al.* 2008). The authors concluded that *F. graminearum* has a high risk for development of resistance to phenamacril and that appropriate precautions against development of resistance in natural populations are needed.

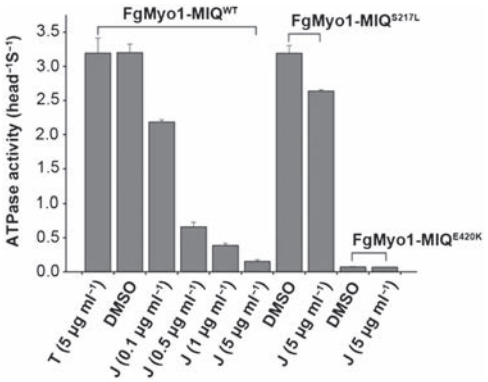

Figure 5 The actin-activated ATPase activity of the wild-type and mutated FgMyo1. An ATP regeneration system was used to determine the inhibition of the actin-activated ATPase activity in FgMyo1-MIQ^{WT}, FgMyo1-MIQ^{S217L}, and FgMyo1-MIQ^{E420K} by phenamacril (JS399-19, abbreviated 'J' on the X axis). Proteins were purified and adjusted to 0.1 μM (Molecular mass of the protein is about 85 KDa). The solvent dimethylsulphoxide (DMSO) and the fungicide tebuconazole (abbreviated 'T' on the X axis) were used as controls. Bars indicate + standard errors from three repeated experiments (Zhang et *al.* 2015).

Resistance mechanism

To determine the mechanisms underlying phenamacril resistance in *F. graminearum*, researchers used a nitrate-non-utilizing mutant (*nit*) as a genetic marker and made genetic crosses between lines differing in sensitivity levels, such as S (sensitive) × HR, MR × HR, and MR × S. The progeny fit a 1:1 segregation ratio of the two parental phenotypes. No segregation was observed in the crosses of S × S or HR × HR. The results suggested that the MR and HR phenotypes in *F. graminearum* were conferred by different allelic mutations within the same locus. In these isolates, resistance to phenamacril was not affected by other genes or cytoplasmic components (Chen et *al.* 2009). When isolates were treated with phenamacril, the transcript abundance level of motility-related proteins the 34 kDa subunit of the ARP2/3 complex and of diatom spindle kinesin1 significantly increased (Hou et *al.* 2013). The genomic DNA sequence of the phenamacril-resistant strain YP-1 was sequenced and analyzed. Compared with the *F. graminearum* reference strain PH-1, 132 genes in YP-1 showed a nucleotide mutation leading to amino acid exchanges. Of those genes, 22 related to actin function in *F. asiaticum*, a major causal agent of Fusarium head blight in China, were sequenced and compared between phenamacril-resistant isolates and their original sensitive wildtype strain 2021. In all resistant strains, mutations occurred in the gene encoding myosin-5 (FGSG_01410). The fact that mutations in myosin-5 confer resistance to phenamacril was confirmed by homologous exchange of the myosin-5 locus between a sensitive and a resistant strain (Figure 3) (Zheng et *al.* 2015). Based on a transcriptome analysis between sensitive and resistant strains, Zhang et *al*. (2015) also found that mutations in FgMyo1 (the same protein as myosin-5) confer resistance to phenamacril. The fluorescent signals at the tip of germ tubes of the wild-

type FgMyo1-GFP diminished dramatically after treatment with 0.5 µg/ml phenamacril for 3 h, while the GFP signals of the resistant strains carrying mutations in the Myo1 protein were not greatly affected by phenamacril treatment (Figure 4), indicating that phenamacril affects localization of the wild-type FgMyo1 but not of the mutated protein. In addition, phenamacril strongly inhibits the ATPase activity of the wild-type FgMyo1 but not of the mutated FgMyo1$^{S217L/E420K}$ (Figure 5) (Zhang et *al.* 2015). Differences in the sensitivity to phenamacril among pathogenic fungi were associated with the homology of their myosin-5 motor domains. The homologies of the *F. graminearum* myosin-5 and *F. verticilioides* and *F. oxysporum* myosin-5 sequence is high, and both species were sensitive to phenamacril (Figure 2). In contrast, the homology of the myosin-5 sequence of *F. graminearum* and *Botrytis cinerea*, *Magnaporthe oryzae*, and *Blumeria graminis* is low, and these three species were insensitive to phenamacril. These results indicated that variation in myosin-5 fully explains differences in the sensitivity to phenamacril.

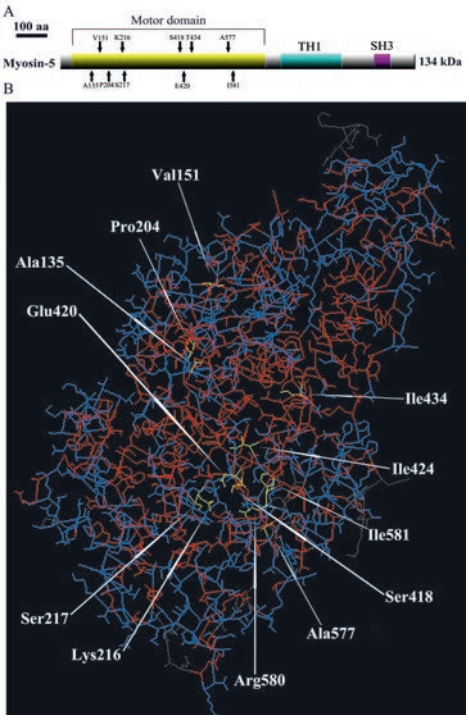

Figure 6 Schematic representation of homology modelling of the *Fusarium asiaticum* myosin-5 motor domain. (A) Sites of Ala135, Val151, Pro204, Lys216, Ser217, Ser418, Glu420, Ile424, Ile434, Ala577, Arg580, and Ile581 mutations are indicated by black arrowheads. The conserved motor domain, myosin tail (TH1), and src homology domain 3 (SH3) are highlighted. (B) Amino acids Ala135, Val151, Pro204, Lys216, Ser217, Ser418, Glu420, Ile424, Ile434, Ala577, Arg580, and Ile581of the myosin-5 motor domain are shown in yellow. Amino acids at these sites have been identified as mutations conferring phenamacril resistance in *F. asiaticum* (Li et *al.* 2016).

Table 2 Frequency and EC_{50} values of phenamacril-resistant mutants obtained from the sensitive *Fusarium asiaticum* strain 2021 (Li et *al.* 2016).

Resistance level[a]	Mutation position	Mutation type	Number of mutants[b]	Resistance frequency (%)	Mutants	EC_{50}
LR	Codon135	A→T	14	17.1	T13,T26,T64,T66,T111,T2-23, T2-25,T2-26,T2-35, T2-38, T2-39,T2-51,T2-75,T2-94	1.88–8.24
	Codon151	V→M	1	1.2	T93	2.17
	Codon204	P→S	1	1.2	T4	1.48
	Codon434	I→M	1	1.2	T2-60	2.95
	Codon577	A→T	1	1.2	T76	3.22
	Codon580	R→G	1	1.2	T54	12.41
		R→H	1	1.2	T86	2.53
	Codon581	I→F	1	1.2	T2-102	3.45
MR	Codon418	S→R	1	1.2	T40	33.96
	Codon424	I→R	2	2.4	T2-89,T2-56	18.54–20.11
	Codon577	A→G	3	3.7	T21,T2-14,T2-76	19.21–28.38
HR	Codon216	K→R	1	1.2	T79	192.05
		K→E	1	1.2	T122	186.16
		S→P	2	2.4	T105,T2-5	149.31–206.54
	Codon217	S→L	27	32.9	T1,T3,T10,T12,T18,T25,T51,T91 T99,T106,T2-1, T2-7, T2-16, T2-18,T2-29,T2-32,T2-37, T2-41,T2-48, T2-67, T2-69, T2-71,T2-85,T2-112, T2-117, T2-125,T2-127	91.17–167.40
	Codon420	E→K	22	26.8	T11,T14,T32,T48,T69,T73,T8, T95,T101,T102,T2-3,T2-9, T2-21,T2-61,T2-78,T2-81, T2-82,T2-86,T2-90,T2-93,T2-98, T2-107	259.41–448.88
		E→G	1	1.2	T50	209.50
		E→D	1	1.2	T112	232.18

[a]Resistance level was based on EC_{50} values: low-level resistance (LR, 1.5-15μg/ml), moderate-level resistance (MR, 15.1-75μg/ml), and high-level resistance (HR, >75μg/ml).

[b]The 82 mutants with the indicated mutation position and type were randomly selected from the 239 resistant mutants.

Genotypes of phenamacril-resistant mutants of *Fusarium asiaticum*

Phenamacril-resistant mutants of *F. asiaticum* were randomly selected and analyzed to determine the relationship between resistance level and mutations occurring in the myosin-5 gene. Of 82 resistant mutants, 25.6, 7.3, and 67.1% showed low resistance (LR), moderate resistance (MR), and high resistance (HR), respectively, to phenamacril as indicated by EC_{50} values (Table 2). LR includes is associated with the A135T, V151M, P204S, I434M, A577T, R580G/H, or I581F mutations. MR strains showed S418R, I424R, or A577G mutations, and HR strains had K216R/E, S217P/L, or E420G/D mutations. Interestingly, all of the mutations were located in the myosin-5 motor domain, and most of the mutations conferring high

resistance occurred at codons 217 and 420, representing the 'core region' of the motor protein (Figure 6) (Li et *al.* 2016). Homology modeling revealed that mutations distant from the 'core region' led to lower levels of resistance.

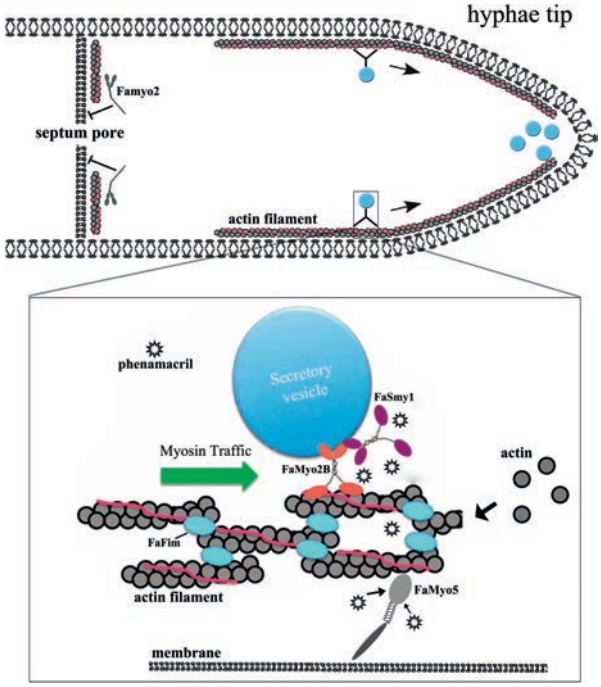

Figure 7 A hyphal tip cell with bilayered membrane. In the top diagram, myosin transports secretory vesicles to hyphal tips along the actin cables in the absence of phenamacril. Famyo2 controls septum development. In the bottom diagram, actin filaments in cables contain an actin-bundling protein (FaFim) and tropomyosin (pink), which maintain filament stability and organization. Myosin-V (FaMyo2B, red) transports FaSmy1 (purple) and secretory vesicles on actin cables to the hyphal tip. FaMyo5 (grey) triggers actin polymerization and movement of vesicles toward the hyphal tip. By binding to FaMyo5 in phenamacril-sensitive strains (the fungicide is indicated by asterisks), phenamacril disrupts transport along the actin cables. In phenamacril-resistant strains, binding of phenamacril to FaMyo5 and, hence, disruption of transport are reduced (Zheng et *al.* 2016).

Mediation of resistance

The mediation of phenamacril sensitivity and resistance by fimbrin, myosin class V myosin2B (FGSG_07469.1), myosin passenger protein Smy1p, and some ncRNAs was verified through disruption, deletion, and over-expression of these heterocaryotic genes. FgFim was considered to be a key protein conferring resistance to the fungicide. The FgFim deletion mutant of the sensitive strain became more sensitive to phenamacril, and the resistance level was lower in the FgFim deletion mutant of the resistant strain than in the resistant strain with FgFim (Zheng

et *al.* 2014). FaMyo2B acted jointly with FaSmy1 to affect resistance to phenamacril in *F. asiaticum* (Zheng et *al.* 2016). A model for phenamacril resistance in *F. asiaticum* was recently presented (Figure 7). According to this model and in the absence of phenamacril, the actin-bundling protein FaFim stabilizes the actin cable; FaMyo5 always triggers Arp2/3 complex-dependent actin polymerization and travels toward the hyphal tip along the actin cable; FaMyo2B and FaSmy1 (the myosin passenger protein) transport secretory vesicles along the actin cable; and Famyo2 maintains cell wall integrity and controls septum development. When a phenamacril-sensitive strain is treated with the fungicide, phenamacril binds to FaMyo5 and inhibits ATPase activity of the FaMyo5 motor domain, thereby reducing actin polymerization and the transport of secretory vesicles along the actin cable. This can greatly disrupt cell function and hyphal growth. Resistance to phenamacril results from mutations in myosin-5, which apparently reduces binding of the fungicide to FaMyo5. While mutations in FaMyo5 result in phenamacril resistance, the disruption of FaMyo2B and deletion of FaSmy1 significantly reduced the phenamacril resistance that had resulted from the disrupted transport of secretory vesicles in the phenamacril-resistant strain (Y2021A).

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