Characterization of the Bull's Eye Rot of Apple in Chile

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Abstract

Soto-Alvear, S., Lolas, M., Rosales, I. M., Chávez, E. R., and Latorre, B. A. 2013. Characterization of the bull's eye rot of apple in Chile. Plant Dis. 97:485-490.

Apple fruit in Chile are primarily produced for export to Asia, Europe, and the United States, which typically requires 15 to 40 days of maritime transportation. Therefore, Chilean apple production must fulfill the sanitization requirements imposed by the receiving countries. Under these circumstances, it was important to clarify the etiology of bull's eye rot that can severely affect 'Cripps Pink' apple and other late-harvest cultivars in Chile. Based on morphological characteristics and the partial sequence analysis of the internal transcribed spacer sequences and β -tubulin genes, *Neofabraea alba* was identified as the causal agent of the bull's eye rot of Chilean apple. These results were

further corroborated using species-specific primers. The incidence of bull's eye rot varied considerably; for instance, in 2009, 0.0 to 58.7% in 38 Cripps Pink orchards surveyed in the relatively arid and humid apple-growing areas of Chile, respectively. There was no evidence for the presence of *N. malicorticis* or *N. perennans*, which are commonly identified as causal agents of bull's eye rot in other apple-producing countries. Altogether, these data suggest that *N. alba* might represent the predominant and possibly the only cause of bull's-eye rot of Chilean apple.

Apple (*Malus domestica* Borkh.) production in Chile, particularly 'Cripps Pink' (Pink Lady) and other late-harvest cultivars, has been affected by bull's eye rot (*Neofabraea* spp.) that usually begins on the lenticels at harvest or during cold storage (0°C) and the transport to market (15). The fruit symptoms consist of circular necrotic lesions that are usually flat or slightly concave and generally dark and firm, causing slow, partial rotting of the fruit. Under humid conditions, acervuli and conidia are produced in a waxy matrix (18). It has been well documented that the primary infection of bull's eye rot occurs in the orchard prior to harvest and remains latent, appearing after 3 months of cold storage (16). In addition to the fruit damage, some *Neofabraea* spp. can produce cankers in the twigs that may act as a source of primary inoculum (9).

Three species of *Neofabraea* have been associated with bull's eye rot in apple worldwide: namely, *Neofabraea alba* (E.J. Guthrie) Verkley (syn. *Pezicula alba* E.J. Guthrie, anamorph *Phlyctema vagabunda* Desm.), *N. malicorticis* H.S. Jacks. (syn. *Pezicula malicorticis* (H.S. Jacks.) Nannf., anamorph *Cryptosporiopsis curvispora* (Peck) Gremmen), and *N. perennans* Kienholz (syn. *P. perennans* (Kienholz) Dugan, R.G. Roberts & G.G. Grove, anamorph *C. perennans* (Zeller & Childs) Wollenw.). Recently, *C. kienholzii* Seifert, Spotts & Lévesque was described as being associated with bull's eye rot in the Pacific Northwest of the United States (17).

In Chile, bull's eye rot was described in stored apple fruit, and *N. alba* was identified morphologically (10). Differences in fruit symptoms, the introduction of nursery stocks, and the importation of apple fruit from countries where different species of *Neofabraea* occur led us to postulate that *Neofabraea* spp. other than *N. alba* could be present in the apple orchards in Chile, as described in other apple-producing countries (8,16–18). The clarification of the

etiology of the post-harvest rots of apple in Chile is of great importance because the apple fruit in Chile are mainly grown for export markets in Asia, Europe, the United States, and Latin American countries where *Neofabraea* spp. can be of quarantine concern. This study expands the results of a previous investigation (10), with the aims of determining the *Neofabraea* spp. causing the bull's eye rot of apple in Chile through morphological and molecular techniques and describing the incidence of bull's eye rot in Cripps Pink apple in the entire apple-production area of Chile.

Materials and Methods

Sources of the isolate collection. In 2005, 10 *Neofabraea* isolates were obtained from infected fruit kept for 3 to 5 months in cold storage (Table 1). In 2008 and 2009, 409 *Neofabraea* isolates were obtained from Cripps Pink apple at harvest in 38 apple orchards in central Chile. These orchards were located along a 650-km north-south axis across a range of diverse climatic zones, with mean annual rainfall of 370 to 1,055 mm, concentrated in the winter months (13). All of the orchards were at relatively low altitudes (Table 2). The incidence of bull's eye rot was estimated in approxi-

Table 1. Isolates of *Neofabraea alba* obtained from apple fruit affected by bull's eye rot in commercial orchards of the Maule Region, Chile, in 2005 and the GenBank accession numbers for the internal transcribed spacer sequences generated

Isolate	Location	Cultivar	GenBank number ^a
012005	Colbún	Cripps Pink	JN937016
032005	Colbún	Cripps Pink	JN937015
062005	San Clemente	Golden Delicious	JN937014
072005	Linares	Royal Gala	JN937009
102005	Sagrada Familia	Cripps Pink	JN937010
112005	San Clemente	Cripps Pink	JN937007
122005	Colbún	Cripps Pink	JN937013
132005	Linares	Scarlet	JN937012
142005	Linares	Scarlet	JN937011
162005	San Clemente	Cripps Pink	JN937008

^a GenBank accession numbers (National Center for Biotechnology Information).

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Accepted for publication 9 October 2012.

mately 400 fruit, randomly selected in each orchard. The fruit were stored for 5 months at $0 \pm 1^\circ\text{C}$ plus 7 days at $20 \pm 2^\circ\text{C}$ to provide favorable conditions for the expression of bull's eye rot symptoms.

The fruit affected by the bull's eye rot were classified as yellow, brown, or dark brown, according to the external color of the diseased tissues. Additionally, the fruit lesions were characterized by the presence or absence of concentric rings and the ease of separating the rotted tissues from the healthy tissues.

Pathogen isolation and identification. Small pieces of tissue (approximately 5 mm long), selected from the margins of the lesions, were cut from surface-disinfected (70% ethanol, 30 s) fruit, placed on potato dextrose agar (PDA) (Difco Laboratories) and incubated at $20 \pm 2^\circ\text{C}$ for 20 days. White to pink, slow-growing colonies, tentatively identified as *Neofabraea* spp., were subcultured on PDA. Pure cultures were obtained by transferring the hyphal tips to PDA and incubating at $20 \pm 2^\circ\text{C}$ for 20 days. The isolates were stored at 4°C .

The isolates ($n = 409$) were identified on the basis of their colony shape, color, aerial growth, and mycelial radial growth in 20-day-old PDA cultures at $20 \pm 2^\circ\text{C}$. The presence of sporulation in the PDA cultures or on infected apple fruit was determined. The acervuli and conidia that developed on the infected apple fruit were characterized morphologically. In addition to the length and width of macroconidia, the presence of microconidia and sporulation in the culture medium were recorded (11). The key and description of Verkley (18) was used.

DNA isolation, polymerase chain reaction amplification, and sequencing. For DNA isolation, each fungal culture was grown on PDA and 0.1 g of a mycelium sample was harvested after 20 days at $20 \pm 2^\circ\text{C}$ (6). The mycelia obtained were treated with 1 ml of DNA extraction lysis buffer (200 mM Tris HCl [pH 7.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate) in an Eppendorf tube, homogenized using a pestle for 5 min, and incubated at 65°C for 60 min with 2 μl of proteinase K prior to centrifugation at $13,000 \times g$ for 5 min at $20 \pm 2^\circ\text{C}$. A 750- μl aliquot of the supernatant was removed to a new Eppendorf tube, mixed with 750 μl of cold isopropanol, incubated at $20 \pm 2^\circ\text{C}$ for 15 min, and then centrifuged at $13,000 \times g$ for 10 min. The pellet was washed with 1 ml of 70% ethanol, dried before resuspension in 100 μl of sterile water plus RNase A (US Biological) at 1 mg/ml, and incubated for 10 min at 37°C . The DNA obtained was stored at -20°C .

The polymerase chain reaction (PCR) was performed using a MyGene Peltier Series thermal cycler (MG96G; Long Gene) in a 50- μl reaction with 0.2 mM dNTPs, 3 mM MgCl_2 , 0.6 μM primers, and 2 units Taq DNA polymerase in $1 \times$ Taq PCR buffer (GoTaq Flexi; Promega Corp.). Approximately 1 to 10 ng of purified DNA was used in each reaction (4).

The PCR amplifications with the DNA obtained from the *Neofabraea* isolates during 2005 were performed using primers UN-UP18S-42 (5'-CGT AAC AAG GTT TCC GTA GGT GAA C-3') and UN-LO28S-22 (5'-GTT TCT TTT CCT CCG CTT ATT GAT ATC-3'), corresponding to the internal transcribed spacer (ITS)1, 5.8S, and ITS2 region (1).

The PCR amplifications of the β -tubulin gene were performed with the *Neofabraea* isolates obtained in 2008 and 2009 using primers Bt-T2m-Up (5'-CAA CTG GGC TAA GGG TCA TT-3') and Bt-LEV-L01 (5'-GTG AAC TCC ATC TCG TCC ATA-3') (4).

The amplification conditions used were 3 min at 96°C ; followed by 35 amplification cycles of 1 min at 96°C , 1 min at 60 or 66°C for β -tubulin and ITS, respectively, and 2 min at 72°C ; and a final extension cycle of 10 min at 72°C . The cycling was followed by a reduction of the temperature to 4°C (4).

The PCR products were purified using the QIAquick purification kit (Qiagen) for ITS sequence and the Favorgen GEL/PCR purification kit (Biotech Corp.) for the β -tubulin gene. The amplicons were sequenced in both directions by GenyTec or MacroGen.

The consensus sequences for ITS and β -tubulin were then obtained using Geneious (v.4.7; Biomatters Ltd. for each sample and deposited in GenBank (National Center for Biotechnology Information [NCBI]). The sequences obtained were aligned with the published *Neofabraea* reference sequences deposited in GenBank (Table 3). The alignments were corrected by visual inspection, eliminating any ambiguous sequences.

In addition, the DNA of 229 *Neofabraea* isolates was amplified according to the procedure described above but by using the species-specific primers proposed for *Neofabraea* spp. (7); Neofab-upTub-100 (5'-TGA TGA GAC CTT CTG TAT GC-3') was replaced with Neo alba-up (5'-TGA TGA GAC CTT CTG TAT CG-3').

Phylogenetic analysis. A phylogenetic analysis was initially performed with 10 ITS sequences (Table 1) and then with 27 selected β -tubulin sequences (Table 4) corresponding to the Chilean isolates; both comparisons were performed using Geneious with 15

Table 2. Incidence of bull's eye rot (*Neofabraea alba*) estimated for 'Cripps Pink' apple produced in different locations in Chile and number of *Neofabraea* isolates obtained in 2008 and 2009

Location	Elevation (m) ^a	Number of orchards ^b	Number of <i>N. alba</i> isolates	Incidence (%) ^c	
				2008	2009
Casablanca	300	1	6	0.0	1.1
Buin	445	1	0	0.0	0.0
Quinta de Tilcoco	320	1	6	0.3	0.0
San Fernando	260–445	3	27	6.9	1.1
Chimbarongo	280–555	6	53	21.3	2.2
Teno	305–330	2	12	48.9	2.2
Rauco	210	1	8	4.4	0.0
Sagrada Familia	135–175	2	16	9.9	0.0
Curicó	220	1	14	1.2	0.0
Molina	270–360	6	77	15.7	2.3
San Clemente	410	1	15	60.8	–
Colbún	260	1	8	44.4	1.3
Villa Alegre	140	1	10	63.4	4.0
Linares	190–395	2	40	61.1	17.3
Longaví	170	2	30	60.1	23.3
Parral	175–210	3	41	48.9	24.4
San Carlos	230	1	10	1.5	4.4
Angol	80	2	26	42.1	1.3
Lumaco	75	1	10	9.2	1.3

^a Meters above sea level.

^b In total, 400 fruit were examined per orchard each year.

^c Average incidence of bull's eye rot by location after 5 months under refrigerated storage at $0 \pm 1^\circ\text{C}$ plus 7 days at ambient temperature ($20 \pm 2^\circ\text{C}$); – indicates no data.

reference sequences from GenBank (Table 3) and *P. cinnamomea* as an outgroup. The reliability levels of the groups were determined by a resampling analysis with 1,000 replicates using the closest neighbor method.

Pathogenicity. The pathogenicity of 180 *Neofabraea* isolates of different geographical origin was examined in mature Cripps Pink apple (18 to 20 lb. of firmness [80 to 89 N]). Apple fruit were surface disinfected (70% ethanol, 30 s) and both sides of the fruit were aseptically wounded using a sterile cork bore before inserting a mycelium plug (7 mm in diameter). The inoculation site was immediately sealed with a petroleum jelly/wax mixture (1:1). Five fruit were used for each isolate. The inoculated fruit and uninoculated controls treated with sterile PDA agar plugs were incubated in humid chambers for 20 days at $20 \pm 2^\circ\text{C}$ prior to the measurement of the diameter of the resulting lesion. Reisolations using PDA were performed from the diseased apple fruit. The re-

sults were analyzed for variance using a completely random design, and the means were separated using Tukey's test ($P < 0.05$). The statistics program Statgraphics plus (v.5.1; Statistical Graphics) was used.

Results

Symptoms and incidence. Symptoms consisted of flat to sunken, firm, spherical lesions, 31.0 ± 12.0 mm in diameter, with well-defined margins and partially composed the fruit. These diseased areas were easily separated from the healthy tissues. Concentric rings were inconsistently observed, the shape of the rotted pulp was either spherical or conical, and the rots were externally brown (69.7%), dark brown (24.9%), or yellow (5.4%).

The incidence of bull's eye rot varied between 0.0 and 76.2% in 2008 and between 0.0 and 58.7% in 2009. The highest disease incidence was obtained in one of three orchards located in Parral in

Table 3. Reference DNA sequences of β -tubulin and internal transcribed spacer (ITS) sequences of isolates of *Neofabraea* spp. obtained from GenBank^a

Species	β -tubulin ^b	ITS ^b	Host	Location
<i>N. alba</i>	AF281452	AF281369	<i>Malus</i>	Pennsylvania, United States
	AF281453	AF281372	<i>Malus</i>	Canada
	AF281454	AF281377	<i>Malus</i>	Canada
	AF281456	AF281366	<i>Malus</i>	Poland
	AF281457	AF141190	<i>Malus</i>	United Kingdom
<i>N. malicorticis</i>	AF281463	AF281379	<i>Malus</i>	Canada
	AF281466	AF281386	<i>Malus</i>	Canada
	AF281467	AF281383	<i>Pyrus</i>	Canada
	AF281469	AF281384	<i>Malus</i>	Washington, United States
	AF281470	AF281388	<i>Malus</i>	Washington, United States
<i>N. perennans</i>	AF281471	AF281389	<i>Malus</i>	The Netherlands
	AF281472	AF281390	<i>Malus</i>	Germany
	AF281473	AF281392	<i>Malus</i>	Canada
	AF281474	AF281395	<i>Malus</i>	United Kingdom
	AF281475	AF281396	<i>Malus</i>	Washington, United States
<i>Pezicula cinnamomea</i>	AF281450	AF281399	<i>Betula</i>	Canada

^a Sequence data used were obtained from de Jong et al. (4).

^b GenBank accession numbers (National Center for Biotechnology Information).

Table 4. Symptoms of bull's eye rot (*Neofabraea alba*) in 'Cripps Pink' apple obtained in the entire apple-production area of Chile and the GenBank accession numbers for the β -tubulin gene sequences

Isolate	Location	Lesion characteristic			GenBank number ^c
		Color	Concentric rings ^a	Shape ^b	
01012008	Casablanca	Brown	+	Circular	JN856022
01022008	Casablanca	Dark brown	+	Circular	JN856023
03012008	Quinta de Tilcoco	Brown	–	Circular	JN856024
04032008	San Fernando	Brown	–	Circular	JN856025
05012008	San Fernando	Brown	+	Conical	JN856026
06022008	San Fernando	Brown	+	Circular	JN856027
07012008	Chimbarongo	Brown	+	Circular	JN856028
09032008	Chimbarongo	Dark brown	+	Conical	JN856029
11022008	Chimbarongo	Dark brown	+	Circular	JN856030
12012008	Chimbarongo	Brown	+	Conical	JN856031
12052008	Chimbarongo	Brown	+	Circular	JN856032
14022009	Teno	Yellow	–	Conical	JN856033
15012008	Rauco	Brown	+	Circular	JN856034
16012008	Sagrada Familia	Yellow	+	Conical	JN856035
18022009	Curicó	Brown	–	Circular	JN856036
20022008	Molina	Brown	–	Circular	JN856037
22022008	Molina	Dark brown	+	Circular	JN856038
24022008	Molina	Yellow	–	Circular	JN856039
25012008	San Clemente	Brown	–	Conical	JN856040
26012008	Colbún	Dark brown	–	Circular	JN856041
27032008	Villa Alegre	Brown	+	Conical	JN856042
28042009	Linares	Dark brown	+	Conical	JN856043
31022009	Longaví	Brown	–	Conical	JN856044
32032009	Parral	Yellow	+	Circular	JN856045
35012008	San Carlos	Dark brown	–	Conical	JN856046
37022008	Angol	Brown	–	Circular	JN856047
38032008	Lumaco	Brown	–	Conical	JN856048

^a With (+) or without (–) concentric rings in the rot.

^b Shape of the internal advance of the rot.

^c GenBank accession numbers (National Center for Biotechnology Information).

the 2008 and 2009 growing season, whereas bull's eye rot was not found in Buin.

Pathogen isolation and identification. In total, 409 *Neofabraea* isolates were obtained from 37 of the 38 Cripps Pink apple orchards sampled in 2008 and 2009 (Table 2); 10 additional isolates were obtained in 2005. These isolates were identified as *N. alba* and were characterized by round, flat colonies having scarce aerial growth that were initially white and turned to pinky-brown, with compact, hyaline, and septated mycelia. A mean colony diameter of 49.0 ± 9.2 mm was obtained on PDA after 20 days at $20 \pm 2^\circ\text{C}$. There was no evidence of sporulation on PDA. Nevertheless, sub-epidermic acervuli of 1.0 ± 0.5 mm in diameter that were creamy and waxy and free of setae were found in the naturally infected fruit. The acervuli contained abundant macroconidia that were unicellular, hyaline fusiform to allantoid, with rounded ends that exhibited oil drops; the mean size ($n = 250$) was between 17.8 ± 1.8 by 2.5 ± 0.5 and 19.0 ± 1.9 by 2.4 ± 0.5 μm . Microconidia were not observed.

Molecular identification. The provisional identification of *N. alba* based on the morphological characteristics was confirmed using ITS and β -tubulin analyses. The ITS sequences of the *Neofabraea* spp. isolates from the apple fruit collected in 2005 in Chile (Table 1) showed >98% similarity when they were aligned with the ex-type GenBank ITS sequences of *Neofabraea* isolates (Table 3). These sequences were deposited in GenBank (Table 1).

The β -tubulin nucleotide sequences of 180 *Neofabraea* isolates collected in 2008 and 2009 had >98% similarity when they were aligned with the ex-types of *N. alba* deposited in GenBank (Table 4).

The identity of 229 isolates of *N. alba* from apple was further confirmed when the species-specific primers (modified as indicated above) were used, generating a 360-bp band.

Phylogenetic analysis. The ITS phylogenetic tree included two well-separated clades. Without exception, the 10 ITS sequences of the *Neofabraea* isolates from Chile were clustered together, along with five known sequences of *N. alba* from GenBank. The ITS

Fig. 1. Phylogenetic relationships using the neighbor-joining method with the internal transcribed spacer sequences of *Neofabraea alba* obtained from DNA isolated from bull's eye rot of apple and the reference sequences of *N. alba*, *N. perennans*, and *N. malicortis* published in GenBank (National Center for Biotechnology Information). *Pezicula cinnamomea* was used as an outgroup.

sequences of the five reference isolates of *N. perennans* and five reference isolates of *N. malicorticis* obtained from GenBank were clustered in the second clade (Fig. 1).

The phylogenetic tree obtained with 27 β -tubulin sequences corresponding to *Neofabraea* isolates from different agroclimatic zones in Chile, which induced relatively different symptoms in apple, and five β -tubulin sequences of the reference *Neofabraea* isolates in GenBank produced three distinctive clades (Fig. 2). Without exception, the 27 β -tubulin sequences of the *Neofabraea* isolates from Chile were clustered together, along with 5 known sequences of *N. alba* from GenBank. The β -tubulin sequences of five reference isolates of *N. perennans* were clustered in the second clade, and the five reference isolates of *N. malicorticis* were placed in the third clade (Fig. 2).

Pathogenicity. Regardless of their geographic origin, 180 isolates were pathogenic in Cripps Pink apple and developed round, brown lesions similar to those found in the natural post-harvest

infections. The mean lesion diameter was 28.0 ± 4.0 mm after 20 days at $20 \pm 2^\circ\text{C}$. The differences in the lesion size among the *Neofabraea* isolates were not statistically significant ($P > 0.05$). The external color of the rots was mostly brown (91.7%), with the presence of concentric rings. Reisolations were successful for 97.0% of the samples.

Discussion

This study was the first attempt to characterize molecularly the species of *Neofabraea* on apple in Chile. Based on the morphological characteristics and partial sequence analysis of the ITS region and β -tubulin gene, *N. alba* was identified as the only causal agent of bull's eye rot in apple in Chile, confirming a previous identification based on morphological characteristics (10).

There was no evidence for the presence of *N. malicorticis* and *N. perennans*, which are commonly found as the causal agents of bull's eye rot in other apple-producing countries in the Northern

Fig. 2. Phylogenetic relationships using the neighbor-joining method with the β -tubulin sequences of 27 isolates of *Neofabraea alba* obtained from bull's eye rot of apple showing differences in symptom expression and collected under different agro-climatic conditions in the entire apple-production zone of Chile. The reference sequences of *N. alba*, *N. perennans*, and *N. malicorticis* were obtained from GenBank (National Center for Biotechnology Information). *Pezicula cinnamomea* was used as an outgroup.

Hemisphere (7,8,20). Therefore, our findings suggest that *N. alba* is the predominant and possibly the exclusive pathogen of bull's eye rot in Chilean apple. The etiological situation in Chile appears to be similar to that observed in South Africa (5) and New Zealand (12), in which only *N. alba* has been reported as the cause of bull's eye rot in apple. The presence of three species of *Neofabraea* has been documented in Europe and Australia, with *N. alba* being the most prevalent species (3,20). In Brazil, only *N. perennans* has been identified (2). In contrast, *N. alba*, *N. perennans*, *N. malicorticis*, and, recently, *C. kienholzii* have been reported in the northwestern region of the United States (17). This information highlights the quarantine importance of *Neofabraea* spp. and the need to prevent their introduction into Chile via imported apple fruit, pear fruit, or nursery plants.

N. alba isolates have been found in other apple-growing areas of the world and are mainly associated with the bull's eye rot of apple (18). The symptoms include a firm, localized spherical decay that is externally brown, with concentric growth rings (8,14,16,18). However, in the present study, *N. alba* was also found to be associated with yellowish lesions without concentric rings. Despite previous symptom description (16), without exception, the diseased tissues of fruit rot caused by *N. alba* were separated easily from healthy tissues. We found that the incidence of bull's eye rot varied considerably each year and between two consecutive growing seasons, which was attributed to differences in weather conditions. For instance, in 2009, an incidence of 0.0 to 58.7% was obtained for the Cripps Pink apple in the relatively arid and humid apple-growing areas in Chile, respectively. Furthermore, Cripps Pink is a late-harvest cultivar that is commonly exposed to rainy periods at harvest, explaining the relatively high bull's eye rot obtained in 2009.

Canker lesions were not observed in the 38 apple orchards surveyed in this study. The presence of cankers on apple twigs has been mainly associated with *N. perennans* and *N. malicorticis* (16). Therefore, the absence of twig cankers reinforces the conclusion that only *N. alba* is found in the apple-growing regions in Chile. However, we cannot rule out the possibility that *N. alba* may be found in a saprophytic stage in dead wood (18) or causing microcankers, as has been reported for pear (11), which would be an important source of primary inoculum for fruit infections.

The morphological identification of *Neofabraea* spp. is difficult. The *N. alba* isolates were characterized by the absence of sporulation in PDA agar medium; however, fusoid to allantoid macroconidia that were slightly curved with relatively pointed ends were observed in the infected apple fruit. Microconidia were absent both in vitro and in vivo. Although these characteristics agree with the morphological description of *N. alba* (18), the DNA sequence comparisons and the ITS and β -tubulin phylogenetic analyses allowed us to verify the identification of *N. alba* in apple in Chile. The results from this study supported previous work suggesting that ITS sequence analyses only allow for the identification of *N. alba*, because it is impossible to separate *N. malicorticis* from *N. perennans* (4). However, the latter species were distinctively separated on the basis of the β -tubulin phylogenetic analysis.

It was impossible to amplify the DNA when the species-specific primers (7) were used, probably because the last two nucleotides of the Neofab-upTub-100 primer do not align with the *N. alba* sequences to be amplified. Because the DNA chain is synthesized in the 5'→3' direction, a free 3'-hydroxyl group must exist to allow the correct initiation of DNA synthesis (19). Therefore, it is of fundamental importance that the terminal 3' position of the PCR primers be homologous with the complementary sequences of the recognition sites flanking the DNA segment to be amplified. To ensure this, Neofab-upTub-100 (5'-TGA TGA GAC CTT CTG TAT GC-3') was modified and replaced by Neo_alba-up (5'-TGA TGA GAC CTT CTG TAT CG-3'), enabling the amplification of the Chilean isolates of *N. alba* and producing a 360-bp band, which agrees with previously reported results (7,11). Therefore, these primers are useful tools for identifying *Neofabraea* spp. associated with bull's eye rot in apple.

Our study demonstrates the lack of intraspecific variation in *N. alba*. The ITS and β -tubulin sequences were examined for 10 and 27 *N. alba* isolates, respectively and no associations between the isolates and their geographical origin and symptom types were found.

In summary, it is possible that bull's eye rot is an emergent disease that is becoming increasingly important because of the commercial introduction of late-harvest cultivars (e.g., Cripps Pink). These cultivars are harvested in the early autumn when frequent rainfalls occur in south-central Chile. It is of interest that the disease prevalence was higher in the zones characterized by higher annual rainfall means, possibly because the rains facilitate the release and dispersal of conidia (9).

Acknowledgments

We thank the apple growers for kindly providing the Cripps Pink apple samples for this study and Laboratorio de Sanidad Vegetal, Universidad de Talca, Chile, for collaboration during this study.

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