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Phenotypic Analyses of Fenhexamid Resistant *Botrytis cinerea* Mutants

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1. Introduction

Grey mould caused by the fungus *Botrytis cinerea* Pers ex Fr. [anamorph of *Botryotinia fuckeliana* (de Bary) Whets] is a major disease of grapes (Elad et al. 2004; Keller et al. 2004). Although biological control of fungal growth is becoming popular on account of environmental concerns (Elmer & Reglinski 2006), generally *B. cinerea* infections are prevented by the application of fungicides. Fenhexamid, a hydroxanilide derivative, is one of the newly developed fungicides that exhibit strong inhibitory activity against *B. cinerea*, owing to its ability to inhibit 3-ketoreductase in the C4-demethylation enzyme complex during ergosterol biosynthesis (Rosslenbroich et al. 2000; Debieu et al. 2001). Fenhexamid is used in many European countries and is considered to be an effective fungicide against *B. cinerea* due to its unique mode of action (Rosslenbroich et al. 2000; Debieu et al. 2001; Baroffio et al. 2003). However, *B. cinerea* wild types exhibiting resistance to fenhexamid were detected shortly after its introduction (Leroux et al. 2002; Baroffio et al. 2003). In Japan, although fenhexamid was registered in 2000, it is not widely used and thus, the existence of fenhexamid resistance in *B. cinerea* has not been reported in Japan.

Molecular-based techniques have been developed to rapidly detect the sensitivity of fungi to several fungicides by taking advantage of point mutations in target genes (Oshima et al. 2002; Paplomatas et al. 2004; Banno et al. 2008, Furuya et al. 2009; Saito et al. 2009). A rapid method for the detection of fungicide resistance is necessary for viticulturists to better understand the incidence of resistance in *B. cinerea* populations in their vineyards. However, it is unknown whether molecular-based techniques for fungicide resistance detection could be applied to the study of fenhexamid resistance in *B. cinerea*. In fenhexamid-resistant field isolates of *B. cinerea*, Fillinger et al. (2008) found various point mutations leading to amino acid substitutions between 195 and 412 in the target protein, 3-ketoreductase, and revealed a relationship between high resistance to fenhexamid and the amino acid substitution at codon 412 in the protein. In the present study, in order to evaluate whether the detection of fenhexamid resistance with molecular-based techniques is possible and whether there are other point mutations in the target gene that are related to the resistance to fenhexamid, we generated 18 mutants showing resistance to fenhexamid by chemical mutagenesis and determined putative point mutations in the *erg27* gene that conferred resistance to fenhexamid.

2. Materials and methods

2.1 *B. cinerea* strains

Two wild type strains of *B. cinerea* (YW01 and YU0622) were collected from the experimental vineyard of the University of Yamanashi, Yamanashi, Japan in 2006. The two wild type strains were used to obtain fenhexamid-resistant mutants. The strains were maintained on potato dextrose agar (PDA, Difco, Detroit, MI) plates and incubated at 23°C until use.

2.2 MNNG treatment

B. cinerea mutation was performed as described by Ziogas et al. (2003) with slight modifications. Briefly, conidia were obtained from 2-week-old *B. cinerea* cultures on PDA plates by washing them with sterile water and filtering them through sterile mesh twice. Conidial concentration was determined with a hemocytometer. Conidial suspension, which contained approximately 10^7 conidia mL^{-1}, was agitated in 10 µg mL^{-1} N-methyl-N-nitro-nitrosoguanidine (MNNG) on a shaker at 23°C for 4 h in the dark and washed twice with sterile distilled water. The conidia were resuspended in water, spread on PDA plate containing 20 µg mL^{-1} fenhexamid and incubated at 23°C for 2 weeks. *B. cinerea* colonies were subsequently transferred and maintained on a PDA plates containing 2.5 µg mL^{-1} fenhexamid. In Total, 18 fenhexamid-resistant mutants were obtained by this method (hereafter referred to as YM01 to YM18).

2.3 Physiological characteristics of *B. cinerea* mutants

The two wild type strains and eighteen mutants, YM01 to YM18, were examined for mycelial growth, spore germination and sensitivity to osmotic pressure on PDA plates. Three 4-mm mycelial plugs for each strain were transferred to the centers of PDA plates (diameter 15 cm) for mycelial growth measurements. Six measurements of the colony diameter for each strain were taken after continuous incubation for 4 days at 23°C in the dark. Mean diameters were calculated with the diameter of plug being subtracted from the mean. Spore germination was determined by counting germinated spores using a microscope following incubation for 6 h on PDA plates in the dark. Sensitivity to osmotic pressure was assessed after incubation for 3 days on PDA plates containing 2.5% KCl as described by Ziogas et al. (2003). Data on mycelial growth, spore germination and sensitivity to osmotic pressure were subjected to analysis of variance using a Dunnett's multiple range test at $P = 0.05$.

2.4 Fungicide sensitivity tests

Fungicide sensitivity tests were performed for the wild type strains and eleven mutants, YM01, YM02, YM03, YM04, YM05, YM06, YM10, YM11, YM12, YM13, YM14. The fungicides used in this study were benzimidazole (thiophanate-methyl; Topjin M 70%; Nippon Soda, Tokyo, Japan), benomyl (Benleto 50%, Sumitomo, Tokyo, Japan), dicarboximide (procymidone; Sumirex 50%; Sumitomo, Tokyo, Japan), fenhexamid (Sigma-Aldrich), fludioxonil (Wako, Osaka, Japan), iprodione (lovral, Bayer CropScience, Tokyo, Japan) and N-phenylcarbamate (diethofencarb, Wako, Osaka, Japan). For each *B. cinerea* strain, mycelial disks (4 mm diameter) were excised from the leading edge growing actively on PDA plates

and transferred to PDA plates (diameter 9 cm) containing each fungicide at various concentrations. After incubation for 3 days at 23°C in the dark, four measurements of the diameters of the mycelial growth on PDA plate were taken from each of three replicates per treatment. Means were calculated with the diameter of the inoculated plug being subtracted from each mean. At least six concentrations each, three replications for each fungicide, were tested to calculate respective fungitoxicity curves. EC_{50} for each fungicide was determined using the dose-response curves after probit analysis.

Sensitivity to fenhexamid for two wild type strains and eighteen mutants of *B. cinerea* was determined by placing the mycelial plug obtained as described above, onto PDA plates (diameter 9 cm) containing 2.5 or 80 µg/µl of fenhexamid. Sensitivity of each strain to fenhexamid was classified as follows: sensitive (S) if there was no growth on the PDA plate containing fenhexamid at the lowest concentration; moderately resistant (MR) if there was growth on the PDA plate containing 2.5 µg/µl of fenhexamid but not at the higher concentration; and highly resistant (HR) if there was growth at all concentrations.

2.5 Virulence and fenhexamid resistance of *B. cinerea* strains on cucumber seedlings

The virulence of *B. cinerea* wild type strains and mutants was determined by examining symptom severity caused by each strain on cucumber seedlings as described by Ziogas et al. (2003) with slight modifications. Cucumber (*Cucumis sativus* cv. Tokiwajibai) seedlings growing in plastic pots for 8-10 days (two seedlings per 7 cm pot) were used at the cotyledon stage. Commercial fenhexamid (Password 50%, Bayer CropScience, Tokyo, Japan) was used for this experiment by dissolving it in sterile water to obtain the appropriate concentration of fenhexamid solution. The cotyledons of cucumber seedlings were sprayed with fenhexamid solution with a hand sprayer and the sprayed seedlings were kept at room temperature until the surfaces of the cotyledons were dry. The center of each cotyledon was punctured with a sterile needle and a 4-mm mycelial plug from the edge of the growing mycelia on PDA plates was placed on the wound. The inoculated plants were incubated at 23°C for 48 h in the dark. Infection incidence was scored by evaluating the lesion of each cotyledon according to the following indices: 0: no infection; 0.5: rot only under inoculum; 1: rot two times bigger than the plug; 2: rot three times bigger than the plug; 3: rot four times bigger than the plug; 4: rot more than five times bigger than the plug (Fig 1). Twenty cotyledons were used for each test.

2.6 DNA sequence of the *erg27* gene in *B. cinerea* mutants

To determine the position of the genetic mutations leading to fenhexamid resistance, we analyzed the DNA sequence of the *erg27* gene encoding 3-ketoreductase in the two wild types strains and the 18 mutants. Genomic DNA was extracted from mycelia according to the protocol described in a previous study (Saito et al. 2009). To determine the sequence of the *erg27* gene, four pairs of oligonucleotide primers were designed as follows: 3kr-F1 (5′- ATGGGATTACCACCATGGGA -3′) and 3kr-R1 (5′- TGCGGAATAAGTGGCGGTAC -3′), 3kr-F2(5′- TGGTCTCCGATTACCTGATG -3′) and 3kr-R2 (5′- TCGTGTGCGAGAACGTAATG -3′), 3kr-F3 (5′- CTTCAAAACAAGCCAAACCC -3′) and 3kr-R3 (5′- GGCTTCCCATCCATCTTACA -3′), and 3kr-F4 (5′- AATCGGAGGAAGAACCAGCA -3′) and 3kr-R4 (5′- TCATTTTTTAACCTTCAAAA -3′).

These four primer pairs amplify the positions between 1234 and 2920 of the *erg27* gene, corresponding to an entire open reading frame (accession no. AY220532) (Albertini et al. 2004). Ten µL of PCR reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.2 mM of each dNTP (TAKARA, Ootsu, Japan), 2 µM of each primer pair, 1.5 U of *Taq* DNA polymerase (TAKARA), and 100 ng of *B. cinerea* DNA was dispensed into a microtube. PCR amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). After an initial incubation at 95ºC for 3 min, *B. cinerea* DNA was amplified for 35 cycles of 95ºC for 30 s; 65ºC for 30 s; and 72ºC for 40 s. The final extension was done at 72ºC for 7 min. The PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining under UV illumination. DNA sequencing of PCR products was performed using an ALFexpress DNA sequencer (GE Healthcare, Piscataway, NJ) with a Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) according to the manufacturer's instructions.

2.7 Statistical analysis

Analyses were made with Ekuseru-Toukei 2006 (Social Survey Research Information Co. Ltd. Japan). Dunnett's multiple range test was used to assess the differences between mycelial growth rate, osmotic sensitivity and spore germination.

3. Results

3.1 Physiological characteristics of *B. cinerea* mutant strains

Eighteen fenhexamid-resistant mutants were obtained by MNNG treatment. All the mutants were derived from wild type strain YW01, except YM03 was derived from wild type strain YU0622. According to our classification of sensitivity to fenhexamid, the two wild type strains, YW01 and YU0622, were classified as sensitive strains (S). Five mutants YM03, YM07, YM08, YM10 and YM11 were classified as highly resistant (HR) and the remaining 13 mutants were classified as moderately resistant (MR) (Table 1). In order to investigate fitness parameters of fenhexamid resistant mutants, mycelial growth rate, sensitivity to osmotic pressure and spore germination were examined for the two wild type strains and eighteen mutants. For mycelial growth rate, seven mutant strains, YM07, YM09, YM11, YM14, YM15 YM16 and YM18, showed an extreme reduction in mycelial growth and were excluded from Dunnett's multiple range tests. Two mutant strains, YM04 and YM08, showed a significant reduction in mycelial growth rate when compared to that of wild type strain YW01 ($P < 0.01$) (Table 1). Three mutant strains, YM04, YM13 and YM17, showed a significant reduction in sensitivity to osmotic pressure and three strains, YM09, YM14 and YM16, in spore germination (Table 1).

3.2 Sensitivity to other fungicides

A study of the sensitivity of 11 representative mutants of *B. cinerea* to other fungicides in comparison with the two wild-type strains did not show any cross-resistance of fenhexamid with benzimidazole, benomyl, diethofencarb, fludioxonil, iprodione and procymidone (Table 2). However, seven mutants, YM01, YM04, YM05, YM10, YM11, YM12 and YM13, showed a higher EC_{50} value for benzimidazole than that of the two wild type strains (Table 2).

Strain	Sensitivity to fenhexamid [a]	Mycelial growth [b] (mean±SE[e])	Sensitivity to osmotic pressure [c] (mean±SE[e])	Spore germination [d] (mean±SE[e])
YW01	S	110.6±2.1[f]	94.5±1.2[f]	94.7±0.8[f]
YU0622	S	109.0±2.5	94.2±0.6	92.2±0.4
YM01	MR	105.4±1.5	95.7±1.2	92.5±1.2
YM02	MR	111.3±1.9	97.3±0.7	96.6±0.7
YM03	HR	109.3±1.5	94.7±1.0	93.7±1.6
YM04	MR	100.9±1.5**	82.7±1.5**	86.8±0.8
YM05	MR	106.1±1.2	93.5±1.4	92.5±0.9
YM06	MR	103.4±0.4	96.0±0.7	93.3±1.2
YM07	HR	18.8±4.0[g]	nd[h]	95.2±1.9
YM08	HR	97.0±1.4**	90.3±1.2	92.3±0.7
YM09	MR	38.4±1.8[g]	nd	65.7±2.8**
YM10	HR	107.0±0.7	94.3±0.9	92.7±2.5
YM11	HR	59.4±2.3[g]	nd	98.8±0.9
YM12	MR	110.4±1.1	97.7±0.8	95.9±1.7
YM13	MR	107.9±1.3	86.5±1.7**	92.5±1.8
YM14	MR	28.5±0.7[g]	nd	55.0±6.7**
YM15	MR	31.3±0.1[g]	nd	81.0±4.6
YM16	MR	5.8±0.6[g]	nd	61.8±6.7**
YM17	MR	116.6±0.5	89.0±0.9*	92.5±2.8
YM18	MR	27.4±2.0[g]	nd	94.6±3.1

Table 1. Characteristics of wild type and mutant strains of *B. cinerea*

[a] S: sensitive, MR: moderately resistant, HR: highly resistant.
[b] Mean colony diameter (mm) measurements after incubation for 4 days (n=4).
[c] Proportion (%) of mycelial growth in the presence of KCl (2.5%) after incubation for 3 days (n=4).
[d] Proportion (%) of germinated conidia after incubation for 6h (n=500).
[e] SE stands for standard error.
[f] Within columns, asterisks represent a significant difference when compared with wild type YW01, according to Dunnett's multiple range test (*P<0.05: **P<0.01).
[g] Data was excluded for Dunnett's multiple range test.
[h] not determined.

Table 2. Fungicide EC_{50} values for wild type and mutant strains of *B. cinerea*.

[a] not determined.

Strain	fenhexamid	benzimidazole	benomyl	diethofencarb	fludioxonil	ip
YW01	0.046	999.05	184.52	0.914	0.029	
YU0622	0.043	954.30	346.38	1.187	0.013	
YM01	30.71	2066.36	169.11	0.973	0.041	
YM02	13.40	1000.63	181.67	1.000	0.031	
YM03	118.49	924.26	312.51	1.184	0.013	
YM04	13.13	2873.76	302.27	1.280	0.012	
YM05	5.84	1584.32	nd	0.758	0.037	
YM06	11.61	657.46	nd	0.843	0.036	
YM10	140.92	3420.99	nd	0.779	0.045	
YM11	170.60	2088.93	nd	1.169	0.028	
YM12	4.64	2130.84	nd	0.810	0.042	
YM13	3.45	3725.21	nd	0.999	0.042	
YM14	39.47	nd[a]	nd	nd	nd	

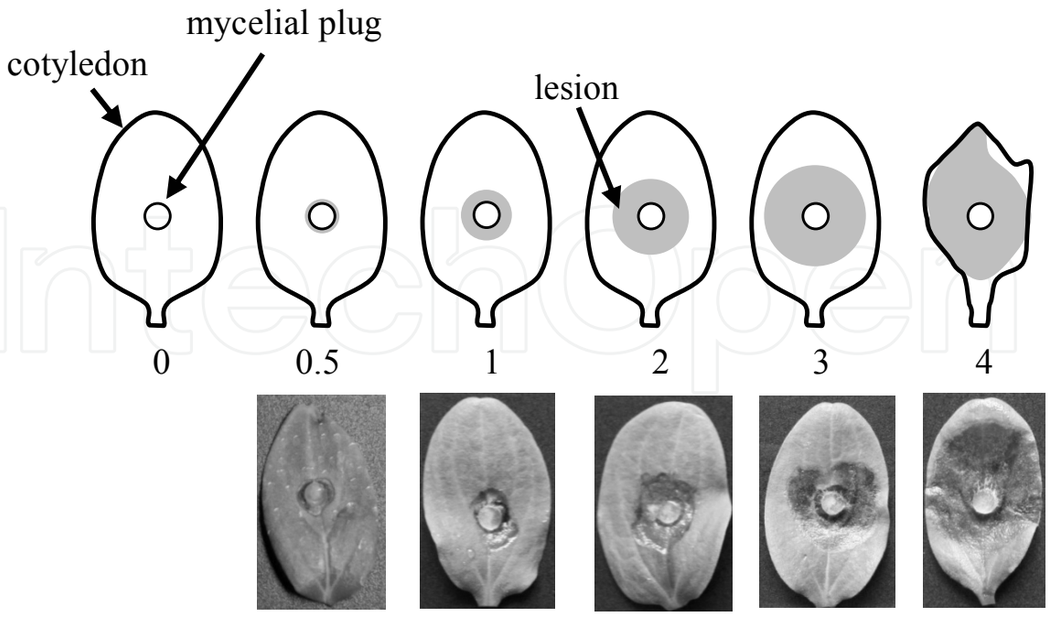

Fig. 1. Scoring key for virulence tests. Lesions on cotyledon of cucumber seedlings were scored according to their sizes. 0: no infection; 0.5: rot only under inoculum: 1: rot two times bigger than the plug; 2: rot three times bigger than the plug; 3: rot four times bigger than the plug; 4: rot more than five times bigger than the plug

3.3 Virulence and fenhexamid response of wild type and mutant strains on cucumber seedlings.

Virulence and fenhexamid response of wild type and 18 mutant strains were examined by monitoring the symptoms on the cotyledon of the cucumber seedlings, according to our evaluation criteria (Fig 1). Virulence studies revealed that seven mutant strains, YM07, YM08, YM14, YM15, YM16, YM17 and YM18, lose their ability considerably to infect cotyledons of cucumber plants (Table 3). However, the other 11 mutants showed virulence similar to the two wild type strains on water treated cotyledons of cucumber seedlings (Table 3). Fenhexamid was effective against the wild type strains but not against the 11 mutants which retain the ability to cause infection. In fact, these eleven mutants were not controlled by fenhexamid at the concentration of 1 000 µg mL^{-1} (Table 3). At the highest concentration of 10 000 µg mL^{-1}, the eleven mutants caused as much of an infection as wild type strains at the lowest concentration of 1 µg mL^{-1} (Table 3).

3.4 Sequence analysis of the *erg27* gene.

To determine the positions of the genetic mutation leading to fenhexamid resistance, we analyzed the DNA sequence of the *erg27* gene encoding 3-ketoreductase in the two wild type strains and 18 mutants. No amino acid substitution was detected in the two wild type strains except a deletion at codon 298 in YU0622 strain (Table 4). We also found the same deletion at codon 298 in YM03, which was derived from YU0622. Various point mutations in the sequenced regions of the *erg27* gene, corresponding to amino acid substitutions between 23 and 516 of the protein, were found in the 18 mutants (Table 4).

Table 3. Virulence tests on cucumber seedlings with wild type and mutant strains of *B. cinerea*

[a] not determined.

fungicide concentration (μ g mL^{-1})	YW01	YU0622	YM01	YM02	YM03	YM04	YM05	YM06	YM07	YM08	YM09	YM10	YM11	YM12	YI
water	4	4	4	4	4	4	4	4	0.50	0.08	4	4	4	4	
fenhexamid															
1	3.90	3.83	4	4	4	4	4	4	0	0	4	4	4	4	
10	3.58	3.24	4	4	4	4	4	4	0	0	4	4	4	4	
100	2.20	2.07	4	4	4	4	4	4	0	0	4	4	4	4	
1000	1.03	0.68	4	4	4	4	4	4	0	0	4	4	4	4	
10000	0.23	0	3.68	3.21	4	3.86	3.68	3.21	nd[a]	nd	3.46	4	2.48	3.39	

		modified residues in 3-ketoreductase protein																						
strain	Fen[a]	G23	C53	T63	K73	V101	L102	H105	K117	K159	K168	L195	T273	P298	S310	K337	I397	I411	F412	H423	A452	Q495	C516
YW01	S																						
YU0622	S													-[b]									
YM04	MR	S																					
YM05	MR	S																					
YM18	MR	S																					
YM13	MR	S	R																				R
YM12	MR			I																			
YM06	MR				E																		
YM16	MR							Y				S						V					
YM09	MR								K											R	P		
YM17	MR									N			A		P								
YM01	MR																V						
YM02	MR																V						
YM14	MR																V						
YM15	MR																V					R	
YM03	HR													-[b]					V				
YM08	HR					A													I				
YM10	HR						Y				E					E			S				
YM07	HR																		S				
YM11	HR																		S				

Table 4. Resistant phenotypes and associated putative mutations in 3-ketoreductase protein.
a Sensitivity to fenhexamid. HR: highly resistant, MR: moderately resistant, and S: sensitive.
b Deletion of one amino acid.

Eleven mutants out of 18 have only one amino acid substitution in the target gene whereas 7 mutants have more than 2 nonsynonymous substitutions (Table 4). The glycine-to-serine mutation at position 23 and the isoleucine-to-valine mutation at position 397, respectively, were revealed to be common in four moderately resistant mutants (Table 4). At position 412, phenylalanine was substituted by isoleucine, valine or serine in the highly resistant mutants, YM03, YM07, YM08, YM10 and YM11 (Table 4). Interestingly, the mutation at position 412 was observed in five mutants that we classified in the previous section as HR (highly resistant).

4. Discussion

Eighteen fenhexamid resistant mutants were successfully obtained after chemical mutagenesis treatment. The result of the physiological characteristics of wild type strains and mutants showed that eleven mutants out of 18 had a fitness penalty, suggesting that the mutation leading to fenhexamid resistance or other unknown mutations would be responsible for some of physiological abnormalities of the mutants, including reduced mycelial growth, sensitivity to osmotic pressure and spore germination. De Guido et al. (2007) obtained 8 fenhexamid resistant mutants by UV-irradiated or unirradiated conidia plated on fenhexamid-containing medium and examined the physiological characteristics of these mutants in comparison with those of wild type strains. According to their results, three mutants out of eight were penalized in mycelial growth (De Guido et al. 2007). In another study, six fenhexamid mutants obtained by chemical mutagenesis did not show any penalties in mycelial growth and osmotic sensitivity but in spore germination (Ziogas et al. 2003). This variability in the data of some characteristics of fenhexamid resistant mutants might be due not only to the acquisition of the fenhexamid resistance but also other mutations in the genome. Although some physiological penalties observed in the mutants could be associated with the acquisition of fenhexamid resistance, we cannot rule out that these may be due to mutations in other part of the fungal genome. Further genetic studies with an appropriate microorganism would be required to elucidate the exact mechanisms responsible.

Virulence tests showed that seven mutants lost their ability to cause infection on cotyledons of cucumber plants. Although the low mycelial growth rate observed in some of these mutants would explain their loss of infection ability, we could not uncover the reason for loss of virulence in the other mutants, such as YM07 and YM17. In the wild type strains, the pathogenic symptoms on the cotyledons were gradually reduced as the concentration of fenhexamid was increased, whereas in eleven mutants, the pathogenic symptoms were constantly observed even when the concentration of fenhexamid reached 10000 μg mL^{-1}. This result suggested that regardless of the fenhexamid resistance level, fenhexamid-resistant *B. cinerea* strains may no longer be susceptible to fenhexamid in field conditions, as the concentration of fenhexamid used to spray vineyards is restricted to less than 750 μg mL^{-1} per application in Japan.

Although Ziogas et al. (2003) reported that the mutation that conferred resistance to fenhexamid was responsible for the increased sensitivity to fludioxonil and iprodione in *B. cinerea*, we didn't detect any cross-resistance relationships among fungicides tested in this study. This contradiction could be attributed to the occurrence of additional mutations in

the relevant gene that conferred tolerance to other fungicides. Resistant mutants were classified into two phenotypic classes in their response to fenhexamid (moderately and highly resistant). The existence of two levels of resistance suggests that distinct mutations in the same locus or in different loci might be involved.

Sequence analysis of the *erg27* gene encoding the target protein of fenhexamid was conducted for 2 wild type strains and 18 mutants. We found a deletion at codon 298 in one of the wild type strain, YU0622 and also in one of the mutants, YM03 which was derived from YU0622. However, this did not appear to have significant effect on virulence, physiological characteristics, or sensitivity to fungicides. Thus, the deletion at codon 298 did not affect the sensitivity to fenhexamid.

Notably five mutants classified as highly resistant, YM03, YM07, YM08, YM10 and YM11, have consistently a point mutation at codon 412. The substitution of phenylalanine at codon 412 with isoleucine, serine or valine has been reported in fenhexamid-resistant *B. cinerea* field isolates (Fillinger et al. 2008). Fillinger et al. (2008) also reported that the point mutation of phenylalanine at codon 412 is responsible for the high resistance of *B. cinerea* field isolates to fenhexamid. In addition, taking into consideration that only one point mutation at codon 412 was found in YM03, YM07 and YM011 in the *erg27* gene, the amino acid substitution at codon 412 might be responsible for high resistance to fenhexamid. In moderately resistant mutants, we detected multiple point mutations in the *erg27* gene, corresponding to amino acid substitutions between position 23 and 516 of the protein. Fillinger et al. (2008) found amino acid substitutions between position 195 and 400 of the protein in field isolates of *B. cinerea* that showed moderate resistance to fenhexamid. It is noteworthy that the moderately resistant mutants, YM04, YM15, and YM18, showed one point mutation at codon 23 where the putative NADPH binding site of the N-terminus of the protein is located (Albertini & Leroux 2004). The glycine-to-serine substitution at codon 23 in those three mutants is responsible for one of the important features of 3-ketoreductase in *B. cinerea*. The 3-ketoreductase gene is conserved in mammals and possesses a putative N-terminal NADP(H) binding site whose conserved structure is GXXXGXG (Peltoketo et al. 1999; Kallberg et al. 2004). In those three mutants, the glycine-to-serine substitution occurred at the second glycine in the site, resulting in GXXXSXG. Interestingly, the glycine at this position is not conserved among 3-ketoreductases in mammals (Peltoketo et al. 1999). Furthermore, in 3-ketoreductase of *Saccharomyces cerevisiae* and *Candida albicans*, asparagine is found at this position instead, resulting in GXXXNXG (Peltoketo et al. 1999). However, the precise mechanism by which the glycine-to-serine substitution at codon 23 of *B. cinerea* 3-ketoreductase induces fenhexamid resistance in *B. cinerea* remains to be determined. The glycine-to-serine substitution may modify the affinity of 3-ketoreductase to fenhexamid. Future studies employing genetic transformation analysis of wild type isolates having a modified *erg27* gene on the NADPH binding site may reveal the relationship between the function of the binding site and fenhexamid resistance in *B. cinerea*. Collectively, a great number of independent point mutation in the *erg27* gene, leading to amino acid substitutions, would be involved in the resistance to fenhexamid. Therefore, it would be impossible to develop molecular-based techniques to detect resistance to fenhexamid.

Ziogas et al. (2003) have reported that the fenhexamid-resistant mutation frequency in *B. cinerea* is high and thus, its inherent resistance risk would be considerable. In the present

study, our findings suggest that the point mutation at codon 412 may confer high resistance to fenhexamid, whereas other amino acid substitutions occurring in 3-ketoreductase might confer moderate resistance, demonstrating that various amino acid substitutions in 3-ketoreductase confer fenhexamid resistance to *B. cinerea*. This may be the reason why *B. cinerea* frequently acquires fenhexamid resistance in the field (Leroux et al. 2002; Barroffio et al. 2003; Ma & Michailides 2005; Esterio et al. 2007; Myresiotis et al. 2007; Kretschmer & Hahn 2008). Therefore, it would be important to use fenhexamid in carefully designed anti-resistance strategies to maintain its effectiveness.

5. Conclusion

Our results suggested that various point mutations in the *erg27* gene that encodes 3-ketoreductase might confer fenhexamid resistance to *B. cinerea*. Due to the various amino acid substitutions in the protein, currently available molecular-based techniques would not be applicable to the detection of fenhexamid resistance in *B. cinerea*. Finally, we need to account for the risk of emerging fenhexamid resistance in *B. cinerea* populations in grapevines.

6. References

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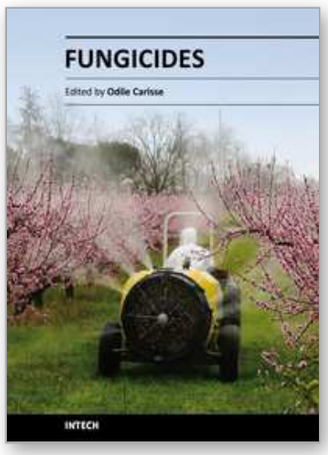

Fungicides

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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