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Occurrence and infective potential of *Colletotrichum gloeosporioides* isolates associated to *Citrus limon* var Eureka

Larralde-Corona Claudia Patricia [a], Ramírez-González María del Socorro [a], Rosas-Hernández Iván [a], De la Cruz-Arguijo Erika [a], De Santiago Carolina [b], Shirai Keiko [b], Narváez-Zapata José Alberto [a,*]

[a] *Instituto Politécnico Nacional, Centro de Biotecnología Genómica. Blvd Del Maestro esq. Elias Piña, Reynosa 88710, Tamaulipas, Mexico*
[b] *Depto. Biotecnología, Laboratorio de Biopolímeros y Planta Piloto de Procesamiento de Desperdicios Orgánicos. Universidad Autónoma Metropolitana Unidad Iztapalapa*

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ABSTRACT

A collection of 37 fungi associated to Italian lemon plants with disease symptoms, was obtained. Ten genera including *Aspergillus, Alternaria, Nigrospora, Lasiodiplodia, Dothideomycetes, Pleurostoma, Setosphaeria, Penicillium, Fusarium* and *Colletotrichum* were identified by using ITS1–5.8S–ITS2, D1/D2 26S and *COX*1 loci. The last three genera were abundant on the damaged fruits, being *Colletotrichum* the more abundant (32.4 %). CaInt2 and CgInt primers support the identity of these isolates as *C. gloeosporioides*. Variability, inferred by rep-PCR and multilocus sequence analysis shows genetic differences among the *C. gloeosporioides* isolates. Infective profile evaluated in *Colletotrichum* isolates shows different leave infection percentages (26 to 60 %). SEM analysis showed mycelium, spores and appressoria on the leaves of selected *Colletotrichum* isolates. Specifically, the AL-05 and AL-13 isolates showed a high chitin deacetylase activity (CDA) peaking at 1.2 U/mg protein in AL-13. This is the first report on *C. gloeosporioides* infecting Italian lemon leaves in Mexico.

Introduction

Citrus trees constitute a fruit crop widely cultivated in tropical and subtropical areas around the world [1,2]. Mexico was the fourth place worldwide in lemon exports in 2017 with 729,650 tons. The Italian lemon (*Citrus limon* var Eureka) is one of the main species produced in Mexico, being the state of Tamaulipas the main producer with around 100,000 tons in 2017 [3]. Citrus crops are severely affected by fungal pathogens, which infect the fruits in pre- and post-harvest stages. Typical pre-harvest diseases are phomosis stem-end rot (*Lasiodiplodia theobromae, Diaporthe citri*), brown rot (*Phytophthora citrophthora*), brown spot (*Alternaria alternata* f. sp. citri) and anthracnose (*Colletotrichum gloeosporioides*) [1]. *Colletotrichum* spp. were recently included in the list of the 10 most important plant pathogenic fungi in the world [4]. Infections by species of *Colletotrichum* can result in several diseases, including anthracnose, fruiting after flowering and postharvest anthracnose [5]. In Mexico, there are reports that *C. acutatum* and *C. gloeosporioides* cause diseases in fruits of Mexican lime (*Citrus aurantiifolia*) [6,7] and grapefruit (*Citrus paradisi*) [8]. Differentiation among *Colletotrichum* species responsible for disease epidemics is critical for developing and implementing effective control strategies [9]. Occurrence, diversity and pathogenicity of *Colletotrichum* spp. associated with *Citrus* trees have been explored by a multi-locus approach in diverse European orchards and gardens, identifying members of the *C. gloeosporioides, C. boninense*, and *C. acutatum* species complexes, and revealed to *C. gloeosporioides* strains as the most virulent specie on these fruits [10]. Similarly, diverse *Colletotrichum* isolates were isolated and identified by a multilocus approach on pomegranate fruits in the southeastern United States. These isolates were grouped within the *C. acutatum* and *C. gloeosporioides* species complexes with differences in its pathogenic profiles level being more aggressive on inoculated fruits or leaves, respectively [11]. According to the above, this study addresses the analysis of the fungal community associated to different plant tissues with disease symptoms in Italian lemon trees (*Citrus limon* var. Eureka) cultivated in Tamaulipas state, Mexico. Specifically, genetic variability and infective profile of representative *Colletotrichum* isolates were analyzed.

* Corresponding author.
E-mail address: jnarvaez@ipn.mx (N.-Z. José Alberto).

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Table 1
Origin and identity of the isolates obtained in different tissues of Italian lemon (*Citrus limon* var. Eureka)

Isolates	Identity	28S/26S (LSU)	ITS (ITS1-5.8S-ITS2)	COX1	Tissue origin
AL-01	*Colletotrichum gloeosporioides*	KC341907.1	KC341946.1	MZ407497	Persistent calyx
Al-02	*Colletotrichum gloeosporioides*	KC341908.1	KC341947.1	NA	Yellowish immature fruits
AL-03	*Colletotrichum gloeosporioides*	KC341909.1	KC341948.1	MZ407498	Leaf with brown lesions
AL-04	*Colletotrichum gloeosporioides*	KC341910.1	KC341949.1	MZ407499	Leaf with brown lesions
AL-05	*Colletotrichum gloeosporioides*	KC341911.1	KC341950.1	MZ407500	Persistent calyx
AL-06	*Colletotrichum gloeosporioides*	KC341912.1	KC341951.1	MZ407501	Yellowish immature fruits
AL-08	*Colletotrichum gloeosporioides*	KC341914.1	KC341953.1	MZ407502	Necrotic leaf
AL-09	*Colletotrichum gloeosporioides*	KC341915.1	KC341954.1	MZ407503	Persistent calyx
Al-10	*Colletotrichum gloeosporioides*	KC341916.1	KC341955.1	MZ407504	Persistent calyx
AL-11	*Colletotrichum gloeosporioides*	KC341917.1	KC341956.1	MZ407505	Leaf with brown lesions
AL-12	*Colletotrichum gloeosporioides*	KC341918.1	KC341957.1	MZ407506	Leaf with brown lesions
AL-13	*Colletotrichum gloeosporioides*	KC341919.1	KC341958.1	MZ407507	Persistent calyx
AL-14	*Fusarium* sp.	KC341920.1	KC341959.1	NA	Persistent calyx
AL-15	*Fusarium* sp.	KC341921.1	KC341960.1	MZ407508	Yellowish immature fruits
AL-16	*Fusarium* sp.	KC341922.1	KC341961.1	NA	Broken fruit
AL-17	*Fusarium* sp.	KC341923.1	KC341962.1	MZ407509	Broken fruit
AL-18	*Fusarium* sp.	KC341924.1	KC341963.1	NA	Yellowish immature fruits
AL-20	*Fusarium* sp.	KC341926.1	KC341965.1	NA	Broken fruit
AL-21	*Fusarium* sp.	KC341927.1	KC341966.1	MZ407510	Dry and broken fruit
AL-22	*Fusarium* sp.	KC341928.1	KC341967.1	NA	Broken fruit
AL-23	*Fusarium* sp.	KC341929.1	KC341968.1	MZ407511	Yellowish immature fruits
AL-24	*Fusarium* sp.	KC341930.1	KC341969.1	NA	Broken fruit
AL-25	*Aspergillus niger*	KC341931.1	KC341970.1	NA	Leaf with brown lesions
AL-26	*Aspergillus niger*	KC341932.1	KC341971.1	MZ407512	Leaf with brown lesions
AL-27	*Aspergillus niger*	KC341933.1	KC341972.1	NA	Leaf with brown lesions
AL-28	*Aspergillus niger*	KC341934.1	KC341973.1	MZ407513	Leaf with brown lesions
AL-29	*Aspergillus niger*	KC341935.1	KC341974.1	MZ407514	Leaf with brown lesions
AL-30	*Aspergillus niger*	KC341936.1	KC341975.1	NA	Dry and broken fruit
AL-31	*Penicillium* sp.	KC341937.1	NA	NA	Dry and broken fruit
AL-32	*Alternaria tenuissima*	KC341938.1	KC341976.1	NA	Yellowish immature fruits
AL-33	*Alternaria tenuissima*	KC341939.1	KC341977.1	NA	Leaf with brown lesions
AL-34	*Setosphaeria*	KC341940.1[a]	KC341978.1	NA	Leaf with brown lesions
AL-35	*Dothideomycetes*	KC341941.1[a]	KC341979.1	NA	Broken fruit
AL-36	*Lasiodiplodia pseudotheobromae*	KC341942.1	KC341980.1	NA	Leaf with brown lesions
AL-37	*Nigrospora oryzae*	KC341943.1	KC341981.1	NA	Dry and broken fruit
AL-38	*Penicillium italicum*	KC341944.1	KC341982.1	MZ407515[b]	Leaf with brown lesions
AL-39	*Pleurostoma richardsiae*	KC341945.1	KC341983.1	NA	Leaf with brown lesions

[a] These isolates received its name according to ITS identity.
[b] This isolate received its name according to *COX*1 identity. NA: No responsive to gene marker amplification.

2. Material and methods

2.1. Study site and sampling strategy

Fungal isolate collection was established in a *Citrus limon* var. Eureka orchard in the Tamaulipas state (N 24° 04.161′ WO 98° 55.712′) of Mexico. This citrus orchard has conditions of controlled humidity by irrigation and with a periodical fungal chemical control by fumigation. Samples were collected from trees with visible disease symptoms. In this orchard, the last fumigation was carried out at least three months previous to sampling. Random sampling was used to survey the fungal diversity from persistent calyxes, leaves with brown lesions, and damaged fruits (yellowish immature, broke, or dry and broken fruits) from eleven trees within an area of 4 km^2.

2.2. Isolation and morphological identification of associated fungi

Fungal isolates were obtained from intracellular (2×2 mm) sections from plant damage tissues (fruits, calyxes, and leaves). These sections were then cut and disinfected with sodium hypochlorite (1% v/v) for 5 min and washed 3 times with sterile distilled water. Then, these were placed on Petri dishes containing Potato-Dextrose Agar (PDA, BD Bioxon, USA) and incubated for 5 days at 29°C. The fungi were subcultured from agar plugs obtained from the edge of the colonies to recover actively growing hyphal tips, to obtain pure isolates. Preliminary morphological identification by sporulation (size and shape of the spores) was used to characterize different fungal species. The selected isolates were kept in PDA at 4°C after its use, and preserved in glycerol 86% (v/v) at -70°C.

2.3. Molecular identification

Fungal isolates were grown in 100 mL of Potato Dextrose broth for five days at room temperature under rotary shaking at 250 rpm. Cells were harvested by filtration through a piece of filter paper and then were washed with distilled water. Fresh biomass (500 mg) was homogenized in liquid nitrogen and transferred to a 1.5 mL tube containing 1 mL of TEN buffer (100 mMTris-HCl, 50 mM EDTA, 500 mMNaCl, pH 8.0) and vortexed for 1 min. After centrifugation for 10 min at 10.000xg at RT, the pellet was re-suspended in 1 mL of TEN buffer and transferred to a fresh 1.5 mL tube to continue with phenol-chloroform extraction method [12]. ITS1-5.8S-ITS2 and D1/D2 LSU regions were amplified using the primers ITS-1 5′TCCGTAGGTGAACCTGCGG3′ and ITS-4 5′TCCTCCGCTTATTGATATGC3′ and NL1 5′GCATATCAATAAGCGGG AGGAAAAG3′and NL4 5′GGTCCGTGTTTCAAGACGG3′, respectively [13, 14]. For the amplification of the cytochrome oxidase subunit 1 (*COX*1) was necessary three primers sets specifically targeted to *Penicillium/Aspergillus* (Pen-F1 5′GACAAGAAAGGTGATTTTTATCTTC3′ and AspR1 5′GGTAATGATAATAATAATAATACAGCTG3′ [15]), *Fusarium* (AHyFu-F 5′CTTAGTGGGCCAGGAGTTCAATA3′ and AHyFu-R 5′ACC TCAGGGTGTCCGAAGAAT3′), and diverse fungal genera, including to *Colletotrichum*, (Pez-F 5′TCAGGRTTAYTAGGWACAGCATTT3′ and Pez-R 5′ACCTCAGGRTGYCCGAAGAAT3′ [16]).

Amplification of the different gene regions was performed in 50 μL of PCR reaction mix containing 5 μL of reaction buffer, 5 units of Taq polymerase (Gibco-BRL, Rockville, MD), 1.5 mM $MgCl_2$, 5μM of each

primer, 120 μM dNTPs and 50 μg of template DNA. Amplification was carried out with an initial denaturation of 5 min at 94 ºC, followed by 30 cycles of 1 min at 94 ºC, 45 s at 58 ºC and 60 ºC for D1/D2 26S and ITS1–5.8S–ITS2 regions, respectively, and 1 min at 72 ºC, with a final extension of 5 min at 72 ºC. *COX*1 amplification (with the different primer sets) was conducted in PCR touchdown conditions with an initial denaturation of 3 min at 94 ºC, followed by 15 cycles of three steps beginning with 30 s at 94 ºC, 45 s at 65 ºC and 1 min at 72 ºC. Alignment temperature dropped 3°C every 3 cycles, ending at 50°C. Finally, an extension step 10 min at 72 ºC was done. Amplicons were confirmed by electrophoresis on 1% agarose gel in TBE buffer (108 gTrisHCl, 55g boric acid, 40 ml of EDTA 0.5mM, pH 8.0 on 1 liter of distilled water). The PCR products were directly sequenced in an automatic sequencer (ABI 377 DNA sequencer, Applied Biosystems, USA) by using the BigDye terminator cycle sequencing ready reaction kit according to the instructions of manufacturer (Applied Biosystems, USA). The nucleotide sequences of each locus were aligned and compared with those at GenBank database. In addition, nucleotide sequences obtained in this study were deposited in the GenBank database, as summarized in Table 1. For each gene, the sequences from different isolates and their closest relatives were aligned. The dendrograms were constructed by an analytical program (MEGA version 10) using the Maximum Likelihood method based on the Tamura-Nei model. The reliability of the clusters was evaluated by bootstrapping with 1000 replicates.

2.4. Molecular differentiation of the Colletotrichum genus

Specific PCR primes CgInt (5′-GGCCTCCCGCCTCCGGGCGG-3′) and CaInt2 (5′-GGGGAAGCCTCTCGCGG-3′) for *C. gloeosporioides* and *C. acutatum*, respectively, were selected. These primers were used in combination with the ITS-4 primer as describes [17] with the *Colletotrichum* isolates of this study and the reference strains Cg-06 for *C. gloeosporioides*, and Ca-93 and Ca-96 for *C. acutatum*, kindly provided by the Laboratory of Plant Biotechnology at CBG–IPN.

Amplification was performed in 25 μL of PCR reaction mix containing 2.5 μL of reaction buffer, 2.5 units of Taq polymerase (Gibco-BRL, Rockville, MD), 1.5 mM $MgCl_2$, 5μM of each primer, 120 μM dNTPs and 50 μg of template DNA. Amplification was carried out with an initial denaturation of 5 min at 94 ºC, followed by 30 cycles of 1.5 min at 94 ºC, 2 min at 55 ºC and 3 min at 72 ºC, with a final extension of 10 min at 72 ºC. Amplicons were visualized by electrophoresis on 1.5 % agarose gel in TAE 1X buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA) using SYBR Green (Invitrogen, USA).

In addition, genetic variability of these *Colletotrichum* isolates was explored by using the rep-PCR analysis. This methodology was selected as it has proven to be effective in identifying fungal species [18]. Rep-PCR amplification was performed in 25 μL of PCR reaction mix containing 2.5 μL of reaction buffer, 5 units of Taq polymerase (Gibco-BRL, Rockville, MD), 5 μM of each primer REPIR-1 5′IIIICGICGICATCIGGC3′ and REP2-I 5′ICGICTTATCIGGCCTAC3′ [19], 1.5 mM of $MgCl_2$, 120 μM of dNTPs and 50 μg of template DNA. Amplification was carried out with an initial denaturation of 5 min at 94 ºC, followed by 30 cycles of 30 s at 94 ºC, 1 min at 47 ºC and 4 min at 65 ºC, with a final extension of 7 min at 72 ºC. PCR products were separated by electrophoresis in an agarose gel (2 %; w/v) at 80 V during 4 h in TAE 1X buffer (40 mM Tris, 20mM acetic acid, and 1mM EDTA), and visualized using SYBR Green (Invitrogen, USA). Rep-PCR bands were analyzed using the PyElph program with a threshold of 10, and a filter parameter of 3. Absence/presence band information was used to build a UPGMA tree to compare the amplification profile of the *Colletotrichum* strains selected.

Genetic variability was also supported in the *C. gloeosporioides* isolates by a concatenated multilocus analysis. For this, the different DNA regions (D1/D2 26S, ITS1–5.8S–ITS2 and *COX*1) were concatenated using the Bioedit software. Then, a dendrogram was constructed using the MEGA version10 program by using the Maximum Likelihood method based on the Tamura-Nei model. Clusters reliability was evaluated by bootstrapping with 1000 replicates. Concatenated sequences (ITS1–5.8S–ITS2; LUXP01000012.1:2474961-2475497, D1/D2 26S; LUXP01000012.1:2474386-2474948 and *COX*1; KR349346.1:2552-3040) of the reference *C. acutatum* strain KC05 were used as outgroup only with aim comparison.

2.5. Infection assay

Infection assays are performed on detached Italian lemon leaves of approximately 5 to 6 cm in length. The leaves were disinfected in 1% sodium hypochlorite solution for 5 minutes, and then washed three times with distilled water. An agar plug (4 mm in diameter) cut from the mycelium grown in PDA for 5 days was placed on the upper part of the leaves. The experiment was carried out with twenty repetitions per treatment. Inoculated leaves were placed in humid conditions inside Petri dishes with a moistened and sterile filter paper at the bottom of the dish. These were incubated in fluorescent light for 7 days at room temperature [20]. The pathogenic potential was recorded by measuring the diameter of the infection in the damaged area of the leaves. Leaves without inoculation (with and without sterile agar plugs) were used as negative controls. In addition, three isolates from avocado were used as biological controls, which includes two *C. acutatum* (Ca-93 and Ca-96) and one *C. gloeosporioides* (Cg-06). Three isolates of this study (Italian lemon) but from different fungal species were also used to compare the pathogenic potential of the *Colletotrichum* isolates. These were *Penicillium digitatum* (AL-38) and two *Fusarium* sp. (AL-20 and AL-21).

2.6. Scanning electron microscopy analysis

Some representative *Colletotrichum* isolates (AL-05, AL-06 and AL-13), according to their infection profile, were selected to further experiments. Leaf infection process of these isolates was observed by Scanning Electron Microscopy (SEM) as follow: Citrus leaves were briefly damaged with a sterile toothpick and inoculated with 20 μL of a concentration of 1×10^5 conidia/mL of the selected *Colletotrichum* isolates. SEM was carried out after 6 days of incubation at 29°C. The samples were prepared by cutting square pieces (0.5×0.5 cm) of the damaged leaves with mycelial growth. Samples were fixed with glutaraldehyde solution (5% v/v) in 0.1 M phosphate buffer pH 7.4 for 24 h at 4°C. Then, osmium tetraoxide was added in 0.1 M phosphate buffer pH 7.4 and allowed to react for 2 h. Then, the samples were dehydrated in ethanol series at concentrations of 30 to 100% in 30 min steps. Finally, samples were dried to a critical point, covered with gold, and observed by SEM (JJSM-5900 LV; JEOL, USA).

2.7. Chitin deacetylase activity

Two *Colletotrichum* isolates (AL-05 and AL-13) with differential infection profiles were selected to explore its chitin deacetylase (CDA) activity. These isolates were cultured in glutamic acid broth [21] for 5 days. Culture medium was then collected and homogenized with 0.1 M potassium phosphate buffer pH 6.5 on a 1:1 (v/v) ratio. Finally, the homogenized solution was filtered through using a 0.45 μm filter to recover the crude enzymatic extract. CDA activity was determined using the method reported by [22] with some modifications. In a typical procedure, 50 μL of the crude enzymatic extract were mixed with 100 μL of 50 mM tetraborate buffer pH 8.5 and 100 μL of ethylene-glycol-chitin, the mixture was incubated at 37°C for 30 min. To stop the reaction 250 μL of 5% (w/v) $KHSO_4$ and 250 μL of 5% (w/v) $NaNO_2$ were added. After 15 min 250 μL of $N_2H_6SO_3$ were mixed, 5 min later other 250 μl of 0.5% (w/v) 3-methyl-2-benzothiazolon hydrazine. The tubes were boiled for 3 min and chilled with cold water. Finally, 250 μL of 0.5 % (w/v) $FeCl_3$ was added. The protein content in the crude extract was determined by Lowry-Peterson assay using serum albumin bovine as standard. The absorbance was read on a DU 650 spectrophotometer (Beckman Coulter,

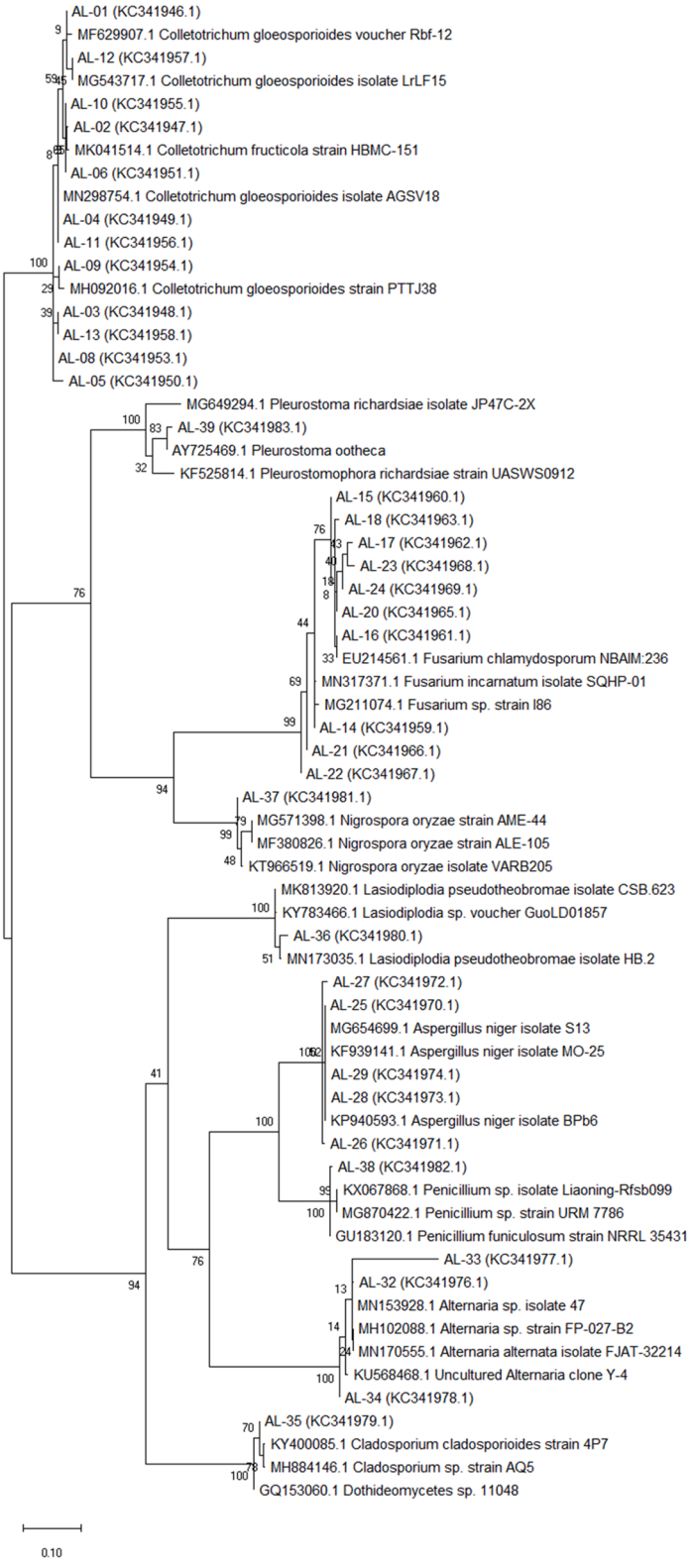

Fig. 1. Phylogenetic dendrogram based on ITS1-5.8S-ITS2 regions from the fungal community of *Citrus limon* var. Eureka. The dendrogram was built using a maximum likelihood method based on the Tamura-Nei model with 1000 bootstrap to infer topology.

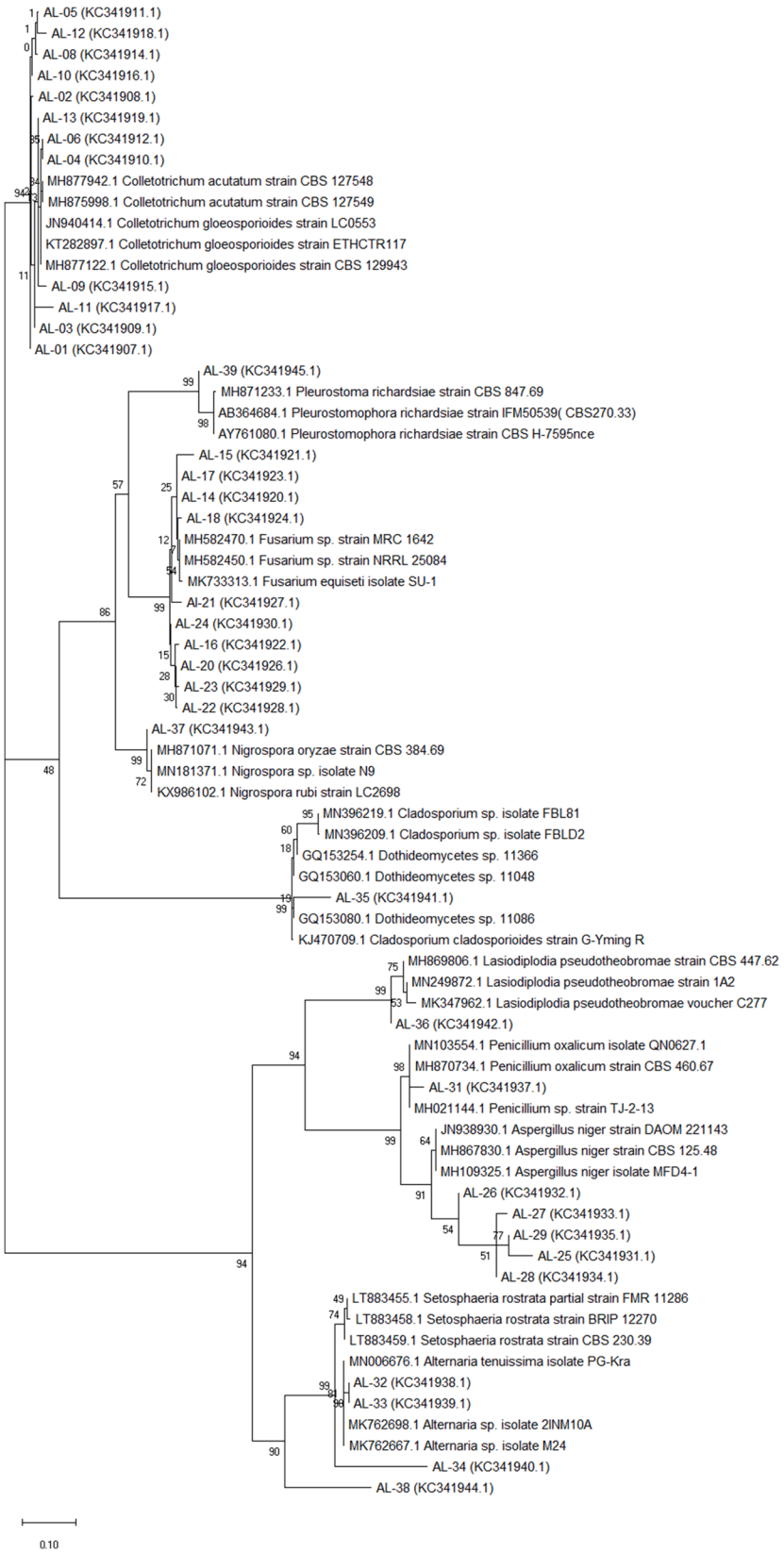

Fig. 2. Phylogenetic dendrogram based on D1/D2 LSU regions from the fungal community of *Citrus limon* var. Eureka. The dendrogram was built using maximum likelihood method based on the Tamura-Nei model with 1000 bootstrap to infer topology.

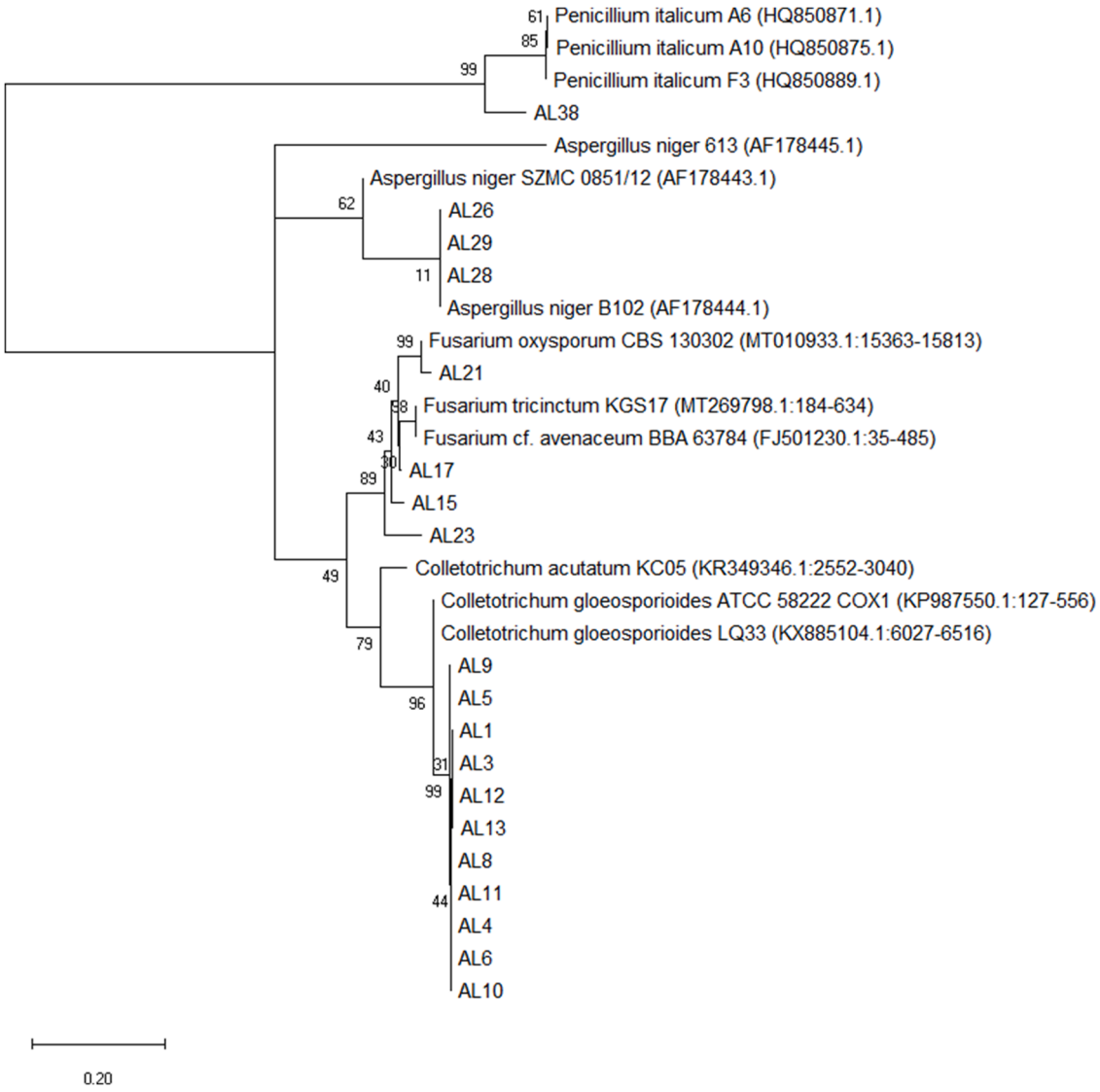

Fig. 3. Phylogenetic dendrogram based on *COX1* gene from the fungal community of *Citrus limon* var. Eureka. The dendrogram was built using a maximum likelihood method based on the Tamura-Nei model with 1000 bootstrap to infer topology.

USA) at 650 nm. A unit of CDA was defined as the amount of enzyme required to release 1 μmol of acetate per minute at standard condition. CDA specific activity was expressed as units per mg of soluble protein.

3. Results

3.1. Fungal isolation and identification

A total of 37 isolates of fungi associated with different tissues of Italian lemon plants were obtained. Particularly, samples of fruits with evident symptoms of anthracnose (yellowish immature, broke, or dry and broke fruits), persistent calyxes, and leaves with brown lesions were considered (Table 1). Most of the fungal isolates were identified by the combined analysis of two gene markers (ITS1-5.8S-ITS2 and D1/D2 LSU regions; Fig. 1 and 2), although some *Fusarium, Penicillium, Aspergillus*, and the most of the *Colletotrichum* isolates were also identified with the *COX*1 gene (Fig. 3). In general, gene markers allowed a reliable identification of most isolates to the genus level, and most of these exhibited in average a high blast identity (99%) with accessions of their assigned specie (Fig. 1; Table 1). The dendrograms presented a reliable separation at the genus level between the different taxa, with a minimum distance between clades of 0.10. In general, the genera identified were *Colletotrichum, Fusarium, Aspergillus, Alternaria, Penicillium, Nigrospora, Lasiodiplodia, Dothideomycetes, Pleurostoma* and *Setosphaeria*. Besides, *Colletotrichum, Fusarium* and *Penicillium* were the more abundant genera in the damaged fruits (Table 1). Phylogenetic analysis performed by separate (Figs. 1–3) show some differences in a few isolates, particularly in the AL-34 and AL-38 isolates, which were identified in this study as *Setosphaeria* spp. and *Penicillium italicum*, based mainly on their ITS1-5.8S-ITS2 and *COX*1 loci, respectively. Selection of this gene marker to make the final identification was based on this region outperformed that of the D2 region in the identification of fungal species [23]. Although most of the isolates were analyzed with at least two gene regions, in some species, there are few reference sequences uploaded to the Genbank, on which case, consistency was not achieved in the identification of these isolates, such as is the case of the *Setosphaeria* and *Dothideomycetes* genera. Importantly, consistency is necessary for the *Fusarium* isolates, as the phylogenetic analysis of the ITS1-5.8S-ITS2, D1 / D2 LSU and *COX*1 (Figs. 1–3) regions did not allow a clear assignment of the species. As *Fusarium* genus was abundant in this study (27 %), and the most frequently occurring on damaged fruit, more efforts are necessary to clarify the specie identity of the *Fusarium* isolates. Only one isolate, AL-31 (*Penicillium* sp.) was identified by using a single gene marker (ITS1-5.8S-ITS2 region) since it was not responsive to D1/D2 LSU and

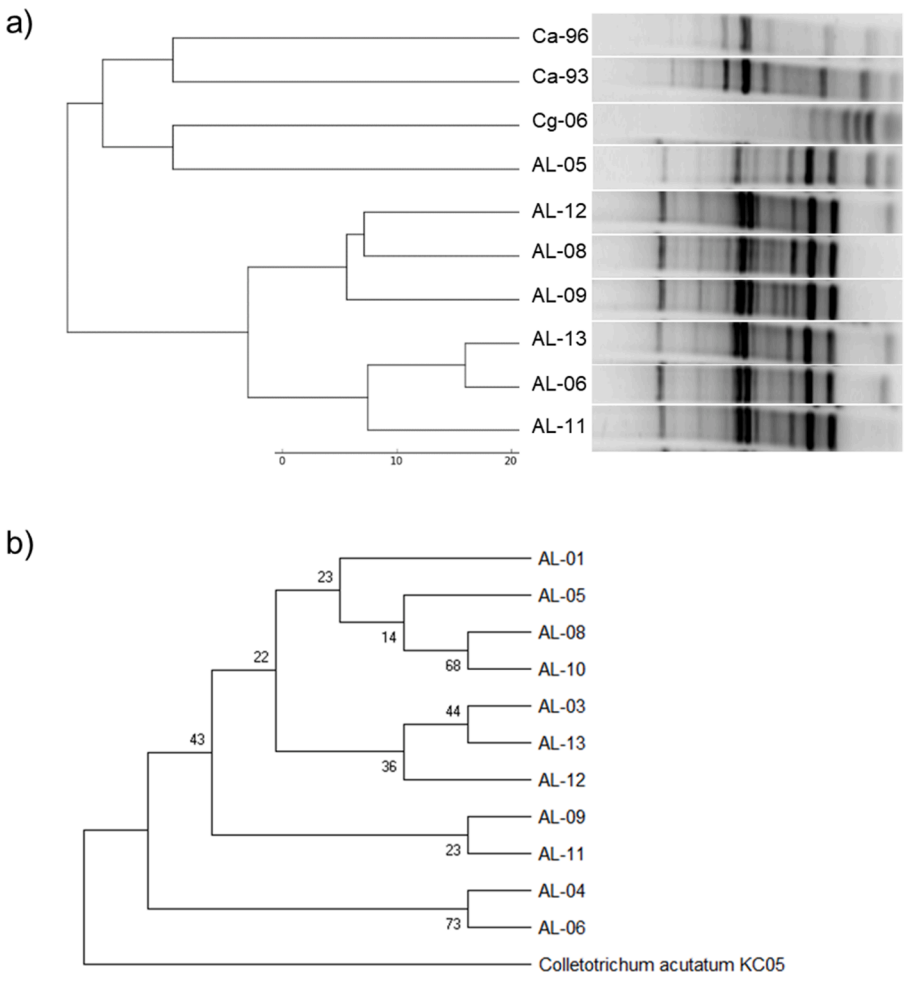

Fig. 4. Genetic variability of the selected *Colletotrichum* isolates in the study inferred by rep-PCR analysis **a**) Left; UPGMA tree from absence/presence band information, right; PCR lanes organized according to UPGMA dendrogram. *C. gloeosporioides* (AL-05, AL-06, AL-08, AL-09, AL-11, AL-12, AL-13) from Italian lemon. *C. gloeosporioides* (Cg-06) and *C. acutatum* (Ca-93 and Ca-96) from avocado. **b**) Multilocus sequence analysis (D1/D2 26S, ITS1–5.8S–ITS2 and *COX*1) in the *C. gloeosporioides* isolates from Italian lemon. Concatenated ITS1–5.8S–ITS2, D1/D2 26S and *COX*1 sequences of the reference *C. acutatum* strain KC05 were used as outgroup.

*COX*1 (Pez F/R primers) primer amplification. Finally, *Colletotrichum* genus was the most abundant in this study (32.4 %) with 12 isolates. The sequences obtained from this genus range between 470 to 625 bp, 518 to 519 bp, and 457 to 620 bp for ITS1-5.8S-ITS2, D1/D2 LSU and *COX*1 loci, respectively. Phylogenetic dendrograms closely grouped these isolates (> 0.1 of distance) in a single clade. ITS1-5.8S-ITS2 and *COX*1 loci group these isolates with *C. gloeosporioides* accessions whereas that D1/D2 LSU dendrogram grouped these with *C. acutatum* and *C. gloeosporioides* accessions (Figs. 1–3). All newick trees are provided as supplementary material.

3.2. Molecular characterization in the Colletotrichum isolates

Colletotrichum isolates of this study were grouped in all the phylogenetic dendrograms, as expected, although they come from different plant tissues, therefore, a subset of seven representative *Colletotrichum* isolates was selected for further analysis. Genetic variability accessed by rep-PCR and multilocus sequence analysis allowed us to support the genetic differences among the *Colletotrichum* isolates (Fig. 4).

Rep-PCR profile showed major differences when isolates of this study were compared with reference *Colletotrichum* strains isolated from avocado (Fig. 4A). The *Colletotrichum* isolates of this study also showed some differences in the rep-PCR profile, although these were minor, being isolate AL-05 the only one that grouped with isolate Cg-06 (Cg) of avocado. Multilocus sequence analysis, considering the concatenated ITS1-5.8S-ITS2, D1/D2 LSU and *COX*1 loci, also showed differences among the *Colletotrichum* isolates of Italian lemon. As with the rep-PCR analysis, the *C. gloeosporioides* isolates were grouped differently to *C. acutatum* (KC05) reference strain (Fig. 4B). Finally, an additional effort for species identification was also conducted on these *Colletotrichum* isolates. For this, a species-specific PCR primers analysis was carried out. CaInt2 and CgInt primers in combination with the ITS-4 primer were used to discriminate between *C. acutatum* and *C. gloeosporioides* species, respectively [17]. Fig. 5A shows the amplification with the CgInt primer in the *Colletotrichum* isolates selected. In general, all isolates exhibited a single 500 bp band according to the previously reported [17]. Similarly, reference strain Cg-06, isolated from *Persea americana*, and identified as *C. gloeosporioides* also exhibited this band. Reference strains Ca-93 and Ca-96, isolated also from *P. americana* and previously identified as *C. acutatum*, did not show any amplification. These same *Colletotrichum* isolates displayed unspecific amplifications when the CaInt2 primer was used, as did reference strain Cg-06. This primer resulted in a specific amplification (~450 bp) in the reference *C. acutatum* strains (Ca-93 and Ca-96), a size band expected according to the previously reported ([17], Fig. 5B) and confirmed these reference *Colletotrichum* strains as *C. acutatum.* According to these results, the *Colletotrichum* isolates of this study were all identified as *C. gloeosporioides.*

3.3. Phenotypic characterization of the Colletotrichum isolates

Molecular characterization showed above suggested that *C. gloeosporioides* isolates presented some genetic differences, particularly the AL-05 isolate, although all these isolates were grouped within the different loci. Therefore, a general phenotypic characterization was conducted for these isolates, which showed similar morphological traits

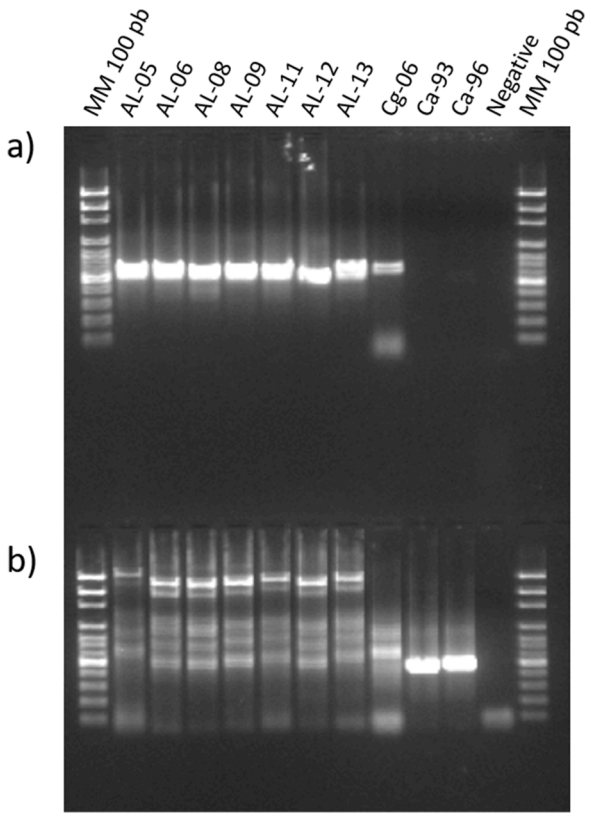

Fig. 5. Discrimination between *C. gloeosporioides* and *C. acutatum* isolates by using species-specific primers. **a**) 1.5% agarose gel showing the specific amplification of the *C. gloeosporioides* strains with the CgInt and ITS4 primers, and **b**) 1.5% agarose gel showing the specific amplification of the *C. acutatum* strains with the CaInt2 primers and ITS4.

such as round-edged orange mycelium and conidia between 4.5 μm and 14.3 μm in size (data no shown). Additionally, and considering that many of these isolates came from fruits with anthracnosis apparent symptoms, an infection analysis on Italian lemon leaves was conducted in the same subset of isolates molecularly analyzed. For this analysis, we also included representative *Fusarium* and *Penicillium* isolates, which were also abundant on these damage fruits (Table 1). In general, all the *C. gloeosporioides* isolates exhibited a basal pathogenic potential, characterized by leaf necrosis (Fig. 6). Radial growth rate, infection diameter and infection percentages are presented in Table 2. All *C. gloeosporioides* and one *Fusarium* tested strains exhibited an infective potential that ranged between 4.4 and 6.3 mm for infection diameter and showed a similar radial growth rate (~250 μm/h), showing no statistically significant differences amongst the *C. gloeosporioides* isolates. The leafs infection percentage varied between the different isolates from 26 to 60 %. Reference *Colletotrichum* strains isolated from avocado were also used for this infection analysis. In general, *Colletotrichum* isolates from Italian lemon were more infective on leaves of this citrus tree, in relation to reference strains. Only the *C. gloeosporioides* strain (Cg-06) of avocado showed infective potential (27 %) on leaves of Italian lemon. Interestingly, both *C. acutatum* strains (Ca-93 and Ca-96) were unable to infect the Italian lemon leaves. This might suggest that exists a difference between the infection mechanisms between the *C. acutatum* and *C. gloeosporioides* species. Infection assay conducted with the representative *P. italicum* (AL-38) isolate showed null infection on Italian lemon leaves, whereas *Fusarium* isolates behaved very differently. Particularly, *Fusarium* (AL-21) isolate showed a high infection percentage (67 %) on these detached Italian lemon leaves. The above indicates that other types of fungi can contribute to the disease symptoms in these citrus trees. More effort must be conducted to clarify the specie identity and the pathogenic potential of the *Fusarium* isolates in combination with the *Colletotrichum* species on the citrus trees.

According to infection assays, two *Colletotrichum* isolates (AL-05 and AL-06) with average infection profiles (~40 %) and the highly infective AL-13 isolate (60 %) were selected to conducted further experiments. Infection colonization documented by SEM analysis shows the mycelial growth for all these *Colletotrichum* isolates at 6 days on surface wound of the lemon leaves (Fig. 7). Isolates AL-05 and AL-06 were less aggressive (sparse mycelia formation, Fig. 7a and c), as was previous inferred in the infection analysis (Table 2). Isolate AL-13 was the more aggressive on leaves, presenting abundant sporulation and formation of appressoria structures (Fig. 7b and d). Finally, isolates AL-13 and AL-05 were selected to analyze their CDA activity, to assess if this correlated with their infective profile (Fig. 8). Results shows that both isolates presented CDA activity, being isolate AL-13 the one with a higher activity peaking to 1.2 U/mg protein after 120 h of incubation, according to the previous SEM and infection profile results. A different profile for CDA activity was observed for isolate AL-05, as it had its peak of activity (0.96 U/mg protein) at 24 h of culture, corresponding to the beginning of the growth phase.

4. Discussion

Fungal endophytic community identified in the current study is similar to the observed in other citrus trees [24]. For example, *Penicillium* and *Dothideomycetes* were often isolated from *Citrus reticulata* cv. Siyahoo [25], and abundant *Alternaria* isolates were recovered from *Citrus sinensis* [26]. Some of these fungi have also been related to different citrus diseases. Species of the genera *Dothiorella, Diplodia* and *Lasiodiplodia* were the most widespread and abundant in commercial citrus orchards of Algeria, being some *Lasiodiplodia* species the most aggressive on citrus shoots highlighting its importance as canker and fruit dieback agents [27]. Similarly, some genera as *Penicillium, Aspergillus, Alternaria, Lasiodiplodia, Fusarium* and *Colletotrichum* have also been associated to skin deterioration of fruits of *Citrus reticulate* [28]. The last two genera (*Fusarium* and *Colletotrichum*) were the most abundant on Italian lemon in the current study. In the current study, *Fusarium* isolates could only be identified at the genus level, even when three loci were analyzed. In this sense, several gene markers have been proposed to resolve closely-related *Fusarium* species and some recent studies point to the use of TEF-1α to support the identification of *Fusarium* species [29]. Therefore, more investigation must be conducted to resolve the species identity of these abundant isolates in the Italian lemon. According to [30] *Fusarium* reads were the most abundant in the acid Mexican lime (*Citrus aurantifolia*) as inferred by a high throughput llumina-MiSeq analysis, which also evidenced the presence of other genera such as *Alternaria, Aspergillus* and *Penicillium*. Isolates of *Colletotrichum* have been widely reported as causal agents of anthracnosis in citrus trees [31]. In the current study, many *Colletotrichum* isolates were obtained from persistent calyxes, which remain attached to the peduncle after the young fruit drop, and from damage fruits [32]. In general, these *Colletotrichum* isolates were phylogenetically grouped with accessions of *C. acutatum* and *C. gloeosporioides*. Identification of *Colletotrichum* species is critical for developing and implementing effective control strategies [9]. The occurrence and pathogenicity of *C. acutatum* and *C. gloeosporioides* species have also been previously detected in diverse citrus trees in Mexico [6–8] and in the southeastern of the United States [11]. Therefore, a further identification by using specie-specific primers was conducted. Particularly, CgInt/ITS4 primer has been useful to distingue to the specie *C. gloeosporioides* from others *Colletotrichum* species [17,33]. This analysis, combined with the phylogenetic dendrograms, allowed the identification of *Colletotrichum* isolates of this study as *C. gloeosporioides*, which supports the previous evidence that this specie is associated with the anthracnose disease in citrus crops [1]. The *Colletotrichum* isolates in this study were closely related, as inferred by using three loci markers. Therefore, the genetic and pathogenic diversity of

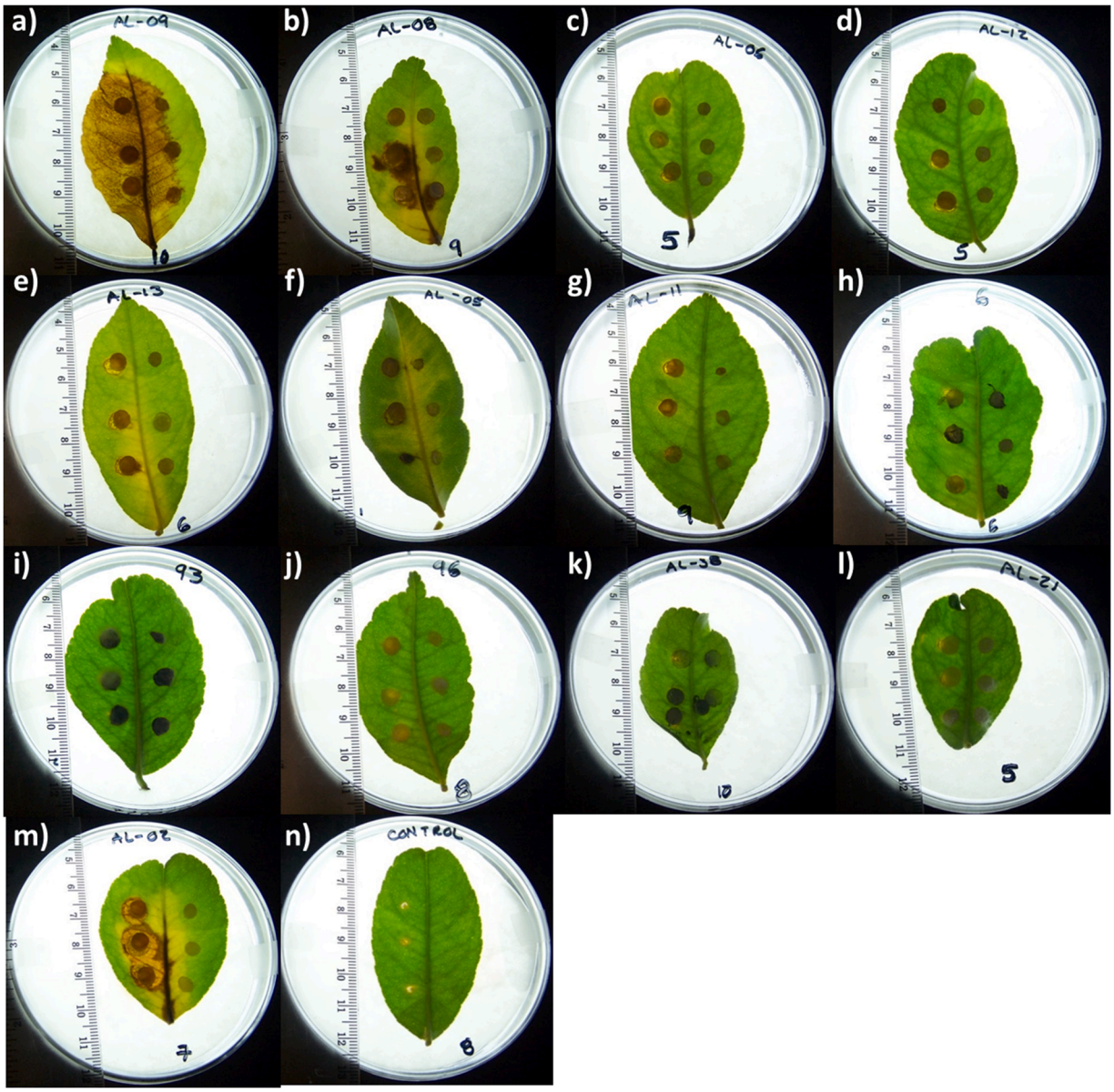

Fig. 6. Infective potential of the *C. gloeosporioides* isolates and other fungal abundant genera on leaves of the Italian lemon (*Citrus limon* var. Eureka). **a**) AL-09, **b**) AL-08, **c**) AL-06, **d**) AL-12, **e**) AL-13, **f**) AL-05, **g**) AL-11, **h**) Cg- 06, **i**) Ca-93, **j**) Ca-96, **k**) AL-38, **l**) AL-21, **m**) AL-20, and **n**) Negative control.

these isolates were evaluated by rep-PCR profiles and multilocus sequence analysis in a sub-set of our isolates, and comparing to other *Colletotrichum* strains obtained from avocado. Regarding, to rep-PCR profiles, similar results have been obtained by using different minisatellite or repeated sequences, comparing *C. gloeosporioides* isolates from almond and avocado, being the banding patterns more diverse for the isolates of this last plant [34]. Similar results, but using arbitrarily-primed PCR, on *Colletotrichum* isolates allowed a differential grouping of *C. gloeosporioides* as compared to *C. capsica* isolates in papaya [33]. Other molecular based fingerprinting techniques, such as RAPD and rDNA- RFLP have also been used on *C. gloeosporioides* isolates from cashew, differentiating them according to their geographical origin, but not being related with their pathogenicity [35].

Regarding multilocus sequence analysis, the *Colletotrichum* strain diversity has also been characterized in pomegranate fruits, where a multilocus sequence analysis allowed the separation of the isolates into two major species complexes; *C. acutatum* and *C. gloeosporioides* [11]. These authors used a different genes combination to the one used in our study, including the locus: ITS, *GAPDH, CHS*-1, and *TUB*2 [11]. In another study, the authors included seven genomic loci (ITS, *GAPDH, ACT, CAL, CHS*-1, *HIS*3 and *TUB*2) that allowed them to identify strains of three major *Colletotrichum* species complexes on orange fruits, identifying *C. acutatum* on citrus in Europe [10]. In the current study, a mitochondrial DNA (*COX*1) locus in combination with two nuclear loci (ITS1-5.8S-ITS2 and D1/D2 LSU) were utilized. In general terms, mitochondrial DNA (mtDNA) mutate more rapidly than nuclear DNA, due to a higher copy number, a greater exposure to reactive oxygen species, and a less efficient DNA repair system [36]. In addition, a previous study, using mitochondrial DNA on the evolution of *C. gloeosporioides* demonstrated that mitogenome sequence can complement prevailing nuclear markers in improving species delimitation accuracy [37]. Similarly, in the current study, the mitochondrial DNA locus (i.e. *COX*1) used as an individual dendrogram or in a multilocus analysis supported the analysis of the genetic delimitation and variability in the *C. gloeosporioides* strains.

Regarding infection potential of the isolates, all *C. gloeosporioides* isolates analyzed exhibited a basal pathogenicity, being the leafs infection percentage the trait that more varied amongst the different isolates.

Table 2
Infective potential characterization for selected fungal isolates from Italian lemon (*Citrus limon* var. Eureka) and avocado (*Persea americana*) strains

Isolate	Identity	Origen	Radial growth rate (μm/h)	Infection ϕ[a] (mm)	Infected leaves (%)
AL-09	*C. gloesporoides*	*Citrus limon*	258	5.4±0.7[b]	53
AL-08	*C. gloesporoides*	*Citrus limon*	261	6.3±0.8[b]	27
AL-06	*C. gloesporoides*	*Citrus limon*	232	5.3±0.6[b]	40
AL-12	*C. gloesporoides*	*Citrus limon*	255	5.2±0.3[b]	30
AL-13	*C. gloesporoides*	*Citrus limon*	241	4.9±0.2[b]	60
AL-05	*C. gloesporoides*	*Citrus limon*	269	4.7±0.2[b]	47
AL-11	*C. gloesporoides*	*Citrus limon*	248	4.4±0.2[b]	33
Cg-06	*C. gloesporoides*	*Persea americana*	168	4.6±0.2[b]	27
AL-20	*Fusarium* sp.	*Citrus limon*	251	5.3±0.4[b]	17
AL-21	*Fusarium* sp.	*Citrus limon*	252	4.4±0.2[b]	67
Ca-93	*C. acutatum*	*Persea americana*	130	0	0
Ca-96	*C. acutatum*	*Persea americana*	164	0	0
AL-38	*Penicillium digitatum*	*Citrus limon*	140	0	0
Negative control				0	0

[a] Mean ± standard error.
[b] No significant differences (ANOVA One-Way, P=0.005) in isolates with infective potential.

In general terms, *Colletotrichum* isolates from Italian lemon were, as expected, more infective on this plant in relation to reference strains isolated from avocado. Pathogenic profile differences among *Colletotrichum* isolates according to their host plant have been documented in several studies. For example, *C. gloeosporioides* isolates from avocado and almond were compared in artificial inoculations, showing that avocado isolates produced various lesions on avocado and almond fruits, whereas the almond isolates infected these fruits at a lower rate [34]. Other study with *C. acutatum* and *C. gloeosporioides* isolates from olive fruits, showed that these isolates also were able to infect other plants, as

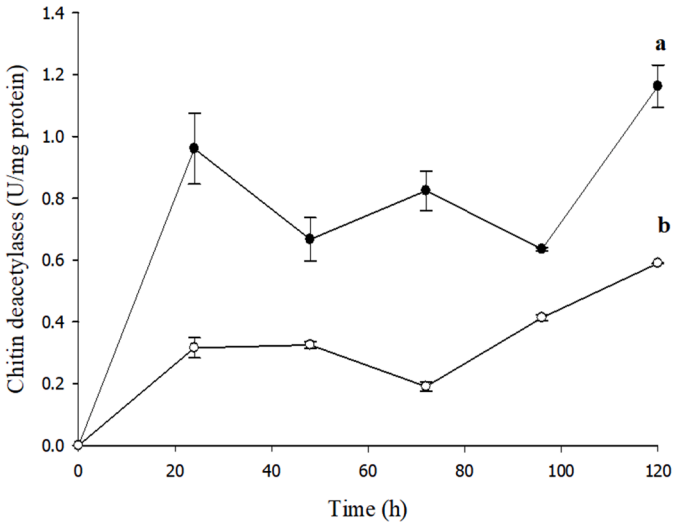

Fig. 8. Determination of the CDA of representative *C. gloeosporioides*; AL-13 (**a**) and AL-05 (**b**).

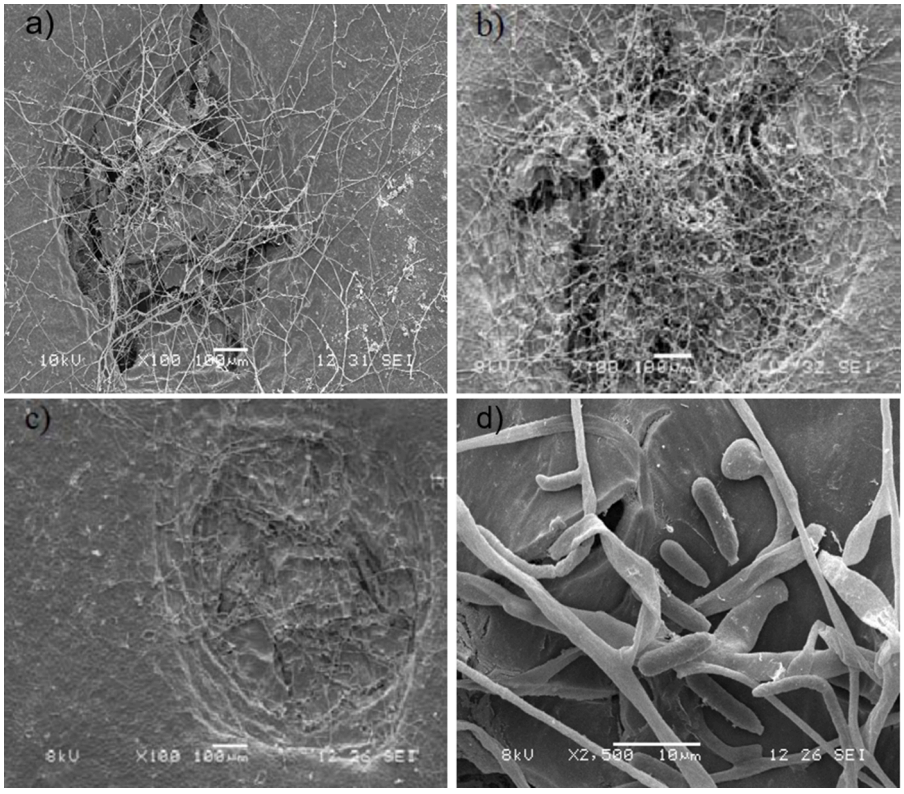

Fig. 7. SEM images of wounded leaves of *Citrus limon* var. Eureka with representative *C. gloeosporioides* isolates showing the mycelial growth at 6 days, and spores and appressoria structures. **a**) AL-06, **b**) AL-13, **c**) AL-05, and **d**) details of AL-13.

strawberry and lupin plants, revealing their cross-infection potential [38]. In this study, the infection assay was also conducted on some representative *Fusarium* isolates, which showed different infection profiles. This fungus was abundant among the isolations of this study. *Fusarium* abundance on citrus trees has been observed by using high throughput sequencing analysis in the roots of acid Mexican lime (*Citrus aurantifolia*) with 46 to 95 % of the total reads [30]. Several fungal pathogens, including *Colletotrichum* and *Fusarium* isolates were collected in infected peel section of diseased fruits in *Citrus reticulate* Blanco (kinnow). Based on physical fruit appearance, authors classified the pathogenic damage in this fruits, suggesting that *Colletotrichum* isolates attack the fruits and produce both pre-harvest and post-harvest problems, whereas other fungal genera, including *Fusarium*, produce damage as a consequence of physical stress by invading the citrus peel, either directly or through already present lesions [28]. More effort must be conducted to clarify the pathogenic potential of the *Fusarium* isolates in combination with the *Colletotrichum* species on Italian lemon.

Infection assays conducted in this study also shows differences among C. *gloeosporioides* isolates from Italian lemon or avocado with regarding to the reference *C. acutatum* strains. This last reference strain did not show any infection potential on the detached lemon leaves. Wound colonization of some selected *C. gloeosporioides* isolates, initially observed in the leaves with necrotic areas, was documented by SEM analysis. *Colletotrichum* species often exhibits a hemibiotrophic colonization strategy, which is characterized by a relatively short biotrophic phase followed of the development of necrotic lesions [39]. SEM analysis of this study also documented the presence of spores and appressoria structures on wounded leaves. Appressoria developed from germinating spores are involved with the cuticle penetration [40]. This results support the reliable identification of these *Colletotrichum* species as C. *gloeosporioides*, since it has been recently documented that *C. acutatum* did not colonize citrus leaves, with or without injury [41]. Finally, CDA activity was identified in two selected *Colletotrichum* isolates. CDA has been previously identified from crude extracts in *C. gloeosporioides* [42]. Our results indicate that the isolates analyzed exhibited their maximal CDA specific activities after 120 h of incubation. Specifically, the less aggressive AL-05 showed an activity peak at 24 h of culture, corresponding to the beginning of the growth phase, which is in agreement with the report of [42] with *C. gloeosporioides* in submerged culture. The more aggressive AL-13 isolate exhibited a gradual increase on CDA activity during the culture. The differences in behavior might be associated to the aggressiveness of the isolate regarding the host modulation on fungal growth, related to pH and production of antagonistic molecules [42]. Moreover, it is worth to note that the production with AL-05 and AL-13 was significantly higher than that found by [43] with *C. gloeosporioides* at pH 6 and 96 h of submerged culture.

5. Conclusion

In the present study, 37 fungal isolates were identified on damaged tissues of Italian lemon trees. These including to the genera *Aspergillus, Alternaria, Nigrospora, Lasiodiplodia, Dothideomycetes, Pleurostoma, Setosphaeria, Penicillium, Fusarium* and *Colletotrichum*. Particularity, these last three genera were abundant on the damage fruits, being the *Colletotrichum* isolates the more abundant with 32.4 % of isolates. In general, ITS1-5.8S-ITS2, D1/D2 LSU and *COX*1 loci were suitable to identify to specie level the most of these isolates, although genera as *Fusarium* and *Dothideomycetes* might require additional molecular analysis to support their species identity. Specifically, *Colletotrichum* isolates identified for further specific-PCR as *C. gloeosporioides*, were closely grouped in the phylogenetic dendrograms, hence their genetic variability was also assessed by rep-PCR and multilocus sequence analysis, including some reference *Colletotrichum* isolates. *Colletotrichum* isolates from Italian lemon exhibited a genetic variability and different rep-PCR profile regarding to reference strains, and isolate AL-05 was the more variable. Infection values on lemon leaves ranged between 26 to 60 %. Although, a *Fusarium* (AL-21) isolate also shows a high infection percentage. *Colletotrichum* isolates from Italian lemon were more infective on leaves of this plant, in relation to reference strains. Infection colonization in some *Colletotrichum* isolates with different infection profile was accessed by SEM analysis showing mycelial growth, spores and appressoria structures on the wounded leaves. Infective mechanism was preliminary analyzed in the two more contrasting (AL-05 and AL-13) isolates by CDA activity showing activity in both isolates, being the aggressive AL-13 isolate the one that displayed the highest CDA activity. This is the first report on *C. gloeosporioides* infecting Italian lemon leaves.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00651.

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