Identification and Characterization of Botrytis Blossom Blight of Japanese Plums Caused by *Botrytis cinerea* and *B. prunorum* sp. nov. in Chile

Enrique E. Ferrada, Bernardo A. Latorre, Juan P. Zoffoli, and Antonio Castillo

First, second, and third authors: Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Vicuña Mackenna 4860, Santiago, Chile; and fourth author: Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Santiago.

Accepted for publication 12 October 2015.

ABSTRACT

Ferrada, E. E., Latorre, B. A., Zoffoli, J. P., and Castillo, A. 2016. Identification and characterization of Botrytis blossom blight of Japanese plums caused by *Botrytis cinerea* and *B. prunorum* sp. nov. in Chile. Phytopathology 106:155-165.

Blossom blight is a destructive disease of plums (*Prunus salicina*) when humid and temperate weather conditions occur in Chile. Disease incidence ranging from 4 to 53% has been observed. Symptoms include light brown petal necrosis, starting as light brown mottles or V-shaped necrosis at the margins of the petals, progressing to the stamen and pistils. In this study, the etiology of blossom blight of plums was determined. High- and low-sporulating isolates of *Botrytis* were obtained consistently from blighted blossoms and apparently healthy flowers of plums. Based on colony morphology, conidial production and molecular phylogenetic analysis, these high- and low-sporulating isolates were identified as *B. cinerea* and *B. prunorum* sp. nov., respectively. Phylogenetic analysis of the genes glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*), heat-

shock protein 60 (*HSP60*), and DNA-dependent RNA polymerase subunit II (*RPB2*) grouped *B. prunorum* isolates in a single cluster, distantly from *B. cinerea* and other *Botrytis* species. The phylogenetic analysis of necrosis and ethylene-inducing protein (*NEP1* and *NEP2*) genes corroborated these results. Analysis of the internal transcribed spacer region and large-subunit (26S) ribosomal DNA and detection of *Boty* and *Flipper* transposable elements, were not useful to differentiate between these *Botrytis* species. Both species were pathogenic on plum flowers and the fruit of plums, apples, and kiwifruits. However, *B. prunorum* was less virulent than *B. cinerea*. These pathogens were re-isolated from inoculated and diseased tissues; thus, Koch's postulates were fulfilled, confirming its role in blossom blight of plums. *B. cinerea* was predominant, suggesting that *B. prunorum* may play a secondary role in the epidemiology of blossom blight in plums in Chile. This study clearly demonstrated that the etiology of blossom blight of plums is caused by *B. cinerea* and *B. prunorum*, which constitute a species complex living in sympatry on plums and possibly on other stone fruit trees.

The Japanese plum (*Prunus salicina* Lindl.) is a very important stone fruit species in Central Chile, currently with over 6,000 ha primarily destined for export as fresh fruit to markets in the United States, Europe, and Asia (www.odepa.cl). Blossom blight has become a very common disease and is often confused with the early stages of the brown rot caused by *Monilinia fructicola* (Winter) Honey and/or *M. laxa* (Aderh. and Ruhl) Honey. However, *Botrytis* spp. have been found to be associated with this disease (Ferrada et al. 2014).

Botrytis blossom blight occurs when cool and wet weather conditions prevail during flowering. The symptoms are characterized by a light brown necrosis of the petals that may affect the entire flowers, which remain attached to the twigs for some time. As it has been described on other hosts, the disease often progresses from fading petals into the rest of the inflorescence (Droby and Lichter 2004). Eventually, gray mold appears on the necrotic tissues under highly humid conditions in the field, and the same symptoms can also be induced in humid chambers under laboratory conditions. A similar disease, green fruit rot (blossom rot or jacket rot) caused by *B. cinerea*, was previously described (Flint 1995). However, the overall colony morphology of the pathogen(s) suggests that more than one species of *Botrytis* are associated with blossom blight of Japanese plums in Chile.

The genus *Botrytis* currently comprises 301 valid species (www.mycobank.org). Most of these species have been identified based on the morphological features and host specificity, but DNA-based

molecular characterization has recently been used to delimit newer *Botrytis* species. Phylogenetic analyses using the sequences of the nuclear genes glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*), heat-shock protein 60 (*HSP60*), and DNA-dependent RNA polymerase subunit II (*RPB2*) have been used to corroborate the identification of *Botrytis* species (Li et al. 2012; Lorenzini and Zapparoli 2014; Zhang et al. 2010a,b; Zhou et al. 2014). Similarly, phylogenetic analyses of the partial sequences of the beta-tubulin gene and *G3PDH* supported the identification of *B. mali* Ruehle (O'Gorman et al. 2008). Furthermore, the necrosis and ethylene-inducing protein (*NEP*) genes 1 and 2 (Staats et al. 2007) could be used to design specific primers to identify some species of *Botrytis* (Grant-Downton et al. 2014; Li et al. 2012; Lorenzini and Zapparoli 2014; Mirzaei et al. 2008; Staats et al. 2007).

A survey conducted in Central Chile from 2012 to 2014 showed that *B. cinerea* and an unidentified *Botrytis* sp. 1 were associated with blossom blight symptoms on Japanese plums (Ferrada et al. 2014, 2015). The colony morphology and scarce conidial production distinguished *Botrytis* sp. 1 from *B. cinerea*. Therefore, the aim of this study was to characterize the Botrytis blossom blight of Japanese plums and to identify *Botrytis* sp. 1 by cultural and morphological characteristics and phylogenetic analysis using the sequences of the nuclear genes mentioned above. The pathogenicity of isolates of *B. cinerea* and *Botrytis* sp. 1 were compared and the relative importance of these species was estimated.

MATERIALS AND METHODS

Sampling locations and fungal isolation. Diseased and apparently healthy flowers were collected during full bloom

Corresponding author: B. A. Latorre; E-mail address: blatorre@uc.cl

(August to September) from six commercial orchards of Japanese plums in six geographic localities in Central Chile (33°29' to 34°39' Lat. S.) during the 2012, 2013, and 2014 growing seasons. In each orchard, 100 diseased and 100 apparently healthy flowers were

collected from at least 10 trees. Flower samples were transported to the laboratory in plastic boxes. Diseased petals showing a light brown necrosis were placed in 2% acidified potato dextrose agar (APDA) with 92% lactic acid at 0.5 ml per liter and incubated for

TABLE 1. Isolates of *Botrytis cinerea* and *B. prunorum* obtained from Japanese plums, nectarine, and peach associated with Botrytis blossom blight in Chile

Season	Isolate	Conidial production ^z	Identity	Host	Origin
2012	Bci-16	HCP	<i>B. cinerea</i>	Plum	Pirque
2012	Bci-35	HCP	<i>B. cinerea</i>	Plum	San Francisco
2012	Bci-32	HCP	<i>B. cinerea</i>	Nectarine	San Francisco
2012	Bpru-8	LCP	<i>B. prunorum</i>	Plum	Pirque
2012	Bpru-21	LCP	<i>B. prunorum</i>	Plum	Pirque
2012	Bpru-49	LCP	<i>B. prunorum</i>	Peach	Graneros
2013	Bci-12	HCP	<i>B. cinerea</i>	Nectarine	Rosario
2013	Bci-14	HCP	<i>B. cinerea</i>	Plum	San Francisco
2013	Bci-15	HCP	<i>B. cinerea</i>	Nectarine	San Francisco
2013	Bci-18	HCP	<i>B. cinerea</i>	Nectarine	Pirque
2013	Bpru-1.2	LCP	<i>B. prunorum</i>	Plum	Rosario
2013	Bpru-1.4	LCP	<i>B. prunorum</i>	Plum	San Francisco
2013	Bpru-1.5	LCP	<i>B. prunorum</i>	Plum	San Francisco
2013	Bpru-1.9	LCP	<i>B. prunorum</i>	Nectarine	Pirque

^z High conidial producer (HCP) isolates were identified as *B. cinerea* and low conidial producer (LCP) isolates were identified as new *Botrytis* species, named *B. prunorum*.

TABLE 2. Species of *Botrytis*, *Monilinia*, and *Sclerotinia*, strains, origin, date, and GenBank accession numbers of the nucleotide sequences used in this study

Species	Strains	Origin	Date	GenBank accession numbers					
				ITS	G3PDH	HSP60	RPB2	NEP1	NEP2
<i>B. aclada</i>	MUCL3106	USA	1961	—	AJ704991	AJ716049	AJ745663	—	—
	PR1006	—	—	AJ716295	AJ704993	AJ716051	AJ745665	—	—
	MUCL8145	Germany	1965	—	—	—	—	AM087059	AM087087
<i>B. byssoides</i>	MUCL94	USA	1923	—	—	—	—	AM087045	AM087079
<i>B. calthae</i>	CBS175.63	USA	1961	AJ716302	AJ704999	AJ716060	AJ745671	—	—
	MUCL1089	Belgium	1960	—	AJ705000	AJ716061	AJ745672	—	—
	MUCL2830	USA	2005	—	—	—	—	AM087031	AM087088
<i>B. caroliniana</i>	CA3	USA	2011	JF777533	JF811586	JF811589	JF811592	JF811594	—
	CB15	USA	2011	JF777531	JF811584	JF811587	JF811590	JF811593	—
	WM4	USA	2011	—	—	—	—	JF811595	—
	—	—	—	—	—	—	—	—	—
<i>B. cinerea</i>	Bci-16	Pirque, Chile	2012	KP091846	KP120864	KP136776	KP120871	—	—
	Bci-32	San Francisco, Chile	2012	KP091847	KP120865	KP136777	KP120872	—	—
	Bci-35	San Francisco, Chile	2012	KP234030	KP120866	KP136778	KP120873	—	—
	Bci-12	Rosario, Chile	2013	KP234031	KP120867	KP136779	KP120874	—	—
	Bci-14	San Francisco, Chile	2013	KP234032	KP120868	KP136780	KP120875	—	—
	Bci-15	San Francisco, Chile	2013	KP234033	KP120869	KP136781	KP120876	—	—
	Bci-18	Rosario, Chile	2013	KP234034	KP120870	KP136782	KP120877	—	—
	B05.10	Germany	1994	—	AJ705002	AJ716063	AJ745674	DQ211824	DQ211825
	MUCL87 ^y	The Netherlands	1928	—	AJ705004	AJ716065	AJ745676	—	—
	SAS56	The Netherlands	2005	AJ716294	AJ705006	AJ716067	AJ745677	—	—
	LB338	The Netherlands	2005	—	—	—	—	AM087029	—
	SAS405	The Netherlands	—	—	—	—	—	AM087028	—
	BC7	The Netherlands	—	—	—	—	—	AM087027	—
	BcB	The Netherlands	2005	—	—	—	—	—	AM087090
	9801	The Netherlands	1998	AJ716304	AJ705007	AJ716068	AJ745679	AM087034	AM087061
<i>B. convoluta</i>	MUCL11595	USA–The Netherlands	1968	—	AJ705008	AJ716069	AJ745680	AM087035	AM087062
<i>B. croci</i>	MUCL436	The Netherlands	2005	—	—	—	—	AM087047	AM087065
<i>B. draytonii</i>	MUCL3865	The Netherlands	2005	—	—	—	—	AM087041	—
	9701	The Netherlands	2005	—	—	—	—	AM087040	AM087072
<i>B. deweyae</i>	CBS134649	Australia	2013	—	—	—	—	HG799527	—
<i>B. elliptica</i>	Be9610	The Netherlands	2005	—	AJ705011	AJ716072	AJ745683	DQ211827	AM087082
	BE9714	The Netherlands	2005	AJ716300	AJ705012	AJ717073	AJ745684	AM087049	AM087089
	BE9401	The Netherlands	2005	—	—	—	—	AM087091	—
	BE0022	The Netherlands	2001	—	—	—	—	AM087050	AM087081
	Be0004	The Netherlands	2005	—	—	—	—	—	DQ211828
<i>B. fabae</i>	CBS109.57	The Netherlands	1957	AJ716303	AJ705013	AJ716074	AJ745685	AM087025	—
	MUCL98	Spain	1929	—	AJ705014	AJ716075	AJ745686	DQ211829	DQ211831
	MUCL7923	The Netherlands	2005	—	—	—	—	—	DQ211832
	1104	Germany	2012	—	—	—	—	—	JX266758
<i>B. fabiopsis</i>	BC-2	China	2006	EU519204	EU519211	EU514482	EU514473	—	—
	BC-13	China	2007	EU563122	EU563109	EU563100	EU563115	—	—

(continued on next page)

^y *B. cinerea* ex-type.

^z *B. mali* neotype.

5 days at 20°C in darkness. White to gray colonies were transferred to fresh APDA, and pure cultures were obtained by subcultivating hyphal tips on APDA. Pure cultures were maintained on APDA at 20°C for further analysis. The apparently healthy flowers were incubated in humidity chambers (80% relative humidity [RH]) for 7 days at 20°C before determining the proportion of necrotic flowers. Diseased flowers were incubated in a humid chamber to facilitate sporulation before examining the presence of *Botrytis* spp. under a stereoscopic microscope, and isolations were performed on APDA.

Cultural characterization and morphological identification. Conidial production was determined initially on APDA. For this purpose, an agar plug (5 mm diameter) was placed individually in the center of 9-cm-diameter Petri dishes containing APDA and were incubated for 7 days at 20°C under 12 h light/dark cycle. Isolates were classified visually as high conidial producers (HCP) and low conidial producers (LCP). To further study conidial production, a subset of seven HCP and seven LCP isolates (Table 1) were arbitrarily selected to quantify conidial production on APDA; King's medium B (KMB), containing per liter 1.5 g of K₂KPO₄·3H₂O, 1.5 g of MgSO₄·7H₂O, 10 ml of glycerol, 17 g of peptone, and 15 g of agar, as proposed by Li et al. (2012); and pea agar medium (PAM), containing per liter 160 g of pea (liquefied for 2 min in 1 liter of distilled water, adjusted to pH 6.0 using HCl), 5 g of sucrose, and 25 g of agar

(Dahmen et al. 1983). HCP and LCP isolates were plated in quadruplicate in 9-cm-diameter Petri dishes containing the respective agar media and incubated for 7 days at 20°C with 12 h dark and 12 h light. To determine total conidial production, each plate was flooded with 10 ml of 0.05% Tween 80 (Sigma Aldrich, MO), and the conidial concentration was estimated with the aid of a hemocytometer under a light microscope.

The morphology and size of the conidia ($n = 40$) and conidiophores ($n = 10$) were studied on PAM incubated for 7 days at 20°C. Sclerotial production was determined on APDA, KMB, and PAM incubated for 7 days at 20°C followed by 30 days at 5°C and 30 days at 20°C. The results were compared with published descriptions of *Botrytis* species (Mirzaei et al. 2008) (www.mycobank.org).

Molecular characterization. DNA was extracted from the same subset of seven HCP and seven LCP isolates as indicated above (Table 1). The DNA extraction was performed from 7-day-old cultures in potato dextrose broth using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA). The internal transcribed spacer (ITS) region, ITS1-5.8S-ITS2 of the nuclear rDNA was amplified using primers ITS4 and ITS5 (White et al. 1990). The large-subunit (LSU) (26S) ribosomal DNA (rDNA) was amplified with primers NL-1 and NL-4 (Kurtzman and Robnett 1997). The partial DNA sequences of

TABLE 2. (continued from preceding page)

Species	Strains	Origin	Date	GenBank accession numbers					
				ITS	G3PDH	HSP60	RPB2	NEP1	NEP2
<i>B. ficariarum</i>	CBS176.63	The Netherlands	2005	–	–	–	–	AM087056	AM087085
	MUCL376	The Netherlands	2005	–	–	–	–	AM087055	–
<i>B. galanthina</i>	MUCL435	The Netherlands	1958	–	AJ705018	AJ716079	AJ745689	–	–
	MUCL3204	The Netherlands	1963	–	AJ705017	AJ716078	AJ745690	–	AM087067
<i>B. globosa</i>	MUCL21514	The Netherlands	2005	–	–	–	–	AM087044	AM087070
	MUCL444	The Netherlands	2005	–	–	–	–	–	AM087071
<i>B. galanthina</i>	MUCL435	The Netherlands	2005	–	–	–	–	AM087057	–
	MUCL3204	The Netherlands	2005	–	–	–	–	AM087058	–
<i>B. hyacinthi</i>	0001	The Netherlands	2005	–	–	–	–	AM087048	AM087066
<i>B. mali</i>	BPI 412756 ^a	USA	2008	–	EF367129	–	–	–	–
<i>B. narcissicola</i>	MUCL2120	The Netherlands	2005	–	–	–	–	–	AM087078
<i>B. paeoniae</i>	0003	The Netherlands	2002	AJ716298	AJ705027	AJ716088	AJ745699	AM087032	AM087064
	MUCL16084	Belgium	1970	–	AJ705028	AJ716089	AJ745700	AM087033	–
<i>B. polyblastis</i>	CBS287.38	The Netherlands	2005	–	–	–	–	AM087039	AM087074
	MUCL21492	The Netherlands	2005	–	–	–	–	–	AM087073
<i>B. porri</i>	MUCL3234	–	1926	AJ716292	AJ705032	AJ716093	AJ745704	AM087060	AM087063
	MUCL3349	Belgium	1963	–	AJ705033	AJ716094	AJ745705	–	–
<i>B. prunorum</i>	Bpru-8	Pirque, Chile	2012	KP234035	KP339979	KP339993	KP339986	KR732657	KP400595
	Bpru-21	Pirque, Chile	2012	KP234036	KP339980	KP339994	KP339987	KR732658	KP400596
	Bpru-49	Graneros, Chile	2012	KP234037	KP339981	KP339995	KP339988	KR732659	KP400597
	Bpru-1.2	Rosario, Chile	2013	KP234038	KP339982	KP339996	KP339989	KR732660	KR425424
	Bpru-1.4	San Francisco, Chile	2013	KP234039	KP339983	KP339997	KP339990	KR732661	KR425425
	Bpru-1.5	San Francisco, Chile	2013	KP234040	KP339984	KP339998	KP339991	KR732662	KR425426
	Bpru-1.9	Pirque, Chile	2013	KP234041	KP339985	KP339999	KP339992	KR732663	KR425427
	<i>B. pseudocinerea</i>	10091	China	2011	JN692379	JN692414	JN692400	JN692428	–
	N11_S_E06	Germany	2012	–	–	–	–	–	JX266759
<i>B. ranunculi</i>	CBS178.63	The Netherlands	2005	–	–	–	–	AM087054	AM087086
<i>B. sinoviticola</i>	GBC-9	China	2009	JN692378	JN692408	JN692394	JN692422	–	–
	GBC-5	China	2010	JN692386	JN692413	JN692399	JN692427	–	–
<i>B. sinoalli</i>	OnionBC59	China	2007	FJ169664	FJ169646	FJ169658	FJ169678	–	–
	OnionBC23	China	2006	EU519203	EU519217	EU514488	EU514479	–	–
<i>Botrytis</i> sp.	B83	Italy	2012	KC191680	KC191677	KC191678	KC191679	KC762944	KC762945
<i>B. sphaerosperma</i>	MUCL21481	The Netherlands	2005	–	–	–	–	AM087042	AM087068
	MUCL21482	The Netherlands	2005	–	–	–	–	AM087043	AM087069
<i>B. squamosa</i>	MUCL1107	USA	1923	–	AJ705037	AJ716098	AJ745710	AM087052	AM087084
	MUCL9112	The Netherlands	1966	–	AJ705038	AJ716099	AJ745711	AM087053	–
<i>B. tulipae</i>	PRI026	The Netherlands	2005	–	–	–	–	AM087051	AM087083
	BT9001	The Netherlands	2000	–	AJ705040	AJ716101	AJ745712	AM087036	AM087075
	BT9830	The Netherlands	2000	AJ716301	AJ705041	AJ716102	AJ745713	–	–
	Bt005	The Netherlands	2005	–	–	–	–	DQ211822	DQ211823
	BT9901	The Netherlands	2005	–	–	–	–	–	AM087076
<i>M. fructigena</i>	9201	The Netherlands	1992	–	AJ705043	AJ716047	AJ745715	–	–
<i>M. laxa</i>	COY1B5	France	1997	AF150674	–	–	–	–	–
<i>S. sclerotiorum</i>	484	The Netherlands	–	–	AJ705044	AJ716048	AJ745716	–	–

G3PDH, *HSP60*, and *RPB2* genes were amplified with the primer pairs G3PDHfor/G3PDHrev, HSP60for/HSP60rev, and RPB2for/RPB2rev, respectively (Staats et al. 2005).

To further characterize isolates of *Botrytis* sp. 1, the variation of the nucleotide sequences of the necrosis and ethylene-inducing protein genes (*NEP1* and *NEP2*) was studied, and these sequences were compared with known *NEP1* and *NEP2* sequences of *Botrytis* spp. available in GenBank. DNA amplifications were performed with primers NEP1for-NEP1revB and NEP2forE-NEP2revE, as previously described (Staats et al. 2007).

Polymerase chain reactions (PCR) were conducted in a thermal cycler (Axygen Biosciences, Union City, CA). Each PCR sample contained 2.5 µl of 10× PCR buffer, 1.0 µl of 25 mM MgCl₂, 0.4 µl of 10 mM dNTPs, 0.4 µl of a 0.5 mM solution of each primer, and 1.2 µl of 1 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) with 5 µl of template DNA in a final volume of 25 µl. A negative control without template DNA was always included. The thermocycling pattern was the same as that described by Staats et al. (2005), but the annealing temperatures were 55.0°C for *G3PDH*, 54.3°C for *HSP60*, 49.0°C for the *RPB2* genes, and 59.0°C for the *NEP1* and *NEP2* genes.

The PCR products were separated on 2% agarose gel in 1× Tris-acetate-EDTA buffer (TAE), stained with GelRed (Biotium Inc., Hayward, CA), and visualized by UV transillumination at 320 nm. The amplified PCR products were purified and sequenced by Macrogen (Macrogen, Geumcheon-gu, South Korea). The sequences were edited using ProSeq v.2.91 (University of Oxford, UK) and aligned using Clustal X 2.0 (Conway Institute, DU, Ireland). A BLAST search was performed against recognized sequences of *Botrytis* species in GenBank (www.ncbi.nlm.nih.gov) database (Table 2).

Phylogenetic analysis. The phylogenetic analysis was performed with molecular evolutionary genetics analysis (MEGA 6) software (Tamura et al. 2011), using maximum parsimony, the heuristic search option, and 1,000 random addition sequence replicates. The bootstrap values were evaluated using 1,000 replicates to test the branch strength. The tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were also recorded. This analysis was performed for the sequences of the genes ITS, *G3PDH*, *HSP60*, *RPB2*, *NEP1*, and *NEP2*. Prior to performing a concatenated analysis of the sequences of the genes *G3PDH*, *HSP60*, and *RPB2*, the congruence of the evolution between the three genes was studied using the partition homogeneity tests in PAUP v.4.0b10 (Sinauer Associates, Sunderland, MA).

The sequences of the *M. fructigena* strain 9201 and the *Sclerotinia sclerotiorum* strain 484 were included as outgroups in the phylogenetic analysis of the sequences of the genes *G3PDH*, *HSP60*, and *RPB2*. *M. fructigena* strain 9201 was used as an outgroup in the analysis of ITS (Table 2). Terminal regions that did not provide data from every sequence were excluded from the analysis.

Detection of transposable elements. The presence or absence of the transposable elements, *Boty* and *Flipper* (Diolez et al. 1995; Levis et al. 1997), was studied in seven HCP and seven LCP isolates (Table 1). The primer pairs used were LTR₉₈ (5'-AGCCTGTAGA ATCACCAACG-3), LTR₇₂₈ (5'-CGGTATTTCTGGTTGGCA-3), F₃₀₀ (5'-GCACAAAACCTACAGAAGA-3), F1550 (5'-ATTCGTT TCTTGGACTGTA-3) for *Boty* and *Flipper*, respectively (Muñoz et al. 2002). The PCR reactions were conducted as explained above. The PCR consisted of 30 cycles, denaturation for 3 min at 95°C, hybridization for 1 min at 48 and 60°C for *Boty* and *Flipper*, respectively, and elongation for 1 min at 72°C. Reactions were completed with a final 15-min elongation step at 72°C.

PCR products were loaded on a 2% agarose gel in 1× Tris-acetate-EDTA buffer (TAE), stained with GelRed (Biotium Inc.) and visualized by UV transillumination at 320 nm.

Effect of temperature on mycelial growth. The effect of temperature on the mycelial growth of seven HCP and seven LCP isolates was determined on APDA. Each 90-mm-diameter Petri dish containing APDA was inoculated with a 4-mm diameter mycelial plug of each isolate and plates were incubated at 0, 5, 10, 15, 20, 25, 30, and 35°C (±1°C) in darkness. The colony diameter in each plate was measured daily until the culture reached the edge of the Petri dish. Isolates were incubated in Velp chambers, and the temperatures inside the Petri dishes were checked with a temperature sensor (HOBO PRO, Onset Computer Corp., MA). The growth rate was estimated as the mean growth obtained at 72 and 96 h of incubation at 25°C in darkness for isolates of *B. cinerea* and *B. prunorum*, respectively. The effect of the *Botrytis* species and temperature on mycelial growth was analyzed by a two-way (*Botrytis* species and temperature) analysis of variance using SigmaStat 3.5 (SPSS, Chicago, IL) with four replicates each with one Petri dish.

Pathogenicity tests. Inoculum suspensions were prepared with conidia obtained from 14-day-old cultures on PAM of the respective *Botrytis* isolates.

Pathogenicity tests were conducted on detached flowers of 'Larry Ann' Japanese plums. Ten detached flowers per isolate were arranged randomly on metal grills, placed in 1-liter polyethylene trays, and sprayed with 100 µl of a 10⁶ conidia/ml suspension per flower of each of seven *B. cinerea* and seven *Botrytis* sp. 1 isolates. Inoculated flowers and the noninoculated controls were incubated for 48 h at 20°C in humid chambers (>80% RH). Each flower was then examined for necrotic petals, and disease severity was expressed as the total number of necrotic petals in the flower. An equal number of flowers treated with sterile distilled water served as controls. Re-isolations from necrotic petals on APDA were attempted. The effect of the *Botrytis* species and isolates on disease severity was analyzed by a two-way (*Botrytis* species and isolates) analysis of variance, and means were separated according to Tukey's test ($P < 0.05$) with 10 replicates each consisting of one flower using SigmaStat.

A separate experiment was conducted on flowers of Japanese plums Larry Ann and Flavor Rich to determine the effects of inoculum concentrations on disease severity. Flowers were inoculated with conidial suspensions at 10⁰, 10², 10⁴, 10⁶, and 10⁸ conidia/ml of *B. cinerea* isolate Bci-16 and *Botrytis* sp. 1 isolate Bpru-1.2, selected among the HCP and LCP *Botrytis* isolates, respectively. Disease severity was determined as the number of blighted petals per flower after incubation for 48 h at 20°C in humid chambers. The effect of the inoculum concentration on disease severity was analyzed by a two-way (*Botrytis* species and inoculum concentration) analysis of variance with four replicates each consisting of 10 flowers using SigmaStat. The relationship between y = number of blighted petals and x = log of inoculum concentration (conidia/ml) was plotted and subjected to regression analysis using SigmaStat.

The ability of seven isolates of *B. cinerea* and seven isolates of *Botrytis* sp. 1 to decay mature fruits ($n = 4$) was studied on mature (13.5% mean soluble solids, MSS) Larry Ann Japanese plums using a conidial suspension (10⁶ conidia/ml) and mycelial plugs (4 mm in diameter) as inoculum. In addition, the ability to decay mature fruits of species other than *P. salicina* was studied on Granny Smith apples (13.9% MSS) and on 'Hayward' kiwifruits (10.5% MSS). The fruits were surface disinfected in 75% ethanol for 5 min and wounded with a sterile core borer prior to insertion of a mycelial plug (4 mm in diameter) underneath the epidermis. Fruits were incubated for 2 to 4 days at 20°C in humid chambers before the diameters of the necrotic lesions were determined. An equal number of non-inoculated but injured fruits were left as controls. The effect of the *Botrytis* species and isolates on the necrotic lesions was analyzed by a two-way (*Botrytis* species and isolates) analysis of variance, and means were separated according to Tukey's test ($P <$

0.05) with four replicates each consisting of one fruit using SigmaStat.

RESULTS

Species and distribution of *Botrytis*. In total, 1,026 isolates of *Botrytis* spp. were obtained from diseased and apparently healthy Japanese flowers between the 2012 and 2014 growing seasons, with 33.9% of the isolates from diseased blossoms and 66.1% from apparently healthy flowers. In addition, one and five isolates were identified on peach and nectarine flowers, respectively. Symptoms started as small light brown mottles on the petals. Lesions expanded rapidly and eventually affected the entire petals. The stamens and pistil eventually also turned brown. AV-shaped necrotic lesion often appeared on petals in the early stage of symptom development. Blighted flowers fell from the trees. Under humid conditions, a gray sporulation appeared on diseased organs. No symptoms were observed on twigs and spurs (Fig. 1).

On the basis of morpho-cultural characteristics and molecular analysis of the DNA, of the 1,026 isolates tested, 90.4% of the isolates were HCP isolates and identified as *B. cinerea*, and 9.6% of the isolates were LCP isolates (*Botrytis* sp.1). *Botrytis* sp. 1 and *B. cinerea* were isolated from diseased blossom and apparently healthy flowers and they were present in all six sampling locations. *Botrytis* sp. 1 was identified as *B. prunorum* sp. nov. in this study.

***B. prunorum* E. E. Ferrada & B. A. Latorre sp. nov.** (Figs. 2 and 3) MycoBank MB814437.

B. prunorum is described here as a new species of *Botrytis* that was commonly isolated along with *B. cinerea* from apparently healthy and blossom blight-affected Japanese plums.

B. prunorum grew 23.2 ± 1.2 mm per day at 25°C, producing white to yellowish colonies, with floccose and tufted aerial mycelia, with scarce sporulation, with a conidial production ranging from 0 to 9.9 conidia/cm² on APDA (Fig. 2). Colonies of *B. cinerea sensu stricto* were white to gray, with less fluffy aerial mycelia and grew at

Fig. 1. Symptoms of *Botrytis* blossom blight in flowers of Japanese plum 'Larry Ann'. **A**, V-shaped necrotic lesion starting at the edge of the petal. **B**, Petal, stamen, and pistil necrosis. **C**, Blighted blossom. **D**, Symptoms on flowers artificially inoculated with conidial suspension of the pathogens; from left to right, *B. cinerea*, *B. prunorum*, and sterile distilled water control.

Fig. 2. Colony morphology of isolates of *Botrytis prunorum* and *B. cinerea* on acidified potato dextrose agar (APDA), King's medium B (KMB), and pea agar medium (PAM) incubated for 7 days at 20°C with a 12 h light/12 h dark cycle.

Fig. 3. Morphology of conidiophores and conidia of *Botrytis prunorum*. **A**, Long septate conidiophore, dark and constricted at the base and hyaline and branched at the apex. **B**, Branched conidiophore with oval to ellipsoidal hyaline to slightly colored conidia. Conidial morphology under scanning electron microscopy of **C**, *B. prunorum* and **D**, *B. cinerea*. Bar = 100 µm (**A**) and 10 µm (**B**, **C**, and **D**).

a rate of 27.4 ± 1.1 mm per day on APDA. Conidial production of *B. cinerea* was significantly ($P < 0.001$) higher than that of *B. prunorum*, ranging from 31.8 to 487.2 conidia/cm² (Fig. 2). Colonies of *B. prunorum* were flat white-creamy to brown in the center on KMB and white floccose with a gray to brown center on PAM, while colonies of *B. cinerea* were gray floccose with uneven growth on KMB and gray, fluffy, and floccose on PAM (Fig. 2).

The composition of the culture media significantly ($P < 0.001$) affected the quantity of conidia produced by both *Botrytis* species, with the highest conidial production on PAM and the lowest on KMB. The interaction between the *Botrytis* species and the culture media was significant ($P = 0.001$).

B. prunorum produced sclerotia at 5°C, but not at 20°C, on APDA and KMB after 30 days, and one of the seven isolates produced few sclerotia on PAM at 20°C. *B. cinerea* produced sclerotia on APDA and PAM at 5 and 20°C. Differences in sclerotial production at 5°C on APDA and PAM were significant (Table 3). The sclerotia of *B. prunorum* and *B. cinerea* were black, ovoid to elongate, and randomly distributed on Petri plates containing either APDA or PAM with a mean length of 2.92 ± 1.07 and mean width of 3.11 ± 1.33 mm for *B. prunorum* and mean length of 5.39 ± 1.49 and mean width of 4.35 ± 1.09 mm for *B. cinerea* (Table 3).

The conidiophores of *B. prunorum* isolates were erect, thick-walled, smooth, septate, subhyaline at the base and the apex and brown in the middle, slightly constricted at the base, gradually tapering toward the apex, irregularly branched toward the apex (Fig. 3A and B), and 508.7 to 1,004.8 µm in length and 10.4 to 14.6 µm in width. The conidia were mostly ovoid, hyaline to slightly colored, smooth, with a slightly protuberant hilum (Fig. 3C) similar to conidia of *B. cinerea* (Fig. 3D). The conidia of *B. prunorum* were 5.9 to 20.8 µm in length and 4.8 to 12.9 µm in width. The length/width ratio was 1.6 ± 0.3 for *B. prunorum* and 1.3 ± 0.03 for *B. cinerea* isolates (Table 3). Chlamydospores and microconidia were not found, and teleomorphs were not present.

Etymology: “*prunorum*” refers to the genus of the host from which the fungus was isolated.

Specimen examined: Chile, San Francisco de Mostazal (33°59'53"S, 70°41'38"W), a dried culture of the isolate Bpru-21 isolated from *Prunus salicina* (Japanese plum) flowers, September 2012, collectors E.E. Ferrada and B.A. Latorre (Holotype BPI893191 [U.S. National Fungus Collection accession number]); CBS140338 initiated from the holotype has been deposited as an authentic ex-type culture. Pirque, Chile (33°40'10"S, 70°35'09"W), a dried culture of the isolate Bpru-1.9 isolated from *Prunus persica* var. *nectarina* (nectarine) flowers, September 2013, collectors E.E. Ferrada and B.A. Latorre (Paratype BPI893192); CBS140337 has been deposited as a living culture of the isolate Bpru-1.9.

Molecular characterization. The ITS1-5.8S-ITS2 regions for all *B. prunorum* isolates and *B. cinerea* isolates were successfully amplified, obtaining 492-bp fragments. In Blast searches of the GenBank database, these fragments, obtained with primers ITS4 and ITS5, were 100% identical to the published sequences of *B. cinerea* isolates deposited in GenBank (Table 2). Similarly, the LSU amplification yielded fragments of 478 bp (Accession numbers KT023582–88 and KT023589–95), which were 99% identical to the published LSU sequences of *B. cinerea* B05.10 deposited in GenBank (Accession number CP009808).

Regions of the *G3PDH*, *HSP60*, and *RPB2* genes for all seven *B. prunorum* isolates and seven *B. cinerea* isolates were amplified, and 886, 976, and 1,093-bp DNA fragments were obtained, respectively. The *G3PDH* DNA sequences obtained for the isolates of *B. prunorum* had 99% identity with that of a reference sequence of *B. cinerea* (strains SAS56 and MUCL87), 98 to 99% identity with that of *B. mali* (strain BPI 412756), and 98 to 99% identity with that of *B. paeoniae* (strain 0003). The same *B. prunorum* isolates were 97% identical to *HSP60* sequences and 97 to 98% identical to *RPB2* sequences of *B. paeoniae*.

All isolates of *B. prunorum* and *B. cinerea* were consistently detected by primers NEP1 for-NEP1revB and NEP2 for-NEP2revE. Amplicons of 687 and 743 bp were obtained for *NEP1* and *NEP2* genes for *B. prunorum*, which showed 87 and 88% identity with *B. cinerea* strain B05.10, respectively. The BLAST analysis of *NEP*

TABLE 3. Morphological characteristics of *Botrytis prunorum* and to *B. cinerea* isolates from Japanese plums

Criteria ^a	<i>B. cinerea</i>	<i>B. prunorum</i>	<i>P</i> value
Growth rate on APDA at 25°C, mm/day	27.4 ± 1.1 ^z	20.3 ± 0.8	<0.001
Conidia production at 20°C on:			
APDA, conidia/cm ²	206.0 ± 174.2	2.1 ± 3.8	0.007
KMB, conidia/cm ²	33.0 ± 27.0	1.7 ± 3.4	0.010
PAM, conidia/cm ²	767.1 ± 247.7	9.9 ± 10.8	<0.001
Conidia length (L):			
Mean, µm	9.6 ± 1.1	11.1 ± 1.6	<0.001
Range, µm	7.4 – 12.8	5.9 – 20.8	
Conidia width (W):			
Mean, µm	7.3 ± 0.7	7.3 ± 0.8	0.966
Range, µm	5.2 – 10.0	4.8 – 12.9	
L:W ratio	1.3 ± 0.03	1.6 ± 0.3	0.054
Conidiophore length:			
Mean, µm	1,172.2 ± 331.9	711.7 ± 104.7	<0.001
Range, µm	464.0 – 2,555.1	315.0 – 1,380.2	
Conidiophore width:			
Mean, µm	12.8 ± 2.0	11.8 ± 2.0	0.231
Range, µm	7.3 – 17.7	6.2 – 20.7	
Sclerotia length at 5°C, mm	5.39 ± 1.49	2.92 ± 1.07	0.002
Sclerotia width at 5°C, mm	4.35 ± 1.09	3.11 ± 1.33	0.109
Sclerotia production at 5°C on:			
APDA, no./cm ²	0.38 ± 0.42	0.18 ± 0.17	0.268
KMB, no./cm ²	0.00 ± 0.00	0.00 ± 0.00	
PAM, no./cm ²	0.25 ± 0.19	0.56 ± 0.66	0.253
Sclerotia production at 20°C on:			
APDA, no./cm ²	0.60 ± 0.49	0.00 ± 0.00	0.008
KMB, no./cm ²	0.00 ± 0.00	0.00 ± 0.00	
PAM, no./cm ²	1.23 ± 0.58	0.02 ± 0.06	<0.001

^a APDA = acidified potato dextrose agar, KMB = King's medium B, and PAM = pea agar medium.

^z Mean values of seven isolates for each species followed by standard deviation.

sequences of *B. prunorum* indicated 91% identity with *B. porri* in *NEP1* (strain MUCL3234) and 93% identity with *B. squamosa* in *NEP2* (strain PRI026). The sequences of the *ITS*, *G3PDH*, *HSP60*, and *RPB2* from *B. prunorum* and *B. cinerea* isolates obtained from Japanese plums, peaches, and nectarines were deposited in GenBank and sequences of *NEP1* and *NEP2* from *B. prunorum* also were deposited in GenBank (Table 2).

Phylogenetic analysis. The phylogenetic analysis, using sequences of the *ITS* gene, grouped *B. cinerea* and *B. prunorum* isolates in two different clusters and various subclusters along with other *Botrytis* species (data not shown).

The phylogenetic analysis, performed separately based on the sequences obtained from the *G3PDH*, *HSP60*, and *RPB2* genes, showed that all *B. prunorum* isolates were grouped together in the same cluster distantly from the cluster where *B. cinerea* isolates were grouped. Other *Botrytis* species such as *B. mali* and *B. paeoniae* were grouped separately.

The partition-homogeneity test performed with the sequences of *G3PDH*, *HSP60*, and *RPB2* was nonsignificant ($P = 0.81$).

Therefore, a maximum parsimony analysis of concatenate *G3PDH*, *HSP60*, and *RPB2* sequences was performed, yielding a single most parsimonious tree (Fig. 4). Based on maximum parsimony analysis, the six most parsimonious trees yielded tree length (TL) = 1038, consistency index (CI) = 0.717, retention index (RI) = 0.895, and recalled consistency index (RC) = 0.642. All isolates of *B. prunorum* were grouped together in a separate clade with 100% bootstrap value distantly related to *B. cinerea* (Fig. 4).

On the basis of the phylogenetic analysis of *NEP1* sequences, *B. prunorum* isolates were grouped in the same clade with 100% bootstrap value, very distantly from *B. cinerea* (Fig. 5). Similarly, sequence analysis of the *NEP2* gene of all *B. prunorum* isolates were grouped in the same clade with 99% bootstrap value, distinct from the *B. cinerea* clade (data not shown). The phylogenetic analysis revealed that sequences of *B. porri* and *B. squamosa*, which

Fig. 4. Phylogenetic analysis of 50 taxa of *Botrytis* spp. in a maximum parsimony tree inferred from the data set based on concatenated DNA sequences of *G3PDH*, *HSP60*, and *RPB2* using MEGA 6.0.

Fig. 5. Phylogenetic analysis of 50 taxa of *Botrytis* spp. in a maximum parsimony tree inferred from the DNA sequence encoding necrosis and ethylene-inducing protein 1 (*NEP1*) among *B. prunorum* isolates and other *Botrytis* species.

were the closest *Botrytis* sequences to *B. prunorum* based on NEP2 BLAST analysis, were grouped distantly from *B. prunorum*.

Detection of transposable elements. The analysis of the presence/absence of the transposable elements *Boty* and *Flipper* (Diolez et al. 1995; Levis et al. 1997) in *B. cinerea* and *B. prunorum* from Japanese plums revealed a 648-bp amplicon, as expected for *Boty*, in two (isolates Bci-14 and Bci-18) of the seven *B. cinerea* isolates and in six (Bpru-8, Bpru-49, Bpru-1.2, Bpru-1.4, Bpru-1.5, and Bpru-1.9) of the seven *B. prunorum* isolates. Evidence for the presence of the transposable element *Flipper* was not obtained.

Effect of temperature on mycelial growth. Mycelial growth was significantly ($P < 0.001$) affected by the *Botrytis* species and temperature. The interaction between *Botrytis* species and temperature was significant ($P < 0.001$). All isolates of *B. prunorum* and *B. cinerea* grew at temperatures between 5 and 30°C, but no growth was observed after 96 h at 35°C. One isolate of *B. cinerea* and five isolates of *B. prunorum* exhibited slight mycelial growth after 96 h at 0°C. The optimum temperature for mycelial growth was estimated to be 25°C for both *B. cinerea* and *B. prunorum*.

Pathogenicity test. When conidial suspension was used as inoculum, all isolates of *B. prunorum* and *B. cinerea* were pathogenic on flowers of Japanese plums Larry Ann (Table 4). Inoculated flowers developed a similar light brown necrosis of the petals that often started as light brown mottles or V-shaped necrosis at the margins of the petals, and necrosis was also observed in the stamens and pistils (Fig. 1D). Isolates of *B. cinerea* caused significantly ($P < 0.001$) higher numbers of blighted petals on flowers than *B. prunorum*, except the isolate Bpru-1.2, indicating that *B. prunorum* was less virulent than *B. cinerea* (Table 4). *B. prunorum* and *B. cinerea* were re-isolated from artificially inoculated flowers. No symptoms were observed on noninoculated flowers.

On flowers of Japanese plums Larry Ann and Flavor Rich, a second order polynomial model best explained the relation between the log inoculum concentration of *B. cinerea* and disease severity (the number of blighted petals per flower) with $y = 4.72x^2 - 15.88x + 28.41$ ($R^2 = 0.99$, $P = 0.007$) and $y = 4.15x^2 - 8.30x + 11.64$ ($R^2 = 0.94$, $P = 0.057$) for Larry Ann and Flavor Rich, respectively

(Fig. 6). For *B. prunorum*, the relationship between the inoculum concentration and the number of blighted petals was best explained by a linear relationship with $y = 5.96x + 5.34$ ($R^2 = 0.79$, $P = 0.044$) and $y = 3.23x + 2.42$ ($R^2 = 0.98$, $P = 0.001$) for Larry Ann and Flavor Rich, respectively (Fig. 6).

All isolates of *B. cinerea* and *B. prunorum* caused dark brown soft decay lesions on mature fruits of Larry Ann plums inoculated with conidia or mycelial plugs. Decay lesion sizes on the fruit inoculated with *B. prunorum* were significantly ($P < 0.001$) smaller than those inoculated with *B. cinerea*, regardless of inoculum type (Table 4). However, when mycelial plugs were used as inoculum, decay lesion sizes did not differ significantly among the isolates of *B. cinerea* and *B. prunorum* (Table 4).

All tested isolates of *B. cinerea* and *B. prunorum* caused dark brown decay lesions on apples and kiwifruits, and decay lesion sizes on the fruit inoculated with *B. prunorum* were significantly ($P < 0.001$) smaller than those inoculated with *B. cinerea* (Table 4). The pathogens were re-isolated from artificial inoculated fruit.

DISCUSSION

In this study, species of *Botrytis* were consistently isolated from blossom blight of Japanese plums in Central Chile, and we confirmed the earlier report of association of *B. cinerea* with this disease (Ferrada et al. 2015) and identified *B. prunorum* sp. nov. as a new *Botrytis* species. Previously, blossom blight caused by *B. cinerea* was reported on plums in California (Ogawa and English 1960) and South Africa (Fourie and Holz 1994).

Blossom blight reduces fruit set, and symptoms resemble those caused by *M. fructicola* and *M. laxa*, which are considered the most important pathogens responsible for this disease on plums and other stone fruit trees around the world (Ogawa et al. 1995). Unlike *Monilinia* infection, *Botrytis* spp. are always restricted to blossoms and never continue to the partially lignified spurs and twigs. The occurrence of this disease varies from year to year.

Disease incidence was estimated to range from 4 to 53% in 2013 and 2014. This variation in prevalence could be explained by differences in the microclimatic conditions (free moisture and temperature) in orchards during bloom stage, as reported previously

TABLE 4. Pathogenicity of isolates of *Botrytis cinerea* and *B. prunorum* on flowers of Japanese plum Larry Ann and mature fruit of Japanese plum, Granny Smith apple, and Hayward kiwifruit

Species	Isolates	Conidial inoculation		Fruit lesions after mycelial inoculation		
		Blighted petals (number) ^x	Plum fruit lesions (mm)	Plum (mm)	Apple (mm)	Kiwifruit (mm)
<i>B. cinerea</i>	Bci-16	5.0 a ^y	19.5	17.5 a	29.8	20.1
<i>B. cinerea</i>	Bci-35	5.0 a	17.8	22.6 a	29.5	22.4
<i>B. cinerea</i>	Bci-32	5.0 a	21.1	17.9 a	29.9	23.9
<i>B. cinerea</i>	Bci-12	5.0 a	31.1	18.9 a	29.5	21.1
<i>B. cinerea</i>	Bci-14	5.0 a	30.4	18.9 a	25.2	25.0
<i>B. cinerea</i>	Bci-15	4.9 a	22.3	23.6 a	32.0	21.6
<i>B. cinerea</i>	Bci-18	5.0 a	29.8	25.7 a	34.1	19.4
Mean		5.0	24.6 A ^z	20.7 a	30.0 A	21.9 A
<i>B. prunorum</i>	Bpru-8	2.4 a	13.0	10.1 a	10.2	10.4
<i>B. prunorum</i>	Bpru-21	1.8 a	13.1	10.9 a	20.1	13.3
<i>B. prunorum</i>	Bpru-49	3.0 ab	13.6	12.9 a	10.6	11.9
<i>B. prunorum</i>	Bpru-1.2	5.0 b	18.5	11.1 a	19.9	12.7
<i>B. prunorum</i>	Bpru-1.4	1.3 a	18.8	10.8 a	20.1	11.4
<i>B. prunorum</i>	Bpru-1.5	1.1 a	16.7	9.9 a	15.0	10.6
<i>B. prunorum</i>	Bpru-1.9	1.3 a	19.4	11.3 a	15.3	10.5
Mean		2.3	16.2 B	11.0	15.9 B	11.6 B
Analysis of variance	df	P	P	P	P	P
Species (S)	1	<0.001	<0.001	<0.001	<0.001	<0.001
Isolates (I)	6	<0.001	<0.001	0.107	0.539	0.041
S × I	6	<0.001	0.339	0.045	0.141	0.285

^x Petals ($n = 50$) were inoculated with 100 µl per flower of a conidial suspension of 10^6 conidia/ml incubated in humid chambers at 20°C for 48 h. Fruits ($n = 4$) were inoculated with a 4-mm-mycelial plug obtained from 7-day-old cultures on acidified potato dextrose agar.

^y Values followed by the same lowercase letter in the same column with the same species did not differ significantly according to Tukey's test ($P < 0.05$).

^z Means followed by the same uppercase letter in each column did not differ significantly according to Tukey's test ($P < 0.05$).

for *B. cinerea* infection of other hosts (Elmer and Michailides 2004; Latorre et al. 2015). However, the relationship between *Botrytis* blossom blight in Japanese plums and weather factors remains to be studied.

Isolates of *B. cinerea* and *B. prunorum* were pathogenic, inducing flower blight and fruit rot on Japanese plums inoculated with conidia or mycelium of the fungi. These pathogens were re-isolated only from inoculated and diseased tissues. Thus, Koch's postulates were fulfilled with *B. cinerea* and *B. prunorum*, confirming its role in the blossom blight of Japanese plums. Further studies are still needed to determine the presence and the role of *B. prunorum* in fruit infection. Given that ascospores of *B. prunorum* were not found, it is most likely that infections may be initiated from air-dispersed conidia in nature that are produced in multiple and very diverse sources of primary inoculum, as described for *B. cinerea* on other hosts (Holz et al. 2004; Williamson et al. 2007). The ability of conidia of *B. cinerea* to germinate and form appressoria on plum flowers has been studied previously (Fourie and Holz 1994). However, the sources of inoculum of *B. prunorum* remain to be determined.

Both *B. cinerea* and *B. prunorum* were isolated consistently from blighted blossoms and apparently healthy flowers. However, *B. cinerea* was always the predominant species that was identified in 91.7 and 89.8% of the isolates obtained from blighted flowers and apparently healthy flowers, respectively. Our results suggest that *B. prunorum* was less fit than *B. cinerea* because of the low proportion of *B. prunorum* isolates. This finding can, at least in part, be explained by the relatively low conidia production, which is the principal inoculum and dispersal structure for *B. cinerea* (Holz et al. 2004), the relatively slow growth rate that reduces the aggressiveness of this pathogen, and the low sclerotial production, which likely play an important role in survival (Coley-Smith 1980).

B. cinerea is a polyphagous and necrotrophic fungus with a wide host range, including over 200 hosts primarily among dicotyledonous plants (van Kan et al. 2014; Williamson et al. 2007). Recently, the capability of *B. cinerea* to internally colonize plants, possibly as an endophyte, has been discussed (Delaye et al. 2013; van Kan et al. 2014). Whether this endophytic life style is also present in *B. prunorum* remains to be determined. Interestingly, *B. cinerea* and *B. prunorum* were found as epiphytes in this study, suggesting that both *Botrytis* species can have a facultative pathogenic behavior. The host range of *B. prunorum* remains to be studied. However, *B. prunorum* was found on blighted flowers of nectarine and peach, and isolates of this species were pathogenic on apple and kiwifruit, suggesting that *B. prunorum* is a nonspecific *Botrytis* species. In addition, *B. prunorum* and *B. cinerea* were consistently isolated from apparently healthy flowers.

The pathogenicity of *B. cinerea* and *B. prunorum* was demonstrated on artificially inoculated Japanese plum flowers and on the fruits of plum, apple, and kiwifruits. However, *B. prunorum* was less virulent than *B. cinerea*. Furthermore, regardless of the *Botrytis* species, the number of blighted petals increased with increasing inoculum concentration, but steeper increases in the number of blighted petals were obtained with *B. cinerea* than *B. prunorum*, and at any given inoculum concentration, the amount of blighted petals was always significantly ($P < 0.05$) higher with *B. cinerea* than *B. prunorum*. These results reinforce the difference in virulence observed between these two species of *Botrytis*.

The occurrence of mycovirus in *B. cinerea* has been studied previously (Castro et al. 2003; Pearson and Bailey 2013), and it has been demonstrated that the presence of mycoviruses and/or the expression of mycoviral genomes conferred the hypovirulence phenotypes to the host fungus (Nuss 2005; Potgieter et al. 2013). Our preliminary research indicated the evidence for the presence of mycovirus in isolates of *B. prunorum*, but additional research is underway to obtain conclusive and definitive results. Therefore, it is likely that the presence of mycoviruses may explain the lower

virulence exhibited by *B. prunorum* isolates. Nevertheless, differences in virulence between *B. cinerea* and *B. prunorum* could also be attributed to differences in the genetic expression of virulence factors.

Isolates of *B. prunorum* were morphologically and phylogenetically distinct from isolates of *B. cinerea*. Our results indicated that isolates of *B. prunorum* can be distinguished from *B. cinerea* by their colony morphology, conidia length, conidiophore length, sclerotia production on PAM at 20°C, and the amount of sporulation on APDA, KMB, and PAM. On average, isolates of *B. prunorum* produced 99% fewer conidia on APDA than isolates of *B. cinerea*.

As previously reported for other *Botrytis* species, the analysis of ITS regions 1 and 2 was not very informative for differentiating *B. prunorum* from *B. cinerea* (Li et al. 2012; Staats et al. 2005; Zhang et al. 2010a,b), nor analysis of the LSU of the rDNA performed according to Kurtzman and Robnett (1997). Therefore, the information obtained with ITS sequences and LSU sequences was insufficient to establish the taxonomic status of *Botrytis* isolates in this study. However, the phylogenetic analyses of the sequences of the nuclear genes *G3PDH*, *HSP60*, and *RPB2*, previously used for the revision of *Botrytis* species (Staats et al. 2005), clearly separated *B. prunorum* from *B. cinerea*. Similarly, the amplification and sequence analysis of *NEP1* and *NEP2* genes (Staats et al. 2007)

Fig. 6. Effect of inoculum concentration of *Botrytis cinerea* and *B. prunorum* on disease severity obtained on flowers of Japanese plum 'Larry Ann' and 'Flavor Rich'. The relationship between y = number of blighted petals and x = log of inoculum concentration (conidia/ml) was analyzed by regression analysis.

supported our delimitation of the isolates of *B. prunorum* as new species, which is distantly related to isolates of *B. cinerea*. Therefore, our data reinforced the usefulness of the *NEP* gene analysis to separate closely related species within the genus *Botrytis*, as previously proposed (Oome and Van den Ackerveken 2014; Staats et al. 2007). On the basis of the phylogenetic analysis, *B. prunorum* was distantly related to the recently described *Botrytis* species on hosts other than Japanese plums, such as *B. fabiopsis* (Zhang et al. 2010a), *B. sinoallii* (Zhang et al. 2010b), *B. pseudocinerea* (Walker et al. 2011), *B. caroliniana* (Li et al. 2012), *B. deweyae* (Grant-Downton et al. 2014), *B. sinoviticola* (Zhou et al. 2014), and *Botrytis* sp. B83 (Lorenzini and Zapparoli 2014).

Isolates of *Botrytis* from grapevines originally classified to group I (*vacuina* isolates) were characterized by the absence of the transposable elements *Boty* and *Flipper* (Fournier et al. 2005). The isolates in group I were recently described as a new cryptic species, *B. pseudocinerea* (Walker et al. 2011). In the present study, *Flipper* was not present in the tested isolates of both *B. cinerea* and *B. prunorum*, but *Boty* was present in some of the isolates of the two species, suggesting the presence or absence of the transposable element was irrelevant for the identification of *B. prunorum*.

NEP genes, first described on *Fusarium oxysporum* (Bailey 1995) and later on species of *Botrytis* (Oome and Van den Ackerveken 2014; Staats et al. 2007), encode necrosis and ethylene-inducing proteins and are considered virulence factors associated with the necrotrophic phase of certain pathogens (Mattinen et al. 2004). Therefore, the presence of the *NEP* genes in *B. prunorum* suggests that the interactions of this pathogen with the host share similar features with *B. cinerea* in terms of a necrotrophic lifestyle in at least part of the life cycle. It has been suggested that necrotrophic plant pathogens need to kill host tissue for nutrition (van Kan 2006).

In conclusion, *Botrytis* blossom blight is an annual threat to plum growers in Chile, and based on our results, a complex of at least two *Botrytis* species was found to be responsible for this disease on Japanese plums. These *Botrytis* species can be differentiated on the basis of PCR amplification and conidial production on APDA and PAM. *B. cinerea* was the predominant pathogen, and *B. prunorum* played a secondary role in the epidemiology of this disease on Japanese plums. Disease management strategies should take into account these pathogens.

ACKNOWLEDGMENTS

We thank J. J. Marois (University of Florida) for his suggestions and help in the editing of this manuscript, and G. A. Díaz (Universidad de Talca Chile), K. Elfar (Pontificia Universidad Católica de Chile), and L. Cottet (Universidad de Santiago, Chile) for their technical help during this project. We thank CONICYT for the doctorate scholarship given to the first author to pursue his postgraduate studies and for the financial support received through Project 7813110002 and to the grant received from “Tesis de la Industria,” CONICYT project 781311002. We also thank DuPont Chile for the financial support provided to pursue this project.

LITERATURE CITED

Bailey, B. A. 1995. Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*. *Phytopathology* 85:1250-1255.

Castro, M., Kramer, K., Valdivia, L., Ortiz, S., and Castillo, A. 2003. A double-stranded RNA mycovirus confers hypovirulence-associated traits to *Botrytis cinerea*. *FEMS Microbiol. Lett.* 228:87-91.

Coley-Smith, J. R. 1980. Sclerotia and other structures in survival. Pages 85-114 in: *The Biology of Botrytis*. J. R. Coley-Smith, K. Verhooff, and W. R. Jarvis, eds. Academic Press, United Kingdom.

Dahmen, H., Staub, T., and Schwinn, F. J. 1983. Technique for long-term preservation of phytopathogenic fungi in liquid nitrogen. *Phytopathology* 73:241-246.

Delaye, L., García-Guzmán, G., and Heil, M. 2013. Endophytes versus biotrophic and necrotrophic pathogens—are fungal lifestyles evolutionarily stable traits? *Fungal Divers.* 60:125-135.

Diolez, A., Marches, F., Fortini, D., and Brygoo, Y. 1995. Boty, a long-terminal-repeat retroelement in the phytopathogenic fungus *Botrytis cinerea*. *Appl. Environ. Microbiol.* 61:103-108.

Droby, S., and Lichter, A. 2004. Post-Harvest *Botrytis* infection: Etiology, development and management. Pages 349-362 in: *Botrytis: Biology, Pathology and Control*. Y. Elad, B. Williamson, P. Tudzynski, and N. Delen, eds. Kluwer Academic Publishers, Netherlands.

Elmer, P. A., and Michailides, T. J. 2004. Epidemiology of *Botrytis cinerea* in orchard and vine crops. Pages 243-272 in: *Botrytis: Biology, Pathology and Control*. Y. Elad, B. Williamson, P. Tudzynski, and N. Delen, eds. Kluwer Academic Publishers, Netherlands.

Ferrada, E. E., Díaz, G. A., Zoffoli, J. P., and Latorre, B. A. 2015. First report of *Botrytis cinerea* causing blossom blight on Japanese plums in Chile. *Plant Dis.* 99:888.

Ferrada, E. E., Latorre, B. A., Díaz, G. A., and Zoffoli, J. P. 2014. Identification of *Botrytis* spp. causing blossom blight in Japanese plums and nectarines in Chile. (Abstr.) *Phytopathology* 104(suppl.):S3.40.

Flint, K. M. 1995. Green fruit rot. Pages 15-16 in: *Compendium of Stone Fruit Diseases*. J. Ogawa, E. Zehr, G. Bird, D. Ritchie, and J. Uyemoto, eds. The American Phytopathological Society, St. Paul, MN.

Fourie, J. F., and Holz, G. 1994. Infection of plum and nectarine flowers by *Botrytis cinerea*. *Plant Pathol.* 43:309-315.

Fournier, E., Giraud, T., Albertini, C., and Brygoo, Y. 2005. Partition of the *Botrytis cinerea* complex in France using multiple gene genealogies. *Mycologia* 97:1251-1267.

Grant-Downton, R. T., Terhem, R. B., Kapralov, M. V., Mehdi, S., and Rodríguez-Enríquez, M. J. 2014. A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*. *PLoS One* 9:6.

Holz, G., Coertze, S., and Williamson, B. 2004. The ecology of *Botrytis* on plant surfaces. Pages 9-24 in: *Botrytis: Biology, Pathology and Control*. Y. Elad, B. Williamson, P. Tudzynski, and N. Delen, eds. Kluwer Academic Publishers, Netherlands.

Kurtzman, C. P., and Robnett, C. J. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the end of the large-subunit (26S) ribosomal DNA gene. *J. Clin. Microbiol.* 35:1216-1223.

Latorre, B. A., Elfar, K., and Ferrada, E. E. 2015. Gray mold (*Botrytis cinerea*), a limiting factor for grape production in Chile. *Cien. Inv. Agric.* 42: 305-330.

Levis, C., Fortini, D., and Brygoo, Y. 1997. Flipper, a mobile Fot1-like transposable element in *Botrytis cinerea*. *Mol. Gen. Genet.* 254:674-680.

Li, X. P., Kerrigan, J., Chai, W. X., and Schnabel, G. 2012. *Botrytis caroliniana*, a new species isolated from blackberry in South Carolina. *Mycologia* 104:650-658.

Lorenzini, M., and Zapparoli, G. 2014. An isolate morphologically and phylogenetically distinct from *Botrytis cinerea* obtained from withered grapes possibly represents a new species of *Botrytis*. *Plant Pathol.* 63:1326-1335.

Mattinen, L., Tshuikina, M., Mäe, A., and Pirhonen, M. 2004. Identification and characterization of Nip, necrosis-inducing virulence protein of *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 17:1366-1375.

Mirzaei, S., Goltapeh, E. M., Shams-Bakhsh, M., and Safaie, N. 2008. Identification of *Botrytis* spp. on plants grown in Iran. *J. Phytopathol.* 156:21-28.

Muñoz, G., Hinrichsen, P., Brygoo, Y., and Giraud, T. 2002. Genetic characterization of *Botrytis cinerea* populations in Chile. *Micol. Res.* 106: 594-601.

Nuss, D. L. 2005. Hypovirulence: Mycoviruses at the fungal-plant interphase. *Nat. Rev. Microbiol.* 3:632-642.

O’Gorman, D. T., Sholberg, P. L., Stokes, S. C., and Ginns, J. 2008. DNA sequence analysis of herbarium specimens facilitates the revival of *Botrytis mali*, a postharvest pathogen of apple. *Mycologia* 100:227-235.

Ogawa, J. M., and English, H. 1960. Blossom blight and green fruit rot of almond, apricot and plum caused by *Botrytis cinerea*. *Plant Dis. Rep.* 44: 265-268.

Ogawa, J. M., Zehr, E. I., and Biggs, A. R. 1995. Brown rot. Pages 7-10 in: *Compendium of Stone Fruit Diseases*. J. Ogawa, E. Zehr, G. Bird, D. Ritchie, and J. Uyemoto, eds. The American Phytopathological Society, St. Paul, MN.

Oome, S., and Van den Ackerveken, G. 2014. Comparative and functional analysis of the widely occurring family of Nep1-like proteins. *Mol. Plant-Microbe Interact.* 27:1081-1094.

Pearson, M. N., and Bailey, A. M. 2013. Viruses of *Botrytis*. *Adv. Virus Res.* 86:249-272.

Potgieter, C., Castillo, A., Castro, M., Cottet, L., and Morales, A. 2013. A wild-type *Botrytis cinerea* strain co-infected by double-stranded RNA mycoviruses presents hypovirulence-associated traits. *Virol. J.* 10:220.

Staats, M., van Baarlen, P., Schouten, A., van Kan, J. A., and Bakker, F. T. 2007. Positive selection in phytoxic protein-encoding genes of *Botrytis* species. *Fungal Genet. Biol.* 44:52-63.

Staats, M., van Baarlen, P., and van Kan, J. A. 2005. Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. *Mol. Biol. Evol.* 22:333-346.

- Tamura, K., Petersen, D., Petersen, N., Stecher, G., Nei, M., and Kumar, S. 2011. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731-2739.
- van Kan, J. A. L. 2006. Licensed to kill: The lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* 11:247-253.
- van Kan, J. A. L., Shaw, M. L., and Grant-Downton, R. T. 2014. *Botrytis* species: Relentless necrotrophic thugs or endophytes gone rogue? *Mol. Plant Pathol.* 15:957-961.
- Walker, A. S., Gautier, A., Confais, J., Martinho, D., Viaud, M., Le Pêcheur, P., Dupont, J., and Fournier, E. 2011. *Botrytis pseudocinerea*, a new cryptic species causing gray mold in French vineyards in sympatry with *Botrytis cinerea*. *Phytopathology* 101:1433-1445.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York.
- Williamson, B., Tudzynski, B., Tudzynski, P., and van Kan, J. A. 2007. *Botrytis cinerea*: The cause of grey mold disease. *Mol. Plant Pathol.* 8: 561-580.
- Zhang, J., Wu, M. D., Li, G. Q., Yang, L., Yu, L., Jiang, D. H., Huang, H. C., and Zhuang, W. Y. 2010a. *Botrytis fabiopsis*, a new species causing chocolate spot of broad bean in central China. *Mycologia* 102:1114-1126.
- Zhang, L., Zhou, Y. J., Li, G. Q., Yang, L., Jiang, D. H., Zhuang, W. Y., and Huang, H. C. 2010b. *Botrytis sinoallii*, a new species of the gray mold pathogen on *Allium* crops in China. *Mycoscience* 51:421-431.
- Zhou, Y. J., Zhang, J., Wang, X. D., Yang, L., Jiang, D. H., Li, G. Q., Hsiang, T., and Zhuang, W. Y. 2014. Morphological and phylogenetic identification of *Botrytis sinoviticola*, a novel cryptic species causing gray mold disease of table grapes (*Vitis vinifera*) in China. *Mycologia* 106:43-56.