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ORIGINAL ARTICLE

Conyza bonariensis as an alternative host for *Colletotrichum* species in Argentina

M. Bonacci[1,2], Á.N. Formento[3], M.C. Morales[4], J. Orlando[4], F. Ibáñez[5], M. Sartori[1,2], M. Etcheverry[1,2], A. Nesci[1,2] and G. Barros[1,2]

1 Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas,, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina
2 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
3 INTA-EEA Paraná, Entre Ríos, Argentina
4 Laboratorio de Ecología Microbiana, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Ñuñoa, Santiago, Chile
5 Instituto de Investigaciones Agrobiotecnológicas, CONICET, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

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Colletotrichum, *Conyza bonariensis*, GAPDH, inoculum reservoir, ITS, soybean.

Correspondence
Germán Barros, Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta Nacional 36, km 601, Río Cuarto, Córdoba, Argentina.
E-mail: gbarros@exa.unrc.edu.ar

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Abstract

Aims: This study investigated the diversity of *Colletotrichum* isolates recovered from *Conyza bonariensis* leaves through the use of morphological characteristics, growth rate, carbon sources utilization and phylogenetic analysis.

Methods and Results: In all, 30 *Colletotrichum* isolates recovered from *C. bonariensis* leaves showing symptoms of disease were included in the present study. Based on the analysis of morphology and sequences, the isolates were distributed into six *Colletotrichum* species complexes. The concatenated alignment of GAPDH and ITS sequences showed that 20 out of 30 isolates were included in four species complexes which comprise the most important pathogens causing anthracnose in soybean or anthracnose and stalk rot in maize: *C. truncatum*, *C. orchidearum*, *C. gloeosporioides* and *C. graminicola*. The remaining 10 isolates were included in the *C. boninense* and *C. destructivum* species complexes or could not be assigned to any complex with the available information.

Conclusion: Weeds belonging to genus *Conyza* are host to soybean and maize potential pathogenic species of *Colletotrichum* and could have a role as inoculum reservoir for cross contamination in the agroecosystem.

Significance and Impact of the Study: The combined use of morphological, kinetics and physiological parameters of growth and phylogenetic analysis in *Colletotrichum* isolates from *Conyza* leaves allowed the detection of species complexes previously not identified in Argentina.

Introduction

The weed *Conyza* Less. belongs to the Asteraceae family and worldwide includes about 100 species. *Conyza* species are native to South America and North America, but over the years have been introduced in different countries of Europe, Africa, Asia and Oceania (Sansom *et al.* 2013). In Argentina, 23 species have been identified and nine are considered endemic (Urdampilleta *et al.* 2005). In recent years, two *Conyza* species have appeared in the Argentinean pampas region as important weeds: *Conyza bonariensis* and *C. sumatrensis*. These plants are characterized by a high number and latency of the seeds, discontinuous germination and effective dispersal mechanisms. These characteristics allow the easy establishment of the weed during crop development, and competition for light and nutrients and space occupation. As a consequence, production costs increase and yield decreases (Ferreira de Silva *et al.* 2013). Several factors have contributed to its rapid spread in the Argentinian pampas region, including wide adoption of no-till crop systems, insufficient and late weed scouting, inadequate crop rotation or soybean

monoculture and ineffective use of herbicides as main management strategy (Papa and Tuesca 2014). Over several years of repetitive management, these weeds have adapted evolutionarily with the soybean crops and the interference phenomenon has caused yield losses in different seasons (Fornarolli *et al.* 2010; Trezzi *et al.* 2015; Diez De Ulzurrun *et al.* 2018). Furthermore, *Conyza* sp. could be a host or reservoir of different pests such as insects or nematodes (Bajwa *et al.* 2016), viruses (Grbelja *et al.* 1988) and also an extensive list of fungi such as *Cercospora* sp., *Phoma* sp., *Fusarium* sp., *Alternaria* sp., *Puccinia* sp., *Septoria* sp. and *Colletotrichum* sp. (Farr and Rossman 2020) that are pathogens in different crops.

One of the investigations in our laboratory showed that foliar diseases caused by fungi were present in *Conyza*. Possible causal agents were identified, and pathogenicity tests were carried out to differentiate saprophytic associations from true fungal pathogens. Among the isolates recovered from *C. bonariensis* leaves, *Colletotrichum* sp. was the most prevalent genus (Bonacci *et al.* 2018). *Colletotrichum* is one of the most relevant pathogenic genera for plants worldwide and has been selected on the top 10 pathogenic fungi of scientific/economic importance (Dean *et al.* 2012). The life cycle of *Colletotrichum* is generally hemibiotrophic; however, it can be also necrotrophic, endophytic or even can have periods of latency (De Silva *et al.* 2017). The genus *Colletotrichum* includes an extensive list of pathogenic species of different crops, cereals, vegetables and fruits (Agrios 2005; Cannon *et al.* 2012). Different species of plant pathogens are mainly characterized for producing anthracnose, with typical lesions of dark colour on leaves, stems or fruits in cereals, pastures or vegetables (Agrios 2005). The genus is organized in species complexes, including approximately 210 currently accepted species. Based on phylogenetic analysis, 14 species complexes were identified within the genus *Colletotrichum* (Jayawardena *et al.* 2016; Damm *et al.* 2019). As with other fungi, characteristics such as conidial morphology, growth rate, colony coloration, pigment production, the presence of setae and sexual cycle have been used for species differentiation (Freeman *et al.* 1998). Some of these characteristics, such as conidial morphology and the presence of setae, are shared among several species of the same complex; however, features such as growth rate and presence of a sexual cycle may vary among isolates of the same species (Hyde *et al.* 2009; Cannon *et al.* 2012; Weir *et al.* 2012). Nowadays, the identification of *Colletotrichum* species combines a multigene approach in fungal taxonomy with traditional diagnostic methods. This means to correlate genotype–phenotype, including morphology, physiology, pathogenicity, secondary metabolites, that is, it points to a polyphasic approach (Cai *et al.* 2009). The population diversity of *Colletotrichum* from previous reports is complicated because of the lack of tools to identify the species and because the traditional methods which were based on morphological differences are now known to be inadequate and inaccurate (Hyde *et al.* 2009). In Argentina, although some studies have been carried out on *Colletotrichum* in extensive crops such as soybean and maize, there is a lack of information regarding the diversity of *Colletotrichum* species from polyphasic approaches. Furthermore, taking into account the high prevalence of *Conyza* sp. in the agroecosystem where soybean/maize rotations are used, we hypothesize that *Conyza* may be a possible reservoir and could provide an inoculum source to infections on soybean and maize. Based on the limited available knowledge on the potential role of weeds in the epidemiology of *Colletotrichum* sp., this study aimed to investigate species diversity in *Colletotrichum* isolates recovered from *C. bonariensis* leaves thorough the use of morphological characteristics, growth rate, carbon source utilization patterns and phylogenetic analysis.

Materials and methods

Colletotrichum isolates

In all, 30 *Colletotrichum* isolates recovered from *C. bonariensis* leaves showing symptoms of disease were included in the present study (Bonacci *et al.* 2018). However, only 14 out of 30 isolates proved to be pathogenic to the weed after Koch's postulates as indicated in Table 1. These isolates were further identified based on macroscopic and microscopic description according to Barnett and Hunter (1998) and deposited in the culture collection of the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto, Argentina. All isolates were maintained as cultures on potato dextrose agar (PDA) at 4°C and as spore suspensions in glycerol 20% w/v at −80°C.

DNA sequencing and phylogenetic analyses

For the production of fungi biomass, fungal propagules were transferred to 50 ml of complete medium (MC) (Correll *et al.* 1987). The flasks were incubated at 25°C during 2 or 3 days in an orbital shaker (150 rev min^{-1}) and them the mycelium was filtrated. The samples were pulverized with liquid nitrogen and the milled tissue saved in an Eppendorf tube. DNA was extracted by means of the cetyltrimethylammonium bromide method (Leslie and Summerell 2006). The amplification of a portion of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 200bp) gene was carried out according to Guerber *et al.* (2003) based on the primers GDF

Table 1 Characteristics of the 30 *Colletotrichum* isolates obtained from *Conyza bonariensis*

Isolate	GenBank accession numbers		Phylogeny	Culture characteristics			Conidia			Carbon source Biolog groups
	ITS	GAPDH	Species complex	Colony colour	Mycelium	Growth rate (mm day)$^{-1\dagger}$	Shape	Length (μm)	Width (μm)	
ER5	MT682970	MT683815	*Colletotrichum truncatum*	Black	Scarce, shallow	6·1 c	Falcate	29·5 ± 2·28	3·5 ± 0·81	A
ER9	MT682972	MT683817	*C. truncatum*	Grey	Abundant, cottony, compact	6·63 b	Falcate	31 ± 2·1	3·72 ± 0·46	A
ER10*	MT682973	MT683818	*C. truncatum*	Grey	Abundant, cottony, compact	6·11 c	Falcate	29·64 ± 1·82	3·48 ± 0·71	C3
ER12	MT682975	MT683820	*C. truncatum*	Grey	Abundant, cottony, compact	6·55 b	Falcate	29·12 ± 1·54	3·64 ± 0·49	C3
ER13	MT682976	MT683821	*C. truncatum*	Grey	Abundant, cottony, compact	6 c	Falcate	29·08 ± 1·61	3·68 ± 0·48	C3
RC63	MT682988	MT683831	*C. truncatum*	Brown	Scarce, shallow	6·21 c	Falcate	28·56 ± 2·22	3·6 ± 0·5	C1
RC69	MT682991	MT683833	*C. truncatum*	Black	Scarce, shallow	5·88 c	Falcate	30·36 ± 1·32	3·76 ± 0·44	C1
RC70*	MT682992	MT683834	*C. truncatum*	Grey	Scarce, shallow	5·41 d	Falcate	30·64 ± 1·63	3·72 ± 0·61	C3
ER4	MT682969	MT683814	*Colletotrichum orchidearum*	Salmon	Scarce, shallow	7 b	Cylindrical	16·3 ± 1·13	5·56 ± 0·56	C2
ER6	MT682971	MT683816	*C. orchidearum*	Salmon	Abundant, cottony, compact	7·58 a	Fusiform	28·5 ± 1·6	4·37 ± 0·72	C2
RC41	MT682984	MT683828	*C. orchidearum*	Salmon	Scarce, shallow	6·98 b	Fusiform	28·8 ± 1·79	4·45 ± 0·73	C3
RC64*	MT682989	MT683832	*C. orchidearum*	White	Abundant, cottony, compact	7·33 a	Cylindrical	16·96 ± 1·79	4·52 ± 0·59	C2
RC98*	MT682998	MT683840	*C. orchidearum*	Salmon	Scarce, shallow	6·03 c	Fusiform	14·72 ± 1·16	2·76 ± 0·44	C3
RC84	MT682993	MT683835	*Colletotrichum graminicola*	Grey	Abundant, cottony, compact	7·78 a	Fusiform	17·56 ± 0·92	4·64 ± 0·57	A
RC85*	MT682995	MT683837	*C. graminicola*	Grey	Abundant, cottony, compact	6·58 b	Fusiform	18·04 ± 0·96	3·08 ± 0·91	A
RC86	MT682994	MT683836	*C. graminicola*	Grey	Scarce, shallow	7·35 a	Fusiform	14·8 ± 1·27	4·28 ± 0·54	A
RC87	MT682996	MT683838	*C. graminicola*	Grey	Abundant, cottony, compact	6·33 c	Fusiform	17·48 ± 0·85	3·88 ± 0·83	A
RC97*	MT682997	MT683839	*C. graminicola*	Grey	Abundant, cottony, compact	6·71 b	Fusiform	17·44 ± 0·93	4·2 ± 0·82	A
ER11	MT682974	MT683819	*Colletotrichum boninense*	Orange	Scarce, shallow	5·1 d	Cylindrical	15·64 ± 0·95	5·84 ± 0·47	B
RC38	MT682981	MT683826	*C. boninense*	White	Abundant, cottony, compact	5·21 d	Cylindrical	15·9 ± 1·89	5·9 ± 0·77	B
RC57*	MT682986	MT683829	*C. boninense*	White	Abundant, cottony, compact	4·98 d	Cylindrical	16·36 ± 0·91	6·68 ± 0·56	B
RC58*	MT682987	MT683830	*C. boninense*	Salmon	Abundant, cottony, compact	4·64 e	Cylindrical	16·8 ± 1·19	6·48 ± 0·5	B
RC26*	MT682979	MT683824	*C. gloeosporioides*	Grey	Abundant, cottony, aerial	7·13 b	Fusiform	14·2 ± 1·08	3·8 ± 0·58	B
RC29	MT682980	MT683825	*C. gloeosporioides*	Grey	Abundant, cottony, aerial	7·4 a	Fusiform	13·48 ± 1·08	3·68 ± 0·69	A
RC17*	MT682977	MT683822	*C. destructivum*	Brown	Scarce, shallow	5·1 d	Fusiform	16·24 ± 1·16	3·76 ± 0·44	B
RC23*	MT682978	MT683823	*C. destructivum*	Black	Scarce, shallow	6·06 c	Fusiform	16·4 ± 1·15	3·8 ± 0·41	C3
RC39	MT682982	MT683827	*C. destructivum*	Brown	Scarce, shallow	5·21 d	Fusiform	16·48 ± 0·82	3·6 ± 0·5	C3
RC40	MT682983		ND	Grey	Abundant, cottony, compact	4·43 e	Cylindrical	7·08 ± 0·91	2·8 ± 0·5	C3
RC55*	MT682985		ND	Salmon	Scarce, shallow	1·64 f	Cylindrical	8·36 ± 1·19	3·16 ± 0·37	C3
RC68*	MT682990		ND	Black	Scarce, shallow	4·32 e	Fusiform	19·64 ± 2·63	4·04 ± 0·35	A

ND, not determined.

*Isolates showing pathogenicity in *C. bonariensis*.

†Within a column, values not sharing a common letter are significantly different ($P < 0·05$).

(5′-GCCGTCAACGACCCCTTCATTGA) and GDR (5′-GGGTGGAGTCGTACTTGAGCATGT) (Templeton *et al.* 1992). The amplification of internal transcribed spacers of the ribosomal RNA coding genes (ITS, 500 pb) was performed according to Peres *et al.* (2008) using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990). PCRs were carried out in a TECHNE Model TC-512 Thermal Cycler (Bibby Scientific, Staffordshire, UK). The amplified DNA fragments were separated by electrophoresis in 1·5% agarose gel containing 1 μg ml^{-1} ethidium bromide in 1X TBE buffer, using a molecular weight marker (DNA ladder 100 bp; Invitrogen, Buenos Aires, Argentina). Fragments were purified by filtration through DNA Wizard Clean-Up Kit (Promega, Madison, WI) and sent for sequencing by Macrogen Korea (Seoul, Rep. of Korea). The sequences were assembled and edited using the program Bioedit ver. 7.1.9 (Hall 1999). Sequence analyses were performed using the algorithm BLASTN (Zhang *et al.* 2000) to identify similarities. Afterwards, sequences were deposited in GenBank, National Center for Biotechnology Information (NCBI) under the accession numbers listed in Table 1. The evolutionary history was inferred using the maximum likelihood method. Bootstrap analysis (100 pseudoreplicates) was performed to assess the confidence on the topology of the original tree. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016). In addition, DNA sequences of representative isolates of different species complexes of *Colletotrichum* obtained from GenBank were included in the molecular phylogenetic analysis.

Morphological analyses

All *Colletotrichum* isolates were grown on PDA (Britania) at 25°C for 7 days in darkness and single-spore isolates were prepared from each culture for morphological and molecular characterization. Three cultures of every isolate were analysed based on growth characteristics including colony colour, mycelium, presence of conidiomata and setae and sporulation. The shape of conidia was determined by randomly examining 25 in each replicate. For size determination, 25 conidia were measured based on the description by Harp *et al.* (2008).

Growth rate assessment

Potato dextrose agar plates were inoculated centrally with an agar plug from 7-day-old fungal cultures using a 5 mm surface-sterilized cork-borer and incubated at 25°C. Colony ratios were measured daily or as required, in two directions at right angles to each other over periods of up to 5 days, or up to when the colony reached a diameter of 90 mm. The growth rates (millimetre per day) were determined by plotting the linear radial mycelial extension rates against time and obtaining the slope of the regression lines. For all isolates, three replicates were included. Data of growth rates were subjected to analysis of variance. When significant differences were observed, mean values were compared by the DGC test ($P < 0·05$) using the program InfoStat version 2012 (Di Rienzo *et al.* 2013).

Carbon source utilization

The *Colletotrichum* isolates were grown on 2% malt extract agar at 25°C with 12-h light photoperiod. The carbon source utilization test was carried out using Biolog FF Micro Plate™, consisting of 96 wells with different carbon compounds. The assay principle is based on the reduction of the tetrazolium salt by the succinate dehydrogenase enzyme (Biolog Inc., Hayward, CA). The inoculum of each isolates was adjusted to an absorbance of 0·2 at 600 nm according to Singh (2009) and 100 μl of suspension was charged into each well. The FF microplates were incubated at 26°C for 10 days. Data collection used a Take3™ Epoch (Biotek Inc., Winooski, VT) reader through Gen5™ Microplate Data Analysis software (Biotek Inc.). The data were taken at 0, 16, 24, 40, 44, 48, 64, 72, 88, 96, 112, 120, 136, 144, 160, 168, 192, 216 and 240 h. For each time, the absorbance was measured at 490 and 750 nm (Tanzer *et al.* 2003). The data were expressed as square root of total absorbance (490 + 750). The Average Well Color Development (Garland and Mills 1991) was calculated for each plate. To obtain a reading time where all the isolates were in the same metabolic phase, and therefore the reading could be compared we calculated the kinetic of colour development through the Gompertz equation (Verschuere *et al.* 1997) with OriginPro8 software (OriginLab Corporation, Northampton, MA). Then, the lag phase (λ) development (μ_m) and maximum absorbance (A) were compared. Finally, RStudio software was used to constructing a heat map with a dendrogram based on Euclidean distance (RStudio Team 2015).

Results

Phylogenetic and morphological analyses

Based on ITS sequence analysis, the 30 *Colletotrichum* isolates were distributed into six *Colletotrichum* species

complexes: *C. boninese, C. destructivum, C. gloeosporioides, C. graminicola, C. truncatum and C. orchidearum* (Fig. 1). The species complexes with the highest number of isolates were *C. truncatum* (8 isolates), *C. orchidearum* (5 isolates) and *C. graminicola* (5 isolates). The concatenated alignment from GAPDH and ITS sequences allowed good resolution within the *Colletotrichum* species complexes. Eight *Colletotrichum* isolates were included in the *C. truncatum* species complex. All of them were grouped with *C. trucatum* CBS 15135 and *C. trucatum* CBS 12757 reference strains (Fig. 2). In this case, all isolates included in the clade showed similar morphological characteristics with dark colonies ranging from black to grey and brown. A distinctive feature of all isolates was the presence of falcate, unicellular, hyaline conidia ending abruptly at the apex. The length and width range of conidia were 29·7 ± 1·9 × 3·6 ± 0·6 μmol l^{-1}, respectively, with a L/W ratio = 8·17 (Table 1). The acervuli were produced on the top of dark brown to black stromata with dark setae observed in all isolates. The *C. orchidearum* species complex included five isolates grouped with reference strains belonging *C. sojae* ATCC 11871 and *C. sojae* CBS 18181 (Fig. 3). Isolates showed salmon to orange coloured colonies on PDA medium and slow sporulation. The conidia were mostly cylindrical with rounded base and in few cases; they were fusiform with truncated bases. The length and width ranges of conidia were 20·9 ± 6·4 and 4·3 ± 1·1 μmol l^{-1}, respectively, with an L/W ratio = 4·84 (Table 1). Setae and acervuli were not observed in all isolates. Five *Colletotrichum* isolates were included within the *C. graminicola* species complex. However, the markers did not allow relating isolates to a particular species within the complex, being the most closely related species represented by *C. jacksonii* MAFF 305460, *C. hanaui* MAFF 305404 and *C. echinochloae* MAFF 511473 reference strains (Fig. 4). The colonies on PDA medium for the five isolates were grey to white with masses of orange conidia in the centre. The sporulation was high to moderate and all the isolates produced fusiform conidia with acute apices, and were quite uniform in length and width: 17·1 ± 1·5 and 4·0 ± 0·9 μmol l^{-1}, L/W, with a L/W ratio = 4·24. Acervuli and setae were never observed (Table 1). Four *Colletotrichum* isolates were included into the *C. boninense* species complex, and all of them showed a high closeness with the specie *C. karstii* (CBS 128550 and CBS 86172 reference strains) (Fig. 5). In the morphological analysis, the colour of the colony on PDA varied from white to pale orange, the mycelium was abundant and cottony and the conidia analysed were cylindrical of uniform size with rounded bases. Acervuli and setae were present in all isolates.

Three *Colletotrichum* isolates were included into the *C. destructivum* species complex, whereas the relationships among them were poorly resolved among phylogenetic species within the complex (Fig. 6). Despite this poor species resolution, morphological characteristics were similar among isolates, that is, dark brown or black colony on PDA medium, scarce mycelium and high sporulation level in acervuli. The conidia were fusiform slightly curved, with slightly acute ends. The length and width range of conidia were 16·4 ± 1·0 and 3·7 ± 0·4 μmol l^{-1}, respectively, with an L/W ratio = 4·4. In the *C. gloeosporioides* species complex, two isolates that showed closeness to the phylogenetic species *C. siamensis*, being grouped with the reference CBS 125969 and ICMP 17795 strains were included (Fig. 7). Regarding the morphological characteristics both strains formed a grey-coloured colony, abundant and cottony mycelium and fusiform conidia with rounded base with a fairly uniform length and width: 13·8 ± 1·1 and 3·7 ± 0·6 μmol l^{-1}, L/W ratio = 3·7. Setae and acervuli were not observed. The remaining three *Colletotrichum* isolates (RC40, RC55 and RC68) could not be assigned to any species complex using concatenated alignment from GAPDH and ITS sequences. These isolates presented variable characteristics regarding their colony colour, mycelial type, shape and size of the conidia and presence of setae and acervuli (Table 1).

Growth rate

The statistical analysis allowed separating six groups of isolates based on their growth rate (Table 1). The *Colletotrichum* isolates within the *C. gloeosporioides* (7·26 mm day^{-1}), *C. orchidearum* (6·99 mm day^{-1}) and *C. graminicola* (6·95 mm day^{-1}) species complexes showed higher average growth, followed by isolates included in the *C. truncatum*, *C. destructivum* and *C. boninense* clades. The lowest growth rates were detected in those isolates that could not be assigned to any species complex, showing growth rates <4·5 mm day^{-1}.

Carbon source utilization

The 30 *Colletotrichum* isolates were evaluated according to their ability to metabolize 95 carbon sources. As shown in Fig. 8, carbon sources were divided into two groups (1 and 2) and each group was subdivided into A, B and C (Table 1). The subgroup 1A includes carbon sources that were not used or were rarely used such as carbohydrates (D-arabitol, β-methyl-D-galactoside, D-melezitose, lactulose, and arbutin), one amine (L-alanine) and one polymer (Dextrine). Conversely, in the subgroup 2C, we found the carbon sources with the highest utilization rate. This subgroup was heterogeneous including carbohydrates (L-sorbose, maltitol, D-xylose), one amino

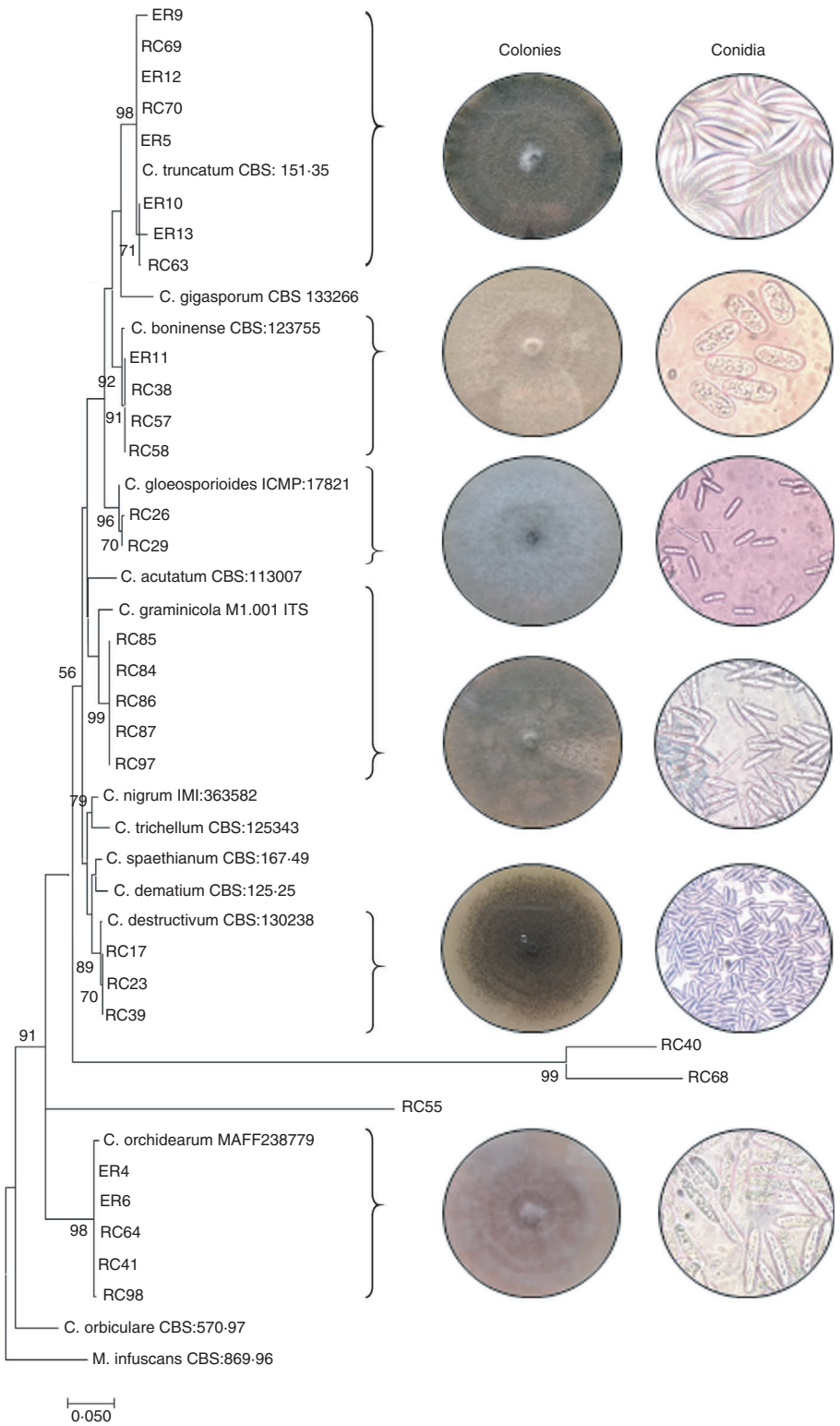

Figure 1 ML phylogenetic tree based on 356 bp of ITS gene sequence. The tree was inferred under the (Kimura two-parameter) substitution model (G = 0·4405). The scale bar represents 0·050 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown. Colonies and conidia features of representative *Colletotrichum* isolates placed in each complex: *C. truncatum* RC69, *C. boninense* RC38, *C. orchidearum* ER6, *C. graminicola* RC97, *C. gloeosporioides* RC26 and *C. destructivum* RC17.

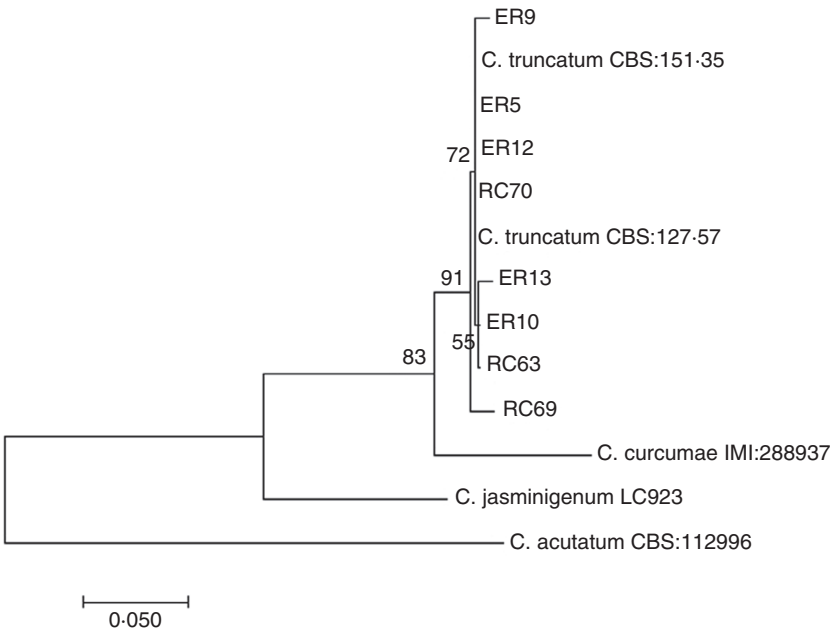

Figure 2 ML phylogenetic tree based on 668 bp of ITS + GAPDH combined gene sequences. The tree was inferred under the (Kimura two-parameter) substitution model (G = 0·3541). The scale bar represents 0·050 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown.

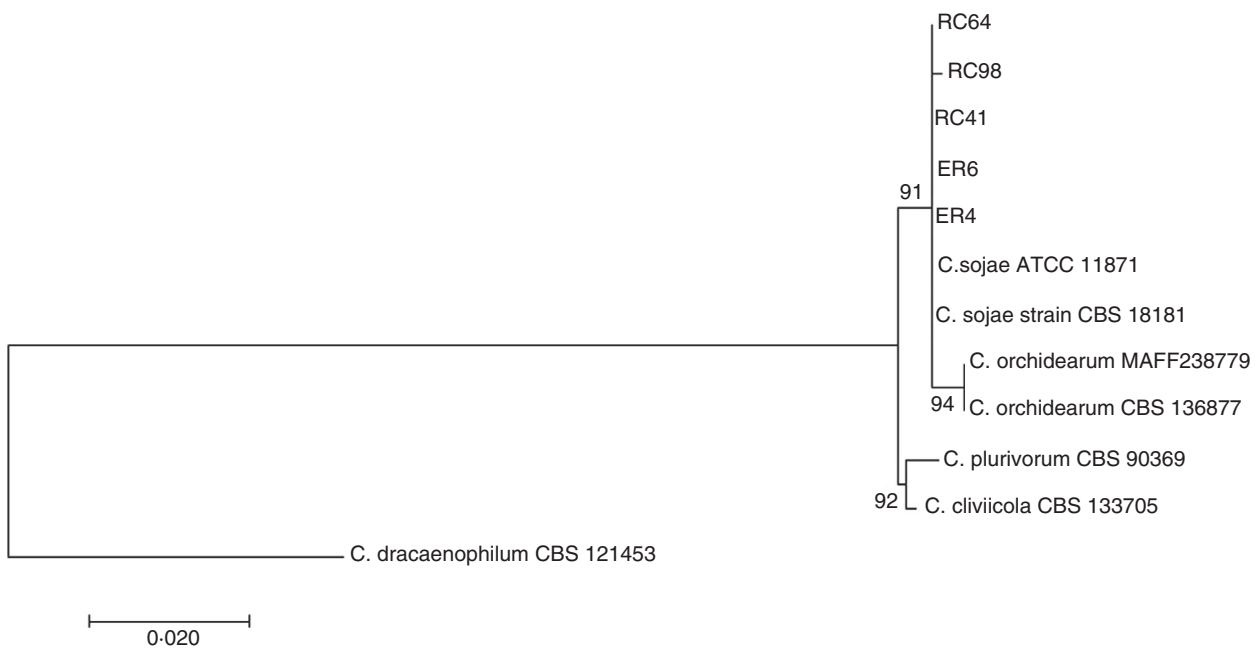

Figure 3 ML phylogenetic tree based on 735 bp of ITS + GAPDH combined gene sequences. The tree was inferred under the (Kimura two-parameter) substitution model. The scale bar represents 0·020 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown.

acid (L-serine), one amide (alaninamide), one carboxylic acid (fumaric acid) and a group integrated by different sources (glycerol, uridine, adenosine, succinic acid monomethyl ester). On the other hand, the *Colletotrichum* isolates were divided according to the catabolic versatility into three clades: A, B and C (Fig. 8).

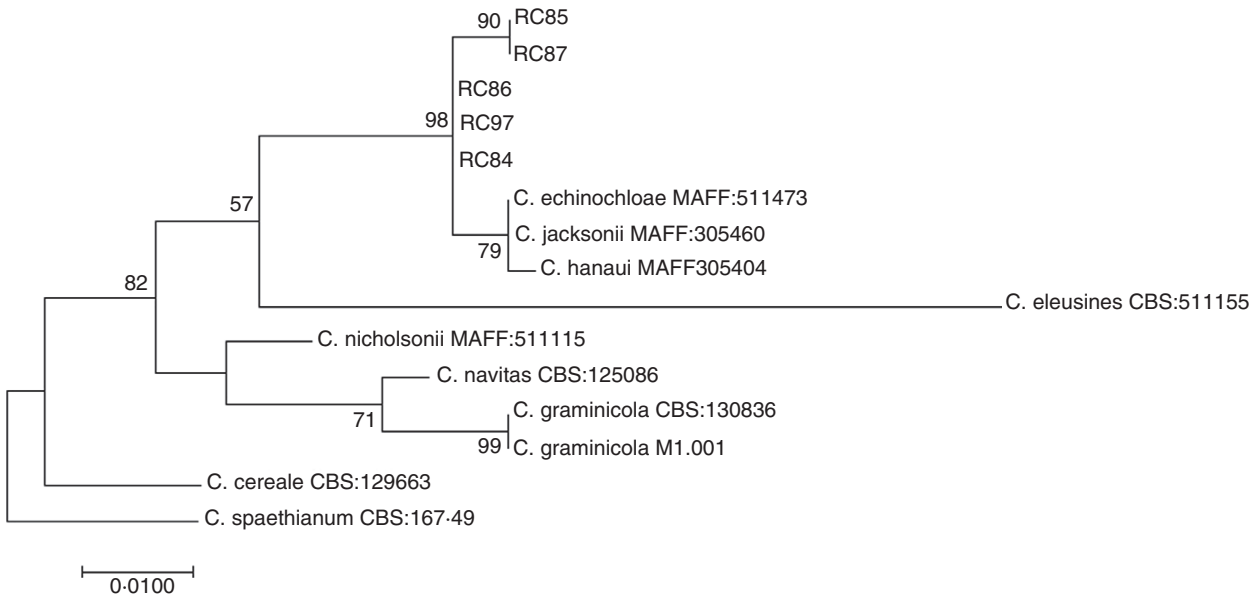

Figure 4 ML phylogenetic tree based on 423 bp of ITS gene sequences. The tree was inferred under the (Kimura two-parameter) substitution model (G = 0·0500). The scale bar represents 0·0100 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown.

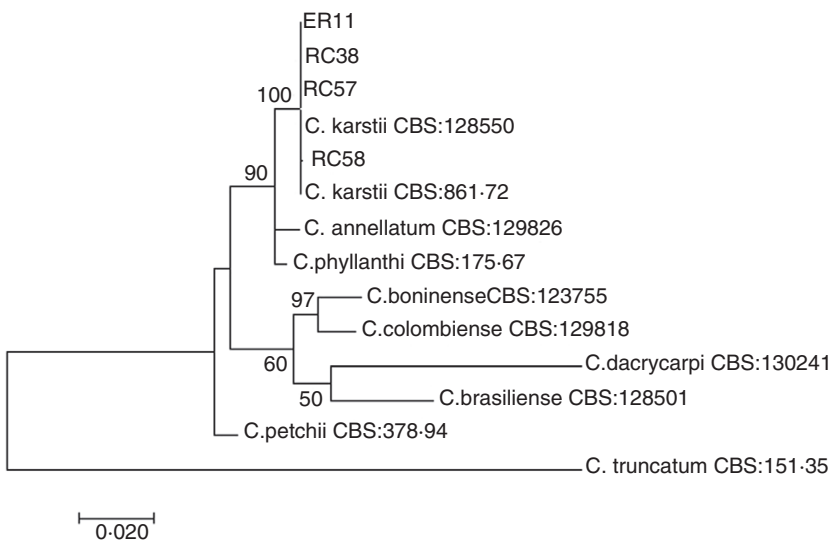

Figure 5 ML phylogenetic tree based on 615 bp of ITS + GAPDH combined gene sequences. The tree was inferred under the (Kimura two-parameter) substitution model (G = 0·1661). The scale bar represents 0·020 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown.

The clade A consisted of nine *Colletotrichum* isolates presenting the highest catabolic versatility, which used carbon sources belonging to group 2. All the isolates belonging to the *C. graminicola* species complex (RC84, RC85, RC86, RC87 and RC97) were included in this clade together with two isolates from *C. truncatum* (ER5 and ER9), one isolate from *C. gloeosporioides* clade (RC29) and one isolate not assigned to any species complex (RC68). The clade B comprised six isolates including all strains belonging to the *C. boninense* species complex (ER11, RC38, RC57 and RC58), one isolate of *C. destructivum* (RC17) and one isolate of *C. gloeosporioides*

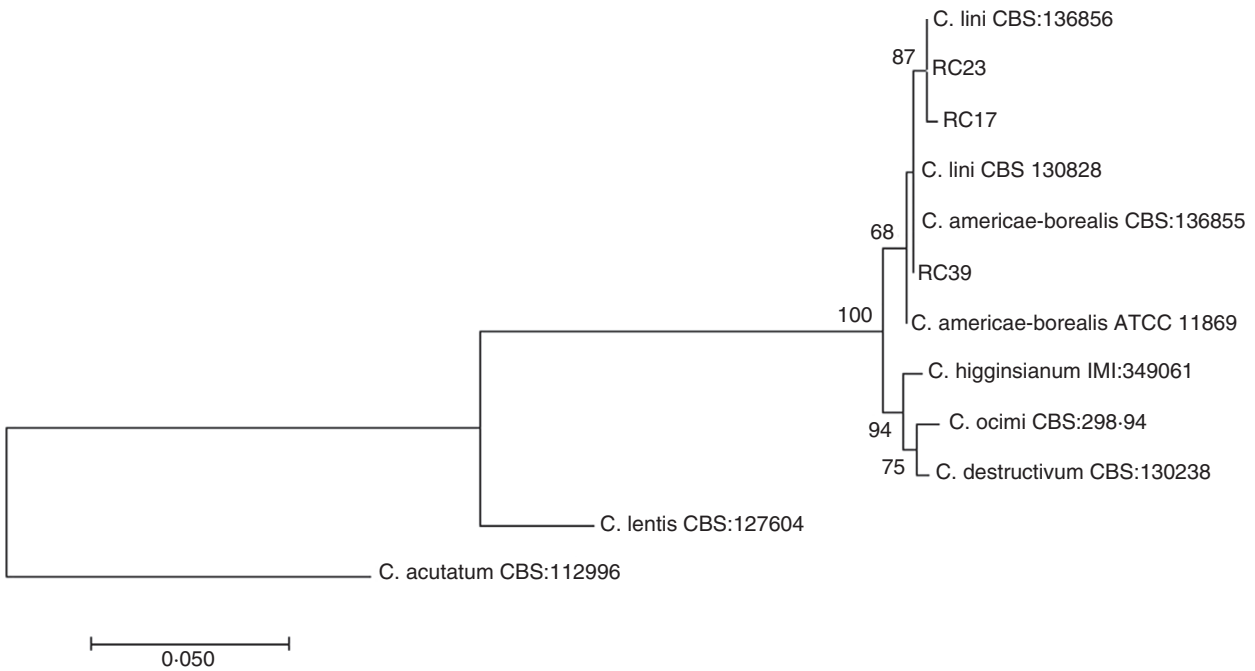

Figure 6 ML phylogenetic tree based on 623 bp of ITS + GAPDH combined gene sequences. The tree was inferred under the (Kimura two-parameter) substitution model (G = 0·5126). The scale bar represents 0·050 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown.

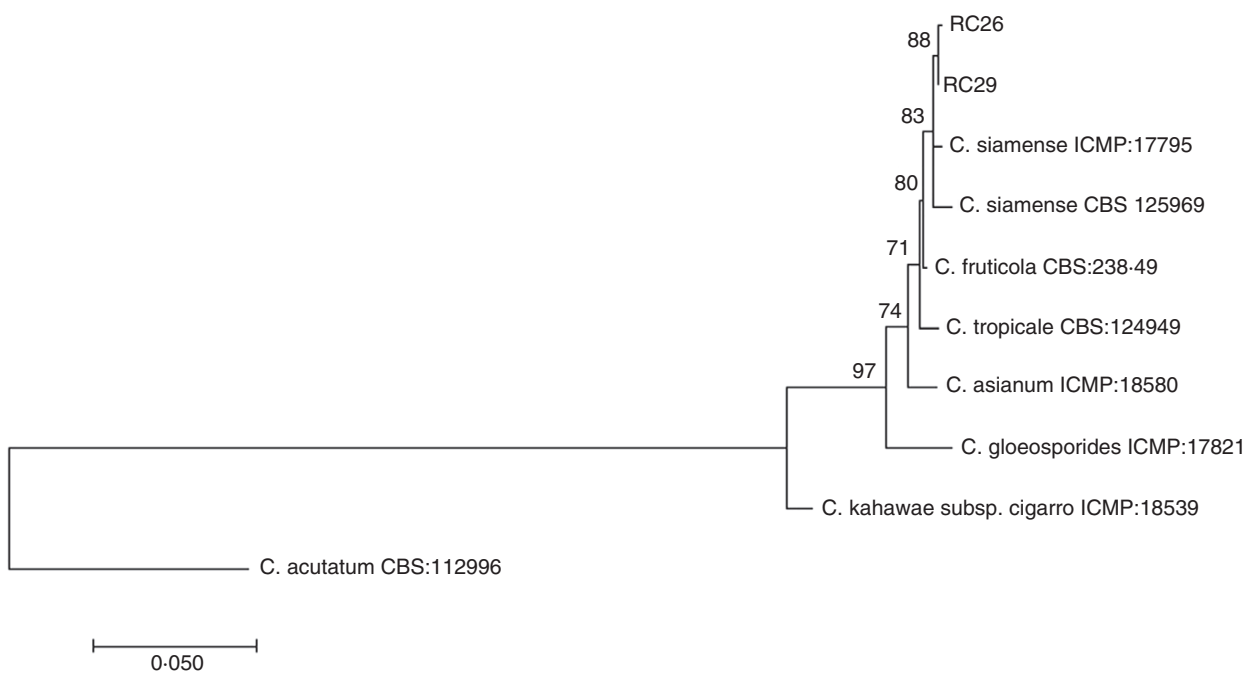

Figure 7 ML phylogenetic tree based on 671 bp of ITS gene sequence. The tree was inferred under the (Kimura two-parameter) substitution model (G = 0·3702). The scale bar represents 0·050 substitutions per site. Bootstrap values (over 50%) for 1000 replicates are shown.

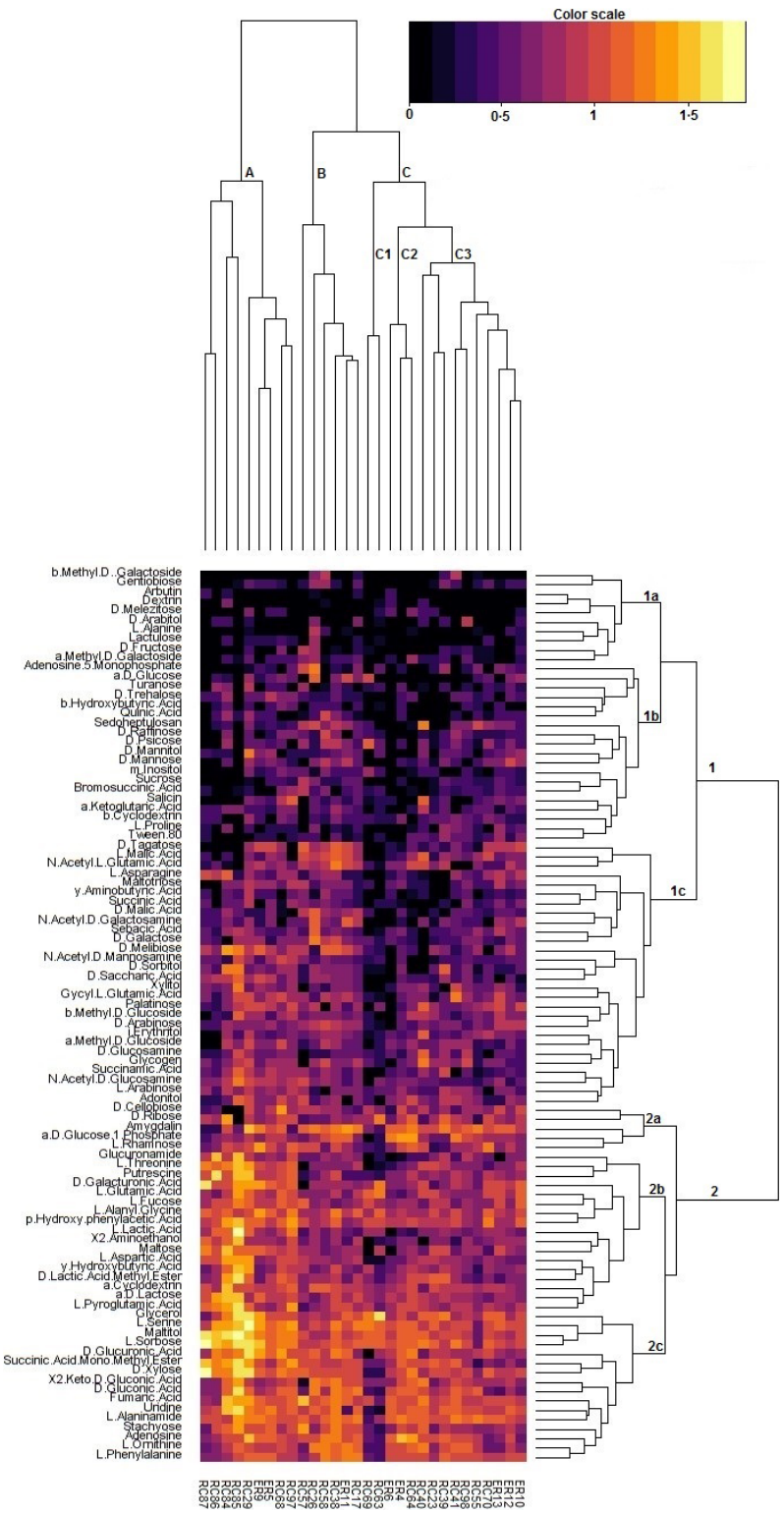

Figure 8 Comparative heat map of *Colletotrichum* isolates in FF Microtiter Plates. The use of carbon sources in the heat map corresponds to the normalized values as square root of the total absorbance (450 nm + 750 nm) at the time closest to the midpoint of the exponential phase. Black to yellow colours is the indication of poor to high growth and substrate utilization by the cultures tested.

(RC26) species complex. The clade C was the most numerous and included isolates belonging to different species complexes that showed less metabolic versatility.

Discussion

Weeds have become one of the main problems in crops grown extensively in Argentina and the *Conyza* genus has greatly expanded in the Argentinian pampas region causing interference in summer crops, mainly soybean. Different species of the *Colletotrichum* genus have broad host ranges and cross infection capacity (Phoulivong *et al.* 2012). Also, they can survive in alternative hosts such as weeds. This allows the pathogen to spread from crops to weeds and vice-versa (Freeman *et al.* 2001; Karimi *et al.* 2019). Previous works carried out in the United States (Roy *et al.* 1994) and Brazil (Duarte *et al.* 2016) have shown associations between *Colletotrichum* sp. and *Conyza* sp. However, there is a lack of knowledge of such associations in Argentina. In this work, we contribute to a better understanding of the *Colletotrichum* diversity that colonizing the weed and we provide background information to estimate the risks associated with agroecosystem in which the alternative hosts are found.

The present work addressed the study of *Colletotrichum* cultures isolated from *Conyza* leaves through the combined use of morphological, physiological and molecular tools. Based on the combination of ITS sequence and the morphological analysis, the *Colletotrichum* isolates could be assigned to at least six species complexes. Jayawardena *et al.* (2016) demonstrated that all the species complexes could be distinguished effectively from each other by using ITS sequence analysis. In contrast, Crouch *et al.* (2009) indicated that the ITS region alone is often unreliable and not enough to resolve the relationships and differences among species. For this reason, in addition to ITS sequence we decided to use GAPDH sequence. Vieira *et al.* (2019) demonstrated that GAPDH had the most considerable barcode gap distance in 7 of the 11 species complexes evaluated. The percentage overlap between intra- and inter-specific distances was <20%, placing it among the best barcode candidates across *Colletotrichum* as a whole. In this work, the concatenated alignment from GAPDH and ITS sequences showed that 20 out of 30 isolates were included in four species complexes which comprise the most important pathogens causing anthracnose in soybean: *C. truncatum, C. orchidearum, C. graminicola* and *C. gloeosporioides*. Eight isolates were included in the *C. truncatum* species complex, displaying morphological and cultural characteristics congruent with previous observations (Damm *et al.* 2009). All the isolates were phylogenetically related to the species *C. truncatum*. Dias *et al.* (2019) evaluated 30 *Colletotrichum* isolates obtained from symptomatic soybean leaves, stems, petioles and pods collected from the Provinces of Entre Ríos, Córdoba and Santa Fe in Argentina. In accordance with our study, all the isolates were identified as *C. truncatum* using molecular tools with a high level of genetic similarity among the Argentinian populations. Rogério *et al.* (2017) obtained similar results, demonstrating that *C. truncatum* was the only species associated with soybean anthracnose in Brazil up to 2007 using the concatenated sequences of the histone (HIS3), GAPDH and ITS regions.

In addition to the *C. truncatum* complex, more recently several species within the *C. orchidearum* complex have been associated with anthracnose in soybean such as *C. cliviae* (= *C. cliviicola*), *C. plurivorum* and *C. sojae* (Barbieri *et al.* 2017; Damm *et al.* 2019). In our study, the five isolates included in the *C. orchidearum* complex grouped with *C. sojae*. However, to confirm this finding, β-tubulin2 (TUB2) and HIS3 sequences should be analysed, taking into account that Vieira *et al.* (2019) found that those sequences are the most powerful genes identified thus far capable of discriminating species within the *C. orchidearum* complex. Moreover, the isolates within this complex presented considerable variability of morphological and physiological characteristics such as size and shape of conidia, colony colour, mycelium and use of different carbon sources. This variability, together with the complex phylogenetic relationships in the *C. orchidearum* complex observed by Damm *et al.* (2019), shows that more studies are needed to resolve the identity of species within this complex.

The *C. graminicola* complex includes *C. graminicola* and 14 closely related species that are only associated with certain grasses (Poaceae) and form a monophyletic clade (Cannon *et al.* 2012). In our study, five isolates could be included with good resolution within the *C. graminicola* complex but the sequences information did not allow associating the *Conyza* isolates with any specific phylogenetic species. Although GAPDH is a good marker for initial diversity screening in *Colletotrichum*, in this species complex it is necessary to incorporate another informative marker such as chitin synthase (CHS-1), actin (ACT) or tubulin (TUB2) according to Jayawardena *et al.* (2016). All the *Conyza* isolates presented uniformity in their morphological characteristics in terms of colony colour, mycelial type and fusiform conidia. Also this species complex displayed the greatest metabolic versatility in the use of different types of carbon sources. *Colletotrichum graminicola* (Ces.) Wils. is recognized in Argentina as an important pathogen in maize causing anthracnose and stalk rot (Díaz *et al.* 2012; Magliano *et al.* 2015) and more studies are necessary to know which phylogenetic species within the *C. graminicola* species complex are associated with these diseases in maize.

Colletotrichum gloeosporioides (Penz.) Penz. and Sacc. (teleomorph = G. cingulata [Stonem.]) is another species associated with anthracnose in soybean (Hartman *et al.* 1999). According to the distinctive morphology and ITS and GAPDH sequences, two *Conyza* isolates could be located within the complex. However, the *C. gloeosporioides* complex is a collective of *C. gloeosporioides* and 37 closely related species and our information is insufficient to delineate species. In this case, a combination of intergenic spacer between DNA lyase and the mating-type locus MAT1-2-1 (APN2/MAT-IGS) and glutamine synthetase (GS) sequences can be used to distinguish the species within this complex according to Liu *et al.* (2015).

The remaining 10 isolates were included in the *C. boninense* and *C. destructivum* species complexes or could not be assigned to any complex with the available information. The *C. boninense* complex is composed of *C. boninense* and 14 related species including pathogens or endophytes. In our study, the four isolates related to this complex showed as distinctive characteristic cylindrical conidia with the morphology described previously by Damm *et al.* (2012), low growth rate and a good ability to use different carbon sources. According to Damm *et al.* (2012), all species within this complex can be recognized just with the analysis of GAPDH sequence. Based on this sequence, we observed that all isolates showed a high sequence similarity with the specie *C. karstii* (bootstrap of 100%). The *C. destructivum* complex is a collective of *C. destructivum* and 14 closely related species that are mainly hemibiotrophic plant pathogens (Damm *et al.* 2014). Three of our isolates were included in this complex exhibiting micromorphology according to that described by Damm *et al.* (2014) with conidia slightly curved and small inconspicuous acervuli. According to Damm *et al.* (2014), all species can be identified by a combination of TUB2 and GAPDH sequences; therefore, the analysis of other sequences that provide more information is necessary to delineate species within the complex.

The daily mycelial growth has been commonly used in the taxonomic analysis of genus *Colletotrichum* (Sutton 1992) and is regarded as a stable and useful feature for interclade distinction for *C. gloeosporioides* and *C. acutatum* (Harp *et al.* 2008; Than *et al.* 2008). Also, differences in growth rate among species have been reported within the same complex such as *C. siamense*, *C. fructicola* and *C. asianum* in the gloeosporioides complex (Prihastuti *et al.* 2009). In this work, we observed different growth rates between clades. In general, *C. gloeosporioides, C. orchidearum* and *C. graminicola* species complexes presented growth rates up to 6·5 mm day^{-1} and *C. truncatum, C. destructivum* and *C. boninense* complexes presented growth rates less than 6·5 mm day^{-1}. Also, different growth rates among isolates within the same species complex have been observed; however, it could be a useful variable to differentiate species in specific cases. For example, Moriwaki and Tsukiboshi (2009) measured a growth rate of 4·5 mm day^{-1} for *C. echinochloae* that can be useful information to discriminate between this species and *C. jacksonii* within the graminicola complex. On the other hand, the three isolates that could not be assigned to a specific complex in our work presented growth rates below 4·5 mm day^{-1}. This distinctive feature, in addition to other sequence analyses, could help further identification.

Numerous investigations evaluated the ability of isolates of *Colletotrichum* to metabolize citrate and tartrate for the characterization and differentiation of species (Varzea *et al.* 2002; Bridge *et al.* 2008; Prihastuti *et al.* 2009). García Muñoz *et al.* (2000) found differences in the utilization of carbon sources between *C. acutatum* and *C. fragariae*, concluding that it can be used to define these taxa as species. In our work, we find different patterns in the utilization of 95 carbon compounds. Some isolates clustered in the same species complex showed similar patterns of carbon sources utilization such as *C. graminicola, C. boninense* and *C. truncatum* (most isolates) complexes, while in other clades there was more variability. Although we found differences in the use of 95 carbon compounds, we did not consider it a determining feature to define taxa due to the complex taxonomy of this genus. However, the phenotypic information obtained in this work will provide a greater understanding in relation to the lifestyle of species in the agroecosystem.

Weeds and wild plants external to crop fields and within them can serve as alternative hosts for pathogen fungi, usually when the economically important host plant is not present (Freeman *et al.* 2001; Bandyopadhyay *et al.* 2006). In previous work, we demonstrated that 13 out of 30 *Colletotrichum* isolates evaluated in this study were able to cause symptoms in the pathogenicity test only in injured *Conyza* leaves (Bonacci *et al.* 2018). This shows that most of *Colletotrichum* isolates have an endophytic or saprophytic relationship with respect to *Conyza*, representing an inoculum reservoir that survives in the agroecosystem in the absence of other crops. The fact that many alternative hosts, including weeds, may not show any typical symptoms of anthracnose disease due to the endophytic nature of *Colletotrichum* could lead growers to underestimate the risk for potential disease outbreaks (Karimi *et al.* 2019). Considering the importance of cross contamination of pathogens and weeds, this is the first report that demonstrates the presence at least six *Colletotrichum* species complexes associated with *C. bonariensis* in Argentina, with four of them related to pathogenic species in soybean and maize. Therefore,

pathogenicity studies are being carried out to assess the virulence of *Colletotrichum* isolated from weeds in different soybean and maize cultivars. In summary, the use of morphological, physiological and molecular tools in a *Colletotrichum* population isolated from *C. bonariensis* allowed the detection of species complexes previously not identified in Argentina. More studies are necessary for reliable species delimitation within some species complexes through the use of additional quality markers for a robust phylogenetic inference. Thus, it will be essential to continue with systematic molecular studies to characterize the *Colletotrichum* populations from *Conyza* to determine the role of this weed as a reservoir of pathogen species.

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Conflict of Interest

No conflict of interest to declare.

Authors' contribution

M.B., Á.N.F. and M.S.: experimental development; M.C.M. and J.O.: carbon source utilization test using Biolog F.F.M.; F.I.: phylogenetic analysis; M.E., A.N. and G.B.: experimental design, manuscript writing.

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