BOSQUE 38(1): 195-201, 2017 DOI: 10.4067/S0717-92002017000100019

NOTAS

Detection of *Colletotrichum pyricola* on urban trees of *Embothrium coccineum* in Chile

Detección de *Colletotrichum pyricola* asociado a árboles urbanos de *Embothrium coccineum* en Chile

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SUMMARY

The genus *Colletotrichum* comprises several species in cryptic complexes that cannot be easily recognizable using morphological and cultural characteristics. As a consequence of the lack of morphological characters suitable for identification, DNA sequence analyses are now typically used as the primary basis in diagnosis and description of new species of *Colletotrichum*. In this study, based on a multi-locus phylogeny analysis, *C. pyricola* was identified on leaves of *Embothrium coccineum* in Chile, corresponding to the first report of this fungus in the country. *Colletotrichum pyricola* is a member of the *C. acutatum* complex, morphologically sharing features with several species of this group, being necessary the combination of morphological and molecular data for its identification. Species of *Colletotrichum* cause diseases in a wide range of hosts, making it important to establish an accurate diagnosis of the species for plant pathology or quarantine purposes.

Key words: *Colletotrichum pyricola, Embothrium coccineum*, anthracnose, morphological characteristics, multi-locus analysis.

RESUMEN

El género *Colletotrichum* comprende varias especies crípticas dentro de complejos que no pueden ser fácilmente identificables usando características morfológicas y culturales. Como consecuencia de la falta de caracteres morfológicos adecuados para la identificación, los análisis de secuencias de ADN son comúnmente usados, en la actualidad, en el diagnóstico e identificación de nuevas especies. En este estudio, mediante análisis filogenético multi-locus se identificó a *C. pyricola* en hojas de *Embothrium coccineum*, correspondiente a primer reporte de este hongo en Chile. *Colletotrichum pyricola* pertenece al complex de *C. acutatum*, morfológicamente comparte características con varias especies de este grupo, siendo necesario la combinación de datos morfológicos y moleculares para su identificación. Especies de *Colletotrichum* causan enfermedades en un amplio rango de hospederos, haciendo importante establecer con precisión el diagnóstico de la especie para fines fitopatológicos o de cuarentena.

Palabras clave: *Colletotrichum pyricola*, *Embothrium coccineum*, antracnosis, características morfológicas, filogenia multi-locus.

INTRODUCTION

Embothrium coccineum J.R. Forst. *et* G. Forst, known by the common names of notro, ciruelillo or fosforito, is a member of the Proteaceae family, endemic of the southern forests of South America whose latitude distribution in Chile extends from Curicó – Linares (35° S) to Tierra del Fuego (55° S) (Rovere *et al*. 2010). Due to its ornamental value, this species has been used in landscaping, park and garden designs, especially in public spaces (Teiller 2008). As a native species and part of the urban trees, it has been object of phytosanitary surveys by the Servicio Agrícola y Ganadero (SAG). In surveillance activities carried out in May 2015, a *Colletotrichum* species associated with leaf spots was detected on *E. coccineum*, in the county of Chillán Viejo, region of Biobío, Chile. The affected tree was an isolated individual showing chlorosis symptoms and partial defoliation of branches. Preliminary results, using specific polymerase chain reaction (PCR) primers, identified the specie as *C. acutatum sensu lato* (Sreenivasaprasad *et al*. 1996, EPPO 2003). Currently it is known that *C. acutatum sensu lato* is a complex comprising about 30 species closely related, sharing hosts and morphological and cultural characteristics that require a multi-locus DNA

analysis for its correct identification (Damm *et al*. 2012). Due to that *C. acutatum sensu stricto* is part of this complex, and as a pathogen is considered a quarantine pest on several agriculture crops in Chile (SAG 2015), it is necessary to establish with precision the diagnostic of the detected species. Therefore, the main objective of this study was to determine the species of *Colletotrichum* found on *E. coccineum*, through the morphological and multi-locus molecular analysis.

METHODS

Samples and isolates. The sample analyzed corresponds to leaves of *Embothrium coccineum* collected in May of 2015, from an isolated tree, located in the urban park Bernardo O'Higgins of Chillán Viejo County (36°51'45'' S, 72°23'46'' W). The leaves with symptoms of anthracnose were left in a wet chamber at room temperature for seven days (18 ± 5 °C). After this period, abundant orange masses of conidia characteristic of the *Colletotrichum* genus was observed, from which monosporic cultures were established in potato dextrose agar (PDA, Merck 110130). One of the monosporic isolates was conserved for morphological and molecular studies, which was stored in the mycology collection of the Regional SAG Chillan Laboratory, with the number SAG:60192-2015.

Morphological analyses. The colony growth, color and texture were evaluated on the PDA culture medium, oatmeal agar (OA) (Smith *et al*. 2002) and synthetic nutrient agar (SNA) (Nirenberg 1976), incubated at 20 °C under alternating cycles of 12 h near UV light and darkness for 10 days. The measuring of the size of conidia and appressoria (n = 30), as well as their morphological description, was completed only in SNA (Damm *et al*. 2012). Additionally, the size measurements of the conidia were completed *in vivo*, collected directly from leaves on *E. coccineum* maintained in a wet chamber. The microscopic preparations of the structures were done with 60 % lactic acid and afterwards were observed with a 100x objective using immersion oil. For the measuring of the structures, the software Piximetre version 5.6 was used.

Molecular analyses. From aerial mycelium growing on PDA, genomic DNA was extracted using the modified protocol of Cooke and Duncan 1997. The internal transcribed spacer (ITS), the intron 2 of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), part of the actin (ACT) and beta-tubulin 2 (TUB2) were amplified with the primer pairs ITS-5 / ITS-4 (White *et al*. 1990), GDF1 / GDR1 (Guerber *et al*. 2003), ACT-512F / ACT-783R (Carbone and Kohn 1999) and TUB2Fd / TUB4Rd (Aveskamp *et al*. 2009), respectively, according to the protocol described by Damm *et al*. (2012). The amplicons obtained were sent to MACROGEN Korea for sequencing in both directions with the primers mentioned before. The sequences ITS, GAPDH, ACT and TUB2 resulting from the sequencing were aligned with another *C. acutatum* species complex belonging to the clade 5 according to Damm *et al*. 2012 (table 1), including *C. orchidophilum* as outgroup, using the algorithm Clustal-W of the software BioEdit 7.2.0. The phylogenetic reconstruction of the concatenated genes was done using criteria of maximum parsimony (MP) with a heuristic search and TBR (tree-bisection-reconnection) with the software PAUP 4.0b10 (Swofford 2003). The support of the internal topology of the phylogenetic tree was completed through the bootstrap analysis with 1,000 iterations.

RESULTS

The *Colletotrichum* isolate SAG:60192 showed different cultural characteristics according to the growth medium. Colonies on PDA were flat with entire margin, moderate grey aerial mycelium and orange mass conidia in the agar surface, with a radial growth of 32 mm in 10 days. Colonies on OA developed more aerial mycelium than that developed by colonies on PDA, but of lighter gray color with orange conidial masses mainly in the center of the dish, exhibiting a radial growth of 30 mm after 10 days. Colonies produced on OA were hyaline, with very low aerial mycelium and radial growth reaching only 26 mm in 10 days. The sizes of the conidia measured on *E. coccineum* leaves were 14,7 [15,8 - 16,2] 17,3 x 4,2 [4,6 - 4,8] 5,3 µm with a relationship L:W= 2,9 [3,3 - 3,5] 3,9 and in the SNA medium 14,9 [15,9 - 16,3] 17,3 x 4,2 [4,5 - 4,6] 4,8 µm with a relationship L:A = 3,1 [3,5 - 3,6] 4 (95 % of confidence). The conidia that formed both on the leaves and in the culture medium were hyaline, smooth-walled, aseptate, straight to cylindrical with one rounded and one acute end. Appressoria, measured on the back side of a plate with SNA medium, registered a size of 6,6 [8,8 - 9,8] 12 x 4,6 [5,6 - 6,1] 7,1 µm with a relationship L:W = 1,1 [1,5 - 1,7] 2,1 (95 % of confidence), with smooth edges and wavy, brown color, mostly oval, rarely irregular or elongated (figure 2). The morphological and cultural characteristics here studied were common to several members of *C. acutatum* species complex, making it not possible to establish a clear differentiation on the basis of these observations.

In the multi-locus phylogenetic analysis (gene boundary in the alignment: ITS: 1-540, GAPDH: 541-800, ACT: 801-1.048, TUB2: 1.049-1539), 1539 characters were processed, including the gaps, of which 94 were parsimony-informative. After a heuristic search using PAUP, six trees were retained. One of them is shown in figure 1 (Length of tree = 130 steps, consistency index (CI) = 0.82, homoplasy index (HI) = 0.18, retention index (RI) = 0.96, rescaled consistency index (RC) = 0.79). In this phylogenetic tree the isolate SAG:60192 can be observed forming a clade with *C. pyricola*, with 99 % bootstrap support for the node (figure 1). The pairwise distance between

Table 1. Species and strain information used in the phylogenetic analyses with NCBI GenBank accessions.

Información de especies y aislados usados en el análisis filogenético con número de accesión de GenBank.

Species of *Colletotrichum*	Culture collection *	Host / Substrate	Country	Accession GenBank No			
				ITS	GAPDH	ACT	TUB2
C. acerbum	CBS 128530	*Malus domestica* Borkh.	New Zealand	JQ948459	JQ948790	JQ949780	JQ950110
C. australe	CBS 116478	*Trachycarpus fortunei* (Hook.) H.Wendl.	South Africa	JQ948455	JQ948786	JQ949776	JQ950106
	CBS 131325	*Hakea* sp.	Australia	JQ948456	JQ948787	JQ949777	JQ950107
C. godetiae	CBS 796.72	*Aeschynomene virginica* (L.) Britton, Sterns *et* Poggenb.	USA	JQ948407	JQ948738	JQ949728	JQ950058
	CBS 131332	*Agrimonia eupatoria* L.	Austria	JQ948429	JQ948760	JQ949750	JQ950080
	CBS 126512	Unknown	Netherlands	JQ948412	JQ948743	JQ949733	JQ950063
	IMI 351248	*Ceanothus* sp.	UK	JQ948433	JQ948764	JQ949754	JQ950084
	CBS 160.50	*Citrus aurantium* L.	Unknown	JQ948406	JQ948737	JQ949727	JQ950057
	CBS 133.44	*Clarkia* (hybrid)	Denmark	JQ948402	JQ948733	JQ949723	JQ950053
	IMI 351253	*Fragaria* × *ananassa*	UK	JQ948421	JQ948752	JQ949742	JQ950072
	CBS 171.59	*Juglans regia* L.	Unknown	JQ948405	JQ948736	JQ949726	JQ950056
	CBS 131331	*Juglans regia*	Austria	JQ948404	JQ948735	JQ949725	JQ950055
	IMI 362149b	*Laurus nobilis* L.	UK	JQ948427	JQ948758	JQ949748	JQ950078
	CBS 129942	*Mahonia aquifolium* (Pursh) Nutt.	Germany	JQ948428	JQ948759	JQ949749	JQ950079
	CBS 198.53	*Malus sylvestris* (L.) Mill.	Netherlands	JQ948432	JQ948763	JQ949753	JQ950083
	CBS 285.50	*Malus sylvestris*	Unknown	JQ948403	JQ948734	JQ949724	JQ950054
	CBS 155.25	Nut shell	Unknown	JQ948410	JQ948741	JQ949731	JQ950061
	CBS 193.32	*Olea europaea* L.	Greece	JQ948415	JQ948746	JQ949736	JQ950066
	CBS 130251	*Olea europaea*	Italy	JQ948413	JQ948744	JQ949734	JQ950064
	CBS 130252	*Olea europaea*	Italy	JQ948414	JQ948745	JQ949735	JQ950065
	CBS 126520	*Parthenocissus sp.*	Netherlands	JQ948426	JQ948757	JQ949747	JQ950077
	CBS 129911	*Podocarpus* sp.	South Africa	JQ948434	JQ948765	JQ949755	JQ950085
	CBS 129912	*Podocarpus* sp.	South Africa	JQ948435	JQ948766	JQ949756	JQ950086
	CBS 129913	*Podocarpus* sp.	South Africa	JQ948436	JQ948767	JQ949757	JQ950087
	CBS 126527	*Prunus avium* L.	UK	JQ948408	JQ948739	JQ949729	JQ950059
	CBS 126522	*Prunus cerasus* L.	Netherlands	JQ948411	JQ948742	JQ949732	JQ950062
	CBS 129934	*Prunus dulcis* (Mill.) D.A. Webb.	Israel	JQ948431	JQ948762	JQ949752	JQ950082

Continue

Table 1 Continued

	IMI 376331	*Prunus* sp.	Norway	JQ948409	JQ948740	JQ949730	JQ950060
	IMI 381927	*Rubus idaeus* L.	Turkey	JQ948438	JQ948769	JQ949759	JQ950089
	CBS 862.70	*Sambucus nigra* L.	Netherlands	JQ948437	JQ948768	JQ949758	JQ950088
	CBS 129951	*Vitis* sp.	UK	JQ948430	JQ948761	JQ949751	JQ950081
	CBS 129917	*Schinus molle* L.	Mexico	JQ948441	JQ948772	JQ949762	JQ950092
	CBS 129809	*Solanum betaceum* Cav.	Colombia	JQ948439	JQ948770	JQ949760	JQ950090
C. johnstonii	IMI 357027	*Citrus* sp.	New Zealand	JQ948443	JQ948774	JQ949764	JQ950094
	CBS 128532	*Solanum lycopersicum* L.	New Zealand	JQ948444	JQ948775	JQ949765	JQ950095
C. kinghornii	CBS 198.35	*Phormium* sp.	UK	JQ948454	JQ948785	JQ949775	JQ950105
C. orchidophilum	CBS 631.80	*Ascocenda* sp.	USA	JQ948152	JQ948482	JQ949473	JQ949803
C. phormii	CBS 102054	*Phormium* sp	New Zealand	JQ948448	JQ948779	JQ949769	JQ950099
	CBS 118201	*Phormium* sp.	New Zealand	JQ948449	JQ948780	JQ949770	JQ950100
	CBS 199.35	*Phormium* sp.	UK	JQ948447	JQ948778	JQ949768	JQ950098
	CBS 118191	*Phormium* sp.	South Africa	JQ948453	JQ948784	JQ949774	JQ950104
	CBS 118197	*Phormium* sp.	New Zealand	JQ948450	JQ948781	JQ949771	JQ950101
	CBS 124953	*Phormium* sp.	Netherlands	JQ948452	JQ948783	JQ949773	JQ950103
	CBS 483.82	*Phormium tenax* J.R. Forst. *et* G. Forst.	New Zealand	JQ948451	JQ948782	JQ949772	JQ950102
	CBS 118194	*Phormium* sp.	Germany	JQ948446	JQ948777	JQ949767	JQ950097
C. pyricola	CBS 128531	*Pyrus communis* L.	New Zealand	JQ948445	JQ948776	JQ949766	JQ950096
C. rhombiforme	CBS 129953	*Olea europaea*	Portugal	JQ948457	JQ948788	JQ949778	JQ950108
	CBS 131322	*Vaccinium macrocarpon* Aiton.	USA	JQ948458	JQ948789	JQ949779	JQ950109

*CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; IMI: Culture collection of CABI Europe UK Centre, Egham, UK.

our isolate SAG:60192 and the CBS reference of *C. pyricola* showed 99.7 % of similarity in the sequenced regions (four nucleotides of difference in the data set). In contrast, pairwise comparisons among the eight other *Colletotrichum* species included in the analysis identified similarities of 90-99 %. The DNA sequences for the ITS, GAPDH, ACT, TUB2 obtained in this study were deposited in GenBank with the accession numbers KU963516, KU963517, KU963518 and KU963519, respectively.

DISCUSSION

In this study, a combination of morphological characteristics and a multi-locus phylogenetic analysis enabled the identification of *Colletotrichum pyricola* on leaves of *Embothrium coccineum*, being this determination the first report of this fungus in Chile. Even though the morphological and cultural characteristics observed in the *Colletotrichum* isolate SAG:60192 correspond to the *C. pyricola*

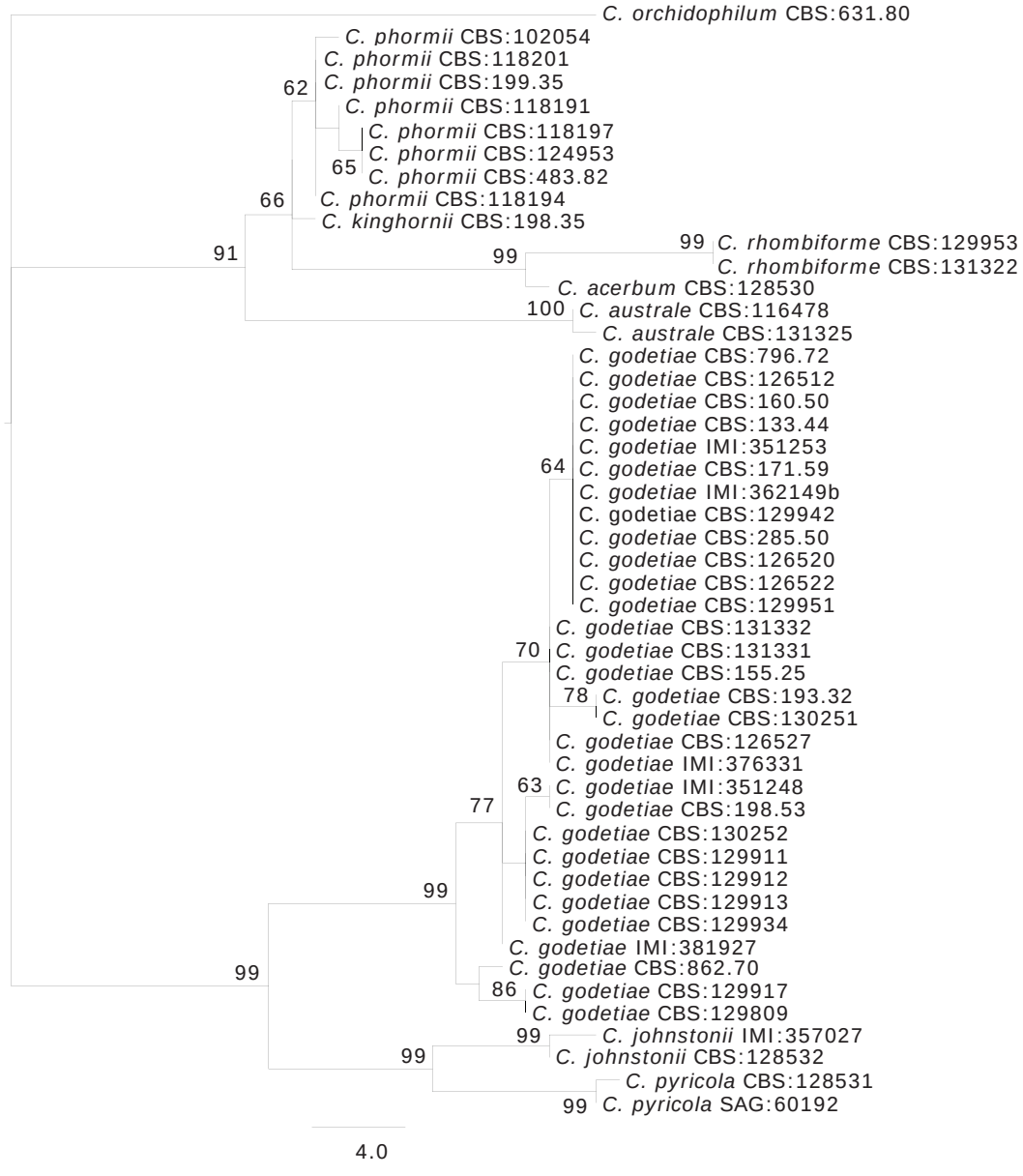

Figure 1. One of the most parsimonious trees obtained from heuristic search of the combined ITS-GAPDH-ACT-TUB2 sequences alignment. The bootstrap values higher than 60 after 1,000 replicates are shown above the nodes. The tree was rooted in *Colletotrichum orchidophilum*.

Uno de los árboles filogenéticos más parsimoniosos obtenidos de una búsqueda heurística del alineamiento ITS-GAPDH-ACT-TUB2. Sobre los nodos se muestran valores de bootstrap mayores a 60, luego de 1.000 replicaciones. El árbol fue enraizado en *Colletotrichum orchidophilum*.

description, their identification can be difficult because of overlapping ranges of conidial size and variation in colony characteristics with other members of the *C. acutatum* species complex. In fact, *C. pyricola* may not be clearly distinguishable from *C. johnstonii* using any morphological or cultural characteristics (Damm *et al.* 2012). To overcome taxonomic problems associated with these traditional identification methods, DNA sequence analyses are now typically used as the primary basis in diagnosis and description of new species of *Colletotrichum* (Bailey 1997, Cannon *et al.* 2012, Liu *et al.* 2016). Based on the multilocus analysis using ITS, GAPDH, ACT and TUB2 sequences, we have identified clearly *C. pyricola*, one of the 29 species known in the complex *acutatum*. Furthermore, this mycological determination has the additional value of discarding the presence of *C. acutatum* sensu stricto, a

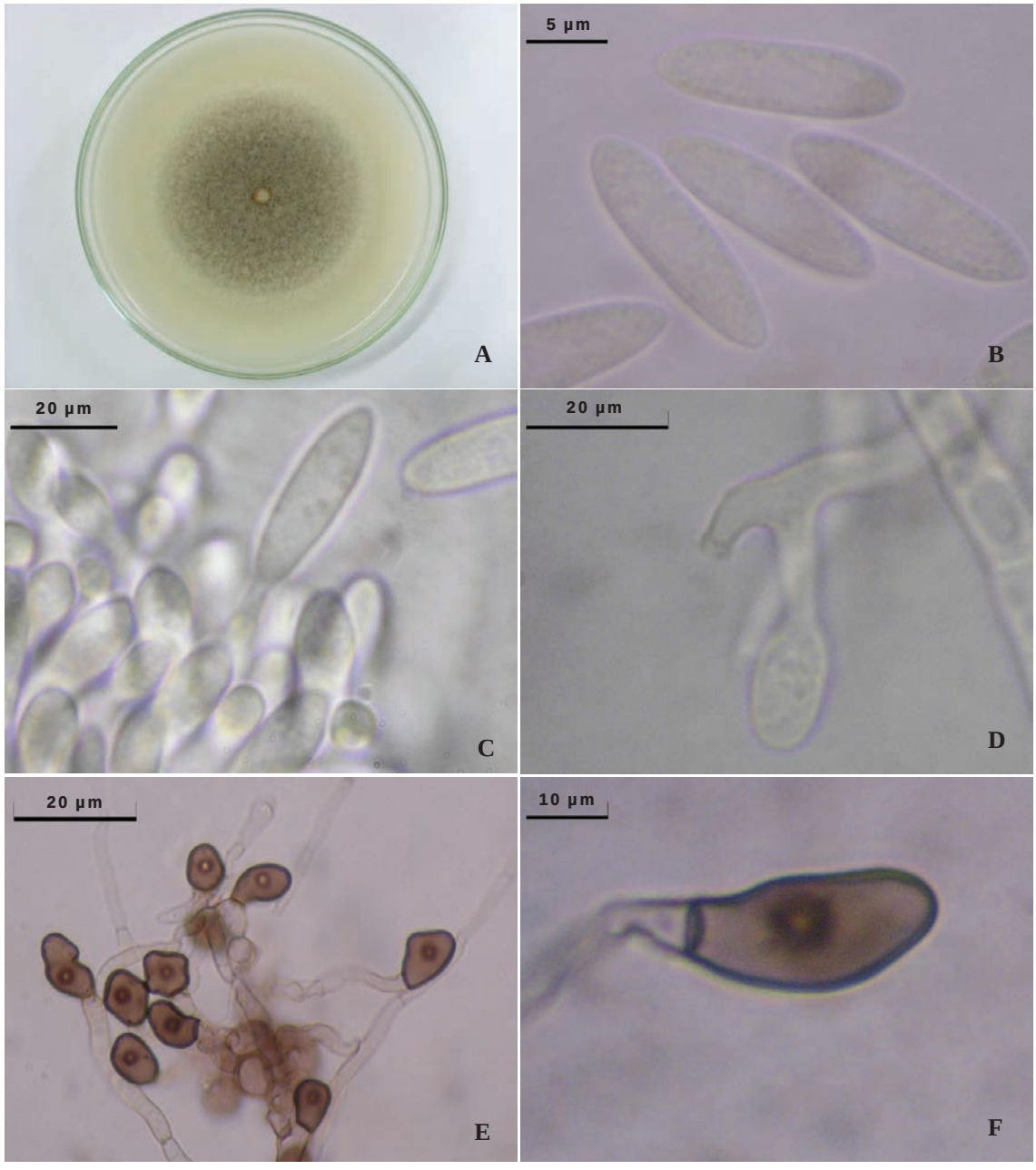

Figure 2. Morphology of *Colletotrichum pyricola* observed for the isolate SAG:60192: A. Colony on OA at 10 days. B. Conidia. C-D. Conidiophores. E-F. Appressoria.

Morfología de *Colletotrichum pyricola* para el aislado SAG:60192: A. Colonia en OA a los10 días. B. Conidia. C-D. Conidioforos. D. Apresorios.

quarantine pest for Chile, which is an important pathogen on strawberry (SAG 2015, CABI 2016).

Colletotrichum pyricola was recently described from fruits rot of *Pyrus communis* L., appearing to be endemic to New Zealand and no other information exists about hosts, distribution and pathological significance (Damm *et al*. 2012). Many species belonging to the genus *Colletotrichum* are causal agents of plant diseases, generally referred to as anthracnose, on a wide range of economically important agricultural crops. For this reason, a future line of research should consider if *C. pyricola* is associated with the genus *Pyrus* or other hosts in Chile, and determine it pathological relevance.

ACKNOWLEDGEMENT

The authors would like to thank Rodolfo Yévenes of the SAG Chillán for sample collection, the technical team of the Unidad de Fitopatología del Laboratorio Regional Chillán for the anonymous work that they performed in this identification, Gloria Cuevas, Head of the Unidad de Fitopatología and María Teresa Illesca, Head of the Laboratorio Regional Chillán, both for granting the necessary facilities to complete this work, and finally, Marcos Béeche of the Division de Protección Agrícola y Forestal of SAG Central, for supporting this technical-scientific dissemination.

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Recibido: 30.03.16
Aceptado: 20.10.16