Short Communication

***Botrytis prunorum* Associated to *Vitis vinifera* Blossom Blight in Chile**Marcela Esterio,[†] Claudio Osorio-Navarro, Claudia Carreras, Madelaine Azócar, Charleen Copier, Verónica Estrada, Mauricio Rubilar, and Jaime Auger

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Abstract

Table grapes are highly susceptible to *Botrytis cinerea* infections during the bloom period. After reaching the flower development stage, *B. cinerea* remains quiescent until berry ripening or gives rise to blossom blight under specific climate conditions. A research study was conducted on the Chilean Central Valley during the 2018–2019 growing season. Flowers of *Vitis vinifera* cv. Thompson Seedless were collected and *B. cinerea* was isolated together to a second and morphologically different species, characterized by white mycelium and low to no sporulation (11.4% of total isolates). Three randomly selected isolates within this population were genetically examined and identified as *Botrytis prunorum* based on a phylogenetic multilocus approach using partial regions of genes *RPB2*, *HSP60*, and *G3PDH* or *NEP1* and *NEP2*. Pathogenicity tests showed that *B. prunorum* infects and causes wilting in healthy table grape flowers. *B. prunorum* isolates were able

to infect Thompson Seedless berries, inducing lesions between 13.11 and 41.53% with respect to the lesion diameter generated by *B. cinerea* B05.10. The fungicide sensitivity was evaluated. The three genetically characterized isolates were sensitive to boscalid and to cyprodinil/fludioxonil mixture with a mean EC₅₀ value of 5.5 µg/ml and 0.065 µg/ml, respectively. However, loss of sensitivity to fenhexamid was determined, with a mean EC₅₀ value of 5.13 µg/ml. Our understanding about blossom blight in *V. vinifera* has been limited to *B. cinerea*. Here we associated *B. prunorum* as a second causal agent of this disease in Chile. This data represents a first approach to the epidemiological characteristics of *B. prunorum* associated with blossom blight in table grapes.

Keywords: *Botrytis prunorum*, table grape, cryptic Botrytis

In the last decade, exhaustive morphological analyses integrated to molecular phylogeny have led to the description of multiple new species within the *Botrytis* genus. As a result, 38 species are included in the *Botrytis* genus to date (Ferrada et al. 2016; Garfinkel et al. 2017; Harper et al. 2019; Hyde et al. 2014; Liu et al. 2016; Rupp et al. 2017; Saito et al. 2016; Zhang et al. 2016; Zhong et al. 2019; Zhou et al. 2014). Among these, *Botrytis cinerea* Pers.:Fr. has the widest host range, affecting more than 1,400 plant species including important agricultural species (Elad et al. 2016). Consequently, it has been listed as one of the fungi with the greatest impact in the world (Dean et al. 2012). Gray mold, caused by *B. cinerea*, is the most important disease in *Vitis vinifera* L., often considered a highly sensitive crop to this fungus. However, a second cryptic species, *Botrytis pseudocinerea*, which also causes gray mold in grapevine, was identified (Walker et al. 2011). *B. cinerea* and *B. pseudocinerea* are morphologically indistinguishable and living in sympatry, but they present different sensitivities to fungicides (Plesken et al. 2015; Walker et al. 2011). This iconic case highlights that adequate disease management requires recognition and fundamental knowledge of all pathogen species in the same ecological niche. In addition, in different geographical areas, novel pathogen-host relationships between *Botrytis* spp. and *V. vinifera* have been identified, but their epidemiological role is still waiting to be elucidated (Acosta Morel et al. 2018; Garfinkel et al. 2017; Harper et al. 2019; Saito et al. 2016; Zhou et al. 2014).

Chile is one of the largest exporting countries of table grapes, with 80 million boxes in the 2018–2019 growing season (ASOEX 2019).

B. cinerea is responsible for devastating effects in the production during pre- and postharvest and is the only causal agent identified in gray mold on table grape in Chile. The bloom period is particularly susceptible to *Botrytis* causing blossom blight disease. All floral organs and tissues are potential targets of *B. cinerea* infection, but the receptacle area in grape flowers has been reported as a predominant infection site (Keller et al. 2003; Viret et al. 2004).

In the 2018–2019 growing season, fungal isolation from healthy and necrotic flowers revealed the presence of two fungal organisms, similar to the situation of *B. cinerea* and the recent described *Botrytis prunorum* species, reported by Ferrada et al. (2016) in Japanese plum in the Central Valley of Chile. Remarkably, *B. prunorum* has been isolated from grape wine bunches in Spain (Acosta Morel et al. 2018).

Fungicide applications are most important on *B. cinerea* control management, but their efficacy is constantly limited by the plasticity of the *Botrytis* genome triggering resistance to fungicide active components (Esterio et al. 2017; Latorre et al. 2015). Thus, the presence and epidemiological characterization of additional pathogens to *B. cinerea* within *V. vinifera* blossom blight is essential to understand how to optimize and improve *Botrytis* control programs.

A *Botrytis* spp. study was conducted in the 2018–2019 growing season from *V. vinifera* cv. Thompson Seedless flowers. Healthy flowers and wilting with early signs of tissue browning were collected during bloom period from a commercial orchard in the Valparaíso Region of the Central Valley of Chile (32°46′48.26″S, 70°41′41.67″W). Thirty bunches were selected from 12 grapevine plants and included in the analysis, at least two aleatory selected bunches were considered per plant; a total of 200 flowers were evaluated for *Botrytis* incidence. The flowers were plated individually in water agar (WA) medium at room temperature and a white to light-gray mycelium was observed after 4 or 7 days. Then, a small fragment of the growing mycelium was transferred to a potato dextrose agar (PDA) medium and incubated in continuous darkness at 20°C. Two types of colonies were observed. The first type presented a mycelium that grew profusely, produced abundant gray sporulation, and was identified provisionally as *B. cinerea* through morphological

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features, but we do not rule out the possibility of *B. pseudocinerea* presence. Nevertheless, a low proportion of white mycelium colonies were obtained (11.4%) in which little to no conidia production was observed. Pure cultures were obtained from a single-spore of *B. cinerea* or by hyphal tips from unsporulating white-colony isolates on PDA medium. The white-colony isolates presented reminiscent characteristics to the novel species *B. prunorum* (Ferrada et al. 2016).

Accordingly, a phylogenetic analysis was conducted with the *V. vinifera* isolates. Genomic DNA was isolated from 7-day-old mycelia using the DNeasy Plant mini kit (QIAGEN) of the three chosen isolates. The isolates were chosen at random from the set of white-

colony isolates. A fragment of *G3PDH*, *TUB2*, *HSP60*, *NEP1*, and *NEP2* genes was amplified by PCR using previously reported conditions (Ferrada et al. 2016) and sent to MACROGEN (U.S.A.) for sequencing. Sequences were aligned by locus using ClustalW in BioEdit sequence alignment editor (Hall 1999). Concatenated *G3PDH*, *TUB2*, and *HSP60* sequences and an independent concatenation of *NEP1* and *NEP2* were used for phylogenetic tree reconstruction with maximum-parsimony analysis using MEGAX (Ferrada et al. 2016; Kumar et al. 2018; Staats et al. 2005, 2007). The sequences used in this analysis are detailed in Table 1, which includes the species within the *Botrytis* genus reviewed by Hyde et al.

Table 1. Details of sequence used in this study

Species	Isolate/strain	GenBank accession number				
		RPB2	HSP60	G3PDH	NEP1	NEP2
<i>Botrytis aclada</i>	MUCL8415	AJ745664	AJ716050	AJ704992	AM087059	AM087087
<i>B. allii</i>	MUCL403	AJ745666	AJ716055	AJ704996	-	-
<i>B. byssioidea</i>	MUCL94	AJ745670	AJ716059	AJ704998	AM087045	AM087079
<i>B. californica</i>	X503	KJ937048	KJ937058	KJ937068	-	-
<i>B. calthae</i>	MUCL1089	AJ745672	AJ716061	AJ705000	-	-
	MUCL2830	-	-	-	AM087031	AM087088
<i>B. caroliniana</i>	CB15	JF811590	JF811587	JF811584	-	-
<i>B. cinerea</i>	MUCL87	AJ745676	AJ716065	AJ705004	-	-
	B05.10	-	-	-	DQ211824	DQ211825
<i>B. convoluta</i>	MUCL11595	AJ745680	AJ716069	AJ705008	AM087035	AM087062
<i>B. croci</i>	MUCL436	AJ745681	AJ716070	AJ705009	AM087047	AM087065
<i>B. deweyae</i>	CBS134649	HG799518	HG799521	HG799521	HG799527	HG799520
<i>B. eucalypti</i>	CERC7160	KX301026	KX301022	KX301018	KX301031	KX301034
<i>B. elliptica</i>	BE9714	AJ745684	AJ716073	AJ705012	AM087049	AM087080
<i>B. euroamericana</i>	CBS141699	KC191679	KC191678	KC191677	KC762944	KC762945
<i>B. fabae</i>	MUCL98	AJ745686	AJ716075	AJ705014	DQ211829	DQ211831
<i>B. fabiopsis</i>	BC-2	EU514473	EU514482	EU519211	-	-
<i>B. ficararum</i>	MUCL376	AJ745688	AJ716077	AJ705016	AM087056	-
	CBS176.63	-	-	-	-	AM087085
<i>B. fragariae</i>	U14_P1	KX429706	KX429692	KX429699	KX429713	KX429720
<i>B. galanthina</i>	MUCL435	AJ745689	AJ716079	AJ705018	AM087057	-
	MUCL3204	-	-	-	-	AM087067
<i>B. gladiolorum</i>	MUCL3865	AJ745692	AJ716081	AJ705020	AM087041	-
	9701	-	-	-	-	AM087072
<i>B. globosa</i>	MUCL444	AJ745693	AJ716083	AJ705022	-	AM087071
	MUCL21514	-	-	-	AM087044	-
<i>B. hyacinthi</i>	MUCL442	AJ745696	AJ716085	AJ705024	AM087048	-
	0001	-	-	-	-	AM087066
<i>B. medusae</i>	B-555	MH732870	MH732866	MH732861	MK211250	MK211255
<i>B. narcissicola</i>	MUCL2120	AJ745697	AJ716087	AJ705026	AM087046	AM087078
<i>B. paeoniae</i>	MUCL16084	AJ745700	AJ716089	AJ705028	AM087033	-
	0003	-	-	-	-	AM087064
<i>B. pelargonii</i>	CBS497.50	AJ745662	AJ716046	AJ704990	AM087030	-
	MUCL1152	-	-	-	-	DQ211834
<i>B. polyblastis</i>	CBS287.38	AJ745702	AJ716091	AJ705030	AM087039	AM087074
<i>B. polyphyllae</i>	YN1	MG431975	MG431978	MG387957	-	-
<i>B. porri</i>	MUCL3234	AJ745704	AJ716093	AJ705032	AM087060	AM087063
<i>B. prunorum</i>	Bpru_21	KP339987	KP339994	KP339980	KR732658	KP400596
	Kw2.2.2	KX196314	KX196316	KX196312	-	-
	BPC1 ^a	MN327624	MN327633	MN327621	MN327627	MN327630
	BPC2 ^a	MN327625	MN327634	MN327622	MN327628	MN327631
	BPC3 ^a	MN327626	MN327635	MN327623	MN327629	MN327632
<i>B. pseudocinerea</i>	10091	JN692428	JN692400	JN692414	-	-
<i>B. pyriformis</i>	SedsarBC-2	KJ543493	KJ543489	KJ543485	-	-
<i>B. ranunculi</i>	CBS178.63	AJ745706	AJ716095	AJ705034	AM087054	AM087086
<i>B. sinoallii</i>	HMAS250008	EU514479	EU514488	EU519217	-	-
<i>B. sinoviticola</i>	GBC-9	JN692422	JN692394	JN692408	-	-
<i>B. sphaerosperma</i>	MUCL21481	AJ745708	AJ716096	AJ705035	AM087042	AM087068
<i>B. squamosa</i>	MUCL1107	AJ745710	AJ716098	AJ705037	AM087052	AM087084
<i>B. tulipae</i>	BT9830	AJ745713	AJ716102	AJ705041	AM087037	AM087077
<i>Monilinia fructigena</i>	9201	AJ745715	AJ716047	AJ705043	-	-
<i>Sclerotinia sclerotiorum</i>	484	AJ745716	AJ716048	AJ705044	-	-

^a This study.

(2014), together with 11 new species reported between 2014 and 2019. The isolates formed a unique and well supported clade with sequences of *B. prunorum* with 100% bootstrap value (Fig. 1A and B). Support for the differences between *B. prunorum* isolates from *Prunus salicina* flowers (Ferrada et al. 2016), *Actidinia deliciosa* kiwifruit (Elfar et al. 2017), and *V. vinifera* flowers were not observed. In addition, the *B. cinerea* clade is topologically distant within the tree. The results show a single lineage formed between white-colony isolates from table grape and *B. prunorum* and consequently it can be identified as this species.

The floral tissue infection was evaluated in order to assess the ability of *B. prunorum* isolates to trigger blossom blight. Two-millimeter mycelial plugs from 5-day-old PDA cultures were used to infect healthy inflorescences previously disinfected using a commercial sodium hypochlorite solution (0.05%, 1 min), rinsed twice with sterile distilled water, and dried under a laminar flow hood. A mycelial plug was inoculated on a rachis primary axis of inflorescence and incubated in a moistened growth chamber at 20°C under a regime of 12-h light and 12-h dark cycle. Three different *B. prunorum* isolates were used for the pathogenicity test. Five replicates and an equal

Fig. 1. Phylogenetic trees generated from maximum-parsimony analysis using sequences of *Botrytis prunorum* from *Vitis vinifera* cv. Thompson Seedless and sequence of 37 *Botrytis* species obtained from GenBank. Bootstrap values are shown. **A**, Tree built with concatenated sequences of *RPB2*, *HSP60*, and *G3PDH* or **B**, with *NEP1* and *NEP2* concatenation. The tree is rooted with *Monilinia fructigena* in **A**. Chilean isolates are highlighted with black circles.

number of control inflorescences inoculated only with agar plugs were included. Each experiment was repeated twice. After 4 to 5 days, mycelium growth on the tissue was observed together with browning and necrosis of the rachis (Fig. 2A) in contrast to the control condition (data not shown). In all pathogenicity tests, the re-isolation of pathogens from inoculated disease sites was conducted to ensure that Koch's rules were completely met. These *in vitro* results revealed the capacity of *B. prunorum* isolates to cause the disease on *V. vinifera* inflorescence and suggest being a potential causal agent of Thompson Seedless blossom blight in Chile.

Flowering is an important stage in the epidemiology of *Botrytis*, as infection at this phenological stage is followed by quiescence. Berries develop normally and disease symptoms are only observed after ripening, when changes in the secondary metabolite profile important in plant defense response occur (Keller et al. 2003; McClellan and Hewitt 1973; Nair et al. 1995; Pezet et al. 2003).

Latent infections are critical for table grape production, triggering substantial fruit loss during harvest and postharvest. The *B. prunorum* epidemiology in table grapes is unknown, but the specific detection reported here for this species in grape flowers raises the

possibility of latent infection. Therefore, *B. prunorum* pathogenicity was evaluated using the three isolates previously characterized genetically (Fig. 1; BPC1, BPC2, and BPC3). Berries of *V. vinifera* cv. Thompson Seedless and cv. Red Globe were used; these cultivars jointly represented 48.1% and 45.2% of total exported production in the 2017–2018 and 2018–2019 seasons, respectively (ASOEX 2019).

Detached healthy berries were surface disinfected in a sodium hypochlorite solution (0.05%, 3 min), washed at least three times with sterile distilled water, and dried in a sterile environment before being used. A 5-mm mycelial plug from 5-day-old PDA cultures was inoculated on each berry and stored on a moistened growth chamber under continuous darkness at 20°C. After 7 days, BCP1, BCP2, and BCP3 were able to colonize and infect Thompson Seedless berries inducing mean lesions of 1.109 mm, 3.512 mm, and 2.603 mm, respectively.

Additionally, the infection level of *B. prunorum* was compared with *B. cinerea* using strain B05.10. Enhanced damage was observed on berries infected by *B. cinerea* with a mean lesion of 8.457 mm, significantly higher than *B. prunorum* (Fig. 2B). In contrast, the level of infection triggered by both species did not show significant

Fig. 2. *Botrytis prunorum* from *Vitis vinifera*: pathogenicity test and fitness parameters. **A**, *B. prunorum* infects flower bunch in vitro (black arrow indicates growing mycelia from tissue). **B**, Infection assay in detached Thompson Seedless and Red Globe berries using BPC1, BPC2, and BPC3 *B. prunorum* isolates. **C**, Sclerotia number, **D**, sclerotia mass, and **E**, sclerotia index of BPC1, BPC2, and BPC3 *B. prunorum* isolates. *B. prunorum* phenotypes were compared with *B. cinerea* B05.10.

Table 2. Fungicide sensitivity of *Botrytis prunorum* isolated from *Vitis vinifera*. Effective concentration causing 50% reduction (EC_{50} value in $\mu\text{g/ml}$) in mycelial growth.

Isolate	Fenhexamid ^a EC_{50}	Boscalid ^b EC_{50}	Cyprodinil/fludioxonil ^c EC_{50}
Cut off value ^d	1	10	1
BPC1	7.89	6.82	0.068
BPC2	1.16	5.71	0.073
BPC3	6.36	3.90	0.053

^a Teldor 50% WP, Bayer CropScience.

^b Cantus 50% WG, BASF SE.

^c Switch 62.5% WG (cyprodinil 37.5%; fludioxonil 25%), Syngenta.

^d Esterio et al. (2017).

differences in Red Globe berries with mean lesions of 0.507 mm, 3.267 mm, and 0 mm for *B. prunorum* BCP1, BCP2, and BCP3, respectively, and 1.261 mm for *B. cinerea* (Fig. 2B). The anatomical characteristics in the Red Globe cultivar led to lower sensitivity to *B. cinerea* infection compared with other table grape cultivars (Mlikota-Gabler et al. 2003). Pathogenicity testing shows that *B. prunorum* cannot overcome these barriers either, suggesting the resistance of Red Globe berries to topical infection by *B. prunorum* isolates studied in the present study. The *Botrytis* spp. survival under adverse field conditions is ensured through sclerotia development. The sclerotia number and sclerotia mass were evaluated after 40 days of growth of a 5-mm mycelial plug from 5-day-old PDA cultures in fresh media under continuous darkness at 20°C. A direct correlation between sclerotia number or sclerotia mass and survival capacity of *Botrytis* has not been shown.

In addition, adjacent sclerotia can be quantified as single sclerotia of larger size generating biases in the results. Therefore, the sclerotia index (number/mass ratio) was also evaluated allowing the normalization and unbiased data collection to evaluate survival structures in *Botrytis* development. Compared with *B. cinerea*, the sclerotia number was reduced in isolates BPC1 and BPC2 of *B. prunorum*, but not in BPC3. No significant differences were observed between the total sclerotia mass of the *B. cinerea* and *B. prunorum* isolates. Consequently, the sclerotia index is equivalent among all the individuals studied.

Fungicide application is the principal *Botrytis* control in the Chilean Central Valley (Esterio et al. 2017; Latorre et al. 2015). In *B. prunorum*, sensitivity to boscalid, the mixture cyprodinil/fludioxonil, and fenhexamid were evaluated in vitro using colony growth (CG) tests according to previously described methodology using the three phylogenetically characterized isolates BPC1, BPC2, and BPC3 (Esterio et al. 2017; Weber and Hahn 2011). Three replicate plates were analyzed by each fungicide concentration for CG experiments (boscalid: 0, 0.05, 0.1, 1, 10, 100; cyprodinil/fludioxonil: 0, 0.01, 0.025, 0.05, 0.1, 0.25; fenhexamid: 0, 0.03, 0.1, 0.3, 1, 3, 10) and measurement of diameter of the resulting colonies was obtained. EC₅₀ values (effective inhibitory dose that trigger half-maximal inhibitory response) were calculated for each isolate using the Minitab version 12 statistical software program. Individually, the three *B. prunorum* isolates were sensitive to boscalid and the mixture cyprodinil/fludioxonil, but not to fenhexamid (Table 2) considering the cutoff values reported by Esterio et al. (2017). Interestingly, natural resistance to fenhexamid has been reported for *B. pseudocinerea* in a pathway independent of the fungicide molecular target, the sterol-3-ketoreductase enzyme (Debieu et al. 2001; Plesken et al. 2015; Walker et al. 2011). The determination of a homologous metabolic mechanism in *B. prunorum* or resistance acquisition as a result of fungicide selection pressure remains to be determined.

The present report is the first one to show blossom blight in *V. vinifera* caused by both *B. cinerea* and *B. prunorum*; accordingly, flowers are the target tissue for both species. This behavior was previously reported in Japanese plums by Ferrada et al. (2016), suggesting a possible evolutionary specialization where both species share the same ecological niche. In grapevines, *B. cinerea* preferentially infects the berry receptacle (Keller et al. 2003); consequently, the *B. prunorum* infection site should be established in order to determine the coexistence or competition of both species.

Cultivated in vitro, both species presented clear morphological differences. While *B. prunorum* presented white colonies with low to no sporulation, *B. cinerea* presented white to gray colonies with abundant gray to dark gray sporulation. *B. prunorum* characteristics are consistent with what was reported for *B. prunorum* isolated from Japanese plum and kiwifruit (Elfar et al. 2017; Ferrada et al. 2016). The limited conidia-forming capacity of *B. prunorum* in artificial growth media has also been reported on other recently discovered *Botrytis* species (Garfinkel et al. 2017; Grant-Downton et al. 2014), highlighting the importance of understanding the interaction between fungus pathogens and their host to sustain their life cycles. Although *B. prunorum* represented a small proportion of total recovered isolates (11.4%), the sclerotia index was not affected, suggesting that the

abundance of this species is not the result of a defect in its ability to develop survival structures. *B. prunorum* is pathogenic in Thompson Seedless berries, but approximately 50% less than *B. cinerea*. This result is consistent with that reported by Acosta Morel et al. (2018), where *B. prunorum* strains showed less aggressiveness in berries and grape leaves compared with *B. cinerea*. Although the damage generated by *B. prunorum* is rather small, it may impact the quality and reception of table grape for export. Additionally, a *B. prunorum* lesion may allow the potential entry of other more aggressive pathogens, including *B. cinerea*.

The study results suggest that *B. cinerea* and *B. prunorum* are part of the so-called *Botrytis* complex in grapevines (Garfinkel et al. 2017; Saito et al. 2016; Zhou et al. 2014). Because both species trigger the same symptoms in floral tissue and fruit infection, an adequate characterization of each species participating in the disease is required to generate appropriate global *Botrytis* control programs.

In addition, *B. prunorum* isolates studied in this report are controlled by boscalid and cyprodinil/fludioxonil, two important fungicides in *Botrytis* control programs; however, reduced fenhexamid sensitivity was detected, in contrast to the observations of Acosta Morel et al. (2018) for *B. prunorum* of wine grapes. In the Central Valley of Chile, during table grape production, fenhexamid is frequently used in the bloom and post bloom period. A second molecule with the same fenhexamid mode of action was recently introduced in the Chilean market for *Botrytis* control, fenpyrazamine (Kimura et al. 2017). Except for fludioxonil, loss of sensitivity to the main fungicide families has been detected in Chile (Esterio et al. 2017). In spite of the low frequency and aggressiveness of *B. prunorum*, the loss of sensitivity to fungicide is a highly relevant aspect in development and disease control in field. Acquisition of resistance to fenhexamide can result from an excessive selection pressure by the fungicide rather than a physiological condition characteristic of this species.

Wang et al. (2018) and Acosta Morel et al. (2018) identified *B. prunorum* isolates in grapevine from the U.S.A. and Spain, respectively, focused on the use of partial sequence of *G3PDH* and *HSP60*. In order to develop adequate control programs, the determination of the population structure of *Botrytis* and the biological characteristics of the species that comprise it must be determined. As shown, this challenge must necessarily be addressed including the study of *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2* to have robust conclusions. The fact that *B. prunorum* persists along the Central Valley of Chile infecting other fruit species, demands an epidemiological review in order to assess its impact mainly on Chilean table grape production.

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