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Identification and Characterization of *Diaporthe ambigua, D. australafricana*, *D. novem*, and *D. rudis* Causing a Postharvest Fruit Rot in Kiwifruit

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Abstract

Diaporthe spp. are important plant pathogens causing wood cankers, blight, dieback, and fruit rot in a wide range of hosts. During surveys conducted during the 2013 and 2014 seasons, a postharvest rot in Hayward kiwifruit (*Actinidia deliciosa*) was observed in Chile. In order to identify the species of *Diaporthe* associated with this fruit rot, symptomatic fruit were collected from seven kiwifruit packinghouses located between San Francisco de Mostazal and Curicó (central Chile). Twenty-four isolates of *Diaporthe* spp. were identified from infected fruit based on morphological and cultural characters and analyses of nucleotides sequences of three loci, including the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), a partial sequences of the β-tubulin, and translation elongation factor 1-α genes. The *Diaporthe* spp. identified were *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis*. Multilocus phylogenetic analysis revealed that Chilean isolates were grouped in separate clades with their correspondent ex-types species. All species of *Diaporthe* were pathogenic on wounded kiwifruit after 30 days at 0°C under normal and controlled-atmosphere (2% O_2 and 5% CO_2) storage and they were sensitive to benomyl, pyraclostrobin, and tebuconazole fungicides. *D. ambigua* isolates were the most virulent based on the lesion length measured in inoculated Hayward and Jintao kiwifruit. These findings confirm *D. ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* as the causal agents of kiwifruit rot during cold storage in Chile. The specie *D. actinidiae*, a common of *Diaporthe* sp. found associated with kiwifruit rot, was not identified in the present study.

The world production of kiwifruit (*Actinidia deliciosa* (A. Chev.) C. F. Liang et A. R. Ferguson) 'Hayward' was estimated at 2,733,795 tons, with a net value of U.S.$ 2.3 billion in 2015 (Belrose, Inc. 2016). Currently, Chile is considered the third largest kiwifruit producer, after Italy and New Zealand. In Chile, over 10,000 ha are planted, with an annual production of 175,381 tons, and the fruit are mainly exported to Europe (39.7%), the United States (18.7%), Latin America (16.8%), and the Far East (11.4%) (Belrose, Inc. 2016). The Chilean kiwifruit is mainly harvested in April and stored for over 4 months at 0°C under either controlled atmosphere (CA) or modified atmosphere packaging alone or in combination with 1-methylcyclopropene (1-MCP).

Fruit softening and decay are the main deterioration factors during storage (Zoffoli et al. 1998). In this sense, Botrytis stem end rot (*Botrytis cinerea* Pers.) is the major pathogen affecting stored kiwifruit in Chile (Latorre 2004; Latorre and Pak 2003) as well as in other kiwifruit-producing countries (Brigati et al. 2003; Manning and Lallu 1997; Michailides and Elmer 2000; Opgenorth 1983; Pennycook 1985; Sommer et al. 1983). However, several studies have reported other postharvest fungal pathogens (Auger et al. 2013; Díaz et al. 2014; Hawthorne et al. 1982; Lee et al. 2001; Li et al. 2016b; Pennycook 1985; Zhou et al. 2015) but with a disease prevalence considerably lower than those caused by *B. cinerea* (Michailides and Elmer 2000). Currently, the following pathogens have been reported causing kiwifruit rots during storage: *Penicillium expansum* Link and *Sclerotinia sclerotiorum* (Lib.) de Bary (Opgenorth 1983; Pennycook 1985; Wang et al. 2015b); Botryosphaeriaceae members such as *Botryosphaeria dothidea* (Moug.) Ces & De Not., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A. J. L. Phillips (Pennycook 1985; Zhou et al. 2015); *Fusarium acuminatum* Ellis & Everh (Wang et al. 2015a); and *Pestalotiopsis microspora* (Speg.) Bat. & Peres (Li et al. 2016a).

Species of the genus *Diaporthe* Nitschke (anamorph: *Phomopsis*) are considered important plant pathogens causing a wide range of diseases such as cankers, leaves spots, blight, dieback, fruit rot, and seed decays (Baumgartner et al. 2013; Elfar et al. 2013; Harris 1988; Petrović et al. 2016; Úrbez-Torres et al. 2013; van Rensburg et al. 2006; Vrandecic et al. 2011). Moreover, Diaporthaceae members are associated with rots in a broad range of hosts worldwide and their importance has been extensively described (Michailides and Thomidis 2006; Petrović et al. 2016; Rosenberger and Burr 1982; Savocchia et al. 2007; Washington et al. 1997; Zhang et al. 2016). Among the species of *Diaporthe* causing kiwifruit rots, *Diaporthe actinidiae* (Lee et al. 2001; Pintos-Varela et al. 2000; Sommer and Beraha 1975), *D. ambigua* (Auger et al. 2013), *D. lithocarpus* (Li et al. 2016b), *D. novem* (Díaz et al. 2014), and *D. perniciosa* (Luongo et al. 2011) have been reported previously in the world. In Chile, *D. ambigua* and *D. novem* were described causing postharvest rots on kiwifruit (Auger et al. 2013; Díaz et al. 2014). However, further studies are needed for a better understanding of the relative role of *Diaporthe* spp. among the fungal postharvest rots found in kiwifruit storage in Chile. This research expanded the results obtained previously in Chile (Auger et al. 2013; Díaz et al. 2014), and it was conducted to (i) characterize, morphologically and molecularly, the *Diaporthe* spp. from kiwifruit and (ii) determine the pathogenicity of *Diaporthe* spp. associated with rots of stored kiwifruit.

Materials and Methods

Samples and symptoms of kiwifruit rot. A survey was conducted in seven commercial packinghouses under normal atmosphere (NA) and CA (2% O_2 and 5% CO_2) storage of decayed 'Hayward' kiwifruit (60 to 120 days at 0°C) from June to August 2013 and from July to September 2014. Sampling included fruit from the main production areas of Chile: San Francisco de Mostazal (33°59′S) and Curicó (34°59′S). Approximately 200 symptomatic decayed kiwifruit were sampled from four bins in each packinghouse in 2013 (n = 1,200 fruit) and 2014 (n = 1,400 fruit). Decayed fruit were placed in a cooler and transported to the laboratory on the same day and stored at 0°C until analysis. The external fruit rot symptoms

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such as gray mold, blue mold, or Sclerotinia rot were visually assessed and recorded and their prevalence determined.

Fungal isolation. All decayed kiwifruit (n = 2600 fruit) were placed inside plastic containers with moist paper towels on the bottom and kept at 20°C for 3 days to facilitate sporulation or development of fruiting bodies. The identification of these fungi was made based on the signs, colony growth in 2% acidified potato dextrose agar (APDA), and morphology of the fungi under a light microscope (Barnett and Hunter 1998). If, after 3 days of incubation, the decayed kiwifruit did not present evidence of signs (*B. cinerea*, *Penicillium* spp., or *S. sclerotiorum*), the isolations were attempted from each kiwifruit showing the above mentioned symptoms. Kiwifruit were cleaned manually (loose hair and debris removed), surface disinfested by spraying 75% ethanol until run-off, and air dried in a laminar flow hood for 5 min. Then, rotten kiwifruit were cut in half longitudinally and three pieces of internal tissue (approximately 5 mm in length) were cut from the margins of decayed and healthy tissues with a sterile scalpel previously flamed for 2 s. The pieces were placed on APDA amended with 0.005% tetracycline, 0.01% streptomycin, and 0.1% Igepal CO-630 (Sigma-Aldrich, Atlanta, GA) (Díaz and Latorre 2014). Plates were incubated at 20°C with a cycle of 12 h of light and 12 h of darkness for 1 to 2 weeks. White and fluffy aerial mycelium colonies with an irregular margin and concentric rings were tentatively identified as *Diaporthe* spp. (Gomes et al. 2013; Udayanga et al. 2011, 2014) and transferred to fresh APDA. Pure cultures were obtained from hyphal tip transfers to APDA and stored in Eppendorf tubes at 0°C.

DNA extraction, polymerase chain reaction conditions, and phylogenetic analyses. Total genomic DNA of 24 *Diaporthe* spp., previously identified by morphological studies, was extracted from fresh mycelium (50 mg) developed in pure cultures on APDA after 7 days of incubation at 20°C using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA). The integrity of fungal DNA was confirmed by electrophoresis in 1.0% agarose gels (run to 60 V for 30 min) in 1.0× Tris-acetate-EDTA (TAE) buffer stained with GelRed (Biotium Inc., Hayward, CA) and visualized under UV light (λ = 302 nm) transillumination. A polymerase chain reaction (PCR) was carried out to amplify three loci: internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2), part of the β-tubulin (BT), and the translation elongation factor 1-α (TEF1) genes. The ITS region was amplified with primers ITS4 and ITS5 (White et al. 1990). Portions of the BT gene and TEF gene were amplified with primers Bt2a and Bt2b and EF1-728F and EF1-986R, respectively (Carbone and Kohn 1999; Glass and Donaldson 1995). The PCR were conducted in a thermal cycler (Axygen Bioscience) using, for each sample, 2.5 µl of 10× PCR buffer, 1.0 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.5 µl of 0.5 mM solution of each primer and 0.2 µl of Taq DNA polymerase (Invitrogen, Carlsbad, CA) at 5 units/µl, and 1 µl of template DNA in a final volume of 25 µl. A control sample without DNA was included. PCR conditions for ITS and BT gene regions followed those indicated by Udayanga et al. (2014), except that the annealing temperatures were adjusted to 55 and 60°C, respectively. The TEF1 gene was amplified following the program indicated by Gomes et al. (2013) but using 51°C for the annealing temperature. PCR products were verified by electrophoresis in 2% agarose gels in 1.0× TAE buffer stained with GelRed (Biotium Inc.) and visualized by UV (λ = 302 nm) transillumination. Amplified products were purified and sequenced in both directions by Macrogene Inc. (Seoul, South Korea; www.macrogen.com/eng/). All sequences were edited using ProSeq v.2.91 (University of Oxford, Oxford, UK) and the ambiguous regions on both sides of the sequences were excluded. Sequences were aligned using Clustal X 2.0 (Conway Institute, Dublin, Ireland). A nucleotide BLAST search was performed against described sequences of *Diaporthe* spp. deposited in the GenBank (https://www.ncbi.nlm.nih.gov/) database (Table 1).

The phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA 5) software (Tamura et al. 2011). Separate phylogenetic analyses were carried out on the basis of the ITS sequences of the rDNA, and sequences for a portion of the BT and TEF1 genes. Gaps were treated as a fifth character weight, and all characters were unordered and of an equal weight. A maximum parsimony (MP) for all analyses was run using the heuristic search option and 1,000 random addition sequence replicates. The bootstrap values were evaluated by using 1,000 replicates to test the branch strength. The tree length (TL), consistency index (CI), retention index (RI), and rescaled CI were also recorded for each analysis. ITS, BT, and TEF1 sequences of known *Diaporthe* spp. available in GenBank were included (Table 1). Sequences of strain CBS 121124 *Diaporthella corylina* (GenBank accession numbers KC343004, KC343972, and KC343730) were included as outgroup in the single and concatenated analysis of ITS, BT, and TEF1 (Table 1).

Morphology and culture studies. In all, 24 isolates were used for morphological observations. Fungal colonies were first identified based on colony and conidial morphology. To induce pycnidia (anamorphic state) and perithecia (teleomorphic state), 4-day-old mycelial plugs (5 mm in diameter) of each of 24 isolates of *Diaporthe* spp. were cultivated on autoclaved alfalfa stems and pine needles placed on 2% water agar (WA), then incubated for 5 days at 20°C prior to incubation at 10°C for 90 days in darkness (Elfar et al. 2013). The presence or absence of pycnidia and perithecia was determined and then examined for the presence of alfa conidia and ascospores, respectively. Morphology and measurements of conidia (n = 20) and ascospores (n = 20) were determined and compared with published descriptions (Gomes et al. 2013; Santos et al. 2011; Udayanga et al. 2011, 2014; van Niekerk et al. 2005; van Rensburg et al. 2006).

Temperature studies. Three isolates of *Diaporthe ambigua* (7-KF, 9-KF, and 18-KF), *D. australafricana* (15-KF, 16-KF, and 19-KF), *D. novem* (1-KF, 3-KF, and 8-KF), and *D. rudis* (10-KF, 11-KF, and 21-KF) were evaluated for mycelial growth on APDA. A 5-mm-diameter mycelial plug (5 days old) in APDA was placed upside down in the middle of 90-mm-diameter Petri dishes, sealed with

Table 1. Isolates of *Diaporthe* spp. obtained from GenBank included in the phylogenetic study

Species	Strain	Host	Reference	GenBank accession number[z]		
				ITS	BT	TEF1
Diaporthe ambigua	CBS 114015 ex-epitype	*Pyrus communis*	Gomes et al. 2013	KC343010	KC343978	KC343736
D. ambigua	UCD2078Te	*Vitis vinifera*	Úrbez-Torres et al. 2013	KF017910	na	KF017892
D. australafricana	CBS 111886 ex-type	*V. vinifera*	Gomes et al. 2013	KC343038	KC344006	KC343764
D. australafricana	CBS 113487	*V. vinifera*	Gomes et al. 2013	KC343039	KC344007	KC343765
D. australafricana	15.2.2(4)	*Vaccinium corymbosum*	Elfar et al. 2013	KC143175	na	KC533440
D. cynaroidis	CBS 122676 ex-epitype	*Protea cynaroides*	Gomes et al. 2013	KC343058	KC344026	KC343784
D. novem	CBS 127270 ex-type	*Glycine max*	Gomes et al. 2013	KC343156	KC344124	KC343882
D. novem	CBS 127271	*G. max*	Gomes et al. 2013	KC343157	KC344125	KC343883
D. rudis	CBS 113201 ex-epitype	*Vitis vinifera*	Udayanga et al. 2014	KC343234	KC344202	KC343960
D. rudis	ER286C	*Acer* sp.	Udayanga et al. 2014	KC843337	KC843183	KC843096
D. vaccinii	CBS 160.32 ex-type	*Oxycoccus macrocarpa*	Gomes et al. 2013	KC343228	KC344196	KC343954
Diaporthella corylina	CBS 121124	*Corylus* sp.	Gomes et al. 2013	KC343004	KC343972	KC343730

[z] ITS = internal transcribed spacer region, BT = portion of the β-tubulin, TEF1 = translation elongation factor 1-α, and na = sequences not available in GenBank.

Parafilm, and incubated for 5 days at 0, 5, 10, 15, 20, 25, 30, 35, and 40 ± 0.5°C in darkness. Colony diameter on each Petri dish was measured in two perpendicular diameters using an electronic caliper Serie 500 absolute Digimatic (Mitutoyo America Corporation, Aurora, IL). Each isolate was seeded in quadruplicate. The experiment was repeated.

The effect of 0°C storage temperature on mycelial growth was further investigated using the three same isolates of each *Diaporthe* sp. grown in APDA. A 5-mm-diameter mycelial plug (5 days old) in APDA was placed in the middle of 90-mm-diameter Petri dishes, sealed with Parafilm, and incubated at 0 ± 0.5°C for 120 days. Colony diameter on each Petri dish was measured after of 30, 60, 90, and 120 days in two perpendicular sides using an electronic caliper. Each isolate was seeded in quadruplicate. The experiment was conducted twice.

Sensitivity of mycelial growth to fungicides. The sensitivity of two isolates of *D. ambigua* (7-KF and 9-KF), *D. australafricana* (15-KF and 16-KF), *D. novem* (1-KF and 3-KF), and *D. rudis* (11-KF and 21-KF) to benomyl (benzimidazole, Benex 500 WP; Arysta, Santiago, Chile), pyraclostrobin (quinone outside inhibitor, Comet 250 EC; BASF, Santiago, Chile), and tebuconazole (demethylation inhibitor, Tebuconazol 250 WP; Agrospec, Santiago, Chile) was assessed on APDA (FRAC 2016). One liter of stock solution was prepared for each active ingredient (a.i.) and appropriate dilutions were added to 1,000 ml of 2% APDA at approximately 50°C. Within 24 h after the plates were prepared, a 5-mm-diameter mycelial plug (5 days old) in APDA was placed upside down in the center of Petri dishes of 2% APDA amended with the respective fungicide at 0.1, 0.5, 1, 5, and 50 μg a.i./ml. Control APDA plates were prepared similarly, adding sterile distilled water instead of the fungicide solution. Plates were incubated for 5 days at 20 ± 0.5°C in the dark. Diameter of the colony on each Petri dish was measured in two perpendicular sides using an electronic caliper. The effective concentration to reduce mycelial growth by 50% (EC_{50}) values were estimated by lineal regression analysis, where X = log concentration and Y = Probit % of the mycelial growth. Each isolate was seeded in quadruplicate. The experiment was conducted twice.

Pathogenicity studies on kiwifruit. Two pathogenicity tests on kiwifruit were performed.

Inoculation with mycelium. One representative isolate of *D. ambigua* (7-KF), *D. australafricana* (13-KF), *D. novem* (1-KF), and *D. rudis* (21-KF) was used. Mature Hayward (6.5% of soluble solids, SS) kiwifruit (n = 600 fruit) harvested from a commercial orchard and stored for 10 days at 0°C were superficially disinfested in 75% ethanol for 2 min, then rinsed in sterile water and air dried for 45 min at 20°C. Kiwifruit were inoculated with a 7-day-old mycelial plug (5 mm in diameter) placed upside down into a fresh wound made on the shoulder of each fruit using a sterile corker borer (5 mm in diameter). The inoculation site was covered with Parafilm. Kiwifruit were wounded and inoculated with a sterile agar plug (5 mm in diameter) for negative controls. Inoculated fruit were placed inside of commercial kiwifruit boxes in three different storage conditions (200 fruit inoculated): (i) at 0°C for 30 days, (ii) at 0°C for 30 days in CA storage (2% O_2 and 5% CO_2), and (iii) at NA at 20°C for 14 days. In all, 50 replicate fruit were inoculated with each isolate of *Diaporthe* for each storage condition. Fruit rot was measured in two perpendicular sides using an electronic caliper. Fungal reisolation was made from symptomatic fruit as previously described to confirm Koch´s postulates. Data were analyzed for variance according to a completely randomized design with five replicates of 10 fruit for each isolate. Means were subjected to Tukey's test ($P < 0.05$) using SigmaPlot 13.0 (Systat Software Inc., San José, CA). The experiment was conducted twice.

Inoculation with conidia. Kiwifruit were surface disinfested and wounded as indicated above. Pycnidia of *D. ambigua* (7-KF and 9-KF), *D. australafricana* (15-KF and 16-KF), *D. novem* (1-KF and 3-KF), and *D. rudis* (11-KF and 21-KF) were induced on alfalfa stems in 2% WA for 90 days at 10°C (Elfar et al. 2013). Pycnidia were collected and processed as described by Kim and Xiao (2006). Concentration of alfa conidial suspension was adjusted to 10^6 conidia/ml. Twenty mature kiwifruit of *A. deliciosa* Hayward (6.5% SS) and *A. chinensis* 'Jintao' (13.9% SS), wounded and not wounded, were inoculated with 20 μl of conidial suspension of each isolate delivered in the wound or on the fruit surface. For control treatment, kiwifruit, wounded and nonwounded, were treated with 20 μl of sterile water. All fruit were placed in cardboard kiwifruit boxes at 0°C for 60 days in NA. Fruit rot was measured in two perpendicular sides using an electronic caliper. Reisolations were conducted from 10 fruit per treatment. The means were separated according to the pairwise multiple comparison Tukey's test ($P < 0.05$) using SigmaPlot 13.0. The experiment was conducted twice.

Results

Symptoms of kiwifruit rot and fungal isolation. Of the 2,600 fruit, 463 kiwifruit sampled showed decayed symptoms associated with fungal species. Out of these symptomatic fruit, 79% was positive for *Botrytis* spp., followed by *Penicillium* spp. (9.9%) and *S. sclerotiorum* (5.6%). However, another 24 fruit developed symptoms different from those caused by *Botrytis*, *Penicillium*, or *Sclerotinia* spp. The symptoms observed on decayed Hayward kiwifruit were associated with *Diaporthe* spp. and included soft rot with brown skin that started at the stem end or middle of the fruit which, in severe cases, affected the entire kiwifruit. Internally, affected fruit showed browning and watery tissues. Sometimes, it was possible to observe an exudate and signs of white fungal mycelium from infected fruit (Fig. 1). On Hayward kiwifruit, infection associated with *Diaporthe* spp. appeared to originate from wounded tissues produced during harvest or packing processing or postharvest. Fruit infected with *Diaporthe* spp. were obtained from three packinghouses and, in two of them, a *Diaporthe* sp. was recovered along with *B. cinerea*. The incidence of Diaporthe rot on kiwifruit was estimated as low (5.2%) from total kiwifruit diseased. In total, 24 *Diaporthe*-like isolates were obtained from decayed fruit. Fungal colonies were white with fluffy aerial mycelia and irregular margins after 3 days at 20°C; concentric rings were observed after 5 days. The formation of spherical and black pycnidia was observed erratically after 30 days at 20°C on APDA.

Phylogenetic analyses. In total, 72 new sequences (24 sequences for each locus) were generated in this study. All Chilean sequences of *Diaporthe* were nearly identical (>98% similarity) to reference

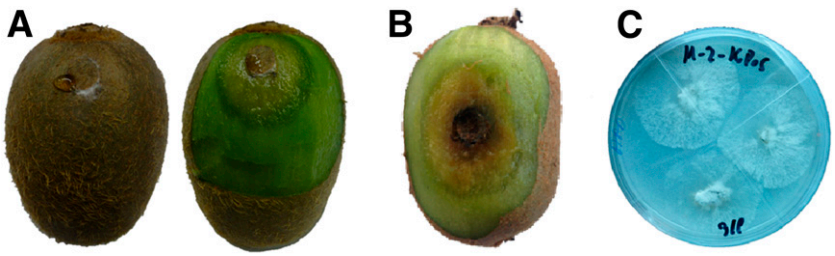

Fig. 1. Pathogenicity on mature kiwifruit Hayward inoculated with *Diaporthe* spp. **A,** Kiwifruit inoculated with a conidial suspension of *Diaporthe novem* after 30 days of incubation at 0°C in controlled atmosphere, showing exudate and signs of white fungi. **B,** Kiwifruit inoculated with a mycelial plug of *D. ambigua* incubated at 0°C for 60 days. **C,** Reisolation of fruit inoculated with *D. australafricana* in acidified potato dextrose agar after 5 days at 20°C.

sequences of ex-types when BLASTed. Several available sequences of known *Diaporthe* specimens, including type specimens, were obtained from GenBank (Table 1). Four alignments included sequences of 24 Chilean isolates of kiwifruit, 4 sequences from known specimens from GenBank (only 2 sequences for BT), and 6 *Diaporthe* sequences from ex-types cultures deposited in GenBank. *Diaporthella corylina* sequences were included as the outgroup (Table 1). All sequences of *Diaporthe* spp. obtained from kiwifruit rot during cold storage in Chile were deposited in GenBank (Table 2).

No major conflicts were detected between single gene phylogenies, indicating that the genes could be combined. MP analysis yielded 10 equally parsimonious trees for the concatenated ITS, BT, and TEF1 analysis (1,154-character dataset, TL = 132 steps, CI = 0.827, RI = 0.962, and composite index = 0.796) (Fig. 2). Six lineages were clearly distinguished. The MP analyses produced two main branches. The upper branch (90% support) grouped three clades (I, II, and III) and the lower branch groups clades IV, V, and VI. *Diaporthe ambigua* and *D. novem* isolates were grouped in clade I and II (100% support), respectively. Isolates of *D. australafricana* and *D. rudis* were grouped in two separated, well-supported clades IV and VI with their corresponding ex-types species (Fig. 2).

Identification and characterization. The 24 isolates of *Diaporthe* obtained from kiwifruit rot produced white to creamy colonies on APDA after 5 days at 20°C. Only nine isolates of *D. ambigua* and two isolates of *D. australafricana* developed pycnidia in 2% APDA after at least 30 days of incubation at 20°C.

Fertile black, globose pycnidia of isolates of *D. ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* were observed on autoclaved alfalfa stems and pine needles in APDA after 90 days of incubation at 10°C. The amount of pycnidia produced by *D. australafricana* and *D. rudis* was high compared against *D. ambigua* and *D. novem* (Table 3). *D. ambigua* produced α conidia that were hyaline, one cell, biguttulate, and ellipsoidal, with obtuse-end dimensions of 6.6 to 7.4 by 2.7 to 3.0 μm. *D. australafricana* produced α conidia that were aseptate, hyaline, biguttulate, and ellipsoidal of 5.4 to 7.2 by 2.2 to 3.2 μm. *D. novem* produced α conidia that were hyaline, one-cell, biguttulate, and oval to cylindrical, with obtuse-end dimensions of 6.4 to 7.7 by 2.2 to 2.9 μm. In *D. rudis*, the α conidia were hyaline, aseptate, smooth, biguttulate, and ellipsoidal, with a subtruncate base measuring 7.3 to 8.4 by 2.3 to 2.6 μm (Fig. 3). The β and γ conidia were absent in the four species of *Diaporthe*.

Perithecia were observed in cultures of *D. ambigua, D. australafricana, D. novem*, and *D. rudis* on autoclaved alfalfa stems and pine needles after 90 days at 10°C. For *D. novem*, perithecia were absent on pine needles (Table 3). The perithecia were dark brown to black, globose, and solitary or aggregate. In *D. ambigua*, the perithecia observed were longer necked (800 to 1200 μm), dark brown to black, and tapered toward the apex, with a red-brown ostiole. Perithecia of *D. australafricana* were filiform, longer necked (250 to 360 μm), and dark brown, and tapered toward the apex, partially covered with white to brown external hyphae at the tip. Perithecia of *D. novem* were black, globose, filiform, and longer necked (800 to 1,500 μm), with external hyphae in the tip. For *D. rudis*, perithecia were black, longer necked (300 to 700 μm), and tapered toward the apex. Only in the isolates of *D. novem* were the perithecia induced when the isolates of *D. novem* were crossed on the alfalfa stem. The asci were clavate, sessile, and eight-biseriate ascospores, hyaline, smooth, fusoid to ellipsoid, medially tapering toward both ends, septate, and widest at the septum, with two to four distinctive guttules, measuring 11.5 to 14.8 by 2.9 to 4.2 μm for *D. ambigua*, 12.8 to 14.3 by 3.2 to 4.5 μm for *D. australafricana*, 9.5 to 12.6 by 2.8 to 3.8 μm for *D. novem*, and 12.5 to 14.7 by 3.0 to 4.3 for *D. rudis* (Fig. 3).

Isolates *D. ambigua* 7-KF (ID BPI 910325), *D. australafricana* 19-KF (ID BPI 910326), *D. novem* 1-KF (ID BPI 910323), and *D. rudis* 11-KF (ID BPI 910324) were deposited as dried cultures in the U.S. National Fungus Collection (United States Department of Agriculture–Agricultural Research Service). Additionally, *D. ambigua* 7-KF (ID RGM 2369), *D. australafricana* 19-KF (ID RGM 2370), *D. novem* 1-KF (ID RGM 2366), and *D. rudis* 11-KF (ID RGM 2367) were deposited as a living culture in The Chilean Microbial Genetic Resources Collection (www.cchrgm.cl/Home.html)

The isolates of *D. ambigua* and *D. novem* grew in a range of temperature between 5 and 35°C, whereas the isolates of *D. australafricana* and *D. rudis* grew between 5 and 30°C. *D. australafricana*, *D. novem*, and *D. rudis* isolates showed an optimal mycelial growth at 20°C, whereas optimal mycelial growth of *D. ambigua* was at 25°C (Fig. 4A). None of the isolates grew when incubated at 0 or at 40°C for 5 days.

All *Diaporthe* isolates grew when stored at 0°C, showing a lineal relation between mycelial growth and cold storage time until 120 days (Fig. 4B). The highest growth rate was obtained by *D. novem* (0.35 mm/day), followed by *D. rudis*, *D. ambigua*, and *D. australafricana* at 0.27, 0.24, and 0.21 mm/day, respectively.

All isolates of *Diaporthe* spp. were sensitive to benomyl, pyraclostrobin, and tebuconazole fungicides in vitro. Data indicated that the range of EC_{50} values for *Diaporthe* spp. was <0.001 to 0.008 mg/liter for benomyl, 0.009 to 0.140 mg/liter for tebuconazole, and <0.001 mg/liter for pyraclostrobin (Table 4).

Pathogenicity studies on kiwifruit. All *Diaporth*e spp. were shown to be pathogenic on mycelium-inoculated kiwifruit in all storage conditions (Table 5). In NA, *D. ambigua* was significantly ($P < 0.001$) more virulent than *D. australafricana* and *D. novem* on inoculated fruit. Kiwifruit inoculated with *D. ambigua* and *D. novem* had longer average lesions (>13.5 mm in CA and >43.6 mm at 20°C) than *D. australafricana* and *D. rudis* during CA storage at 0°C for 30 days or under NA storage at 20°C for 15 days (Table 5).

Conidial inoculation showed all *Diaporthe* spp. to be pathogenic based on the necrotic lesions caused on kiwifruit stored for 60 days at 0°C. Unwounded kiwifruit inoculated with *Diaporthe* spp. isolates remained symptomless. *D. ambigua* isolates were the most aggressive ($P < 0.05$), producing the largest lesions (over 31.0 mm) on Hayward and Jintao fruit (Fig. 5). The infection caused by *D. novem* was classified

Table 2. Isolates of *Diaporthe* spp. obtained from kiwifruit rot during cold storage in Chile, deposited in GenBank, and used for phylogenetic study in this work

Species, isolate	Symptoms on fruit	GenBank accession number[z]		
		ITS	BT	TEF1
Diaporthe ambigua				
6-KF	Stem-end rot	KJ210025	KJ210039	KY046390
7-KF	Lateral rot	KJ210026	KJ210040	KY046391
9-KF	Stem-end rot	KJ210028	KJ210042	KY046392
13-KF	Stem-end rot	KJ210032	KJ210046	KY046393
17-KF	Stem-end rot	KX999704	KY046373	KY046394
18-KF	Lateral rot	KX999705	KY046374	KY046395
20-KF	Lateral rot	KX999706	KY046375	KY046396
22-KF	Stem-end rot	KX999708	KY046376	KY046397
24-KF	Stem-end rot	KX999709	KY046377	KY046398
D. australafricana				
15-KF	Stem-end rot	KX999701	KY046370	KY046387
16-KF	Stem-end rot	KX999702	KY046371	KY046388
19-KF	Lateral rot	KX999703	KY046372	KY046389
D. novem				
1-KF	Stem-end rot	KJ210020	KJ210034	KY046380
2-KF	Lateral rot	KJ210021	KJ210035	KY046381
3-KF	Stem-end rot	KJ210022	KJ210036	KY046382
4-KF	Lateral rot	KJ210023	KJ210037	KY046383
5-KF	Stem-end rot	KJ210024	KJ210038	KY046384
8-KF	Stem-end rot	KJ210027	KJ210041	KY046385
14-KF	Lateral rot	KJ210033	KJ210047	KY046386
D. rudis				
10-KF	Lateral rot	KJ210029	KJ210043	KY046399
11-KF	Stem-end rot	KJ210030	KJ210044	KY046400
12-KF	Lateral rot	KJ210031	KJ210045	KY046401
21-KF	Stem-end rot	KX999707	KY046378	KY046402
23-KF	Stem-end rot	KX999710	KY046379	KY046403

[z] ITS = internal transcribed spacer region, BT = portion of the β-tubulin, and TEF1 = translation elongation factor 1-α.

as second most virulent (over 26.4 mm) in both cultivars, followed by the group of *D. australafricana* and *D. rudis* (Fig. 5). Negative controls in both wounded and unwounded fruit did not produce lesions and no fungi were reisolated. All inoculated kiwifruit produced symptoms similar to those observed in decayed fruit in packinghouses (Fig. 1). Koch's postulates were fulfilled by reisolating each *Diaporthe* isolate only from the symptomatic fruit. Isolates were reidentified on the basis of their cultural and morphological characteristics (Fig. 1).

Discussion

Molecular and morphological studies revealed the presence of four Diaporthaceae species causing kiwifruit rot in Chile, including *D. ambigua*, *D. australafricana*, *D. novem*, and *D. rudis*. This study constitutes the first attempt to characterize *Diaporthe* spp. obtained from kiwifruit rots during cold storage and the first report of *D. australafricana* and *D. rudis* causing postharvest rots in kiwifruit worldwide. Moreover, this work confirmed the findings by Auger et al. (2013) and Díaz et al. (2014), who identified *D. ambigua* and *D. novem* causing kiwifruit rots in Chile during storage. Previously, *D. ambigua* and *D. actinidiae* have been reported in the United States (Sommer and Beraha, 1975), South Korea (Lee et al. 2001), and New Zealand (Hawthorne et al. 1982) affecting kiwifruit during postharvest. Additionally, other *Diaporthe* spp. have been described causing rots in kiwifruit such as *D. lithocarpus* and *D. perniciosa* in China and New Zealand, respectively (Hawthorne et al. 1982; Li et al. 2016b). Interestingly, the pathogen *D. actinidiae*, which is a species of *Diaporthe* commonly found in other countries causing kiwifruit rots (Hawthorne et al. 1982; Lee et al. 2001; Pintos-Varela

Table 3. Development of perithecia and pycnidia of *Diaporthe* spp. growing on autoclaved alfalfa stem and pine needle placed on 2% water agar (WA) incubated first at 20°C for 5 days prior to incubating at 10°C for 90 days[z]

	Perithecia (*n*)		Pycnidia (*n*)	
Species	**Stem**	**Needle**	**Stem**	**Needle**
Diaporthe ambigua	26	4	9	5
D. australafricana	78	38	13	10
D. novem	3	0	8	13
D. rudis	90	40	17	9

[z] Data shown are means obtained from three isolates of *D. ambigua*, three isolates of *D. australafricana*, five isolates of *D. novem*, and three isolates of *D. rudis*. In *D. novem*, only perithecia were observed when isolates were crossed in alfalfa stem on WA media.

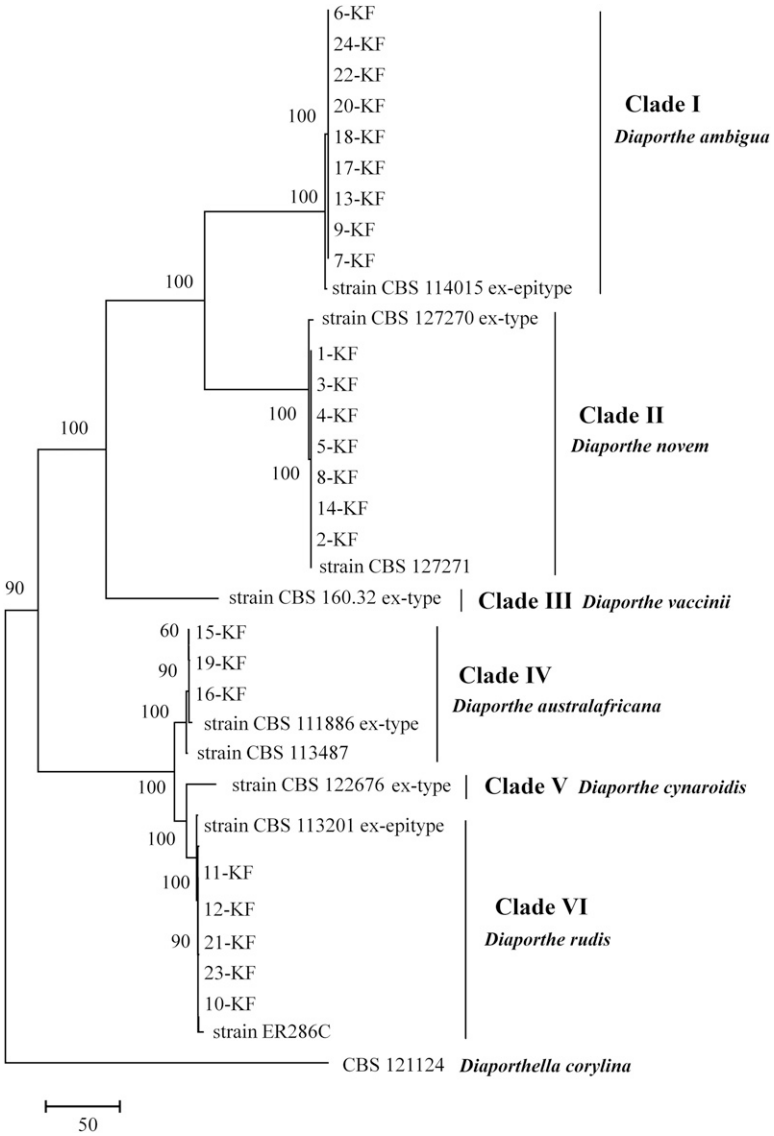

Fig. 2. Phylogram obtained from maximum-parsimony combined analysis of the internal transcribed spacer region of rDNA, part of the β-tubulin, and translation elongation factor 1-α sequences. Bootstrap support values from 1,000 replicates >50% are reported at the nodes. Values were obtained with Molecular Evolutionary Genetics Analysis software, version 5.0. Code number (1-KF to 24-KF) are *Diaporthe* isolates obtained from kiwifruit rot in Chile; other codes are strains plus the accession number from GenBank.

et al. 2000; Sommer and Beraha 1975), was not identified in this work. Therefore, it is possible that this fungal postharvest pathogen has not yet been introduced into Chile.

The Chilean isolates of *D. ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* had conidial and ascospore dimensions similar to those described previously for each species (Elfar et al. 2013; Santos et al. 2011; Udayanga et al. 2014; van Rensburg et al. 2006). This work also confirm that *D. ambigua*, *D. australafricana*, and *D. rudis* are homothallic, easily forming perithecia in alfalfa stems incubated for at least 90 days with an initial incubation at 20°C followed by a final incubation at 10°C in darkness (Elfar et al. 2013; Udayanga et al. 2014; van Niekerk et al. 2005). However, *D. novem*, a heterothallic species, required a sex-compatible isolate to induce the sexual structures (Gomes et al. 2013). Accordingly, with the present results, the morphological characterization alone was insufficient to identify the species of *Diaporthe* from stored kiwifruit, making it necessary to characterize them molecularly.

The concatenated ITS, BT, and TEF1 phylogenetic analysis allowed the clear identification of the four species of *Diaporthe* isolated from kiwifruit rots in this study, as stated by Udayanga et al. (2014). Moreover, the multilocus analysis was particularly useful to separate the *D. australafricana/D. rudis* complex; TEF1 phylogenetic analysis performed alone grouped *D. australafricana* and *D. rudis* together. These results were in agreement with those described previously by Udayanga et al. (2014) but differed from another study suggesting that different species of the genus *Diaporthe* can be separated using TEF1 sequences (Santos et al. 2011). Several studies have proposed phylogenetic analyses of at least two or three gene sequences for the discrimination of *Diaporthe* spp., including the TEF1 gene (Gomes et al. 2013; Santos et al. 2011; Udayanga et al. 2012, 2014). Furthermore, Udayanga et al. (2014) used five gene regions, including ITS, BT, TEF1, actin, and calmodulin, to reassess species in *Diaporthe*. In contrast to two evolutionarily close species (*D. australafricana* and *D. rudis*), the ITS, BT, and TEF1 sequence analysis clustered the sequences of Chilean isolates *D. ambigua* and *D. novem* in very distant clades (Santos et al. 2011; Udayanga et al. 2014; van Rensburg et al. 2006). Based on the concatenate analysis performed in this study, all Chilean isolates of *Diaporthe* spp. from kiwifruit rots were clustered with the ex-type or ex-epitype of *D. ambigua*, *D. australafricana*, *D. novem*, and *D. rudis*. Undoubtedly, the recent work of Gomes et al. (2013), Santos et al. (2011), Udayanga et al. (2012, 2014), and van Rensburg et al. (2006) has greatly assisted in the discrimination of species closely related at the molecular level in *Diaporthe*.

The species of *Diaporthe* found in this study exhibited different growth-temperature profiles, with an optimum at 20°C (*D. australafricana, D. novem*, and *D. rudis*) or 25°C (*D. ambigua*); and, interestingly, *D. ambigua* was the only species that clearly grew at 35°C. All *Diaporthe* spp. grew at 0°C for at least 30 days, demonstrating their capacity to infect kiwifruit during cold storage. These results confirmed those previously reported for *D. australafricana* and *D. ambigua* isolates obtained from blueberry in Chile (Elfar et al. 2013).

This study revealed that *Diaporthe* spp. obtained from kiwifruit rots were sensitive to benomyl, pyraclostrobin, and tebuconazole, suggesting that Diaporthe rot can be prevented chemically; however, these fungicides are registered in Chile for wound protection during pruning in winter. Moreover, Chilean protection programs for Botrytis end rot use well-known registered botryticides such as boscalid,

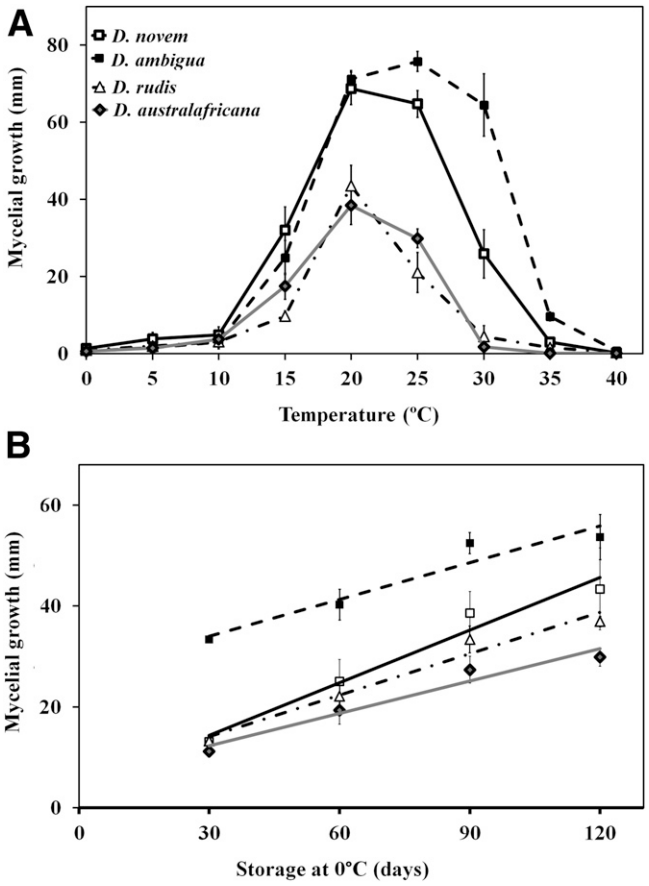

Fig. 4. Effect of the temperature on mycelial growth (in millimeters) of three isolates of *Diaporthe ambigua* (dashed black line), *D. australafricana* (gray line), *D. novem* (black line), and *D. rudis* (segmented-point black line). **A,** On acidified potato dextrose agar (APDA) after incubation for 5 days between 0 and 40°C and **B,** on APDA after of 120 days at 0°C. Vertical bar = standard deviation.

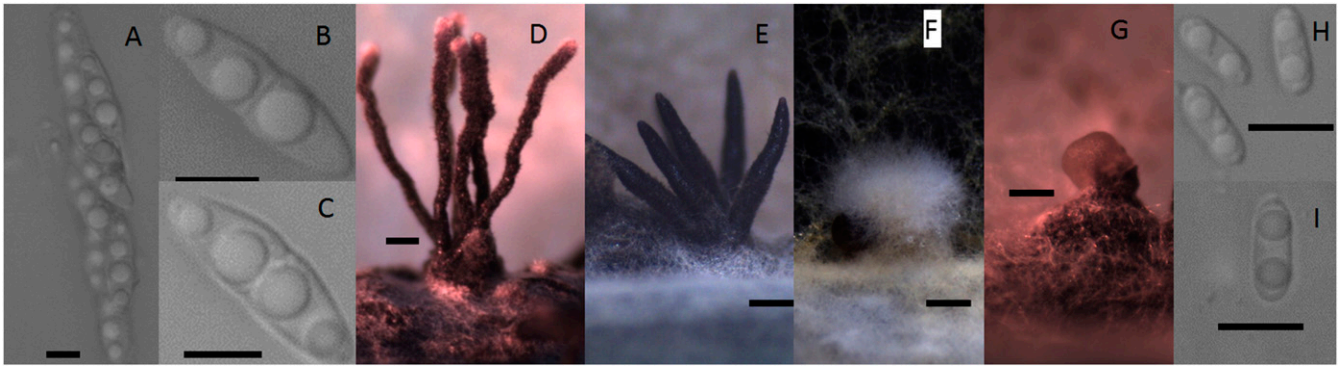

Fig. 3. Morphological features of Chilean isolates of *Diaporthe* spp. obtained from kiwifruit rot during cold storage. **A,** Ascus of *Diaporthe novem*; **B,** hyaline, smooth, fusoid ascospore of *D. australafricana*; **C,** hyaline, smooth, two-cell, tetraguttulate ascospore of *D. rudis*; **D,** aggregate of perithecia with sparse external hyphae covered the tip of *D. australafricana*; **E,** aggregate of perithecia of longer neck, black and tapered toward the apex of *D. ambigua*; **F,** sporulation of pycnidia of *D. novem*; **G,** sporulation of pycnidia of *D. ambigua*; **H,** one-celled, hyaline, and biguttelate α conidia of *D. novem*; **I,** one-celled, hyaline, fusiod, and biguttelate α conidia of *D. australafricana*. Scale bars: A, B, C, H, and I = 5 μm and D, E, F, and G = 100 μm.

Table 4. In vitro sensitivity of *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* isolates to benomyl, pyraclostrobin, and tebuconazol[z]

	EC_{50} (mg/liter)		
Species, isolate	**Benomyl**	**Pyraclostrobin**	**Tebuconazole**
D. ambigua			
7-KF	<0.001	<0.001	0.113
9-KF	0.008	<0.001	0.034
D. australafricana			
15-KF	<0.001	<0.001	0.019
16-KF	<0.001	<0.001	0.004
D. novem			
1-KF	<0.001	<0.001	0.109
3-KF	0.001	<0.001	0.140
D. rudis			
11-KF	<0.001	<0.001	0.030
21-KF	<0.001	<0.001	0.009

[z] Isolates were obtained from symptomatic decayed Hayward kiwifruit stored for at least 60 days at 0°C. EC_{50} = effective concentration to reduce mycelial growth by 50%.

Table 5. Pathogenicity of *Diaporthe* spp. on Hayward kiwifruit after storage under normal atmosphere (NA) for 30 days at 0°C or 14 days at 20°C or under controlled atmosphere (CA; 2% O_2 and 5% CO_2) for 30 days at 0°C

	Necrotic lesion (mm) for conditions of storage[y]		
	0°C for 30 days		20°C for 14 days
***Diaporthe* spp.**	**NA**	**CA**	**NA**
Diaporthe ambigua	23.4 a	16.4 a	45.8 a
D. australafricana	15.1 c	10.1 b	35.8 b
D. novem	18.6 b	15.4 ab	43.6 a
D. rudis	…	…	36.5 b
Control[z]	2.1 d	1.5 c	2.7 c

[y] Means followed by the same letters in each column did not differ significantly according to Tukey's pairwise multiple comparison test ($P = 0.05$). The reisolation from inoculated kiwifruit was 100% for all fruit inoculated with each *Diaporthe* sp.

[z] Control treatment consisted of inoculation with a sterile agar plug; the reisolation was negative on acidified potato dextrose agar.

iprodione, or tebuconazole that are applied during blossom to prevent build up of *Botrytis* spp. on floral senescent tissues in the orchard or for decreasing *Botrytis* populations during postharvest handling of fruit (Latorre and Pak 2003; Pennycook 1985). However, the rate of recent fungicide use in Chilean kiwifruit orchards is low for these fungicides, in contrast to the practice of kiwifruit growers in New Zealand and the United States, who made at least two fungicide treatments (Michailides and Elmer 2000; Pennycook 1985). Currently, fungicide treatments are not allowed to be used as postharvest treatments of stored kiwifruit in Chile. Therefore, fungicides sprayed during blossom or before harvest should be evaluated to prevent infection against Botrytis end rot and Diaporthe rot during storage in Chile.

A very important agronomic practice is the curing of kiwifruit, which delays cooling treatment of fruit. Growers commonly use curing in order to reduce Botrytis end rot (Ippolito et al. 1994; Pennycook and Manning 1992). Immediately after harvest, the fruit are placed in open storehouses (allowing wind flow) with a mean temperature of 10 to 15°C for at least 24 h to accelerate the cicatrisation of the picking wound and then decrease the probability of colonization by *Botrytis* spp. and other fungi (Ippolito et al. 1994; Latorre and Pak 2003; Michailides and Elmer 2000; Pennycook and Manning 1992). Based on the results of this study, both practices (botryticides and curing) could reduce Diaporthe rot in kiwifruit. Nevertheless, the value of the curing practice to prevent Diaporthe rot in addition to Botrytis end rot remain to be studied.

All tested isolates from all four *Diaporthe* spp. were pathogenic on artificially inoculated kiwifruit maintained in cold storage (0 ± 0.5°C) under CA and NA. The *Diaporthe* spp. were infectious when kiwifruit was inoculated with a conidial suspension but only in injured fruit. It is important to emphasize that conidial suspensions of *Diaporthe* spp. were not able to infect unwounded kiwifruit. Thus, minimizing wounds on the fruit by careful harvest and postharvest would reduce the presence of Diaporthe rot on Chilean kiwifruit. This study found significant differences in virulence among *Diaporthe* spp., in agreement with previous works (Baumgartner et al. 2013; Úrbez-Torres et al. 2013; van Niekerk et al. 2005). In the present study, *D. ambigua* was the most virulent species, followed by *D. novem*. Previously, Elfar et al. (2013) found that isolates of

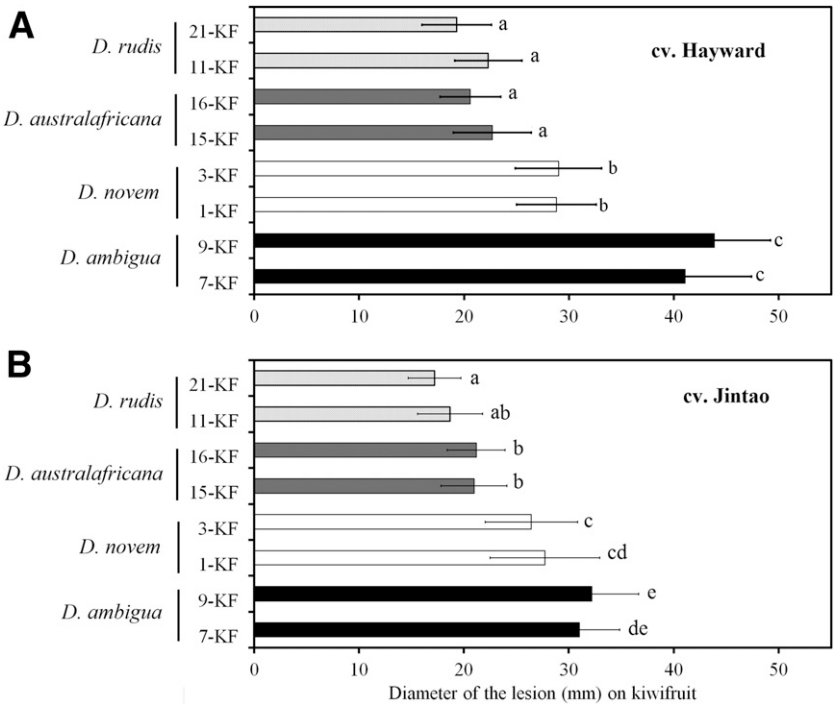

Fig. 5. Diameter of the lesion (in millimeters) caused by isolates of *Diaporthe ambigua*, *D. australafricana*, *D. novem*, and *D. rudis* on mature kiwifruit inoculated with 20 µl of conidial suspension (10^6 conidia/ml) and stored for 60 days at 0°C. **A,** *Actinidia deliciosa* Hayward and **B,** *A. chinensis* Jintao.

D. australafricana were more virulent than *D. ambigua* isolates on blueberry fruit. In this sense, inoculations with *D. amygdali* and *D. viticola* were shown to be more virulent than *D. ambigua*, causing lesions on green shoot of grapevines (van Niekerk et al. 2005). Similar results were obtained by Úrbez-Torres et al. (2013), where *D. viticola* was most virulent than *D. ambigua*, causing lesions on the wood in several cultivars of grapevine.

In the present work, the presence of Diaporthe rot in kiwifruit was low, because Botrytis end rot (*Botrytis* spp.) is the most important fungal disease in postharvest kiwifruit in Chile and worldwide where kiwifruit is cultivated (Brigati et al. 2003; Elfar et al. 2017; Latorre and Pak 2003; Manning and Lallu 1997; Michailides and Elmer 2000; Opgenorth 1983; Pennycook 1985). Although *D. ambigua, D. australafricana, D. novem*, and *D. rudis* were found at a low frequency causing kiwifruit rot during cold storage, their frequency could increase because *D. ambigua* and *D. australafricana* are commonly found associated with cordon dieback, the most important disease on kiwifruit in Chile (Díaz et al. 2016). Therefore, further research is needed to understand the role of *Diaporthe* spp. as fungal trunk pathogens and their relationship to fruit infection causing fruit rot during storage.

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