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Phenotypic and genetic characterization of Chilean isolates of *Botrytis cinerea* with different levels of sensitivity to fenhexamid

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Summary. Forty three Chilean *Botrytis cinerea* isolates of different fenhexamid sensitivities, obtained from table grapes, were phenotypically analyzed and sequenced for the *erg27* gene that encodes the 3-ketoreductase enzyme. Fifteen isolates were highly resistant to fenhexamid (HydR3$^+$) with conidial germination EC_{50} values >5 μg·mL^{-1} and colony growth EC_{50} values >2 μg·mL^{-1}. Five isolates had slight to moderate resistance levels (HydR3$^-$) with conidial germination EC_{50} values between 0.7 and 2.6 μg·mL^{-1} and colony growth EC_{50} values between 0.4 and 3 μg·mL^{-1}. Twenty-three isolates were fenhexamid sensitive (HydS) (conidial germination and colony growth EC_{50} values <0.1 μg·mL^{-1}). Resistance to anilinopyrimidine (phenotype AniR1), benzimidazole (phenotype BenR1) and dicarboximide fungicides (phenotype ImiR1) was common among isolates tested. When HydR3$^-$ and HydR3$^+$ sequences were compared with fenhexamid-resistant French isolates, it was verified that all the HydR3$^+$ had a modification in the C-terminal at position 412 of the protein, close to the putative transmembrane domain responsible for fenhexamid resistance. The HydR3$^-$ isolates showed six specific amino acid changes in the sequenced region of the *erg27* gene, between positions 199 and 408 of the protein, with three of these described for the first time.

Key words: grapevine, grey mould, *Vitis* sp., *erg27* gene, HydS, HydR3$^-$, HydR3$^+$ phenotypes.

Introduction

Botrytis cinerea is a pathogen that causes grey mould of grapevine, resulting in important economic losses both at domestic and world level. Grey mould control in Chile is carried out using cultural and agrochemical disease management methods. The chemical control programs are based on the use of fungicides of various groups (dicarboximides, anilinopyrimidines, phenylpyrroles, carboxamides and, mainly, hydroxyanilides), with four to six fungicide applications each season, and at least one of these applications made with fenhexamid.

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Fenhexamid (hydroxyanilide) acts by inhibiting the 3-ketoreductase (Debieu *et al*., 2001), one of the components of the enzymatic complex involved in C-4 demethylation during the ergosterol biosynthesis. Ergosterol is the main sterol present in *B. cinerea* and most filamentous fungi, with the exception of Oomycota (Kuck and Vors, 2007). The *erg27* gene, encoding the 3-ketoreductase enzyme, has various mutations related to different levels of fenhexamid resistance. In this manner, fenhexamid-resistant *B. cinerea* isolates have been described and previously classified into three categories: HydR1, HydR2 and HydR3 (Albertini *et al*., 2002; Leroux *et al*., 2002; Albertini and Leroux, 2004; Leroux, 2004). The HydR3 category has been subdivided into HydR3$^+$ and HydR3$^-$ (Fillinger *et al*., 2008). The isolates belonging to the HydR1 category, classified according to the allelic correspondence of gene Bc-*hch*

to Botrytis Group 1 (*Botrytis pseudocinerea*), have not been detected in Chile, but they were found in France (Albertini *et al*., 2002) and show fenhexamid-resistant phenotypes even when not previously exposed to the fungicide (Fournier *et al*., 2003, 2005). The HydR2 isolates classified according to the allelic correspondence of gene Bc*hch* to Botrytis Group 2 (*Botrytis cinerea* [*sensu stricto*]), have been reported in Germany and Japan (Albertini *et al*., 2002), but not in France (Leroux *et al*., 2006). These isolates have moderate fenhexamid resistance only at the mycelial growth level (Leroux *et al*., 2002), and no mutations in the *erg27* gene were detected (Albertini and Leroux, 2004).

The first HydR3 isolates were detected in years 2002 in Germany and 2004 in France. They belong to the *Botrytis cinerea* (*sensu stricto*) species and have moderate to high fenhexamid resistance levels, both for mycelial growth and conidial germination (Albertini *et al*., 2002; Leroux *et al*., 2002). Sequencing analyses indicate the presence of various mutations in the *erg27* gene leading to various amino acid substitutions in the coded protein. The HydR3$^+$ isolates, characterized by their high levels of resistance, showed a substitution at position F412 of the protein (Albertini and Leroux, 2004; Fillinger *et al*., 2008) which is responsible for fenhexamid resistance (Fillinger *et al*., 2008). The HydR3$^-$ isolates have moderate to high levels of fenhexamid resistance and various single point mutations in the *erg27* gene, corresponding to the following amino acid changes: L195F, V309M, A314V, S336C, N369D, L400F or L400S (Fillinger *et al*., 2008).

Fenhexamid is one of the specific botrycides that has been widely used in Chilean table grapes since 2000, providing excellent results for control of grey mould caused by *B. cinerea*. However, since 2006 some field-collected isolates of the pathogen showed slight to moderate fenhexamid resistance (Esterio *et al*., 2007). Hence, surveys at national level were carried out to verify the severity of the problem, and to ensure the long term effectiveness of the fungicide through adequate field use and correct timing in fungicide spray schedules.

The aim of the present study was to geneticaly and phenotypicaly characterize Chilean *B. cinerea* isolates with different fenhexamid sensitivity levels recovered from table grape vineyards subjected to an intense chemical control schedules with at least two fenhexamid sprays per season. In addition, the sensitivity levels to dicarboximides, benzimidazoles and anilinopyrimidines was determined for the isolates with HydS, HydR3$^-$ and HydR3$^+$ phenotypes. The presence of possible mutations in the gene *erg27* was also determined and these were compared to those detected in French *B. cinerea* isolates.

Materials and methods

Isolates and culture media

The isolates were recovered from flowers and berries of the grapevine cultivar Thompson Seedless during the 2007–2008 growing season (Table 1). The isolates were grown on malt yeast agar medium (20 g L^{-1} malt extract, 5 g L^{-1} Bacto yeast extract, 12.5 g L^{-1} agar) under constant white light until conidiation.

Sensitivity to fenhexamid and other fungicides

The fenhexamid sensitivity was evaluated *in vitro* using germ tube elongation (GTE) and colony growth (CG) tests. For GTE tests, a phosphate based synthetic medium (PG; 10 g L^{-1} glucose, 2 g L^{-1} K_2HPO_4, 2 g L^{-1} KH_2PO_4, 12.5 g L^{-1} agar) was used. Petri dishes containing PG medium with different fenhexamid concentrations (0; 0.1; 0.3; 1; 3 and 10 μg mL^{-1}) were incubated with 300 μL of a conidial suspension containing 2×10^5 conidia mL^{-1}, and maintained at 20°C in darkness for 24 h. Length of the germ tubes was measured for 20 conidia of each isolate per concentration. Isolates were classified as resistant when germ tube elongation was not affected and length was similar to the nil fenhexamid control. In addition, the response to benzimidazoles (carbendazim), N-phenylcarbamates (diethofencarb), dicarboximides (iprodione, vinclozolin) and anilinopyrimidines (pyrimethanil) was evaluated only in GTE tests (Table 2).

In the CG tests, Petri dishes containing Sisler synthetic medium (2 g L^{-1} KH_2PO_4, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 10 g L^{-1} glucose, 2 g L^{-1} yeast extract and 12.5 g L^{-1} agar) (Leroux *et al*., 1999) amended with various concentrations of fenhexamid (0; 0.03; 0.1; 0.3; 1; 3 and 10 μg mL^{-1}) were seeded with 4-day-old mycelium plugs and then kept for 4–6 days at

Table 1. Chilean *Botrytis cinerea* isolates used in this study.

Phenotype [a]	Isolate	Year	Locality	Plant phenological stage [b]	GTE [c] EC_{50} µg mL^{-1}	CG [d] EC_{50} µg mL^{-1}
$HydR3^{+}$						
	A1-R3	2006	Ovalle	Full bloom	7.6	2.3
	A2	2006	Ovalle	Full bloom	>10	>10
	LGP1	2006	Ovalle	Full bloom	>10	2.3
	22F	2006	Lampa	Ripe berries	5.3	6.2
	21D-R3	2006	Lampa	Ripe berries	>10	>10
	21E-R3	2006	Lampa	Ripe berries	10	7.4
	21F	2006	Lampa	Ripe berries	>10	8.2
	CC22C	2007	Lampa	Ripe berries	5.9	>10
	CC19F-R3	2007	Lampa	Ripe berries	>10	5.5
	CR22B-R3	2007	Lampa	Full bloom	>10	n.a.
	CR50F-R3	2007	Lampa	Full bloom	>10	>10
	CHTS12A	2007	Buin	Full bloom	>10	7.6
	CHTC11I	2007	Buin	Full bloom	>10	3.0
	ANP2C	2007	El Olivar	Full bloom	>10	8.8
	CHANP1B	2007	El Olivar	Full bloom	>10	8.3
$HydR3^{-}$						
	184	2006	San Fco de Mostazal	Full bloom	0.7	0.4
	CC54F	2006	Lampa	Ripe berries	1.2	0.8
	AC6G	2007	El Olivar	Ripe berries	2.3	3.0
	ANP4E	2007	El Olivar	Full bloom	1.6	2.3
	CHAC6J	2007	El Olivar	Full bloom	2.6	1.5
HydS						
	46C	2006	Punitaqui	Ripe berries	<0.1	<0.1
	47C	2006	Punitaqui	Ripe berries	<0.1	<0.1
	3E	2006	Coltauco	Ripe berries	<0.1	<0.1
	23F	2006	San Felipe	Ripe berries	<0.1	<0.1
	18D	2006	Catemu	Ripe berries	<0.1	0.1
	21D	2006	Lampa	Ripe berries	<0.1	<0.1
	2A	2006	Hacienda Chada	Ripe berries	<0.1	<0.1
	5F	2006	El Olivar	Ripe berries	<0.1	<0.1
	1E	2006	Nancagua	Ripe berries	<0.1	<0.1
	PC12D	2007	Catemu	Ripe berries	<0.1	<0.1
	CC22A	2007	Lampa	Ripe berries	<0.1	<0.1
	CC22B	2007	Lampa	Ripe berries	<0.1	<0.1
	CR22B	2007	Lampa	Full bloom	<0.1	<0.1
	CR22T	2007	Lampa	Full bloom	<0.1	<0.1
	CR50F	2007	Lampa	Full bloom	<0.1	<0.1
	CR50H	2007	Lampa	Full bloom	<0.1	0.1
	AC3D	2007	El Olivar	Ripe berries	<0.1	<0.1
	ANP6E	2007	El Olivar	Full bloom	<0.1	n.a.
	188	2007	San Fco de Mostazal	Full bloom	<0.1	<0.1
	ANP3F	2007	El Olivar	Full bloom	<0.1	<0.1
	ANP3H	2007	El Olivar	Full bloom	<0.1	<0.1
	AC1E	2007	El Olivar	Ripe berries	<0.1	<0.1
	AC3G	2007	El Olivar	Ripe berries	<0.1	0.1

[a] The fenhexamid phenotypes are classified as HydS (sensitive); $HydR3^{-}$ (slightly to moderately resistant); $HydR3^{+}$ (highly resistant).
[b] The phenological stage are described according to BBCH scheme.
[c] 50% inhibition of germ tube elongation (GTE) after 24 hours.
[d] 50% inhibition of colony growth (CG) after 5 days.
n.a., Not analyzed.

Table 2. Fungicides and threshold concentrations used for discriminating sensitive from resistant isolates in a germ tube elongation (GTE) test.

Chemical group	Reference fungicide	GTE threshold concentration (μg mL^{-1})
Methyl-benzimidazole carbamates	Carbendazim	1.0
N-Phenyl-carbamates	Diethofencarb	10.0
Dicarboximides	Iprodione	2.5
	Vinclozolin	5.0
Anilino-pyrimidines	Pyrimethanil	1.0

20ºC in darkness. Diameters of resulting colonies (CG) and the germ tube elongation tests (GTE) were measured for three replicate plates of each fenhexamid concentration.

For the GTE and CG tests, EC_{50} values for each isolate were calculated using the Minitab - Version 12 statistical software program.

Erg27 gene amplification and sequencing

Five-day-old mycelium grown in glucose liquid medium (10 g L^{-1} glucose, 500 mL L^{-1} potato broth) was lyophilized prior to DNA extraction, carried out as described by Bainbridge *et al*. (1990) with the modifications of Muñoz *et al*. (2002). The PCR mix used to amplify the *erg27* gene was composed of 50–100 ng of genomic DNA, 5 μL PCR 10× buffer, 1 μL of dNTPs 10 mM, 0.1 μL of 100 μM of each primer, erg27BEG (5'-TGGGATTAC-CACCATGGGAGACAAGTG-3') and erg27END (5'-CAATGGTTCCGCATTTCTTTGCCTCCC-3'), 0.3 μL Titanium Taq (Clontech Inc., Paolo Alto, USA) in a total volume of 50 μL. The PCR conditions were the following: initial preheating (2 min at 95°C), 35 cycles composed of denaturation at 95°C for 15 sec and annealing plus polymerization at 68°C for 1 min, with a final extension step (68°C for 2 min).

The amplified PCR products were sequenced on both strands with the primers erg27BEG and erg27END. The HydS - B05.10 isolate was used as a control. The sequencing edition and alignment were made using the CodonCode Aligner program (CodonCode Corporation, Dedham, MA).

Results

Sensitivity to fenhexamid

According to the EC_{50} values obtained for the 43 isolates analyzed (Table1), 20 of them showed resistance to fenhexamid, and among these 15 isolates corresponded to the $HydR3^+$ phenotype (highly resistant) (GTE EC_{50} 5.3 to >10 μg mL^{-1}; CG EC_{50} 2.3 to >10 μg mL^{-1}). Only five isolates corresponded to the phenotype $HydR3^-$ (intermediate resistance), with GTE EC_{50} values 0.7 to 2.6 μg mL^{-1}, and for and CG EC_{50}s of 0.4 to 3 μg mL^{-1}. None of the isolates corresponded to the HydR1 and HydR2 phenotypes discriminated by Albertini and Leroux (2004). The HydS isolates had GTE and CG EC_{50} values of ≤0.1 μg mL^{-1}.

The $HydR3^+$ isolates were also resistant to dicarboximides (87%), anilinopyrimidines (40%), and benzimidazoles (100%). The $HydR3^-$ isolates were also resistant to dicarboximides (80%), anilinopyrimidines (20%) and benzimidazoles (100%). Among HydS isolates, 52% were resistant to dicarboximides, 61% were resistant to anilinopyrimidines 57% were resistant to benzimidazoles.

Erg27 gene amplification and sequencing

The sequencing of the *erg*27 gene of the fenhexamid resistant Chilean *B. cinerea* isolates (15 $HydR3^+$, 5 $HydR3^-$) showed that 14 of the highly resistant isolates ($HydR3^+$) presented the replacement of phenylalanine with serine in position 412 of the protein, and only in one isolate was the amino acid substituted by valine. In addition,

two other mutations were detected for some of these isolates: P238S and N369D. In the HydR3$^-$ isolates, three amino acid changes were detected in the protein, in agreement with Fillinger *et al*. (2008), corresponding to P238S, N369D, and S336A. In addition, in the HydR3$^-$ isolates three new amino acid changes were detected that have not been described, at position 199 from isoleucine to leucine, at position 408 from tyrosine to serine and a deletion of proline in position 298 of the protein (Table 3).

Discussion

Fenhexamid sensitivity analyses through GTE and CG tests for Chilean *B. cinerea* isolates allowed detection of two of the four fenhexamid-resistant phenotypes previously described (Leroux *et al*., 1999; Albertini *et al*., 2002; Ziogas *et al*., 2003; Albertini and Leroux, 2004; De Guido *et al*., 2007; Fillinger *et al*., 2008). The HydR3$^+$ isolates had resistance levels from 50-fold to greater than 100-fold, while the HydR3$^-$ isolates showed resist-

Table 3. Resistance phenotypes and aminoacidic substitutions in *Botrytis cinerea* isolates in the 3-ketoreductase enzyme coded by the *erg27* gene.

Phenotype	Isolate	*B. cinerea* isolate[a]						
		I199 [b]	P238 [c,d]	P298 [b]	S336 [c]	N369 [c]	Y408 [b]	F412 [c,e]
HydR3$^+$								
	LGP1							S
	CR22BR3							S
	TC11ICH							S
	A1R3							S
	CC19FR3		S					S
	21ER3		S					S
	22F		S					S
	CR50FR3		S					S
	CC22C		S			D		S
	21F		S			D		S
	21DR3			-				S
	ANP2C			-				S
	TC12ACH			-				S
	ANP1BCH			-				S
	A2			-				V
HydR3$^-$								
	184	L		-				
	CC54F		S			D	S	
	AC6JCH			-	A			
	ANP4E			-	A			
	AC6G				A			

[a] I, Isoleucine; L, Leucine; P, Proline; S, Serine; A, Alanine; N, Asparagine; D, Aspartic Acid; Y, Tyrosine; F, Phenylalanine; V, Valine; (-), deletion.
[b] Mutations identified in this study.
[c] Mutations previously described by Fillinger *et al*. (2008).
[d] Mutations previously described by Albertini and Leroux (2004).
[e] Mutation previously described by Albertini and Leroux (2004), (substitution of phenylalanine to isoleucine)

ance levels between 7 and 26-fold.

Our results show that the fenhexamid-resistant isolates were also resistant to fungicides in the benzimidazole (phenotype BenR1) and dicarboximide groups (phenotype ImiR1). The global and the particular Chilean situations indicate that benzimidazole-resistance is generalized and stable in the time (Auger and Esterio, 1997) although the use of this fungicide group has been discontinued at field level. Regarding resistance to dicarboximides, resistant strains of the pathogen have developed due to the repeated usage of these fungicides, especially in zones where grey mould epidemics are severe due to local highly predisposing weather conditions (Auger and Esterio, 1997). The presence of dicarboximide-resistant isolates is associated to losses of efficacy to this group, which in turn has been attributed to high disease incidence (Beever *et al*., 1989). Moreover, a high percentage of anilinopyrimidine-resistant isolates (AniR1 phenotype) was detected. Although the resistance mechanism to this group is not known with certainty, *B. cinerea* isolates with moderate to high resistance levels have unstable behavior. This could explain why these isolates have not been selected as fast as benzimidazole and dicarboximide-resistant isolates (Leroux, 2004).

DNA sequencing of the *erg*27 gene in isolates displaying two fenhexamid-resistant phenotypes ($HydR3^-$ and $HydR3^+$) indicated a relationship between the detected phenotypes and single nucleotide polymorphisms. In most $HydR3^+$ isolates, a major mutation caused a substitution of phenylalanine to serine at position 412 in the 3-ketoreductase enzyme; phenylanalanine was replaced by valine in only one isolate. Despite the identical sequence detected in the isolates, the EC_{50} values varied, as for the isolates 22F and CC22C, both carrying the F412S substitution. These isolates gave GTE EC_{50} values of 5.3 and 5.9 μg mL^{-1}, respectively, less than the analogue values of the other $HydR3^+$ isolates. This indicates that natural variation among *B. cinerea* isolates may explain some differences in their responses to fenhexamid.

In the $HydR3^-$ isolates, at least six different point mutations were detected. Three of these have not been described before, and this is the first report of these mutations in Chilean *B. cinerea* isolates. Mutation I199L, S336A are close to conserved domains in the protein. I199L affects the NAGI motif of unknown function whereas the alanine at position 336 is next to the lysine 337 of the active site.

The mutations N369D and the deletion at position 298 are located in domains of unknown function in the 3-ketoreductase enzyme. The mutation Y408S, not been previously described, is located in the possible transmembrane domain, and is therefore potentially modifying the protein-membrane anchoring.

The *erg27* gene is highly susceptible to mutations as evidenced by the high diversity of $HydR3^-$ phenotype populations. However, these mutations only confer weak to moderate resistance levels at low frequencies (Fillinger *et al*., 2008). These findings suggest that $HydR3^-$ phenotypes are "counter-selected", perhaps because of their low resistance levels or because of a potential fitness cost, or for both reasons. The three new identified mutations were all detected in $HydR3^-$ phenotypes, except the proline deletion at position 298 of the protein which was common to few $HydR3^+$ phenotypes. This possibly indicates only that mutations conferring the $HydR3^+$ phenotype may impact fenhexamid efficacy in the field.

This is the first Chilean study confirming the presence among *Botrytis cinerea* isolates, of multiple resistance to different fungicide groups which are important on field control of grey mould. The results obtained has provided important basic knowledge of the genetic basis of fungicide resistance, and also indicates that appropriate resistance management strategies are required to ensure continued effectiveness for botrytis chemical control.

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