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

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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} in Chilean mutant 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 dirctions 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, myceliuml growth, and sclerotium development. Consequently, field problems associated with loss of efficacy of fenhexamid have 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 of the isolates 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 Valparaíso 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}

KH_2PO_4, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} $(NH_4)_2$ SO_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) 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. EC_{50} 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 (10^6 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 *erg27*^{F412V}, 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 EC_{50}. The isolates were then classified as sensitive (Fen^S) or resistant (Fen^R) 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 Fen^S, 72 isolates were obtained in 2014–2015 and 6% were Fen^S, and 24 isolates were obtained in 2015–2016 and 13% were Fen^S (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 Fen^S (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

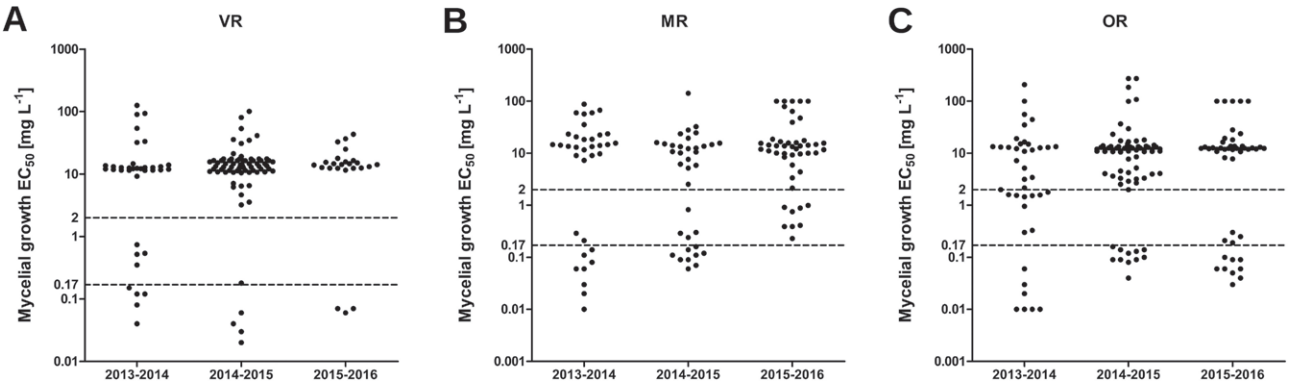

Figure 1. Sensitivity to fenhexamid of *Botrytis cinera* isolates obtained in the 2013-2014, 2014-2015 and 2015-2016 growing seasons from three regions of the Chilean Central Valley: Valparaíso Region (VR; N = 132), Metropolitan Region (MR; N = 118), and O'Higgins Region (OR; N = 158). Fenhexamid effective concentration (mg L^{-1}) was evaluated by EC_{50} value (effective concentration that reduces mycelial growth by 50%). Isolates were considered as low resistance when 0.17 mg L^{-1} > EC_{50} ≥ 2mg L^{-1} and high resistance when the EC_{50} ≥ 2 mg L^{-1}; bothof these sensitivity limits are shown by dashed lines.

1). Resistance to fenhexamid was classified as low when 0.17 mg L^{-1} > EC_{50} ≥ 2 mg L^{-1} and high when EC_{50}≥ 2 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. cinerea* population analysed in this study increased with time and this occurred in the three geographical regions under study.

Genetic characterization of Erg27 *in* Botrytis cinerea *isolates*

Mutations in wild type *Erg27* allele in *B. 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; Amiri and Peres, 2014). In particular, mutations in 412 codon of *Erg27* trigger high resistance to this fungicide (Fillinger *et al*., 2008; Debieu and Leroux, 2015; Fillinger and Walker, 2016).

The *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 (*erg27*^{F412S}) and 58% the isoleucine substitution (*erg27*^{F412I}) at position 412, and only 13% of total isolates maintained phenylalanine at the 412

Table 1. Sensitivity to fenhexamid of *Botrytis cinerea* isolates from Central Chile, from Valparaíso Region (VR), Metropolitan Region (MR) and O'Higgins Region (OR).

Region	Season	Total isolates	% S[a]	% LR[b]	% HR[c]	*Mean* EC_{50}[d]	*Min* EC_{50}[e]	*Max* EC_{50}[f]
	2013–14	36	11.11	11.11	77.78	19.16	0.04	125.50
VR	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 (EC_{50} < 0.17 mg L^{-1}).
[b] Frequency occurrence (%) of low-resistant isolates (0.17 mg L^{-1} < EC_{50} ≤ 2.0 mg L^{-1}).
[c] Frequency occurrence (%) of high-resistant isolates (EC_{50} > 2.0 mg L^{-1}).
[d] Mean EC_{50} (mg L^{-1}).
[e] Minimum value of EC_{50} (mg L^{-1}).
[f] Maximum value of EC_{50} (mg L^{-1}).

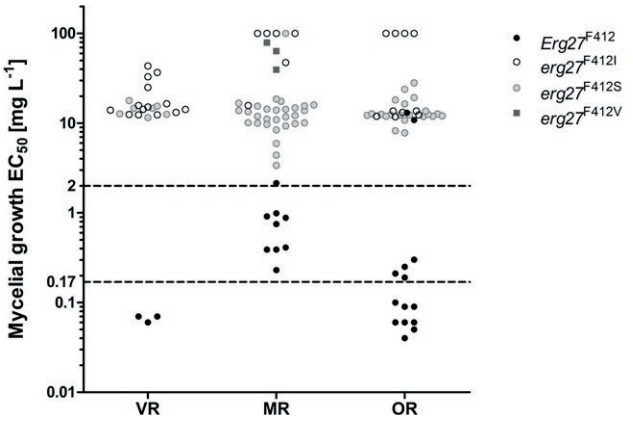

Figure 2. Fenhexamid sensitivity levels of *Botrytis cinerea* isolates carrying a mutation *Erg27*^{F412} from Valparaíso Region (VR; N =24), Metropolitan Region (MR; N = 47) and O'Higgins Region (OR; N = 46) in the 2015-2016 growing season, based on EC_{50} values (effective concentration that reduced mycelium growth by 50%). Sensitivity limits are shown by dashed lines: low resistance = 0.17 mg L^{-1} > EC_{50} ≥ 2 mg L^{-1} and high resistance = EC_{50}≥ 2 mg L^{-1}.

position (*Erg27*^{F412}) (Table 2, Figure 2). As expected, all isolates from this region carrying the mutations in the 412 position of *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 *erg27*^{F412S} was at frequency of 62%, and *erg27*^{F412I} at 13%, while 19% of the isolates had no mutation in *Erg27*^{F412} (Table 1, Figure 2). Three isolates (6%) carried a non-common mutation of valine instead phenylalanine at 412 position (*erg27*^{F412V}, 6%) (Table 1, Figure 2). All the highly resistant isolates in this population had mutations in codon 412 of *Erg27*, as expected. However, nine isolates with no mutation in *Erg27*^{F412} showed some resistance to fenhexamid, suggesting that mutations in other positions of *Erg27* or on another gene could be responsible for the resistance.

In the 46 isolates from OR, 48% carried the *erg27*^{F412S} mutation and 22% the *erg27*^{F412I} mutation, exhibiting high resistance to fenhexamide (Table 1, Figure 2). In this case, 30% of the isolates had no mutation at *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 *Erg27*^{F412} raised the possibility of another codon of *Erg27* being mutated and conferring resistance to fenhexamid. To answer this, the sequence of the *Erg27* gene was scrutinized to identify other mutations. Five other mutations were found in the *Erg27* gene, including *erg27*^{L195F}, *erg27*^{P238S}, *erg27*$^{\Delta 298}$, *erg27*^{R330P} and *erg27*^{N369D}. These mutations were found in different combinations, in the 117 isolates analyzed from

Table 2. Numbers of *Botrytis cinerea* isolates of *Erg27* genotype at the 412 position, obtained from Central Chile, from Valparaíso Region (VR), Metropolitan Region (MR) and O'Higgins Region (OR).

Region	Total isolates	*Erg27*^{F412}		*erg27*^{F412I}		*erg27*^{F412S}		*erg27*^{F412V}	
		%[a]	Mean EC_{50}[b]	%[a]	Mean EC_{50}[b]	%[a]	Mean EC_{50}[b]	%[a]	Mean EC_{50}[b]
VR	24	13	0.07	58	19.85	29	14.99	-	-
MR	47	19	0.79	13	77.18	62	17.90	6	60.59
OR	46	30	1.81	22	47.65	48	13.90	-	-

[a] Frequency occurrence of genotype in percentage
[b] Mean EC_{50} (mg L^{-1}) for genotype of *Erg27* at the 412 position.

the three regions. *erg27*^{P238S}, *erg27*$^{L195F/\Delta 298}$ and *erg27*$^{\Delta 298/R330P}$ were present in isolates that lacked mutation in position 412 of *Erg27* and were resistant to fenhexamid (Figure 3A), suggesting that these mutations could lead to resistance to fenhexamide. However, *erg27*$^{\Delta 298}$ by itself possibly did not affect resistance to this fungicide. Similarly, *erg27*^{N369D} combined with *erg27*$^{P238F/N369D}$ did not give resistance to fenhexamid, although *erg27*^{P238S} by itself correlated with fenhexamid resistance. Mutations in other positions were also detected in the isolates carrying *erg27*^{F412S}or *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* Erg27

Growth parameters and virulence were analyzed to evaluate the performance of fenhxamid resistant isolates from VR, OR, and MR carrying mutations in the 412 position of the *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 *Erg27*^{F412} and *erg27*^{F412S}, *erg27*^{F412I} or *erg27*^{F412V} at the three growing temperature tested (Figure 4A, 4B and 4C).

Development of sclerotia as survival structures is essential for overwintering of *B. 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 *Erg27*^{F412}, *erg27*^{F412S} or *erg27*^{F412I} at 5°C, but at this temperature, the restriction of sclerotium develop-

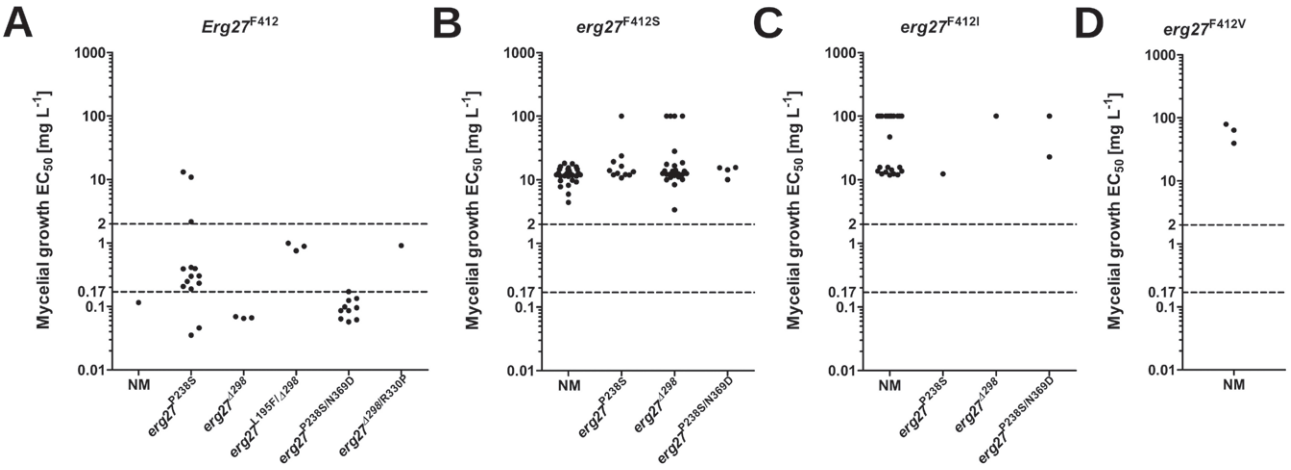

Figure 3. Sensitivity to fenhexamid in *Botrytis cinerea* isolates *Erg27*^{F412} (A), *erg27*^{F412S} (B), *erg27*^{F412I} (C) and *erg27*^{F412V} (D) carrying additional mutations is shown based on EC_{50} values (effective concentration that reduces mycelium growth by 50%). Five other mutations were detected: *erg27*^{L195F}, *erg27*^{P238S}, *erg27*$^{\Delta 298}$, *erg27*^{R330P} and *erg27*^{N369D}. Dashed lines indicate sensitivity limits: low resistance = 0.17 mg L^{-1} > EC_{50} ≥ 2 mg L^{-1} and high resistance = EC_{50}≥ 2 mg L^{-1}.

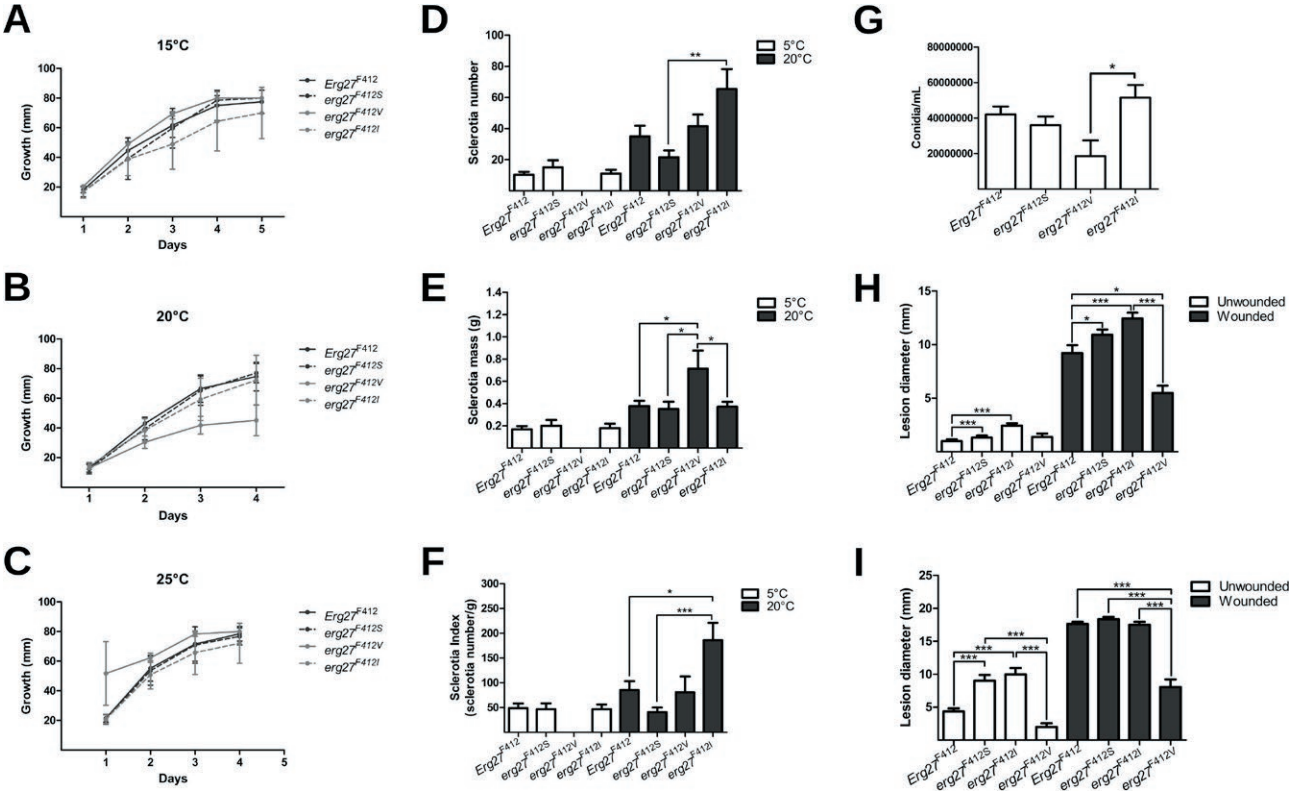

Figura 4. Comparation on fitness parameters between *Erg27*^{F412}, *erg27*^{F412S}, *erg27*^{F412I} and *erg27*^{F412V} isolates of *Botrytis cinerea*. Radial mycelium growth was evaluated at 15°C (A), 20°C (B) or 25°C (C). Sclerotium development was measured using numbers of sclerotia (D), sclerotium 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 (Figur 4D, 4E and 4F). At 20°C, *erg27*^{F412S} produced fewer of sclerotia than *erg27*^{F412I}, 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).

Conidium production was investigated to establish the propagation capacity of isolates carrying different *Erg27* mutations at optimal temperature for *B. cinerea* development. No differences in conidium 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 4G), indicating that this mutation impaired conidium development.

The infection capacity of the *Erg27* mutants was measured on detached 'Thompson Seedless' grape berries using six isolates of each genotype. On unwounded berries incubated at 0°C, *erg27*^{F412I} isolates developed larger rot lesions than berries inoculated with *Erg27*^{F412}, *erg27*^{F412S} or *erg27*^{F412V} isolates. In wounded berries, *erg27*^{F412S} and *erg27*^{F412I} generated larger lesions compared to the *Erg27* ^{F412} genotype, while *erg27*^{F412V} produced smaller lesions than the *Erg27* ^{F412} genotype isolates (Figure 4H). At 20°C, unwounded berries inoculated with *erg27*^{F412I} or *erg27*^{F412S} isolates developed enhanced infection damage compared with *Erg27*^{F412}. However, the necrotic damage observed in wounded berries infected by *erg27*^{F412S} or *erg27*^{F412I} did not differ from that caused by *Erg27*^{F412} isolates. In contrast, *erg27*^{F412V} isolates were less aggressive than *Erg27*^{F412} isolates in both unwounded and wounded berries (Figure 4I). Together, these results indicate that growth in the *erg27*^{F412S}, *erg27*^{F412I} and *erg27*^{F412V} isolates was not affected, although they carried a mutation in *Erg27*, and isolates carrying *erg27*^{F412S} and *erg27*^{F412I} were of increased aggressiveness on both unwounded and wounded berries.

DISCUSSION

Table grape vines are cultivated mostly in the Central Valley of Chile because this region has favorable agroecological conditions for grape production. Grape vineyards in Chile are threatened by *Botrytis* outbreaks due to the frequent cool springs and wet weather conditions. Therefore, fungicides are commonly applied to vineyards in this region. Development of resistance to fungicides has been observed in *B. cinerea* in this area, endangering the ability to control gray mold (Latorre *et al*., 2015; Esterio *et al*., 2017).

In the present study *B. cinerea* populations collected from 'Thompson Seedless' table grape vineyards from three regions of Central Chile were shown to have reduced sensitivity to fenhexamid. This reduced sensitivity increased progressively in the the three successive growing seasons tested. Nevertheless, fenhexamid is still one of the main fungicides regularly used in the local *B. cinerea* control programmes, with at least two applications of this chemical in each growing season.

Botrytis cinerea resistant isolates to fungicides with unisite modes of action have been widely reported in the last 10 years, after intensive application programmes, accompanied by reduced fungicide efficacy (van den Bosch *et al*. 2015; Fillinger and Walker, 2016). The *Erg27* mutation in position 412 is one of the most common changes linked to fenhexamid resistance (Fillinger *et al*., 2008; Billard *et al*., 2012; Debieu and Leroux, 2015). We reported isolates carrying *erg27*^{F412S}, *erg27*^{F412I} and *erg27*^{F412V} mutations; these have been previously reported in *B. cinerea* isolated from fields in France, Germany and the United States of America, and have been associated with high fenhexamid-resistance levels (Grabke *et al*., 2013; Amiri and Peres, 2014; Rupp *et al*., 2017). 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 ($EC_{50} \geq 2$ mg L^{-1}). The *erg27*^{F412I} and *erg27*^{F412V} genotypes showed the greatest EC_{50} values in each population, while *erg27*^{F412S} presented the lowest EC_{50} 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* population.

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*$^{L195F/\Delta 298}$ and *erg27*$^{\Delta 298/ R330P}$. Isolates carrying mutations *erg27*^{L195F} and *erg27*^{R330P} are the first reported in Chile. Particularly, *erg27*^{R330P} associated with moderate resistance to fenhexamid has not been previously reported (Debieu and Leroux, 2015). The presence of *erg27*^{P238S} and *erg27*^{N369D} together suppressed resistance to fenhexamid more than in isolates carrying *erg27*^{P238S} alone. However, the level of resistance to fenhexamid remained unchanged in strains *erg27*^{F412S} and *erg27*^{F412I} when

erg27^{N369D} 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 *erg27*^{F412S} produced a second functional change within the *Erg27* sequence, in contrast to *erg27*^{F412I} and *erg27*^{F412V}.

Mutations in position 412 of *Erg27* have been previously reported to reduce isolate performance (Billard *et al*. 2012)**.** However, the isolates *erg27*^{F412I} and *erg27*^{F412S}, 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 *erg27*^{F412I} and *erg27*^{F412S} isolates, in contrast to previous reports that showed growth retardation in fenhexamid-resistant strains (Billard *et al*., 2012; Saito *et al*., 2014). *erg27*^{F412I} and *erg27*^{F412S} *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 *erg27*^{F412I} and *erg27*^{F412S} 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 *erg27*^{F412I} and *erg27*^{F412S} (Billard *et al*., 2012). Isolates carrying *erg27*^{F412V} 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 *Erg27* 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 *Erg27* genotype in *B. cinera* 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 fenehxamid-resistant *B. cinera* 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.

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