

Neofusicoccum spp. Associated with Stem Canker and Dieback of Blueberry in Chile

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ABSTRACT

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Blueberry (*Vaccinium* spp.) plantings have significantly increased in Chile during the last decade and, currently, over 10,700 ha are cultivated throughout the country. Among other diseases, stem canker and dieback has been frequently observed in commercial plantations with incidences between 15 and 45%. The aim of this study was to identify and characterize *Neofusicoccum* spp. causing stem canker and dieback of blueberry in Chile. Three species, *N. arbuti*, *N. australe*, and *N. parvum*, were identified based on colony and conidia morphology, and nucleotide sequence analysis of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2). These *Neofusicoccum* spp. were found alone or coexisting with *Pestalotiopsis* spp., *Truncatella* spp., or *Phomopsis* spp. Koch's postulates showed all *Neofusicoccum* spp. isolated from infected plants to be pathogenic when inoculated on blueberry fruit and twigs using both mycelia and conidia suspension. All blueberry cultivars tested, including, Brigitta, Bluecrop, Brightwell, Duke, Elliott, Misty, and O'Neal, were susceptible to *Neofusicoccum* spp. infection. Pathogenicity tests showed *N. parvum* to be the most virulent species and Elliott to be the most susceptible cultivar. This report represents the first description of *N. arbuti*, *N. australe*, and *N. parvum* as canker-causing agents on blueberry in Chile.

Blueberry (*Vaccinium* spp.), with over 10,700 ha planted and 30,000 t of production in 2008, has become an economically important crop during the last decade in Chile. Blueberry production is mainly exported to the United States and Europe. Blueberry commercial plantations in Chile extend throughout a north-south axis of approximately 1,400 km in length under very different soil and environmental conditions (3,27).

Several viral, bacterial, and fungal diseases have been described on blueberry worldwide (6,14). Among these, stem canker and dieback of blueberry is a primary factor limiting both longevity and production. To date, more than 250 *Botryosphaeria* spp., including species of *Neofusicoccum*, are known to occur worldwide and have been described as plant pathogens, saprophytes, or endophytes of both cultivated and native plants (5,13,37).

Botryosphaeriaceae spp. have been associated with canker and dieback symptoms in a broad range of different peren-

nial fruit crops worldwide, and their importance as canker-causing and fruit rot agents has been extensively recognized (2,7,18,19,22–24,28–30,34,37,39,43,47). *Botryosphaeria corticis* and *B. dothidea* have been reported to cause twig and stem dieback, leaf spots, and fruit rots on blueberry and have been responsible for devastating disease outbreaks in the United States (23). *B. dothidea* and *B. ribis* were reported in blueberry plantations located in the VII and X regions in southern Chile in 2003 (11,16). Blueberry fruit are currently grown in a wide geographic zone of Chile under very different agricultural and environmental conditions that may favor the development of new diseases. Canker and stem dieback has been commonly observed during the past few years in Chile and disease incidence varies between 15 and 45%. However, no comprehensive effort to identify the canker-causing agents occurring in Chile has been reported. The purpose of this study was to identify and characterize the *Neofusicoccum* spp. associated with canker and stem dieback of blueberry in Chile by means of morphological features, DNA analyses, and pathogenicity studies.

MATERIALS AND METHODS

Sampling locations and fungal isolation. Symptomatic stems (1 and 2 years

old) were collected from eight commercial blueberry plantations, including northern high bush (*Vaccinium corymbosum*) (cvs. Aurora, Bluegold, Brigitta, Duke, Elliott, and Liberty) and southern high bush (*V. corymbosum* × *V. darrowi*) (cv. Misty) blueberry cultivars. Plantations were located in the Metropolitan Region (33°16' S), VI Region (34°00' S), and X Region (40°45' S) of Chile (Table 1). Samples were surface disinfected in 0.5% NaOCl for 2 min in the laboratory. Small fragments (5 mm²) of necrotic tissue were cut from the margins of the stem lesions after peeling of the bark and plated on potato dextrose agar (PDA) acidified with 96% lactic acid (APDA) at 0.5 ml/liter. Cultures were incubated in the dark at 20°C until colonies were observed. Pure cultures were obtained by hyphal tips from the margin of the suspected *Neofusicoccum* colonies, which were subcultured on fresh APDA and maintained at 20°C.

Characterization and identification of fungal isolates. Identification of the fungal colonies was first based on colony and conidia morphology. In order to enhance conidia formation, 4-day-old mycelium plugs from the different fungal isolates were cultivated on autoclaved *Pinus radiata* needles aseptically placed on 2% water agar. Cultures were incubated at 25°C for 7 to 10 days under continuous UV light. After sporulation, length, width, color, and the presence or absence of septa of 50 conidia were determined for each *Neofusicoccum* isolate using an optical microscope (Olympus Cx31 provided with an Evolution LC camera) (Table 2). The results were compared with previous descriptions (10,17,30,44).

Morphological characterization was corroborated by molecular analysis. Genomic DNA from 1-week-old cultures from eight *Neofusicoccum* isolates was extracted using the protocol previously described (8). Internal transcribed spacer (ITS) primers ITS1 and ITS4 were used to amplify the ITS1-5.8S-ITS2 region (49). The polymerase chain reaction (PCR) was performed in a thermocycler (MiniCycler; MJ Research Inc., Watertown, MA) using 50 µl of reaction mixture composed of DNase-free water (17.0 µl); 1× PCR buffer solution (Tris HCl) containing 50 mM MgCl₂, 0.25 mM dNTPs, bovine se-

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rum albumen at 1 mg ml⁻¹, 0.2 µM primer ITS4, 0.2 µM primer ITS1, *Taq* DNA polymerase (Invitrogen, Brazil) at 1 U µl⁻¹, and 1 µl of genomic DNA. After an initial denaturation for 2 min at 94°C, 40 PCR cycles were performed at 94°C for 30 s, followed by annealing at 55°C for 45 s and elongation at 72°C for 90 s, with a final elongation step at 72°C for 5 min (32,37,38). PCR products were separated by electrophoresis (Model 250 EX Life Technologies, GIBCO BRL Electrophoresis Power Supply, and HU10W SCIE-PLAS, England) (80 V for 50 min) in a 1.5% agarose gel in 10× Tris-acetate-EDTA UltraPure buffer solution (0.4 M Tris, 0.05 M acetic acid, and 0.01 M EDTA, pH 7.85). For each electrophoresis, a 100-bp ladder marker (Invitrogen, Carlsbad, CA) (0.5 µl of marker and load buffer solution) was used. The profiles obtained were visualized under UV light (UV Tran-

silluminator UVP, Upland, CA) after staining the gel with 1% ethidium bromide for 10 to 15 min. PCR products were separated and purified using AxigenPrep PCR (Axigen), and DNA concentrations were estimated using a Nanodrop (ND 1000-UV/Vis spectrophotometer; Nanodrops Technologies, Inc.). Each PCR product was sequenced in both directions using Macrogen (Macrogen, Korea). The results were manually aligned using an ITS ribosomal DNA (rDNA) gene sequence according to Chromas (Technelysium Pty Ltd., Australia).

Sequences of *Neofusicoccum* spp. from Chile were compared with exotype species from previous studies available in GenBank (25; Table 3). The alignment was corrected by visual inspection and any ambiguously aligned characters were deleted. Phylogenetic analysis was run for the ITS dataset using PAUP (version

4.0b10; Sinauer Associates, Inc., Publishers, Sunderland, MA). *B. dothidea* was used as outgroup. Maximum parsimony was performed using the heuristic search option (branch swapping NNI) and 1,000 random addition sequences replicates. Bootstrap values were evaluated using 1,000 replicates to test branch strength. Tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were also recorded. Resulting trees were printed in PAUP (version 4.0b10). ITS *Neofusicoccum* sequences from Chile were deposited into GenBank (25). *Neofusicoccum* spp. isolated from blueberry in Chile were deposited in CABI Genetic Resource Collection (Surrey, UK).

Effect of temperature on mycelial growth. In order to determine optimum growth temperature for the different *Neofusicoccum* isolates, four replicates of a 5-

Table 1. Host and origin of *Neofusicoccum* isolates obtained in this study from commercial blueberry (*Vaccinium* spp.) plantations in Chile during 2005 and 2006

Isolates ^x	Origin		Host	Cultivar	IMI no. ^y
	Locality	South latitude			
<i>N. arbuti</i>					
B03-07 ^z	Río Negro, Osorno, X Region	40° 45'	<i>Vaccinium corymbosum</i>	Aurora	395833
B04-07	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Aurora	...
B05-07	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Bluegold	...
B06-07	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Brigitta	...
B09-07 ^z	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Brigitta	395834
B62-07 ^z	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Elliott	...
<i>N. australe</i>					
B1-05 ^z	Rapel, VI Region	34° 00'	<i>V. corymbosum</i>	Duke	...
<i>N. parvum</i>					
B1-06	Lampa, Santiago, RM	33° 16'	<i>V. corymbosum</i> × <i>V. darrowi</i>	Mysti	...
B02-07	Nancagua, VI Region	34° 40'	<i>V. corymbosum</i>	Brigitta	395832
B4.2-06 ^z	Rapel, VI Region	34° 00'	<i>V. corymbosum</i>	Duke	395831
<i>Neofusicoccum</i> sp.					
B01-07	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Liberty	...
B08-07	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Brigitta	396598
B2.1-07	Río Negro, Osorno, X Region	40° 45'	<i>V. corymbosum</i>	Liberty	...

^x *Neofusicoccum* spp. from blueberry in Chile were determined based on colony and conidial morphology and DNA analyses.

^y Isolates deposited at the International Mycological Institute (IMI) CABI Genetic Resource Collection, Surrey, UK.

^z Isolates used for phylogenetic analysis.

Table 2. Conidial characteristics of *Neofusicoccum* spp. isolated from blueberry (*Vaccinium* spp.) in Chile

Isolates	Size (µm) ^y	Mean ± SD (µm) ^z	Length:width ratio ± SD ^z
<i>Neofusicoccum arbuti</i>			
B03-07	(19.1–)22.9–28.7 × (5.4–)7.1–8.8	23.6 ± 2.0 × 7.2 ± 0.7	3.3 ± 0.4
B04-07	(16.3–)19.5–27.6 × (6.3–)8.4–10.6	26.5 ± 1.7 × 7.6 ± 0.7	3.5 ± 0.4
B05-07	(23.3–)25.3–29.3 × (6.6–)7.7–9.3	25.6 ± 1.5 × 7.9 ± 0.7	3.3 ± 0.3
B06-07	(21.9–)26.7–30.5 × (6.3–)6.5–9.5	26.5 ± 1.7 × 7.6 ± 0.7	3.5 ± 0.4
B09-07	(20.4–)25.4–31 × (4.8–)6.9–9.3	25.5 ± 2.1 × 7.1 ± 0.9	3.6 ± 0.6
B62-07	(19.1–)23.7–26.9 × (6.2–)7.5–8.03	24.3 ± 1.5 × 7.0 ± 0.5	3.5 ± 0.4
<i>N. australe</i>			
B1-05	(17.1–)20.1–23.6 × (7.5–)9.5–10	20.4 ± 1.4 × 8.8 ± 0.7	2.1 ± 0.3
<i>N. parvum</i>			
B1-06	(16.1–)19.9–21.9 × (7.4–)9.3–10.9	19.3 ± 1.4 × 9.4 ± 0.8	2.3 ± 0.3
B02-07	(19.1–)23.3–24.8 × (6.3–)7.8–10.8	21.7 ± 1.3 × 8.0 ± 0.9	2.8 ± 0.4
B4.2-06	(12.2–) 22.2 × (5.2–)8.4–9.7	17.5 ± 2.2 × 8.1 ± 0.7	2.2 ± 0.3
<i>Neofusicoccum</i> sp.			
B01-07	(19.3–)25.8–28.7 × (5–)7.5–8.8	24.9 ± 2.2 × 7.4 ± 0.8	3.4 ± 0.6
B08-07	(20.9–)26.3–28 × (6.5–)7.4–9.2	25.0 ± 1.5 × 7.8 ± 0.9	3.2 ± 0.3
B2.1-07	(20.7–)23.3–30.6 × (5–)7.1–8.1	24.7 ± 1.9 × 6.6 ± 0.7	3.8 ± 0.5

^y Minimum size shown between parentheses followed by mode and maximum size in length and width of 50 conidia were recorded for each *Neofusicoccum* isolate from blueberry in Chile.

^z SD = standard deviation.

mm-diameter mycelial plug from seven isolates of the different *Neofusicoccum* spp. were cultured on APDA and incubated separately from 0 to 40°C (±0.5°C) at 5°C intervals. The effect of the temperature on colony growth was determined by measuring the radial growth of the mycelia after 4 days of incubation in the dark. This experiment was repeated twice.

Pathogenicity tests. Pathogenicity of *Neofusicoccum arbuti*, *N. australe*, and *N. parvum* (Table 4) was first studied in mature apple cv. Granny Smith and kiwifruit cv. Hayward. A second pathogenicity test was conducted on lignified 2-year-old detached stems from blueberry plant cvs. Brigitta and O'Neal. Fruit and detached stems were inoculated using 5-mm-diameter mycelial plugs from 7-day-old cultures on APDA. Fruit surfaces were disinfected using 0.5% NaOCl and 0.05% ethanol for 60 s and injured with the aid of a cork borer before inoculation. Concurrently, the same fruit were also inoculated on the uninjured skin, opposite to the wounded side. An equal number of fruit equally treated but using sterile agar plugs were left as control. To avoid rapid dehydration, the inoculation site was covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI). Fruit were incubated at 20°C in humid chambers with 100% relative humidity (RH) as determined with an RH microsensors (StowAway RH). The size of necrotic lesions was determined after 7 to 10 days of incubation.

The pathogenicity of *N. parvum* B01-06, B4.2-06, and B02-07, which were the most aggressive isolates in apple and kiwifruit fruit, was repeated on wounded detached blueberry stems (cv. O'Neal) using separately mycelial plugs and 15 µl of a conidial suspension (10⁶ conidia ml⁻¹). Inoculated stems were incubated for 25 days in humid chamber at 20°C.

A pathogenicity test was performed on 2-year-old potted blueberry cvs. Brigitta and O'Neal. Stems were surface disinfected with 0.5% NaOCl for 60 s before making a tangential cut (5 mm in length) on the bark. A 5-mm-diameter mycelial plug was taken from 5-day-old cultures on APDA and inserted underneath the bark. To avoid rapid dehydration, the inoculation sites were covered with Parafilm for 3 days. Plants were incubated under semi-shaded conditions. The length of the necrotic lesion was determined 25 days after inoculation.

To compare the effect of mycelial and conidial inoculations, detached stems of blueberry cv. O'Neal were inoculated with *N. parvum*, which was shown to be the most virulent isolate in the previous experiment. For this purpose, 2-year-old stems (20 cm in length and with a similar diameter) were surface disinfected, injured as indicated above, and then inoculated with either a 5-mm mycelium plug or 15 µl of 10⁶ conidia ml⁻¹ obtained from pycnidia that were crushed in sterile water. All stems were vertically distributed in humid

chambers (100% RH) and incubated at 20 to 22°C for 25 days before determining the length of the canker lesion developed. An equal number of injured stems treated with sterile water or sterile agar plugs were used as control.

To reisolate the causal agent, small pieces (5 mm in length) of diseased tissue were cut from the margin of the necrotic lesions from fruit and stems and were plated on APDA and incubated for 7 days at 20°C. Identity of the reisolated fungus was verified by morphological characters.

Blueberry cultivar susceptibility. Susceptibility of blueberry cvs. Brightwell, Brigitta, Bluecrop, Duke, Elliot, Misty, and O'Neal to infection by *Neofusicoccum* spp. was studied using 2-year-old potted plants under 50% shade. Mycelial plugs from 7-day-old cultures on APDA of *N. australe* (B1-05) and *N. parvum* (B1-06 and B4.2-06), which were the most virulent isolates in previous pathogenicity tests (Table 5), were used to inoculate lignified stems (2.7 to 5.1 mm in diameter) as previously described. An equal number of injured stems treated with noncolonized agar plugs were left as control. All inoculation sites were covered with Parafilm to avoid rapid dehydration, and plants were incubated in semi-shaded conditions (18 to 25°C) for 15 days before determining the length of canker development. This test was repeated on the same blueberry cultivars using 15 µl of a conidial suspension (10⁶ conidia ml⁻¹) of *N. parvum*, which was one of the most virulent and frequently isolated species. All plants were kept in a greenhouse (80 to 100% RH) at 20 to 25°C for 25 days before determining the length of the lesions.

Sensitivity to fungicides. The sensitivity of *N. parvum* isolates B1-06 and B4.2-06 to boscalid (Cantus 50 WG; BASF, Santiago, Chile), chlorothalonil (Hortyl 50 F; Agrícola Nacional SACI, Santiago, Chile), cyprodinil (Vangar 50; WP Syngenta Crop Protection, Santiago Chile),

Table 3. *Neofusicoccum* isolates from GenBank used for phylogenetic analyses

Species	GenBank accession no.	Origin	Host
<i>Neofusicoccum arbuti</i>	UW01 ^z	United States	<i>Arbutus menziesii</i>
<i>N. australe</i>	CMW 6837 ^z	Australia	<i>Acacia</i> sp.
<i>N. corticosae</i>	CPC 12926 ^z	Australia	<i>Eucalyptus carticola</i>
<i>N. mediterraneum</i>	CPC 13137 ^z	Greece	<i>Eucalyptus</i> sp.
<i>N. parvum</i>	96-29 ^z	New Zealand	<i>Malus</i> sp.
<i>N. vitifusiforme</i>	CAP 227	Italy	<i>Olea europaea</i>

^z Extype isolates.

Table 4. Pathogenicity of *Neofusicoccum* spp. obtained from blueberry in fruit of Granny Smith apple, Hayward kiwifruit, and blueberry stems^z

Species	Apple fruit (mm)		Kiwifruit fruit (mm)		Blueberry stems (mm)		df	Apple		Kiwifruit	
	Mycelia	Conidia	Mycelia	Conidia	Brigitta	O'Neal		F	P	F	P
<i>Neofusicoccum arbuti</i>											
B03-07	34.3 b	nd	19.0 bcde	nd	13.7 b	12.5 b
B09-07	34.7 b	nd	16.5 bc	nd	14.0 b	11.3 b
B62-07	30.0 b	nd	14.00 b	nd	13.3 b	11.3 b
<i>N. australe</i>											
B1-05	28.3 b	nd	23.5 de	nd	26.0 bc	17.5 b
<i>N. parvum</i>											
B1-06	43.5 bc	43.2 b	23.0 de	18.7 b	44.0 cd	63.9 c
B4.2-06	55.7 c	50.5 b	20.5 cde	22.0 b	nd	nd
B02-07	33.7 b	41.0 b	24.50 e	20.0 b	95.5 d	70.7 c
Control											
Noninoculated	0.0 a	0.0 a	0.0 a	0 a	0 a	0 a
Analysis of variance											
Isolate (I)	6	5.05	0.006	10.44	<0.001
Methods (M)	1	443.13	<0.001	1,841.02	<0.001
I × M	6	4.65	0.001	10.54	<0.001

^z Fruit and stems were inoculated with a 5-mm mycelial plug. Fruit were also inoculated with 15 µl of a 10⁶ conidia/ml conidial suspension. Results of fruit inoculations were obtained after 7 to 10 days and stem inoculations were obtained after 25 days of incubation. Means followed by different letters in each column are significantly different according to the Tukey's test ($P = 0.05$); nd, not determined.

iprodione (Rovral 4 F; Bayer Crop Science, Santiago, Chile), fludioxonil (Scholar 230 SC; Syngenta Crop Protection), and pyraclostrobin (Comet 250 SL; BASF) was studied in vitro.

Small fragments (5 mm in length) of mycelium were placed in quadruplicate 90-mm-diameter petri plates containing APDA emended with the respective fungicides at 1 $\mu\text{m liter}^{-1}$. Each fungicide was suspended in sterile distilled water and added to autoclaved agar media after cooling the media to approximately 60°C. All cultures were incubated at 25°C for 48 h before determining the radial growth of the mycelium.

The median effective concentration (EC₅₀) of fludioxonil and iprodione, the most effective fungicides in the previous experiment, was determined. Each isolate was seeded in quadruplicate on APDA containing the respective fungicide at 0, 0.025, 0.05, 0.1, 0.5, 1.0, 1.5, and 2.0 $\mu\text{m liter}^{-1}$. All cultures were incubated at 25°C for 48 h before determining the radial growth of the mycelium. Results are expressed as the control efficiency (E) according to the following equation: $E = (A - B)/A \times 100$, where A is the diameter of the fungus colony in control plates and B is the colony diameter obtained in APDA plates emended with the respective fungicide.

Design and statistical analysis. The effect of temperature on mycelia radial growth and the study of mycelial growth rate, obtained among *Neofusicoccum* spp. in 48 h at 25°C, were completely randomized with four replicates, using a petri plate as an experimental unit. Pathogenicity tests performed in fruit and stems were completely randomized using a fruit or a twig as experimental units. Tests performed on apple and kiwifruit were completely randomized with 7 × 2 (isolates × inoculation method) factorial arrangement of treatments.

The relative susceptibility of blueberry cultivars to *Botryosphaeriaceae* spp. was studied according to complete block design with a 7 × 3 (seven cultivars × three isolations) factorial arrangement of treatments.

ments, with four replicates of one plant each.

Data were subjected to analysis of variance and means were separated according to Tukey's test using SAS (SAS Institute, Cary, NC).

The EC₅₀ of iprodione and fludioxonil was estimated by linear regression analysis between $y =$ percent mycelial growth inhibition and $x =$ log of the concentration of each fungicide. Prior the analysis, the y values were transformed using the Probit scale (SAS PROBIT).

RESULTS

Field survey and fungal isolations.

Diseased plants in commercial plantations

were characterized by the presence of reddish-brown necrotic lesions that extended from the base of the stem to the crown during spring and summer. Symptoms were often observed only in one side of the plants. Bud necrosis, twig dieback, and eventual death of stems were also observed. Internal lesions were characterized by vascular discoloration of the xylem in diseased branches (Fig. 1).

Neofusicoccum spp. were isolated in 6 of 12 surveyed blueberry plantations (Metropolitan Region, Region VI, and Region X; Table 1). In four of those six blueberry plantations, only *Neofusicoccum* spp. were isolated. In the other two plantations, *Neofusicoccum* spp. were co-isolated with

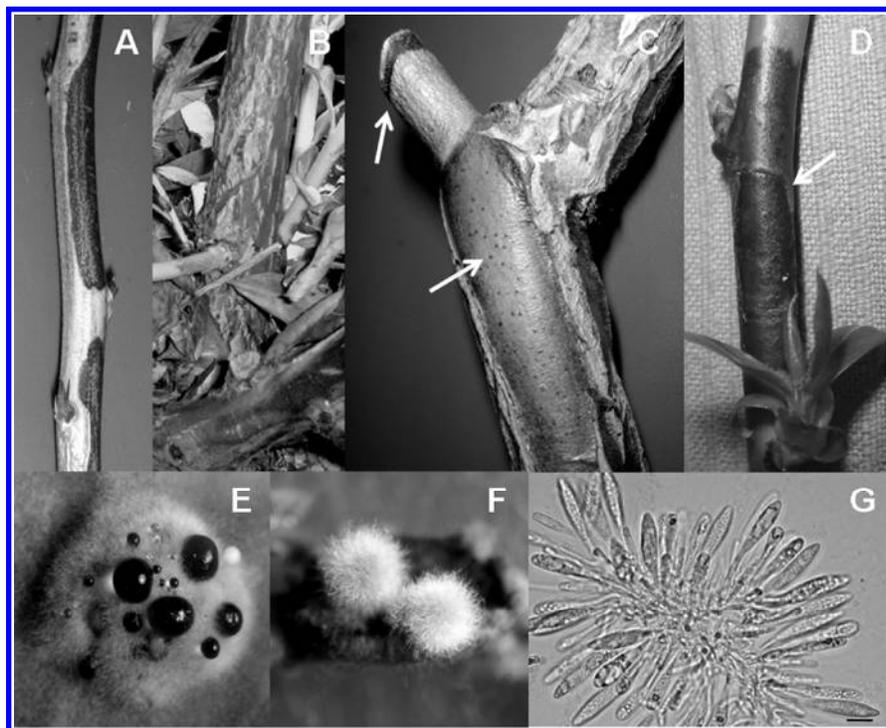


Fig. 1. Symptoms of canker and dieback of blueberry (*Vaccinium* spp.) caused by *Botryosphaeriaceae* spp. **A–C,** Symptoms observed in commercial blueberry plantations in Chile. **A,** Partially necrotic stem. **B,** Necrosis observed in the basal portion and crown of blueberry plants. **C,** Canker associated with pruning wound. Arrows show the pruning wound and pycnidia subcortical. **D,** Canker lesion obtained after stem inoculations. **E,** Pycnidia multiloculate on acidified potato dextrose agar medium. **F,** Single pycnidia on pine aciculae. **G,** Conidiophores and conidia of *Neofusicoccum arbuti*. Bar = 10 μm .

Table 5. Relative susceptibility of blueberry (*Vaccinium* spp.) after stem inoculations with mycelia of *Neofusicoccum australe* and *N. parvum*, and conidia of *N. parvum*²

Isolate	Length of lesion on blueberry cultivars (mm)								df	F	P
	Elliott	Brigitta	O'Neal	Brightwell	Duke	Bluecrop	Misty	Mean			
Mycelia inoculation											
<i>N. australe</i> B1-05	33.5	26.0	12.5	13.1	17.5	13.0	11.5	18.2 b
<i>N. parvum</i> B1-06	120.7	44.0	63.9	47.5	35.7	31.2	26.0	52.7 a
<i>N. parvum</i> B4.2-06	24.0	21.0	11.2	12.0	10.5	18.2	17.7	16.4 b
Mean	59.4 A	30.3 AB	29.2 AB	24.2 AB	21.2 B	20.8 B	18.4 B
Analysis of variance											
Cultivars (C)	6	8.9	<0.001
<i>Neofusicoccum</i> spp. (N)	2	61.0	<0.001
C × N	12	1.9	0.057
Conidia inoculation											
<i>N. parvum</i> (B1-06)	37.5 A	10.0 C	16.2 ABC	31.2 AB	12.7 BC	9.7 C	11.7 BC

² Means followed in each line and column by different letters are significantly different according to Tukey's test ($P = 0.05$). Data were transformed to $\log(x + 1)$ before analysis, but untransformed data are also presented.

species of *Pestalotiopsis*, *Phomopsis*, and *Truncatella*.

Characterization and identification.

All isolates grew on APDA and developed white colonies that turned greenish or gray to black after 15 days. Of 13 isolates, 4 developed abundant aerial mycelia and 3 had scarce aerial growth. All isolates produced globular, black, solitary, or stromatic pycnidia on APDA or on *P. radiata* needles. Pycnidia were also observed on autoclaved blueberry stems as well as on the blueberry, apple, and kiwifruit fruit. Pycnidia produced hyaline conidia that were fusiform to ellipsoid and were borne on short hyaline conidiophores. Conidia were liberated in the form of whitish cirrus under humid conditions. Based on colony and conidia morphology, four different fungal groups were differentiated (Table 2).

The first fungal group formed white to olive-green colonies which developed moderate aerial growth. They produced small pycnidia dispersed on APDA after 20 days of incubation. Conidia were fusiform, hyaline, smooth, and guttulate in young cultures, with a truncated base, and occasionally dark and biseptate with age. Mean size was between $23.6 \pm 2.0 \times 7.2 \pm 0.7$ and $26.5 \pm 1.7 \times 7.6 \pm 0.7 \mu\text{m}$, with a length:width (L:W) ratio between 3.2 ± 0.4 and 3.6 ± 0.6 (Table 2). This type of conidia was especially prominent on pycnidia developed on *P. radiata* needles. Stromatic black pycnidia with abundant aerial mycelia were produced on APDA (Fig. 2). All of these characters were in concordance with the description of *N. arbuti* (15).

The second fungal group formed slightly white to gray mycelia colonies and a characteristic yellow pigmentation was observed at the center of young colonies on APDA plates. Fluffy and cottony aerial mycelium developed in the center of the colonies and acquired a dark gray color with age. The underside of the colonies turned dark. Small black pycnidia covered with abundant mycelia developed after 15 days on *P. radiata* needles. Conidia were unicellular, fusiform, and hyaline, with a thin cell wall, with a mean size of $20.4 \pm 1.4 \times 8.8 \pm 0.7 \mu\text{m}$ with L:W ratio of 2.1 ± 0.3 (Table 2). Microconidia were not observed (Fig. 2). All of these characters were in concordance with the description of *N. australe* (10,20,37).

The third fungal group formed abundant aerial mycelia on APDA that were initially white to grayish but turned dark gray to olive green after 10 days at 25°C. The mycelium exhibited rapid growth on APDA covering the petri dishes (90 mm) in 48 h. Colonies stained the back of the culture media dark brown. Small, dark pycnidia covered with scarce mycelia were produced on *P. radiata* needles. Conidia from 15-day-old cultures were unicellular, hyaline, and ellipsoidal, with a truncated base, with mean size fluctuating between

$17.5 \pm 2.2 \times 8.1 \pm 0.7$ and $21.7 \pm 1.3 \times 8.0 \pm 0.9 \mu\text{m}$, with a L:W ratio of 2.2 ± 0.3 to 2.8 ± 0.4 (Table 2). Biseptate ellipsoidal conidia that were partially light brown appeared with a darker middle cell after 30 days (Fig. 2). All these characters were in concordance with the description of *N. parvum* (29–31).

The fourth fungal group formed olive-green colonies with white margins, and moderate aerial mycelium was observed in the center of the colonies on APDA. The mycelium gradually turned dark green. The reverse of the cultures was darkly stained. Black pycnidia covered with scarce mycelia were produced on APDA.

The conidia were unicellular, hyaline, thin-walled, and fusiform to ellipsoid shaped with a truncated base, with mean size varying from $24.7 \pm 1.9 \times 6.6 \pm 0.7$ to $25.0 \pm 1.5 \times 7.8 \pm 0.9 \mu\text{m}$, with a L:W ratio between 3.2 ± 0.3 and 3.8 ± 0.5 (Table 2). Occasionally, monoseptate conidia were observed (Fig. 2). This fungal group was identified as a *Neofusicoccum* sp.

The provisional identification of *N. arbuti*, *N. australe*, and *N. parvum* based on morphological characteristics was confirmed using molecular analyses. ITS sequences of *Neofusicoccum* spp. from blueberry from Chile (Table 1) were aligned with extype GenBank ITS sequences of

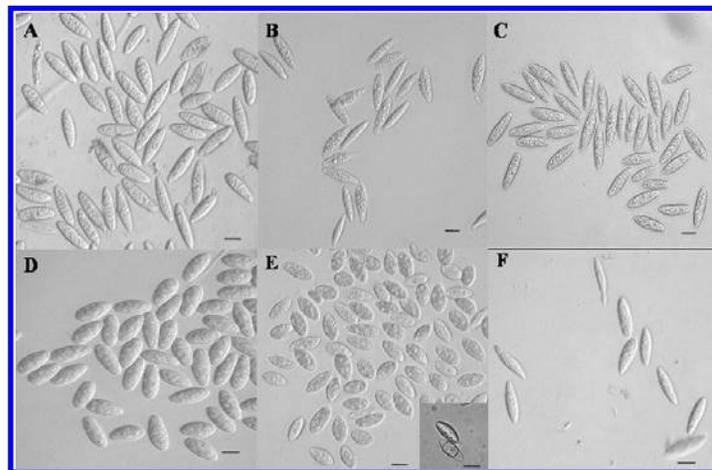


Fig. 2. Conidia of *Neofusicoccum* spp. obtained from blueberry in Chile. **A–C**, Conidia variability of *Neofusicoccum arbuti*; **D**, *N. australe*; **E**, *N. parvum* showing young, nonseptate fusiform conidia and a detail of old biseptate and fusiform conidia; **F**, *Neofusicoccum* sp. Bars = 10 μm .

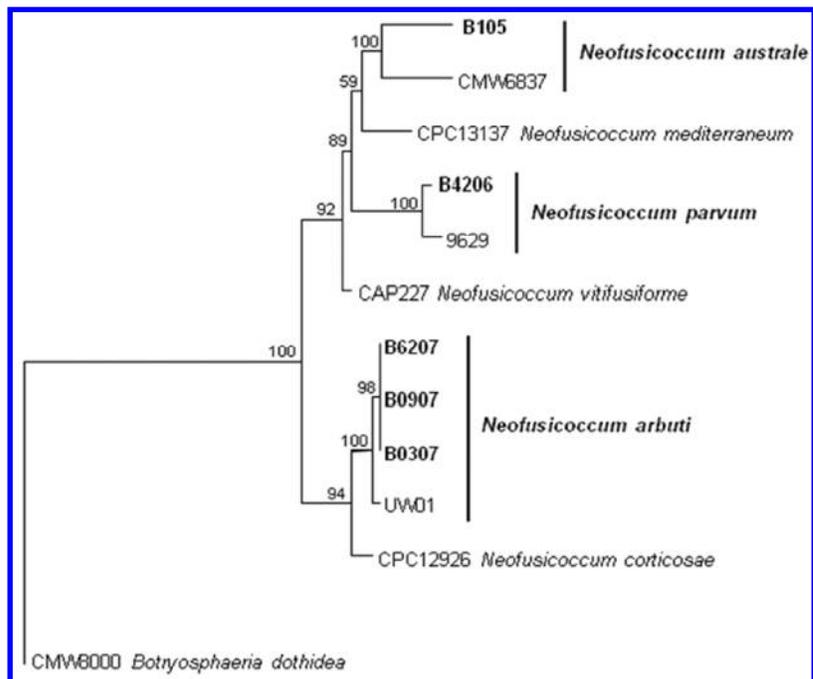


Fig. 3. One of 26 equally most parsimonious trees with bootstrap values from the 5.8S ribosomal DNA gene and flanking internal transcribed spacer (ITS)1 and ITS2 regions of *Neofusicoccum* spp. and outgroup *Botryosphaeria dothidea* isolate CMW8000. Tree length = 66, consistency index = 0.894, retention index = 0.837, rescaled consistency index = 0.748, and homoplasy index = 0.106. Isolates in bold represent *Neofusicoccum* isolates from blueberry from Chile.

Neofusicoccum isolates from other hosts (Table 3). Of the 530 nucleotides analyzed, 21 were parsimony informative. Maximum parsimony analyses of the ITS region resulted in 26 most parsimonious trees, 1 of which is shown (Fig. 3). *Neofusicoccum* isolates from blueberry from Chile showed sequences nearly identical to the extype isolates from other hosts from GenBank. *N. arbuti*, *N. australe*, and *N. parvum* isolates from Chile fell into three different, well-defined clades supported with 100% bootstrap values (Fig. 3).

Effect of temperature on mycelial growth. The analysis of variance showed a significant effect ($P < 0.001$) of temperature on mycelial growth. All isolates of *Neofusicoccum* grew at a range of temperatures from 10 to 35°C but optimum mycelial radial growth was observed at 25°C. Isolates of *N. parvum* grew slightly at 5°C but no mycelial growth was observed at 0°C. *N. arbuti* and *N. australe* did not grow at 5°. None of the *Neofusicoccum* spp. grew at 40°C. *N. parvum* exhibited the fastest mycelial growth at 30°C among all *Neofusicoccum* spp.

Significant differences ($P < 0.001$) in mycelial growth rate were obtained between isolates at 25°C. Differences in mycelial growth between *N. parvum* (85.0 mm per 48 h) and *N. australe* (76.6 mm per 48 h) were statistically significant (Fig. 4).

Pathogenicity tests. All *Neofusicoccum* spp. used in this study were pathogenic on apple and kiwifruit fruit when inoculated with mycelia. Pathogenicity of *N. parvum* on apple and kiwifruit fruit was also determined using conidial suspension. Post-inoculation symptoms on apple and kiwifruit were characterized by the development of a soft and watery rot that eventually covered the entire fruit. Superficially, white mycelia and black pycnidia developed after 7 days. *Neofusicoccum* isolates

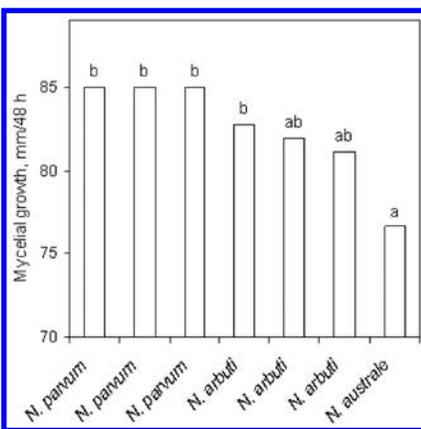


Fig. 4. Growth rate obtained for isolates of *Neofusicoccum* spp. after of 2 days of incubation at optimal temperature (25°C). Isolates were *Neofusicoccum australe* (B1-05), *N. arbuti* (B09-07, B62-07, and B03-07), and *N. parvum* (B1-06, B4.2-06, and B02-07). Means followed by different letters are significantly different according to Tukey's test ($P = 0.05$).

and inoculation method (wounded and unwounded fruit) had a significant effect ($P = 0.006$ and $P < 0.001$, respectively) on the size of the rot lesions obtained. The interaction between the inoculation method and the isolates was significant ($P < 0.001$). No symptoms were observed on noninoculated controls (Table 4).

All isolates were pathogenic on 2-year-old blueberry stems. Reddish-brown canker lesions from 13.3 to 95.5 and 11.3 to 70.7 mm in length were observed on blueberry cvs. Brigitta and O'Neal, respectively. Internally, a light-brown vascular necrosis was observed. Development of black pycnidia, which eventually liberated conidia in the form of whitish cirrus, was observed from infected stems (Fig. 1). Pathogenicity differences among isolates were significant ($P < 0.05$), with *N. parvum* being the most virulent species and *N. arbuti* the least virulent isolates (Table 4).

Both conidia and mycelia inoculation methods proved to be equally pathogenic on 2-year-old wounded blueberry cv. O'Neal stems when inoculated with *N. parvum*. However, the extent of necrotic discoloration obtained was considerably larger using conidial suspension than mycelial inoculations (Table 6).

In all pathogenicity tests, reisolutions from necrotic stems were successful, and the same isolates with morphology identical to those used for inoculations were consistently recovered from diseased fruit and diseased blueberry stems.

Blueberry cultivar susceptibility. Based on analysis of variance, there was a significant effect between cultivars ($P < 0.001$) and the length of the cankered lesions from the different *Botryosphaeriaceae* spp. on 2-year-old wounded stems when inoculated with mycelium. However, the interaction between cultivars and *Neofusicoccum* spp. was not significant ($P = 0.057$). Canker length was significantly different ($P < 0.05$) depending on the cultivar used. Among all cultivars tested, Elliot was shown to be the most susceptible; Brightwell, O'Neal, and Brigitta were moderately susceptible; and Misty, Bluecrop, and Duke were the least susceptible cultivars. With the exception of Brigitta, a similar trend was obtained when the same cultivars were inoculated with conidia of

N. parvum isolate B1-06, which proved to be highly virulent in previous experiments (Table 5).

Sensitivity to fungicides. Regardless of the isolate used, mycelia of *N. parvum* were highly sensitive to fludioxonil, resulting in complete mycelial growth inhibition at 1 $\mu\text{m ml}^{-1}$. Isolates B1-06 and B4.2-06 exhibited 100 and 50% mycelial growth inhibition, respectively, with iprodione at 1 $\mu\text{m/ml}$. The relationship between percent mycelial growth inhibition and log of the fungicide concentration was explained linearly ($R^2 > 0.91$), and the EC_{50} values for fludioxonil and iprodione fluctuated between 0.019 and 0.022 mg ml^{-1} and 0.021 and 0.2 mg ml^{-1} , respectively.

DISCUSSION

This study represents the first attempt to determine the presence and diversity of *Neofusicoccum* fungi associated with canker and stem dieback symptoms in the major blueberry-producing areas of Chile. Three different *Neofusicoccum* spp. were identified to be associated with stem canker and dieback of blueberry in Chile, including *N. arbuti*, *N. australe*, and *N. parvum*. These fungi were isolated mostly during spring (November to March) on cvs. Aurora, Brigitta, Bluegold, Duke, Liberty, and Misty in a wide geographic zone that varied along a gradient from north to south from relatively dry to humid conditions (mean <436 to <1,542 mm of annual rainfall) (26). However, it is interesting that *N. arbuti* was isolated only in southern Chile (Table 1), characterized by wet and cool weather conditions (26), while *N. australe* and *N. parvum* were isolated from plantings located in a warm and rather dry area of central Chile. Symptoms were reproduced after stem inoculations of blueberry. Reisolation of the causal agents from necrotic lesion development confirmed that these *Botryosphaeriaceae* spp. are pathogenic on blueberry. However, the relative importance of these species in the epidemiology of the stem canker and dieback of blueberry remain to be determined.

The symptoms obtained were similar to those described in blueberry-growing areas of United States caused by *B. corticis* and *B. dothidea* (23). Although *B. dothidea* was reported previously on blueberry in

Table 6. Pathogenicity on detached stems of O'Neal blueberry using mycelia and conidia of *Neofusicoccum parvum* after 25 days of incubation in a humid chamber at 20 to 25°C^z

Species	Isolates	Blueberry stems inoculated with	
		Mycelia (mm)	Conidia (mm)
<i>N. parvum</i>	B02-07	20.3 c	55.0 b
<i>N. parvum</i>	B1-06	13.5 b	75.0 b
<i>N. parvum</i>	B4.2-06	14.5 b	48.7 c
Noninoculated	...	0.0 a	0.0 a

^z Mycelia = plug 5 mm in diameter and conidia = 15 μl of a conidial suspension adjusted to 10^6 conidia/ml. Means followed by different letters in each column are significantly different according to Tukey's test ($P = 0.05$).

Chile, this species was not confirmed in this study (11).

The colony characteristics, conidia morphology, and sequence analysis of the ITS1-5.8S-ITS2 of the nuclear rDNA confirmed the presence of *N. arbuti*, *N. australe*, and *N. parvum*. These identifications were based on anamorphic characteristics and were in agreement with previous descriptions (10,15,20,38,40,47).

The shape, size, presence of septum, and pigmentation of conidia in combination with molecular data can be useful for identifying species of *Botryosphaeriaceae* isolated from blueberry, especially because the teleomorph stage was not found. However, conidial characters in *Neofusicoccum* spp. must be interpreted carefully because of their inherent variability. For instance, the presence of biseptated conidia with a dark-brown middle cell in cultures older than 30 days was a morphologically distinctive characteristic of *N. parvum*. However, the conidia were relatively larger than the conidia dimensions reported in other studies for *N. parvum*. It is possible that the differences in conidial size could be due to differences in the culture conditions used in this study. In a similar manner, a highly distinctive characteristic of *N. australe* was the slight yellow pigmentation observed in young colonies of APDA (29–31,45). However, the yellow pigmentation also characterizes young colonies of *N. luteum* (20). Therefore, DNA analysis appears to be necessary for the identification of *Neofusicoccum* spp.

To our knowledge, this is the first report of *N. arbuti*, *N. australe*, and *N. parvum* associated with diseased blueberry plants in Chile. Presence of *B. ribis* was reported previously in blueberry plantations located in the VIII, IX, and X administrative regions of Chile (16). The presence of *B. dothidea* and *B. corticis* was reported on blueberry in the United States (23), but *N. arbuti*, *N. australe*, and *N. parvum* have not been found in other blueberry-growing areas. Considering that blueberry was introduced primarily from the United States, it is interesting that *B. corticis* has not been found in Chile yet. It is possible that only clean plant material was introduced and plant quarantine measures were successful in avoiding its introduction.

Previous studies have associated *Botryosphaeriaceae* spp. as important pathogens causing wood and canker symptoms in other perennial crops in Chile and elsewhere (1,2,4,12,19,24,30,46). Therefore, it is possible that more than one species of *Botryosphaeriaceae* can be associated with the stem canker of blueberry, as has been found in grapevines and other crops throughout the world (36,45,47,48). Nevertheless, considerable differences in virulence were obtained among *Botryosphaeriaceae* spp. identified on blueberry in this study. *N. parvum* was the most aggressive species. It was interesting that a consider-

able and consistent variability in virulence were obtained with *N. parvum* on stem inoculations of seven blueberry cultivars (Table 5). Further research is needed to verify these differences in virulence and the possible significance in the development of canker diseases.

Optimal temperature for mycelial growth was 25°C. This temperature was lower than the optimal temperatures previously reported for *N. australe* and *N. parvum* (21,35,47). This could suggest a possible adaptation of these species to the relatively cool conditions that characterize the central valley of Chile during the spring and summer months when most of the blueberry are planted.

The pathogenicity of these species in fruit other than blueberry suggests that they are not host specific and agrees with previous reports (9,22,42,43). The necessity of a wound to initiate infection and the presence of cankers associated with pruning wounds suggest that pruning, frost damage, hail, mechanical injury, and other physical damages may favor the development of this disease under natural conditions, as has been suggested in other fruit crops (7,36).

The susceptibility of blueberry cultivars to some *Botryosphaeriaceae* spp. has been reported in previous studies (9,23,33,41,43). For instance, O'Neal has been shown to be resistant to stem canker caused by *B. corticis* but is susceptible to *B. dothidea* in the United States. In this study, Misty, Bluecrop, and Duke were the least susceptible cultivars to *N. parvum* in greenhouse experiments. However, field tests need to be performed in order to establish a final conclusion.

In Chile, blueberry plantations have considerably increased in recent years and are cultivated in very different soil and climatic conditions (3). This situation can partially explain the relatively rapid increase of these pathogens that may be disseminated from nurseries because evidence of the presence of *Botryosphaeriaceae* spp. in nursery plants was found in this study. Field diagnosis of the causal organism is difficult because symptoms caused by *Botryosphaeriaceae* spp. resemble those caused by *Pestalotiopsis* and *Phomopsis* spp., which sometimes coexist in the same plant (14). Pruning wounds can be an important avenue for penetration, as has been previously suggested on other fruit crops (30). Although fungicide treatments against this disease on blueberry are not currently recommended, the high sensitivity of *N. parvum* to fludioxonil and iprodione opens the possibility of developing a fungicide treatment to protect pruning wounds against these pathogens.

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