Reduced fitness cost and increased aggressiveness in fenhexamid-resistant *Botrytis cinerea* field isolates from Chile

Marcela ESTERIO*, Claudio OSORIO-NAVARRO, Madelaine AZÓCAR, Charleen COPIER, Mauricio RUBILAR, Lorena PIZARRO+, Jaime AUGER

Departamento de Sanidad Vegetal, Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago, Chile

* Current affiliation: Instituto de Ciencias Agroalimentarias, Animales y Ambientales (ICA3), Universidad de O’Higgins, Ruta 90 km 3, San Fernando, Chile

*Corresponding author. E-mail: mesterio@uchile.cl

Summary. Disease management programmes in Chilean table grape vineyards use the hydroxyanilide fenhexamid as a pivotal fungicide for *Botrytis cinerea* control. However, fenhexamid-resistant populations of this pathogen have progressively increased in vineyards under fungicide use. *Botrytis cinerea* isolates were collected in ‘Thompson Seedless’ vineyards under fenhexamid control programmes (>two sprays per season) from three regions of Central Chile, during the 2013–2014, 2014–2015 and 2015–2016 growing seasons. Focusing on the 2015–2016 growing season when the greatest level of resistance was measured, only 8% of recovered isolates were sensitive to fenhexamid with 92% of isolates exceeding the sensitivity threshold for mycelium growth. All fenhexamid resistant isolates analyzed carried a mutation in the *Erg27* gene, which encodes for 3-keto reductase (3-KR) enzyme. The largest proportion of isolates presented a single-point mutation, leading to a substitution of phenylalanine by serine or isoleucine in the 412 residue of 3-KR (*erg27*F412S, 27% ; *erg27*F412I, 48%). Substitution by valine in this position was observed in a lower proportion of isolates (*erg27*F412V, 2%). In contrast to a previous report indicating high fitness cost in isolates carrying *erg27*F412S or *erg27*F412I, mycelium growth and sclerotia development under different restrictive temperatures were not affected compared to wildtype *Erg27*F412 isolates. At 0°C, *erg27*F412S and *erg27*F412I generated larger lesions than *erg27*F412V and *erg27*F412 isolates in wounded and unwounded berry assays. Another five mutations were detected in low-resistance *Erg27*F412 isolates; one was a previously unreported mutation: *erg27*R330P. This study has demonstrated a significant loss of sensitivity to fenhexamid, limited fitness cost and high aggressiveness levels (*erg27*F412S and *erg27*F412I) in field isolates carrying *Erg27* mutations, giving directions for the design of *Botrytis* control programmes based on fenhexamid.

Keywords: *Botrytis* fitness cost, *Erg27* mutations, resistance, increased virulence.
INTRODUCTION

Gray mold (caused by Botrytis cinerea Pers.: Fr.) is the most economically important disease in Chilean table grape production. Botrytis cinerea infection is favoured under wet conditions with temperatures below 22°C; it is a cool-season disease. Environmental conditions between late winter and spring in Chile usually provide the requirements for B. cinerea infection in the table grape growing area, causing blossom blight during the bloom period at the beginning of the season. Botrytis cinerea infections may also remain latent (Keller et al., 2003; Viret et al., 2004), leading to disease appearance after harvest either during storage or after purchase by consumers.

Control of B. cinerea on diverse crops is commonly achieved with combinations of pesticide and agronomic practices. Agronomic practices alone cannot prevent the disease in central Chile, so chemical treatments must be applied (Esterio et al., 2011). Because of the epidemiological traits of B. cinerea, disease forecasting models are not commonly used. Instead, treatments are applied at fixed phenological plant stages: bloom, bunch closure, veraison, and pre-harvest. However, more sprays may be scheduled under specific weather events that increase the risks of disease outbreaks. Among the wide range of fungicides registered for use against B. cinerea, fenhexamid, a hydroxyanilide derivate, has become a key component of gray mold management in Chilean table grape vineyards.

The sterol-3-ketoreductase enzyme (3-KR) encoded by the Erg27 gene is the biological target of fenhexamid. This enzyme is required for C4 demethylation during ergosterol biosynthesis (Debieu et al., 2001). Inhibition of 3-KR leads to ergosterol depletion and accumulation of cytotoxic-ergosterol precursors, triggering defects in central cellular processes (Akins, 2005). Resistance to fenhexamid in B. cinerea has been reported in vineyards from Europe and the United States of America (Fillinger and Walker, 2016), and is linked to several mutations in the Erg27 gene. High level of resistance occurs in isolates carrying single point mutations in codon 412 (Fillinger et al., 2008).

Since the introduction of fenhexamid in 1999, this fungicide has been widely used to B. cinerea control in table grape vineyards in Chile, being applied mainly during the grapevine bloom period. Fenhexamid resistance was reported in B. cinerea isolates from the Central Valley of Chile in the 2006–2007 growing season (Esterio et al., 2007; Esterio et al., 2011). Therefore, alternation of fungicides with different modes of action has been the strategy widely used for B. cinerea chemical control, in order to reduce the selection pressure.

Acquisition of high-level specific resistance to fenhexamid in B. cinerea has been described in isolates carrying mutation in codon 412 of Erg27, and has been associated with important decreases in pathogen fitness, including reduced conidium germination, mycelium growth, and sclerotium development. Consequently, field problems associated with loss of efficacy of fenhexamid has not been reported to date (Ziogas et al., 2003; De Guido et al., 2007; Billard et al., 2012). In recent years, however, loss of sensitivity to fenhexamid has progressively and persistently increased in table grape fields in central Chile (Esterio et al., 2017). In order to maintain and promote fenhexamid effectiveness, the fitness cost of fenhexamid-resistance isolates from central Chile must be determined, and these should be included during construction of comprehensive and updated B. cinerea control programmes. For this purpose, B. cinerea isolates were recovered from six ’Thompson Seedless’ vineyards managed with at least two fenhexamid applications per growing season to: (i) assess their sensitivity to fenhexamid; (ii) determine their Erg27 genotype; and (iii) evaluate fitness parameters.

MATERIALS AND METHODS

Botrytis cinerea isolation and culture media

Botrytis cinerea isolates were recovered during the 2013–2014, 2014–2015 and 2015–2016 growing seasons, from grapevine flowers collected at the full bloom and berries with 16.5° Brix stages, from six cv. Thompson Seedless vineyards located in the Chilean Central Valley, covering the three most important table grape production areas of Valparaiso Region (VR), Metropolitan Region (MR) and O’Higgins Region (OR). These vineyards had been undergoing field programmes with high fungicide pressure, being sprayed at least twice with fenhexamid per growing season. Botrytis cinerea single-conidium cultures isolated from these three regions were grown on malt yeast agar (20 g L⁻¹ malt extract, 5 g L⁻¹ Bacto yeast extract, 12.5 g L⁻¹ agar) maintained at 20°C in constant darkness until conidiation. In total, 132 isolates from VR, 118 from MR, and 158 from OR were used in this study.

Fenhexamid sensitivity assay

Fenhexamid sensitivity was evaluated in vitro using colony growth tests. Colony growth tests were made on plates containing Sisler synthetic medium (2 g L⁻¹
KH₂PO₄, 1.5 g L⁻¹, K₂HPO₄, 1 g L⁻¹, (NH₄)₂SO₄, 0.5 g L⁻¹, MgSO₄·7H₂O, 10 g L⁻¹ glucose, 2 g L⁻¹ yeast extract and 12.5 g L⁻¹ agar) (Leroux et al., 1999) supplemented with different concentrations of fenhexamid (0; 0.03; 0.1; 0.3; 1; 3 and 10 mg L⁻¹). Four-day-old mycelium plugs were seeded on the plates, and the cultures were then kept for 5 days at 20°C in darkness. Colony growth was determined by measuring the diameter of the resulting colonies. Three replicate plates were analyzed for each fenhexamid concentration in colony growth experiments. EC₅₀ values (effective inhibitory dose that gave half-maximum inhibitory responses) were calculated for each isolate using the Minitab Version 12 statistical software program.

Erg27 genotyping: amplification and sequencing

Botrytis cinerea genomic DNA was isolated from 7-day-old mycelia using the DNeasy Plant mini kit (QIAGEN). A fragment of the Erg27 gene was amplified using primers erg1800down and erg27End, which amplify a 1052 pb fragment, previously described by Fillinger et al. (2008). The PCR mix was composed of 50-100 ng genomic DNA, 1X GoTaq® Green Master Mix (Promega) and 0.2 μM each primer; 25μL volume was completed with nanopure water (Promega). The PCR product was purified and used for sequencing (Macrogen). Identification of Erg27 genotypes was performed by alignment of the sequences using BioEdit software (Hall, 1999).

Pathogenicity test

To assess the pathogenicity on grape berries B. cinerea isolates were inoculated onto wounded and unwounded berries of ‘Thompson Seedless’, at harvest stage based on soluble solids content (16.5°Brix). The berries were washed in 1% sodium hypochlorite solution for 0.5 min, rinsed twice with sterile distilled water and allowed to dry under a laminar flow hood. Subsequently, a 10 μL droplet of B. cinerea isolate suspension (10⁶ conidia mL⁻¹) was inoculated on the surface of each unwounded or wounded berries. Wounding was made by puncturing each berry with a sterile needle to a depth of 2 mm. Inoculated berries were incubated at 0 or 20°C in sealed humidity chambers (80% relative humidity) for 4 days and the diameter of the gray mold lesion on each berry was measured. Eighteen berries were used for each Erg27 genotype separated into three replicates. The experiment was repeated twice independently, firstly using table grape berries from seasons 2016–2017 and and second from 2018–2019.

Evaluations of colony growth, conidium production and sclerotium development

Six isolates for each identified Erg27 genotype were used in this study, including wild type (no mutations in the Erg27 gene). The exception was for erg27F412V, where only three isolates were found. In each case, 4-days-old non-sporulating mycelium plugs grown in Potato Dextrose Agar (PDA) were transferred onto a fresh PDA plate for phenotype evaluation. Three plates were used for each genotype in two independent experimental repetitions.

Mycelium radial growth was evaluated for 4 or 5 days in continuous darkness, under three temperature conditions: 15, 20 or 25°C. Conidium production was evaluated after 17 days of continuous colonial growth in darkness at 20°C. For each evaluation, total sporulating mycelium was recovered in a vial with 15 mL of sterile water, which was stirred, and conidia concentration was determined using a haematocytometer. For sclerotium development, plates were incubated for 40 days in darkness at 5 or 20°C. Number and mass of sclerotia in each plate were recorded, and the Sclerotium Index was defined as the ratio of total number of sclerotia to total sclerotium mass per plate.

Statistical analyses

Statistical analyses were carried out using ANOVA and the Bonferroni post hoc test in InfoStat software (Di Rienzo et al., 2015).

RESULTS

Sensitivity of Botrytis cinerea isolates to fenhexamid

Sensitivity to fenhexamid of each isolate was evaluated through the mycelium growth EC₅₀. The isolates were then classified as sensitive (Fen⁵) or resistant (Fen⁸) to fenhexamid, using the recommended cutoff value for field applications of fenhexamid (0.17 mg L⁻¹; Teldor-Bayer). Thirty-six isolates were obtained from VR vineyards in the 2013–2014 season and 11% of these were Fen⁵, 72 isolates were obtained in 2014–2015 and 6% were Fen⁵, and 24 isolates were obtained in 2015–2016 and 13% were Fen⁵ (Figure 1A, Table 1). In the 2013–2014, 2014–2015 and 2015–2016 seasons, 35, 36, and 47 isolates from the MR vineyards were analyzed, and 23%, 25%, and 0% of them were Fen⁵ (Figure 1B, Table 1). Of the isolates from OR vineyards 40, 72, and 46 isolates were obtained in the seasons 2013–2014, 2014–2015 and 2015–2016, respectively, among them 18%, 15% and 19% presented sensitivity to the fungicide (Figure 1C, Table 1).
Resistance to fenhexamid was classified as low when \(0.17 \text{ mg L}^{-1} > \text{EC}_{50} \geq 2 \text{ mg L}^{-1}\) and high when \(\text{EC}_{50} \geq 2 \text{ mg L}^{-1}\), considering the cutoff value described by Fillinger et al. (2008). The frequency of fenhexamid-resistant and highly resistant isolates in the \(B. \text{cinerea}\) population analysed in this study increased with time and this occurred in the three geographical regions under study.

Genetic characterization of \(\text{Erg27}\) in \(B. \text{cinerea}\) isolates

Mutations in wild type \(\text{Erg27}\) allele in \(B. \text{cinerea}\) isolates from the field and laboratory-generated strains have been associated with different ranges of loss of sensitivity to fenhexamid (Fillinger et al. 2008; Esterio et al. 2011; Grabke et al. 2013; Amirri and Peres, 2014). In particular, mutations in 412 codon of \(\text{Erg27}\) trigger high resistance to this fungicide (Fillinger et al., 2008; Debieu and Leroux, 2015; Fillinger and Walker, 2016).

The \(\text{Erg27}\) genotypes of isolates from the three regions (2015–2016 season) were evaluated in order to find a genetic factor associated with resistance to fenhexamid. Of a total of 24 isolates from the VR region, 29% carried a serine substitution (\(\text{erg27}^{F412S}\)) and 58% the isoleucine substitution (\(\text{erg27}^{F412I}\)) at position 412, and only 13% of total isolates maintained phenylalanine at the 412

| Region | Season | Total isolates | % S\(^a\) | % LR\(^b\) | % HR\(^c\) | Mean \(\text{EC}_{50}\)\(^d\) | Min \(\text{EC}_{50}\)\(^e\) | Max \(\text{EC}_{50}\)\(^f\) |
|--------|--------|----------------|---------|---------|---------|----------------|----------------|----------------|
| VR     | 2013–14| 36             | 11.11   | 11.11   | 77.78   | 19.16          | 0.04            | 125.50         |
|        | 2014–15| 72             | 5.56    | 1.39    | 93.05   | 16.19          | 0.02            | 100.70         |
|        | 2015–16| 24             | 12.50   | 0.00    | 87.50   | 15.73          | 0.06            | 43.26          |
|        | 2013–14| 35             | 22.86   | 5.71    | 71.43   | 18.67          | 0.01            | 87.08          |
| MR     | 2014–15| 36             | 25.00   | 11.11   | 63.89   | 12.75          | 0.06            | 142.30         |
|        | 2015–16| 47             | 0.00    | 17.39   | 82.61   | 23.43          | 0.23            | 100.00         |
|        | 2013–14| 40             | 17.50   | 22.50   | 60.00   | 16.41          | 0.01            | 208.10         |
| OR     | 2014–15| 72             | 15.28   | 1.39    | 83.33   | 21.80          | 0.04            | 273.60         |
|        | 2015–16| 46             | 19.15   | 8.51    | 72.34   | 19.05          | 0.03            | 100.00         |

\(^a\) Frequency occurrence (%) of sensitive isolates (\(\text{EC}_{50} < 0.17 \text{ mg L}^{-1}\)).  
\(^b\) Frequency occurrence (%) of low-resistant isolates (\(0.17 \text{ mg L}^{-1} < \text{EC}_{50} \leq 2.0 \text{ mg L}^{-1}\)).  
\(^c\) Frequency occurrence (%) of high-resistant isolates (\(\text{EC}_{50} > 2.0 \text{ mg L}^{-1}\)).  
\(^d\) Mean \(\text{EC}_{50}\) (mg L\(^{-1}\)).  
\(^e\) Minimum value of \(\text{EC}_{50}\) (mg L\(^{-1}\)).  
\(^f\) Maximum value of \(\text{EC}_{50}\) (mg L\(^{-1}\)).
position (\(\text{Erg27}^{F412}\)) (Table 2, Figure 2). As expected, all isolates from this region carrying the mutations in the 412 position of \(\text{Erg27}\) were highly resistant to fenhexamid, and the isolates without mutation in this codon were fenhexamid sensitive. From the MR region, 47 isolates were tested. Substitutions \(\text{erg27}^{F412S}\) was at frequency of 62%, and \(\text{erg27}^{F412I}\) at 13%, while 19% of the isolates had no mutation in \(\text{Erg27}^{F412}\) (Table 1, Figure 2). Three isolates (6%) carried a non-common mutation of valine instead phenylalanine at 412 position (\(\text{erg27}^{F412V}\), 6%) (Table 1, Figure 2). All the highly resistant isolates in this population had mutations in codon 412 of \(\text{Erg27}\), as expected. However, nine isolates with no mutation in \(\text{Erg27}^{F412}\) showed some resistance to fenhexamid, suggesting that mutations in other positions of \(\text{Erg27}\) or on another gene could be responsible for the resistance.

In the 46 isolates from OR, 48% carried the \(\text{erg27}^{F412S}\) mutation and 22% the \(\text{erg27}^{F412I}\) mutation, exhibiting high resistance to fenhexamid (Table 1, Figure 2). In this case, 30% of the isolates had no mutation at \(\text{Erg27}^{F412}\); two of these isolates showed high resistance to the fungicide and four had low resistance.

The low and high resistance in isolates from the MR and OR regions that lacked mutations in \(\text{Erg27}^{F412}\) raised the possibility of another codon of \(\text{Erg27}\) being mutated and conferring resistance to fenhexamid. To answer this, the sequence of the \(\text{Erg27}\) gene was scrutinized to identify other mutations. Five other mutations were found in the \(\text{Erg27}\) gene, including \(\text{erg27}^{L195F}\), \(\text{erg27}^{238S}\), \(\text{erg27}^{298F}\), \(\text{erg27}^{330P}\) and \(\text{erg27}^{N369D}\). These mutations were found in different combinations, in the 117 isolates analyzed from the three regions. \(\text{erg27}^{238S}\), \(\text{erg27}^{L195F}/\text{A298}\) and \(\text{erg27}^{298F/ R330P}\) were present in isolates that lacked mutation in position 412 of \(\text{Erg27}\) and were resistant to fenhexamid (Figure 3A), suggesting that these mutations could lead to resistance to fenhexamid. However, \(\text{erg27}^{L298}\) by itself possibly did not affect resistance to this fungicide. Similarly, \(\text{erg27}^{N369D}\) combined with \(\text{erg27}^{P238S/N369D}\) did not give resistance to fenhexamid, although \(\text{erg27}^{238S}\) by itself correlated with fenhexamid resistance. Mutations in other positions were also detected in the isolates carrying \(\text{erg27}^{F412S}\) or \(\text{erg27}^{F412I}\) (Figure 3B-D). However clear correlations between their presence and fenhexamid resistance were not detected, indicating that mutations in position 412 are more relevant for fenhexamid resistance.

**Evaluation of growth parameters and virulence of the Botrytis cinerea isolates carrying mutations in \(\text{Erg27}\)**

Growth parameters and virulence were analyzed to evaluate the performance of fenhexamid resistant isolates from VR, OR, and MR carrying mutations in the 412 position of the \(\text{Erg27}\) gene. Mycelium growth was evaluated under suboptimal (15°C) and optimal temperature conditions (20 or 25°C). No differences in mycelium radial growth were observed among field isolates with non-mutated \(\text{Erg27}^{F412}\) and \(\text{erg27}^{F412S}\) or \(\text{erg27}^{F412I}\) at the three growing temperature tested (Figure 4A, 4B and 4C).

Development of sclerotia as survival structures is essential for overwintering of \(B. \text{cinerea}\) inoculum in the field. Therefore, sclerotium development was evaluated in two contrasting temperature conditions: 5 or 20°C. Numbers of sclerotia, sclerotia masses and sclerotia indices (ratio of numbers to masses) were quantified. No statistically significant differences were observed between \(\text{Erg27}^{F412}\), \(\text{erg27}^{F412S}\) or \(\text{erg27}^{F412I}\) at 5°C, but at this temperature, the restriction of sclerotium develop-

**Table 2. Numbers of Botrytis cinerea isolates of \(\text{Erg27}\) genotype at the 412 position, obtained from Central Chile, from Valparaiso Region (VR), Metropolitan Region (MR) and O’Higgins Region (OR).**

| Region | Total isolates | \(\text{erg27}^{F412}\) | \(\text{erg27}^{F412S}\) | \(\text{erg27}^{F412I}\) | \(\text{erg27}^{F412V}\) |
|--------|----------------|----------------|----------------|----------------|----------------|
| VR     | 24             | 13             | 0.07           | 19.85          | 29             |
| MR     | 47             | 19             | 0.79           | 77.18          | 62             |
| OR     | 46             | 30             | 1.81           | 47.65          | 48             |

* Frequency occurrence of genotype in percentage
* Mean \(\text{EC}_{50}\) (mg L\(^{-1}\)) for genotype of \(\text{Erg27}\) at the 412 position.
Figure 3. Sensitivity to fenhexamid in *Botrytis cinerea* isolates *Erg27*F412 (A), *erg27*F412S (B), *erg27*F412I (C) and *erg27*F412V (D) carrying additional mutations is shown based on EC50 values (effective concentration that reduces mycelium growth by 50%). Five other mutations were detected: *erg27*L195F, *erg27*P238S, *erg27*Δ298, *erg27*R330P and *erg27*N369D. Dashed lines indicate sensitivity limits: low resistance = 0.17 mg L⁻¹ > EC₅₀ ≥ 2 mg L⁻¹ and high resistance = EC₅₀ ≥ 2 mg L⁻¹.

Figure 4. Comparison on fitness parameters between *Erg27*F412, *erg27*F412S, *erg27*F412I and *erg27*F412V isolates of *Botrytis cinerea*. Radial mycelium growth was evaluated at 15°C (A), 20°C (B) or 25°C (C). Sclerotium development was measured using numbers of sclerotia (D), sclerotium mass (E) and Sclerotia Index (F), evaluated at 5°C or 20°C. Conidium production was also evaluated (G). Wounded and Unwounded detached table grape berries were used to evaluate aggressiveness levels at 0 or 20°C in *Erg27* mutant isolates (H, I). Asterisks indicate significant differences ( * P < 0.05; ** P < 0.01; *** P < 0.001)
ment did not occur with erg27^F412V isolates (Figure 4D, 4E). At 20°C, erg27^F412I produced fewer of sclerotia than erg27^F412V, but equivalent numbers to Erg27^F412 and erg27^F412V (Figure 4D). The sclerotium mass was greater in erg27^F412V compared to other isolates carrying mutant alleles (Figure 4E). Evaluation of the sclerotium indices showed greater values in strains carrying erg27^F412I (Figure 4F).

Conidion production was investigated to establish the propagation capacity of isolates carrying different Erg27 mutations at optimal temperature for B. cinerea development. No differences in conidion production were observed between erg27^F412S, erg27^F412I and isolates carrying the wild type Erg27. However, significantly fewer conidia were produced by erg27^F412V isolates (Figure 4D, 4E). Together, these results indicate that growth in wounded berries infected by erg27^F412V isolates was not reduced by fenhexamid-resistant isolates in other isolates carrying mutant versions of Erg27 were predominant, including erg27^F412I from VR and erg27^F412S from MR and OR. Strong correlations were observed between the presence of mutations at codon 412 of Erg27 and high resistance to fenhexamid (EC50 ≥ 2 mg L^-1). The erg27^F412I and erg27^F412V^* genotypes showed the greatest EC50 values in each population, while erg27^F412S presented the lowest EC50 among the mutants. This indicates that this mutation conferred less resistance to fenhexamid. In all the Chilean regions analyzed in this study, progressive increases of the resistant isolates were detected over the three growing seasons assessed, demonstrating the effects of constant fungicide pressure on B. cinerea populations.

In addition to high fenhexamid resistance related to mutation in Erg27, particularly in the 412 position, we detected other mutations that produced moderate levels of resistance in other Erg27 codons: erg27^P238S, erg27^L195F and erg27^L195F associated with moderate resistance to fenhexamid has not been previously reported (Debieu and Leroux, 2015). The presence of erg27^P238S and erg27^L195F together suppressed resistance to fenhexamid more than in isolates carrying erg27^F412V alone. However, the level of resistance to fenhexamid remained unchanged in strains erg27^F412S and erg27^F412I when
**erg2**

**erg2** was also present, suggesting that changes close to the 3-KR transmembrane domain were more relevant in the interaction between fenhexamid and 3-KR. Our data also suggest that the presence of **erg2** produced a second functional change within the **erg2** sequence, in contrast to **erg2** and **erg2**.

Mutations in position 412 of **erg2** have been previously reported to reduce isolate performance (Billard et al., 2012). However, the isolates **erg2** and **erg2**, identified in the present study grew similarly to fenhexamid-sensitive strains at 15°C, 20°C and 25°C. Sclerotium development and conidium production were also not affected in **erg2** and **erg2** isolates, in contrast to previous reports that showed growth retardation in fenhexamid-resistant strains (Billard et al., 2012; Saito et al., 2014). **erg2** and **erg2** B. cinerea isolates were more pathogenic, particularly in unwounded grape berries at all the temperatures tested. It is possible that the low effects on fitness and the increase in infection capacity observed in **erg2** and **erg2** were due to accumulation of additional mutations that conferred adaptive advantages for survival under high fungicide selection pressure (Ishii, 2015), overcoming the negative effect reported in strains carrying **erg2** and **erg2** (Billard et al., 2012). Isolates carrying **erg2** exhibited fitness costs, producing few conidia and possessing only minor increases in infection capacity. This suggests that this mutation may be rare in the field B. cinerea populations, being found only three times in isolates obtained in the present study.

Amino-pyrazolinone fenpyrazamine was recently introduced as a Botryticide for gray mold control in Chile. Fenpyrazamine, like fenhexamid, targets 3-KR (Kimura et al., 2017), but **erg2** changes associated with resistance to fenpyrazamine have not been studied. Fenpyrazamine could potentially control fenhexamid-resistant isolates by inhibiting 3-KR, targeting the enzyme independently of the amino-acid at position 412. Therefore, experiments determining fenpyrazamine efficacy on fenhexamid-resistant isolates are required, to provide a basis for restructuring chemical control strategies to reduce occurrence of highly resistant B. cinerea populations.

The present research has highlighted the prevalence of fenhexamid resistance linked to the **erg2** genotype in B. cinerea populations isolated from ‘Thompson Seedless’ vineyards treated with this fungicide in the Central Valley of Chile. These results show an overall reduction of fitness in fenhexamid-resistant B. cinerea isolates, suggesting the appearance of adapted strains resistant to this fungicide. This poses serious risks for field control of gray mold in table grape production in Chile.

**ACKNOWLEDGEMENTS**

This research was financially supported by Grant FIA PYT-2016-0243. We thank Veronica Estrada for technical support.

**LITERATURE CITED**

Amiri A., Peres N.A., 2014. Diversity in the **erg2** Gene of *Botrytis cinerea* field isolates from strawberry defines different levels of resistance to the hydroxyanilide fenhexamid. *Plant Disease* 98(8): 1131–1137.

Akins R.A., 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Medical Mycology* 43(4): 285–318.

Billard A., Fillinger S., Leroux P., Lachaise H., Beffa R., Debieu D., 2012. Strong resistance to the fungicide fenhexamid entails a fitness cost in *Botrytis cinerea*, as shown by comparisons of isogenic strains. *Pest Management Science* 68(5): 684–691.

Debieu D., Bach J., Hugon M., Malosse C., Leroux P., 2001. The hydroxyanilide fenhexamid, a new sterol biosynthesis inhibitor fungicide efficient against the plant pathogenic fungus *Botrytisfuckeliana* (*Botrytis cinerea*). *Pest Management Science* 57(11): 1060–1067.

Debieu D., Leroux P., 2015. Sterol Biosynthesis Inhibitors: C-4 Demethylation. In: *Fungicide Resistance in Plant Pathogens* (H. Ishii, D.W. Hollomon, ed.) Springer, Japan, 217–232.

De Guido M.A., De Miccolis Angelini R.M., Pollastro S., Santomauro A., Faretra F., 2007. Selection and genetic analysis of laboratory mutants of *Botryotinia fuckeliana* resistant to fenhexamid. *Journal of Plant Pathology* 89: 203–210.

Di Rienzo J.A., Casanoves F., Balzarini M.G., González L., Tablada M., Robledo C.W., 2015. *InfoStat*. *InfoStat Group, FCA, Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar*

Esterio M., Auger J., Ramos C., García H., 2007. First report of fenhexamid resistant isolates of *Botrytis cinerea* on grapevine in Chile. *Plant Disease* 91(6): 768.

Esterio M., Muñoz G., Ramos C., Cofré G., Estévez R., ... Auger J., 2011. Characterization of *Botrytis cinerea* isolates present in Thompson Seedless table grapes in the Central Valley of Chile. *Plant Disease* 95(6): 683–690.

Esterio M., Copier C., Román A., Araneda M.J., Rubilar M., ... Auger J., 2017. Frequency of fungicide-resistant *Botrytis cinerea* populations isolated from
'Thompson Seedless' table grapes in the Central Valley of Chile. Ciencia e Investigación Agraria 44(3): 295–306.

Fillinger S., Leroux P., Auclair C., Barreau C., Al Hajj C., Debieu D., 2008. Genetic analysis of fenhexamid-resistant field isolates of the phytopathogenic fungus Botrytis cinerea. Antimicrobial Agents and Chemotherapy 52 (11): 3933–3940.

Fillinger S., Walker A.S., 2016. Chemical Control and Resistance Management of Botrytis Diseases. In: Botrytis – the Fungus, the Pathogen and its Management in Agricultural Systems (S. Fillinger, Y. Elad, ed.) Springer, Switzerland, 189–216.

Grabke A., Fernández-Ortuño D., Schnabel G., 2013. Fenhexamid resistance in Botrytis cinerea from strawberry fields in the Carolinas is associated with four target gene mutations. Plant Disease 97(2): 271–276.

Hall T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Ishii H., 2015. Stability of Resistance. In: Fungicide Resistance in Plant Pathogens (H. Ishii, D.W. Hollomon, ed.) Springer, Japan, 35–48.

Keller M., Viret O., Cole M., 2003. Botrytis cinerea infection in grape flowers: defence reaction, latency and disease expression. Phytopathology 93: 316–322.

Kimura N., Hashizume M., Kusaba T., Tanaka S., 2017. Development of the novel fungicide fenpyrazamine. Journal of Pesticide Science 42(3): 137–143.

Latorre B.A., Elfar K., Ferrada E.E., 2015. Gray mold caused by Botrytis cinerea limits grape production in Chile. Ciencia e Investigación Agraria 42(3): 305–330.

Leroux P., Chapeland F., Desbrosses D., Gredt M., 1999. Patterns of cross-resistance to fungicides in Botryotinia fuckeliana (Botrytis cinerea) isolates from French vineyards. Crop Protection 18: 687–697.

Rupp S., Weber R.W., Rieger D., Detzel P., Hahn M., 2017. Spread of Botrytis cinerea strains with multiple fungicide resistance in German horticulture. Frontiers in Microbiology 7: 2075.

Saito S., Cadle-Davidson L., Wilcox W.F., 2014. Selection, fitness, and control of grape isolates of Botrytis cinerea variably sensitive to fenhexamid. Plant Disease 98(2): 233–240.

van den Bosch F., Paveley N., Fraaije B., van den Berg F., Oliver R., 2015. Evidence-Based Resistance Management: A Review of Existing Evidence. In: Fungicide Resistance in Plant Pathogens (H. Ishii, D.W. Hollomon, ed.) Springer, Japan, 63–76.