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RESEARCH NOTE

Frequency of fungicide-resistant *Botrytis cinerea* populations isolated from 'Thompson Seedless' table grapes in the Central Valley of Chile

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Abstract

M. Esterio, C. Copier, A. Román, M.J. Araneda, M. Rubilar, I. Pérez, and J. Auger. 2017. Frequency of fungicide-resistant *Botrytis cinerea* populations isolated from 'Thompson Seedless' table grapes in the Central Valley of Chile. Cien. Inv. Agr. 44(3): 295-306. During the 2013 and 2014 growth seasons, 526 single spore isolates of *Botrytis cinerea* were collected from naturally infected 'Thompson Seedless' table grape flowers from fifteen orchards in three regions of the Central Valley of Chile. The isolates were tested for resistance to azoxystrobin, boscalid, fenhexamid, fludioxonil and pyrimethanil. Among the 526 isolates, 106 (20.15%) were sensitive to all fungicides tested; from north to south, the frequency of sensitive isolates in the regions of Valparaiso, Metropolitana and O'Higgins ranged from 48.15% to 21.1% and 5.88%, respectively. Four hundred and twenty isolates (79%) showed resistance to single or multiple fungicides, 134 (25.4%) were simultaneously resistant to azoxystrobin and pyrimethanil. No fludioxonil-resistant isolates were found, indicating that fludioxonil has great potential for gray mold control in table grapes in Chile. From sixty randomly selected *B. cinerea* isolates, only the azoxystrobin-resistant isolates carried the G143A point mutation; according to the cytochrome b (*cyt b*) gene structure, the third intron Bcbi-143/144 was only detected in the azoxystrobin-sensitive isolates. The H272R and H272Y point mutations in the succinate dehydrogenase subunit B (*sdhB*) gene were associated only with the boscalid-resistant isolates. The F412S and F412V point mutations were found in the sequenced *erg27* gene of randomly selected fenhexamid-resistant isolates. These results contribute to the knowledge of *B. cinerea* fungicide resistance for table grape vine crops in Central Chile, particularly for the development of multiple-resistance and the associated resistance mechanisms of azoxystrobin, boscalid and fenhexamid-resistant isolate populations. Anti-resistance strategies are discussed in a general manner.

Keywords: Anilino-pyrimidines, gray mold, multiple-resistance, phenylpyrroles, quinone outside inhibitors, sterol biosynthesis inhibitors class III, succinate dehydrogenase inhibitors.

Introduction

Grape vines, one of the major Chilean fruit crops, are mostly cultivated in the Central Valley because of its favorable regional agroecological conditions. The vast majority of table grapes grown in Chile are exported to the northern hemisphere. *Vitis vinifera* cv. 'Thompson Seedless' ('Th. Seedless') ranks as the second most important commercial grape variety, with a total crop value of US$ 234 million (ODEPA, 2016). However, since most table grapes are trained onto overhead arbors

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and due to the frequent changes to cool and wet weather conditions in the last growing seasons, 'Th. Seedless' table grape growers and fruit export traders have been affected by important pre- and postharvest Botrytis gray mold outbreaks. In fact, grape production decreased from 170,194 tons in 2012 to 132,465 tons in 2014 (ODEPA, 2016). In Chile, *B. cinerea* has caused blossom blight during the bloom period and fruit rot during the pre- and postharvest periods (Latorre *et al*., 2002).

The application of fungicide sprays from blooming until harvest is the essential measure taken to reduce Botrytis infection-related losses. However, *B. cinerea* can develop resistance to commonly used fungicides, an ability that is partly due to its relatively large genetic diversity and enormous capacity for asexual reproduction by means of conidia (Leroux *et al*., 2002).

Resistance to recently introduced fungicides, such as anilino-pyrimidines (APs), phenylpyrroles (PPs) and sterol biosynthesis inhibitors class III (SBIs-III; hydroxyanilides), has also been reported (Leroux *et al*. 2002; Moyano *et al*., 2004; Weber, 2010). Moreover, field resistance to novel succinate dehydrogenase inhibitor (SDHI) fungicides was detected soon after their introduction (Bardas *et al*., 2010). Molecular characterization of *B. cinerea* isolates that were sensitive or resistant to single-site fungicides showed the involvement of major genes of the fungus and a strong association between resistant phenotypes and point mutations (single nucleotide polymorphisms, SNPs) (De Miccolis Angelini *et al*., 2012). These results have been observed for several groups of fungicides, including SDHIs (Yin *et al*., 2012), SBIs-III (Fillinger *et al*., 2008) and quinone outside inhibitors (QoIs) (De Miccolis Angelini *et al*., 2012; Ishii *et al*., 2009).

During the last decade, pyrimethanil, azoxystrobin, boscalid, fenhexamid and fludioxonil fungicides have been used extensively to control gray mold disease in table grapes in Chile, and fungicide resistance of *B. cinerea* has been reported (Esterio *et al*., 2007, 2012, 2015; Latorre *et al*., 2002; Piqueras *et al*., 2014). We hypothesized that the frequency of multiple-resistance in *B. cinerea* populations would increase; thus, it would be crucial to monitor such frequency in table grape orchards to evaluate the risk of infection for specific areas and growing conditions and elaborate appropriate anti-resistance strategies. Therefore, the objectives of the present study were as follows: i) to screen the sensitivity of *B. cinerea* populations isolated from 'Th. Seedless' table grapes to azoxystrobin, boscalid, fenhexamid, fludioxonil and pyrimethanil and assess their resistance frequencies to these fungicides and ii) to investigate the point mutations associated with QoI, SDHI and SBI-III resistance in azoxystrobin-, boscalid- and fenhexamid-resistant isolates.

Materials and methods

Collection of B. cinerea isolates.

To detect fungicide resistance in *B. cinerea,* 'Th. Seedless' naturally infected flowers were sampled during the 2013 and 2014 growing seasons from fifteen orchards in three regions of the Central Valley of Chile (Valparaiso, Metropolitana and O´Higgins). Two hundred flowers (n=50/plate) per orchard were incubated for 3–5 days on water agar medium at 25 °C. Mycelia from the colonized flowers were transferred to potato dextrose agar medium and incubated at 22 °C under a diurnal regime (12-h light/12-h dark light cycle). Pure cultures were transferred to malt extract agar (MEA) medium and incubated at 20 °C. *B. cinerea* isolates were then single-spore cultured on MEA medium, and mycelia were stored in 15% glycerol at -80 °C.

In vitro fungicide assays.

In vitro responses of *B. cinerea* isolates to azoxystrobin, boscalid, fenhexamid, fludioxonil and pyrimethanil were determined. The EC_{50} values

(the effective concentrations that inhibited the conidial germ tube growth by 50% relative to the control) of fenhexamid (Teldor® 50% WP, Bayer CropScience AG, Monheim, Germany) and fludioxonil (Scholar® 23% SC, Syngenta S.A., Monthey, Switzerland), were determined by spotting aliquots (20 µl) of suspension containing 5×10^5 conidia ml^{-1} on 1% MEA medium, either without or amended with increasing concentrations of fungicide: 0, 0.01, 0.1, 1, 10 and 100 µg ml^{-1}. For the QoI fungicide azoxystrobin (Quadris®, 25% CS, Syngenta S.A., Monthey, Switzerland), the alternative oxidase inhibitor salicylhydroxamic acid (SHAM, Sigma-AldrichLaboratories Inc., St. Louis, USA) was added to the MEA medium at a concentration of 100 µg ml^{-1}. For the pyrimethanil (Scala® 40% SC, Bayer CropScience AG, Monheim, Germany) assay, 0.5% sucrose agar medium was used instead of the MEA medium to exclude amino acids, and for the SDHI fungicide boscalid (Cantus® 50% WG, BASF SE, Ludwigshafen, Germany), 0.5% yeast extract agar medium was used to prevent the interference of sugars with the assay (Weber and Hahn, 2011). For each isolate and fungicide concentration tested, growth was measured after 16 h using a 6331 Nauborn Wetzlar microscope (Will®, Germany) fitted with a 10x objective and an eyepiece reticule at 100x final magnification. Each test was performed 2–3 times. The EC_{50} values were calculated by regressing the relative inhibition of conidial germination against the $\log_{10}$-transformed fungicide concentrations. To measure cross-resistance between pairs of fungicides, the correlation coefficients (*r*) of the EC_{50} values of all fungicides were calculated. The EC_{50} values were $\log_{10}$ transformed before analysis. All statistical analyses were conducted using InfoStat statistical software V. 2014 (Di Rienzo *et al*., 2014). The fungicide resistance or sensitivity of the isolates was tested as described previously using the following discriminatory fungicide concentrations (per ml): 100 mg of azoxystrobin supplemented with SHAM, 10 mg of boscalid, 1 mg of fenhexamid, 1 mg of fludioxonil and 10 mg of pyrimethanil (Weber and Hahn, 2011).

Genetic characterization.

SNPs were searched in specific key genes of resistance to QoIs, SDHIs and SBI-IIIs in *B. cinerea* isolates. For all assays, DNA was extracted from mycelium as previously described by Veloukas *et al*. (2011).

Analysis of a partial sequence of the cytochrome b (cyt b) gene from azoxystrobin-resistant (Azo^R) and azoxystrobin-sensitive (Azo^S) isolates.

The PCR primers cytb-BcF and cytb-BcR (Table 1) were used to amplify a *cyt b* gene fragment containing the codons 137 (carrying the G137R mutation) and 143 (carrying the G143A mutation) from randomly selected Azo^R and Azo^S *B. cinerea* isolates. The primers cytb129-F and cytb129-R (Table 1) were used to amplify a 978 bp fragment of the *cyt b* gene containing the codon 129 (carrying the F129L mutation). Additionally, as a specific diagnostic tool, the allele-specific PCR primers BcAR-F and BcAR-R (Table 1) were used to amplify a 260 bp fragment containing the G143A mutation only on Azo^R *B. cinerea* isolates. The PCR amplifications were conducted using the parameters described by Jiang *et al*. (2009). The PCR products were examined by electrophoresis on a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer (De Miccolis Angelini *et al*., 2012).

DNA sequence analysis of the succinate dehydrogenase subunit B (sdhB) gene from boscalid-resistant (Bos^R) and boscalid-sensitive (Bos^S) isolates.

A fragment containing codons 225 and 272 in the *sdhB* gene was amplified from randomly selected Bos^R and Bos^S *B. cinerea* isolates using the IpBcBeg and IpBcEnd2 primers (Table 1), and the PCR products were sequenced and compared with the *sdhB* gene reference sequence (Gene ID: 5428850, GenBank). For the detection of each of the five polymorphisms, a primer-introduced

Table 1. PCR primers used in this study

Gene	Primer pair	Sequences (5' – 3')	PCR product (bp)
*erg*27	Fnx.42	GGTATTCCTCCGATTGTGGA	1156
	Fnx.1197	TAAAGGCATCAGCTCGTGTG	
	Fnx.844	GAGCTGAGATCTTGGGGATG	1164
	Fnx.2007	TGTGTATGATGTACGGCCAAC	
cyt b	BcAR-F	GGCAAATGTCACTGTGAGC	260
	BcAR-R	ACCATCTCCATCCACCATACCT	
	cytb-BcF	TAAAGTGGTATAACCCGACGG	1768/564
	cytb-BcR	CCATCTCCATCCACCATACCT	
	cytb129-F	GCATAAAGCATTGGGGCTAA	978
	cytb129-R	CCGTCTGGCGTCACTATAAAT	
sdhB	IpBcBeg	CCACTCCTCCATAATGGCTGCTCTCCGC	953
	IpBecEnd2	CTCATCAAGCCCCCTCATTGATATC	
	H272L	GGCAGCTTTGGATAACAGCATGAGTTTGTACAGAGATC	120
	H272R	GGCAGCTTTGGATAACAGCATGAGTTTGTACAGATGGC	120
	H272Y	GGCAGCTTTGGATAACAGCATGAGTTTGTACAGATAT	120
	H272-rev	GCCATTTCCTTCTTAATCTCCGC	--
	N230I-fw	GACCCAGCACCAGAAGGAAAAG	150
	N230I-rev	GATAGCTGGTCCCAAGTACTCCTCACGG	
	P225F-fw	GTATTCTCTGCGCATGCTGCTCGACATCAAGC	144
	P225-rev	AAGCTGCCTTACGTTCTTCC	

restriction analysis polymerase chain reaction (PIRA-PCR) technique was used (Veloukas *et al*., 2011). For this purpose, the PCR products were amplified with primer pairs P225F-fw and P225-rev, N230I-fw and N230I-rev, H272L-fw and H272-rev, H272R-fw and H272-rev, and H272Y-fw and H272-rev (Table 1) and digested with the enzymes *HindIII, BamHI, BglII, HhaI*, and *EcoRV*, respectively. Digestion solutions consisted of 5 µl of PCR product, 0.5 unit of each enzyme and 1 unit of the respective enzyme buffer in a total reaction volume of 10 µl. Digestions were performed overnight at 37 °C. The PCR and digested product were separated by electrophoresis on a 2% agarose gel in 1× TAE buffer and visualized after SYBR® Safe DNA gel staining under UV light (Veloukas *et al*., 2011).

Analysis of erg27 gene sequences from fenhexamid-resistant (Fen^R) and fenhexamid-sensitive (Fen^S) isolates.

Randomly selected Fen^R and Fen^S *B. cinerea* isolates were analyzed for specific key genes of resistance to SBI-IIIs. PCR amplification was performed using two specific primer pairs to amplify partially overlapping fragments of the *erg27* gene of *B. cinerea*: Fnx.42 and Fnx.1197, and Fnx.844 and Fnx.2007 (Table 1). PCR amplifications were conducted using the parameters described by De Miccolis Angelini *et al*. (2014).

All PCR products (Azo^R, Bos^R and Fen^R) were sequenced by an external service (Macrogen USA

Corp, DNA Sequencing Service, Maryland, USA), and the DNA sequence analysis was performed using the software Vector NTI suite 7.

Results

Collection of B. cinerea *isolates.*

A total of 526 monosporic *B. cinerea* isolates were obtained from the fifteen 'Th. Seedless' orchards sampled (Valparaiso n=171; Metropolitana n=180; O'Higgins n=175).

Fungicide resistance frequency distribution among locations.

Fungicide resistance monitoring of the 526 isolates, based on discriminatory concentrations of azoxystrobin, boscalid, fenhexamid, fludioxonil and pyrimethanil, identified 14 resistant phenotypes. Among the 526 isolates, 106 (20.15%) were sensitive to all fungicides tested. Resistance to single fungicides was distributed as follows: 35 (6.65%) isolates were resistant to azoxystrobin, 14 (2.66%) to boscalid, 20 (3.8%) to fenhexamid, and 35 (6.65%) to pyrimethanil. Resistance to two fungicides was distributed as follows: 134 (25.4%) isolates were resistant to azoxystrobin and pyrimethanil, 29 (%5.5%) to azoxystrobin and fenhexamid, 10 (1.9%) to azoxystrobin and boscalid, 8 (1.5%) to pyrimethanil and fenhexamid, and 6 (1.14%) to pyrimethanil and boscalid, and only one (0.19%) isolate was resistant to fenhexamid and boscalid. Resistance to three or more fungicides was distributed as follows: 68 (12.9%) isolates were resistant to fenhexamid, azoxystrobin and pyrimethanil, 27 (5.1%) to boscalid, azoxystrobin and pyrimethanil, and 8 (1.52%) to azoxystrobin, boscalid, fenhexamid and pyrimethanil (Fig. 1). Among the three regions in Central Chile from north to south, Valparaiso (n=171), Metropolitana (n=180) and O'Higgins (n=175), the frequencies of sensitive isolates to all fungicides were 48.15%, 21.11% and 5.88%, respectively. From north to south, the frequency of resistant isolates to azoxystrobin, boscalid, fenhexamid and pyrimethanil ranged from 31.6% to 86.3%, 11.1% to 18.85%, 26.3% to 36.5%, and 30.4% to 73.14%, respectively. No fludioxonil-resistant isolates were found among the 526 isolates tested.

To investigate the point mutations associated with QoI, SDHI and SBI-III resistance for azoxystrobin, boscalid and fenhexamid, 30 each of Azo^R, Azo^S, Bos^R, Bos^S, Fen^R and Fen^S *B. cinerea* isolates were randomly selected.

Molecular characterization of a partial sequence of the cyt b gene from randomly selected Azo^R and Azo^S B. cinerea isolates.

Two types of *cyt b* gene fragments were obtained from the PCR amplification with the primers cytb-BcF and cytb-BcR in all the *B. cinerea* isolates tested. Fourteen (23.3%) isolates amplified a *cyt b* gene fragment of 1768 bp containing four introns (Bcbi-67/68, Bcbi-131/132, Bcbi-143/144 and Bcbi-164), and 46 (76.66%) isolates amplified a *cyt b* gene fragment of 564 bp containing three introns (Bcbi-67/68, Bcbi-131/132 and Bcbi-164). After the PCR products were sequenced, the G143A mutation was detected only in the 564 bp fragment of the Azo^R *B. cinerea* isolates. This point mutation is known to affect azoxystrobin sensitivity in many fungal species. None of the Azo^R isolates tested carried the Bcbi-143/144 intron in the *cyt b* gene. None of the 1768 bp fragments contained the G143A mutation. The allele-specific primers BcAR-F and BcAr-R produced a 260 bp fragment only in Azo^R *B. cinerea* isolates, indicating that they all carried the G143A point mutation (Fig. 2). The G137R and F129L point mutations were not detected in any of the *B. cinerea* isolates tested.

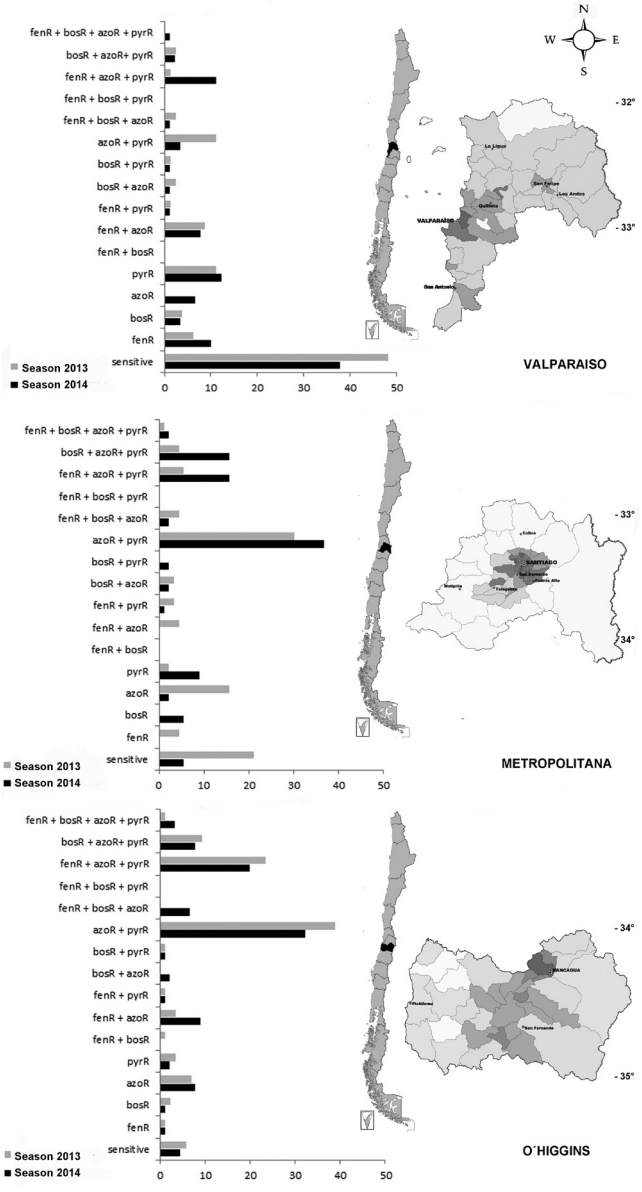

Figure 1. Frequency distribution of the different *B. cinerea* phenotype populations isolated from fifteen 'Thompson Seedless' orchards in three regions of the Central Valley of Chile.

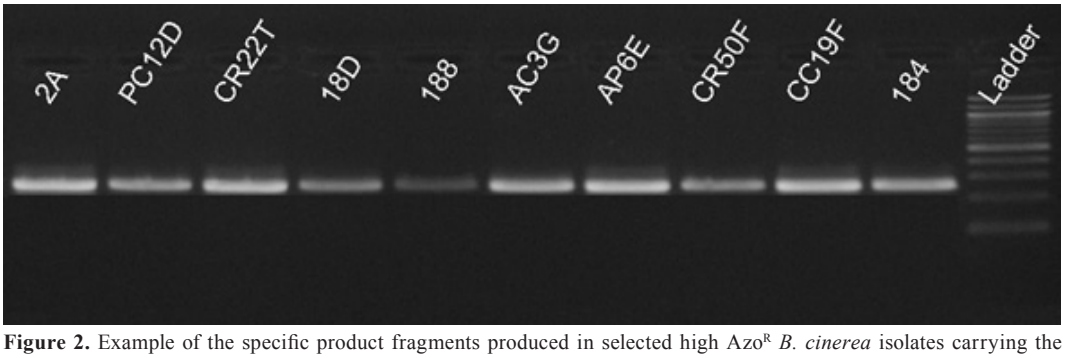

Figure 2. Example of the specific product fragments produced in selected high Azo^R *B. cinerea* isolates carrying the G143A point mutation (260 bp). L=100 bp DNA ladder (New England Bio-Labs, Beverly, MA).

Molecular characterization of the DNA sequence of the sdhB gene from randomly selected Bos^R and Bos^S B. cinerea *isolates.*

The analysis of the DNA sequence of the *sdhB* gene from Bos^R *B. cinerea* isolates showed that the CAC codon (histidine) changed to TAC (tyrosine) in 12 Bos^R isolates (H272Y) and to CGC (arginine) in 18 Bos^R isolates (H272R). Except for the two point mutations at position 272 in Bos^R isolates, all 60 isolates analyzed had identical deduced amino acid sequences in the *sdhB* gene. All PCR reactions with the forward primers H272R and H272Y and the common reverse primer H272-rev amplified a 120 bp product. The digestion of the PCR products amplified by primer pair H272R-fw/H272-rev with *Hha*I resulted in two fragments of 85 and 35 bp only for isolates possessing the H272R mutation. The digestion of the PCR products amplified by primer pair H272Y-fw/H272-rev with *Eco*RV resulted in two fragments of 85 and 35 bp only for isolates that lacked the H272Y mutation, whereas in isolates with the H272Y mutation, the PCR products remained undigested. Results showed that H272R was the predominant mutation associated with Bos^R *B. cinerea* isolates.

Molecular characterization of erg27 gene sequences from randomly selected Fen^R and Fen^S B. cinerea *isolates.*

Two SNPs in the *erg27* gene sequence were found only in Fen^R *B. cinerea* isolates that were always associated with the replacement of a phenylalanine with serine (F412S, 90%) or valine (F412V, 10%) in the position 142. No mutations were associated with Fen^S *B. cinerea* isolates.

Cross-resistance relationships.

With the exception of fludioxonil, the correlation coefficients of the EC_{50} values for all other fungicide combinations were medium to high, ranging from 0.48 (azoxystrobin vs. pyrimethanil, Valparaiso region) to 0.91 (fenhexamid vs. pyrimethanil, Valparaiso region), and were significant ($P<0.001$) (Table 2).

Discussion

Among the three regions in Central Chile from north to south (from Valparaiso to O´Higgins), the frequency of sensitive isolates decreased from 48.15% to 5.88% (Fig. 1). In addition, 19.77% of the isolates tested were resistant to one fungicide, 35.74% were resistant to two, 22.81% were resistant to three, and only 1.52% were resistant to four fungicides. Similar results were found by Latorre and Torres (2012), in 214 *B. cinerea* isolates collected from commercial vineyards with previous use of anilino-pyrimidines, DMIs, and hydroxyanilides.

Table 2. Cross-resistance patterns among azoxystrobin, boscalid, fenhexamid and pyrimethanil in *Botrytis cinerea* isolates from 'Thompson Seedless' orchards

Region	Fungicide	Fungicide							
		azoxystrobin		boscalid		fenhexamid		pyrimethanil	
		r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Valparaíso	azoxystrobin	-	-	0.53	<0.0001	0.63	<0.0001	0.48	<0.0001
	boscalid	-	-	-	-	0.88	<0.0001	0.87	<0.0001
	fenhexamid	-	-	-	-	-	-	0.91	<0.0001
Metropolitana	azoxystrobin	-	-	0.8	<0.0001	0.79	<0.0001	0.7	<0.0001
	boscalid	-	-	-	-	0.87	<0.0001	0.68	<0.0001
	fenhexamid	-	-	-	-	-	-	0.84	<0.0001
O´Higgins	azoxystrobin	-	-	0.85	<0.0001	0.87	<0.0001	0.86	<0.0001
	boscalid	-	-	-	-	0.69	<0.0001	0.58	<0.0001
	fenhexamid	-	-	-	-	-	-	0.86	<0.0001

Among the three regions, the frequency of multiple-resistant *B. cinerea* phenotypes increased from 30.40% in Valparaiso to 81.70% in O´Higgins (Fig. 1), indicating that the risk of development of multiple resistance in *B. cinerea* found in table grape populations in Central Chile is higher where the environmental conditions are more favorable for Botrytis infection. In O´Higgins, the most cool and wet of the three regions, it is common for table grape growers to apply 6-7 fungicide sprays per season. In Valparaiso, the most warm and dry of the three regions, only 4-5 applications are required. These differences modulate the selective pressure exerted by the fungicides on the *B. cinerea* populations in the different regions.

Six of 10 *B. cinerea* isolates collected in the three regions showed resistance to azoxystrobin. In the sensitivity test, all 336 Azo^R isolates showed high resistance to azoxystrobin, ranging from 102 to >1000 μg ml^{-1}. Similar results were reported by Yin *et al*. (2012).

Among the 60 randomly selected isolates, only the Azo^R isolates harbored the G143A point mutation, in agreement with many previous studies (Yin *et al*., 2012). None of the Azo^R isolates tested carried the Bcbi-143/144 intron; the presence of this intron in the *cyt b* gene prevents the occurrence of G143A-mediated resistance (Grasso *et al*., 2006; Yin *et al*., 2012) in many fungal species. Moreover, as only 23.3% of the 60 *B. cinerea* isolates contained the Bcbi-143/144 intron, this low percentage indicates that *B. cinerea* populations from 'Th. Seedless' in Chile hold a high inherent risk of development of resistance to QoI fungicides.

In the present study, we detected an unpredictably high frequency of pyrimethanil-resistant *B. cinerea* isolates: five out of 10 *B. cinerea* isolates collected showed resistance to pyrimethanil with EC_{50} values from 10.2 to 62.1 μg ml^{-1}. Among the anilino-pyrimidine fungicides, cyprodinil was used for a limited number of seasons for gray mold control in table grapes in Chile, until resistant populations emerged. Latorre *et al*. (2002) reported that 38.5% of the *B. cinerea* population was resistant to anilino-pyrimidines in vineyards following four fungicide applications in a two-year period. Afterwards, in a survey conducted between 2007 and 2011, the frequency of anilino-pyrimidine-resistant isolates increased from 45% to 77.4% (Latorre and Torres, 2012). Even though in the last decade, Switch® (cyprodinil + fludioxonil) has been the most widely used botryticide in table grape Botrytis control, it is likely that selection for cyprodinil resistance has occurred in Chile despite the presence of fludioxonil in the sprayed fungicides. On the other hand, it would also be due to the use of other anilino-pyrimidine fungicides registered for gray mold control in table grapes.

The resistance to AP fungicides is stable and without fitness costs because it does not affect the vital components of the fungal cells; several studies have documented the existence of strong cross-resistance between AP fungicides in *B. cinerea* (Zhang *et al*., 2009).

Fenhexamid has been available for use on table grapes and vegetable crops since 1999 and has become a key component of gray mold disease management in Chile. Despite the fact that *B. cinerea* strains with reduced sensitivity to fenhexamid have been detected in Chile, it did not lead to a total field control failure (Esterio *et al*., 2007, 2012).

Four of 10 *B. cinerea* isolates collected in the three regions showed resistance to fenhexamid, and in the sensitivity test, the EC_{50} value of 149 Fen^R isolates ranged from 1.25 to 299 μg ml^{-1}. After partial sequencing of the *erg27* gene in the randomly selected Fen^R *B. cinerea* isolates, 90% (n=27) possessed the F412S substitution, and 10% (n=3) possessed the F412V substitution. The F412S and F412V mutations had already been reported in Chilean *B. cinerea*-resistant isolates from table grapes (Esterio *et al*., 2012). Reduced survival in the field has been reported for fenhexamid-resistant strains. The effect of F412 mutations in the high Fen^R *B. cinerea* isolates would impact

their survival capacity, limiting their dispersal and persistence, particularly when overwintering under field conditions (Billard *et al*., 2012).

Boscalid is a recently introduced member of SDHIs, which are effective in controlling diseases caused by *B. cinerea*. In the present study, two out of 10 *B. cinerea* isolates collected in the three regions showed resistance to boscalid. The EC_{50} values ranged from 15.04 to >1000 µg ml^{-1}. Our results showed that H272R was the predominant mutation associated with the randomly selected Bos^R *B. cinerea* isolates; 60% (n=18) possessed the H272R substitution and 40% (n=12) possessed the H272Y substitution, in agreement with previous studies conducted on grapevines where *sdhB* H272Y and *sdhB* H272R were the most frequent mutations in *B. cinerea* populations treated with boscalid (Esterio, 2014; Veloukas *et al*., 2011; Yin *et al*., 2011). In Champagne vineyards (Walker *et al*. 2013), due to the availability of other classes of botryticides and the limited use of SDHIs, boscalid resistance frequency has not become widespread, and the emergence of the moderately resistant mutants *sdhB* H272Y and *sdhB* H272R has been favored over more resistant mutants.

In Chile, Piqueras *et al*. (2014) reported a high boscalid resistance in Botrytis isolates associated with the H272L point mutation. Esterio *et al*. (2015) reported the detection of the H272R/Y/L and P255L/H point mutations in table grapes at frequencies of 52.8%, 35.42%, 2.1%, 6.25% and 4.16%, respectively. The introduction of new SDHI fungicides in Chile, such as fluopyram, may modify the structure of local *B. cinerea* populations. In fact, no cross-resistance has been detected between pyridine-carboxamides (e.g., boscalid) and pyridinyl-ethyl-benzamides (e.g., fluopyram) in *sdhB* H272Y and *sdhB* H272R mutants; this new family of fungicides may therefore be very useful for the control of these prevalent mutants (Veloukas and Karaoglanidis, 2012).

In the present study, azoxystrobin and pyrimethanil resistance frequencies in *B. cinerea* isolates collected from the three regions were only 7.0% (35 out of 526 isolates), but the frequency of the double-resistant Azo^R+Pyr^R phenotype reached 25.4% (134 out of 526 isolates). The development of multiple-resistance to chemically unrelated fungicides has been reported in different *B. cinerea* populations (Weber, 2011).

On the other side, in the Metropolitana and O´Higgins regions, the detected frequencies of the multiple-resistant *B. cinerea* phenotypes Azo^R+Pyr^R+Fen^R and Azo^R+Pyr^R+Bos^R were only 10.56% and 10%, and 21.7% and 8.5%, respectively. The frequencies of these two *B. cinerea* multiple-resistant phenotypes (Azo^R+Pyr^R+Fen^R and Azo^R+Pyr^R+Bos^R) were much lower than the dual Azo^R+Pyr^R *B. cinerea* phenotype frequency, the Azo^R+Pyr^R+Bos^R phenotype being the least frequent in both regions. Additionally, in the present study, a very low multiple-resistant Azo^R+Pyr^R+Fen^R+Bos^R phenotype frequency was detected (Fig. 1). These results suggest that the increase in the fitness cost observed in this study in the *B. cinerea* multiple-resistant phenotypes may be due to the simultaneous presence of the *erg27* (F412S or F412V) and/or *sdh*B (H272R/Y) gene mutations (Fig. 1). Veloukas *et al*. (2014) reported that most of the isolate groups with multiple-resistance to SDHIs and QoIs, associated with different *sdhB* mutations and the G143A mutation, suffered significant fitness costs.

In the present study, no fludioxonil-resistant isolates were detected among the 526 isolates tested using inhibition growth assays, and the EC_{50} values ranged from 0.01 to 0.27 µg ml^{-1}. These results indicate that fludioxonil still holds a high potential for *B. cinerea* control in table grapes in Chile. Similar results were found by Myresiotis *et al*. (2007), who did not find fludioxonil-resistant isolates among the *B. cinerea* populations resistant to azoxystrobin and pyrimethanil, while few fludioxonil-resistant populations from strawberry fields were observed by Fernández-Ortuño *et al*. (2013). High levels of fludioxonil resistance have only been detected in laboratory mutants and were linked to a decrease

in fitness, probably due to the polygenic control of the resistance, as shown by Vignutelli *et al*. (2002), or the reduced competitive ability of the fludioxonil-resistant strains compared with the wild-type strains (Ziogas and Kalamarakis, 2001). The development of fludioxonil resistance in the field might have detrimental effects on the survival of the pathogen.

Maintaining the effectiveness of fludioxonil, fenhexamid and boscalid against *B. cinerea* by delaying resistance development, and delaying resistance evolution to QoIs and APs by limiting the number of fungicide applications in table grapes for gray mold control programs in the Central Valley of Chile, should be required for the successful control of the disease. Resistance monitoring is therefore essential to detect the emergence of new resistant phenotypes on the 'Th. Seedless' *B. cinerea* populations.

To maintain the effectiveness of the available fungicides for the future, strict resistance management strategies are required. These include good crop management and sanitation measures to reduce the risk of gray mold infection, such as suitable use of nitrogen fertilizers, restraint of canopy humidity through manipulations, adequate cluster thinning, and removal of infected fruits and inoculum reservoirs. To limit selection for resistance, rotation between fungicides of different classes and limitation of treatments with the same class of compounds to one per season should be mandatory, with a possible exception for fludioxonil, which has a low risk of specific resistance. However, these rules are often broken by table grape growers, and fungicides are often used excessively. One of the reasons the rules are broken is the maximum number of detectable pesticide residues on the fruit that are imposed by international retailers, which limits the available options for fungicide rotations. Furthermore, fludioxonil and SDHIs are sold in Chile mixed with other fungicides, restricting the flexibility to optimize the spraying programs. The availability of quick cultivation-based and molecular tests allows a rapid evaluation of resistance, including the prevalence of resistance mutations before and after treatments, to help devise locally adapted resistance management programs.

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Resumen

M. Esterio, C. Copier, A. Román, M.J. Araneda, M. Rubilar, I. Pérez, y J. Auger. 2017. Frecuencia de poblaciones de *Botrytis cinerea* resistentes a fungicidas en uva de mesa 'Thompson Seedless' en el Valle Central de Chile. Cien. Inv. Agr. 44(3): 295-306. Durante las temporadas de crecimiento 2013 y 2014, se colectaron 526 aislados de *Botrytis cinerea* desde flores naturalmente infectadas, en quince huertos de uva de mesa 'Thompson Seedless', de tres regiones del Valle Central de Chile (Valparaíso, Metropolitana y O'Higgins). Se evaluó la sensibilidad de todos los aislados colectados a: azoxystrobin, boscalid, fenhexamid, fludioxonil y pyrimetanil. 106 aislados (20,15%) fueron sensibles a todos los fungicidas evaluados con una frecuencia (norte a sur) de 48,15%, 21,1% y 5,88%, respectivamente. Cuatrocientos veinte aislados fueron resistentes a uno o varios fungicidas, de los cuales, 134 (25,4%) fueron simultaneamente resistentes a azoxystrobin y pyrimetanil. No se detectaron aislados resistentes a fludioxonil, lo que indica que este fungicida mantiene un adecuado potencial de control de la pudrición gris en uva de mesa en los huertos muestreados. De 30 aislados de *B. cinerea* resistentes a azoxystrobin (Azo^R) y 30 sensibles a azoxystrobin (Azo^S), arbitrariamente seleccionados, solo

los aislados Azo^{R} portaban la mutación G143A y de acuerdo a la estructura del gen *cyt b*, el tercer intron Bcbi 143/144 solo fue detectado en los aislados Azo^{S}. De 30 aislados de *B. cinerea* resistentes a boscalid (Bos^{R}) y 30 sensibles a boscalid (Bos^{S}), arbitrariamente seleccionados, las mutaciones H272R and H272Y se encontraron asociadas al gen *sdhB* solo en los aislados Bos^{R}. La secuenciación parcial del gen *erg27* en los 30 aislados resistentes a fenhexamid (Fen^{R}), permitió detectar, solo en los aisladdos FenR, las mutaciones F412S y F412V. Los resultados de este estudio constituyen una contribución al conocimiento de la resistencia de *B. cinerea* a los principales fungicidas utilizados en el control de la pudrición gris en uva de mesa en el Valle Central de Chile, particularmente en lo que se refiere a la predominancia de las mutaciones asociadas a poblaciones de *B. cinerea* resistentes.

Palabras clave: Anilinopirimidinas, fenilpirroles, inhibidores de la biosíntesis del esterol clase III, inhibidores de succinate dehidrogenasa, inhibidores externos de quinona, pudrición gris, resistencia multiple.

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