

Identification of *Colletotrichum* species causing anthracnose on Tahiti lime, tree tomato and mango

Identificación de las especies de *Colletotrichum* causantes de antracnosis en lima Tahití, tomate de árbol y mango

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ABSTRACT

In Colombia, citrus, tree tomato and mango crops are likely to suffer considerable losses from anthracnose caused by several *Colletotrichum* species, which were identified by the present study on infected organs of the three fruit crops, sampled in different regions of the country. Identification was based on their morphological and molecular characteristics, as well as on fungicide (benomyl and copper hydroxide) sensitivity and pathogenicity tests. The latter assessed infectivity on both the original hosting crop and the other two crops (crossed infection), by putting the fungi in contact with organs taken from the three fruit crops. Molecular identification of the *Colletotrichum* species was carried out through amplification of rDNA ITS regions by means of *C. gloeosporioides* (CgInt) and *C. acutatum* (CaInt2) specific primer PCR combining the use of ITS4 universal primer. The results indicate that *C. acutatum* is the infectious agent in Tahiti lime and tree tomato, and so is *C. gloeosporioides* in mango. Although *C. acutatum* is the infectious agent in two different fruit species, the strains proved to be specific of their original hosts.

Key words: *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, tropical fruit, diseases.

RESUMEN

En Colombia los cultivos de cítricos, tomate de árbol y mango presentan elevadas pérdidas en su producción a causa de la antracnosis ocasionada por varias especies del género *Colletotrichum*. Por esta razón, en este estudio se identificaron las especies de *Colletotrichum* de los tres frutales, provenientes de varias zonas productoras del país, mediante sus características morfológicas, patogénicas, moleculares y de sensibilidad a los fungicidas benomil e hidróxido de cobre. Para la prueba de patogenicidad se colocó el hongo en contacto con órganos desprendidos de los tres frutales, para evaluar la infectividad de cada aislamiento sobre su hospedero de origen, y sobre las otras dos especies, a fin de detectar infección cruzada. La identificación molecular de las especies de *Colletotrichum* se realizó mediante amplificación de secuencias de las regiones ITS del ADN ribosomal por medio de PCR, utilizando cebadores específicos de *C. gloeosporioides* (CgInt) y de *C. acutatum* (CaInt2), en combinación con el iniciador universal ITS4. De acuerdo con los resultados obtenidos, se determinó que *C. acutatum* es el agente causal de la antracnosis en lima Tahití y en tomate de árbol, en tanto que *C. gloeosporioides* lo es en mango. A pesar de ser el agente causal de la enfermedad en dos especies frutales diferentes, *C. acutatum* mostró especificidad hacia su hospedero de origen.

Palabras clave: *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, frutas tropicales, enfermedades.

Introduction

Colletotrichum induced anthracnose is considered an important disease in Colombian fruit crops, due to the considerable losses it determines. In Tahiti lime (*Citrus latifolia* Tanaka), it is featured by mainly attacking the flowers, determining total fruit rotting and premature dropping (Agostini *et al.*, 1992), and lowering productivity by 50% in the citrus growing regions of the country (Osorio, 2000).

In tree tomato (*Solanum betaceum* Cav.), anthracnose directly affects the fruit, producing oily stains that turn black as they grow in size. In commercial crops that receive continuous fungicide applications, losses range from 10 to 25% of the harvested fruit. When management measures are not efficient, losses can go up to 80 or even 100% of the harvest. This is, therefore, the most expensive of all the crop's issues, sometimes determining its abandonment or substitution (Tamayo, 2001).

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In mango, (*Mangifera indica* L.) anthracnose mainly attacks inflorescences and fruits (both during ripening and post harvest), occasionally affecting young leaves. In physiologically mature fruits, the disease is featured by causing black or brown superficial damage (Arauz, 2000), determining losses that go up to 35% of the harvested fruit (Páez, 1995).

Controlling *Colletotrichum* is still a deficient task, as is our knowledge about the basic aspects of its biology such as the infectious agent and its genetic variability, inoculum dispersal and host specificity (one single *Colletotrichum* species has been found causing crossed infection on several hosts).

C. gloeosporioides and *C. acutatum* are the two species that have been commonly found in anthracnose infected fruit crops. Their identification is therefore a fundamental criterion in the development of more efficient control measures, as far as it allows better knowledge of the pathogen's epidemiological behavior (Freeman *et al.*, 1998). However, due to their morphological variability, the ample range of their hosting crops, and the wide variety of their cultured isolates, they are partially difficult to identify by traditional taxonomic methods, which must then be complemented with molecular techniques (Andrade *et al.*, 2007; Whitelaw-Weckert *et al.*, 2007).

C. gloeosporioides and *C. acutatum* specific oligonucleotides have been widely used in differentiating these two species by means of PCR (Freeman *et al.*, 1998, 2001; Peres *et al.*, 2002b; Afanador *et al.*, 2003; Sanabria, 2007). The clear identification of the infectious agent allowed by this technique has sometimes led to discarding crossed infection hypotheses (i.e. those stating that just one fungal species accounts for infecting different fruit crops) (Freeman and Katan, 1997).

In this framework, the objectives of the present work were to identify, by means of molecular and conventional identification techniques, the *Colletotrichum* species that are associated to anthracnose in Tahiti lime, tree tomato and mango in the main productive regions of Colombia; and to explore the possibility of crossed infection taking place between these fruit species.

Materials and methods

Three hundred and fifty one Merck PDA grown *Colletotrichum* isolates were obtained from anthracnose affected organs (Tahiti lime flowers, and mango and tree tomato

fruits), collected in different productive provinces³ of Colombia (Tab. 1). The morphology of the colonies and fungal structures was registered after 10 days of inoculation at 23°C. The isolates were included and documented in the Colombian *Colletotrichum* Collection, with an internal code in the corresponding data base of the Laboratory of Phytopathology of Corpoica, at Centro de Investigación Tibaitata⁴. Each of the isolates was purified through monosporic culturing and then preserved in filter paper embedded in a 20% glycerol solution.

TABLE 1. Origin and number of the *Colletotrichum* spp. isolates obtained for the study.

Fruit crop	Province	No. of isolates obtained
Tahiti lime	Santander	26
	Meta	26
	Coffee zone	70
Tree tomato	Antioquia	40
	Cundinamarca	68
Mango	Tolima	45
	Cundinamarca	76

Morphological analysis

Out of the above mentioned 351 isolates, 60 (20 of each fruit crop) were randomly chosen for their morphological analysis, which, after 5 days of growing at 25°C, consisted in registering color, general aspect, edge morphology and growth mode of the cultured colonies.

The morphological analysis of the conidia was carried out on 80 spores from each isolate. They were classified in three classes, according to their morphology: 0 (conidia rounded on both ends); 1 (1 round, 1 sharp ended conidia); and 2 (both end sharpened conidia) (Sutton, 1992).

Length and width of each conidia were additionally measured. The obtained results were statistically analyzed through a Ward algorithm conglomerate analysis (respective maximum and minimum inter and intra group variation), applied with a version 9.1.3 SAS® software package.

The mycelium obtained from the PDA and micro cultures was described through observing and registering length and general features of the terminal hyphae (Barnet and Barry, 2003) on trypan blue lactophenol optic microscope (40x) preparations. A statistical analysis of such morphometrical data was carried out by means of analysis of variance.

³ In the original in spanish "departamentos", which are the administrative units of the country. Translator's note.

⁴ Tibaitata Research Center. Translator's note.

Benomyl and copper hydroxide susceptibility tests

In order to determine fungicide susceptibility of the pathogens, a hundred 0.5 cm agar discs were taken from equal number of randomly chosen isolates of each fruit crop (for a total of 300 isolates), to be grown in solid culture media containing either benomyl or copper hydroxide as fungicides. The benomyl medium was prepared with a 2 $\mu\text{g mL}^{-1}$ solution of the product dissolved in PDA. After 72 h of incubation in the darkness at 27°C, colony diameter was measured and compared to that of a non-fungicide added medium. The (copper hydroxide) selective medium contained 42 mg of metallic copper (kocide 2000: 53.8% of $\text{Cu}(\text{OH})_2$) plus 300 mg of streptomycin sulfate per liter of PDA. After incubation, and under the same conditions of the benomyl test, color and diameter of the colonies were compared to those of the control test. The results of the two tests were interpreted according to the classification chart shown in Tab. 2.

TABLE 2. Classification of the *Colletotrichum* species according to fungicide sensitivity tests (Abang *et al.*, 2003).

Selective medium	Benomyl medium	Classification
FGO (Fast growing olive):	Susceptible	<i>C. gloeosporioides</i>
SGO (Slow growing orange):	Tolerant	<i>C. acutatum</i>
SGG (Slow growing gray):	Susceptible	<i>C. gloeosporioides</i>
FGS (Fast growing salmon):	Susceptible	<i>C. gloeosporioides</i>

Pathogenicity tests

Pathogenicity tests were carried out by inoculating randomly chosen isolates on: 1) organs of the original hosting crop (20, 31 and 30 respective Tahiti lime, tree tomato and mango colonies; and 2) organs of the other two crops (9, 18 and 18 respective Tahiti lime, tree tomato and mango colonies), with the aim of determining crossed infection.

The lime flowers were taken from active plantations, packed in air containing plastic bags, and transported in a styrofoam cool box. Once in the laboratory, 320 petals were chosen to be disinfected in a 0.5% sodium hypochlorite (NaOCl) solution for 45 s, and then washed with water and aspersed with a 70% alcohol solution.

After disinfection, they were inoculated with 0.5 mL of a water suspension containing 1×10^6 isolate spores per mL. For the original host infection tests, a completely random experimental design was applied to measure anthracnose symptom manifestation. The necessary conditions for the development of the disease were provided by placing the petals into a moist chamber (90% RH) at $12 \pm 2^\circ\text{C}$ for 12 h. Regarding tree tomato, 128 green big fruits were taken from active plantations of the 'Common red' variety. After hav-

ing been packed in individual paper bags and transported to the laboratory in a plastic styrofoam cool box, they were disinfected in a 2.6% NaOCl solution for 30 s, and washed and aspersed with a 70% alcohol solution. Agar discs taken from the *Colletotrichum* spp. isolates (1×10^6 spores/mL) were then placed at two points on each fruit. Again, a completely randomized design with four repetitions per isolate was applied to measure anthracnose symptom manifestation. The fruits were incubated in moist chambers (90% RH) at $20 \pm 2^\circ\text{C}$ for 30 d.

Finally, 124 mango fruits at first maturation stages were obtained from active plantations of 'hilacha' variety. They were harvested and packed in individual paper bags, which were in turn placed into plastic ones, and then transported to the laboratory in a styrofoam cool box. The disinfection protocol was the same as for mango, with the only difference that NaOCl immersion took one minute, after which the fruits were washed with sterile distilled water and aspersed with the same alcohol solution. Then, except for the spore concentration of the agar discs, which was 1×10^5 spores/mL, the inoculation process was also the same, and so was the experimental design. Moist chamber incubation took 14 d at $25 \pm 2^\circ\text{C}$, during which anthracnose symptoms were daily registered.

For the crossed infection tests, fruits and petals were collected, transported and disinfected in the same mode described above, but remaining in the moist chambers for 72 h (lime), 96 h (tree tomato), and 28 d (mango). The data from both tests were treated with a SAS® completely randomized design analysis applying the chi-square dependency test ($P \leq 0.05$) for the hypotheses that *Colletotrichum* spp. isolates are capable of reproducing anthracnose symptoms in: 1) their original hosting crops, and 2) the other two studied crops.

Molecular determination of the species

Identification of the species of the pathogen was carried out on 293 of its monosporic isolates, which had been grown in V8® liquid medium at 28°C and 130 rpm for 8 d. Mycelium from such cultures was macerated for DNA extraction and purification, which followed the method proposed by Kelemu *et al.* (1997; 1999). The solution was adjusted to a final concentration of 20 $\text{ng } \mu\text{L}^{-1}$ for amplification of rDNA ITS region sequences by means of PCR with species specific primers. Using *C. gloeosporioides* CgInt (5'-GGCCTCCCGCTCCGGGCGG-3') and *C. acutatum* CaInt2 (5'-GGGGAAGCCTCTCGCGG-3') specific primers, and in combination with ITS4 universal primer (Freeman *et al.*, 2000; Afanador *et al.*, 2003), such procedure allowed identifying the species.

In determining *C. acutatum*, amplification took place in a final volume of 20 μ L containing Promega® Taq Polymerase buffer, 1.5 mM of MgCl₂, 200 μ M of each dinucleotide, 0.3 μ M of each primer, 1 unit of Taq Polymerase enzyme, and 40 ng of DNA. The amplification profile consisted of an initial cycle of 5 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C, and a final extension of 7 min at 72°C.

ITS4 and *Cgint* primers were used in the identification of *C. gloeosporioides*. Amplification started with an initial denaturation at 95°C for 5 min, followed by 40 amplification cycles (30 s denaturation at 95°C, 65°C annealing for 30 s, and a 1 min extension at 72°C), plus a final extension cycle of 7 min at 72°C.

In estimating size of the amplified products a kb DNA Ladder marker with molecular weight of 1, ranging between 10,000 and 250 bp was used. Positive controls consisted in DNA taken from the following CIAT isolates: TOM 021 (*C. acutatum*) (Afanador *et al.* 2003), and 1613 (*C. gloeosporioides*) (Kelemu *et al.*, 1997; Kelemu *et al.*, 1999). 120-COL (*C. lindemuthianum*) (Tamayo *et al.*, 1995) was used as negative control.

Results and discussion

Morphology of colonies and conidia

Most of the isolates coming from citrus crops formed grey and salmon cottony colonies. Tree tomato isolates

formed orange colonies with a rather flattened mycelium, and grey or white bottom color. Although mango isolate colonies showed wider variety, abundant green, white, grey or orange cottony mycelium was dominant, sometimes showing luxuriant orange conidial masses with grey or white bottom color (Fig. 1).

From the results, it can be said that lime and tree tomato isolate colonies showed typical *Colletotrichum* colors, coinciding with Simmonds' (1965) and Von Arx's (1957) descriptions of *C. acutatum*. In turn, mango isolate colonies corresponded to descriptions of *C. gloeosporioides* published by Sutton (1980) and Baxter (1983). However, the morphology of *Colletotrichum* colonies varies within and among groups, depending on culture medium, substrate and temperature, among other factors (Contreras, 2006).

The analysis of conidial morphology showed that one single colony may contain two different types of spores. Some of the Tahiti lime isolates gave rise to classes 0 and 1 conidia. Isolates coming from tree tomato showed several types (0, 1 and 2; or 0 and 1). On the other hand, most of the 20 mango isolate colonies grew class 1 conidia, and a few of them exhibited classes 0 and 1 (Fig. 2).

Mango isolates stood out by exhibiting bigger average conidia (21.5 μ m), whereas tree tomato and lime ones proved to be smaller. Conglomerate data analysis of these features showed great variability.

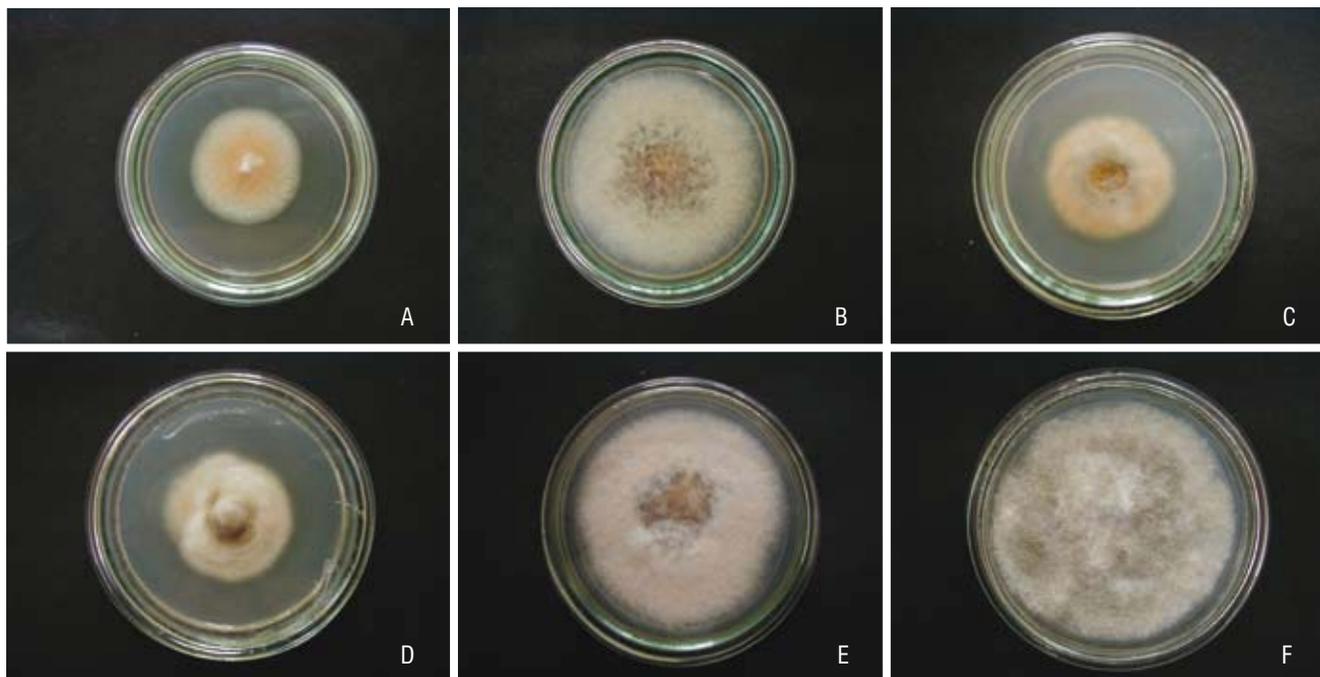


FIGURE 1. Characteristics of the *Colletotrichum* colonies in PDA. A and B: Tahiti lime isolates; C and D: tree tomato isolates; E and F: mango isolates.

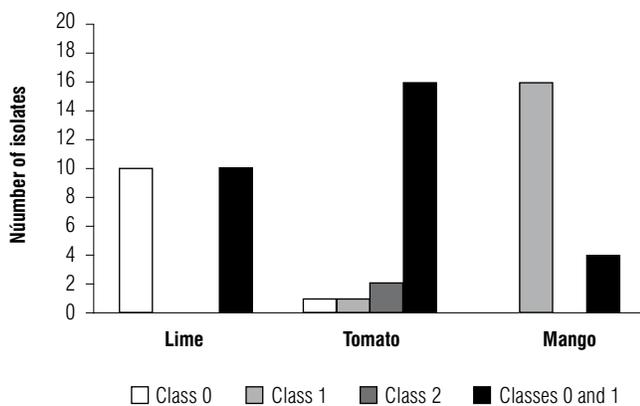


FIGURE 2. Tahiti lime, tree tomato and mango isolates, classified according to conidial morphology.

PDA grown hyphae were observed to be hyaline, with defined septa, sometimes exhibiting cytoplasmic contents, and sometimes intercellular spaces. Microscopic characterization of the micro-cultured hyphae of the different fruit crop isolates showed no significant differences in the Anova test ($P \leq 0.05$). This is, therefore, a character of little use in differentiating these species.

Fungicide susceptibility

The results obtained through the benomyl and copper hydroxide tests allowed identifying the 100 mango isolates as *C. gloeosporioides* (Tab. 3), due to their high growth rate, grey and salmon color in the selective medium, and susceptibility to benomyl (Agostini and Timmer, 1992). In turn, the 100 tree tomato isolates were identified as *C. acutatum* for presenting tolerance to benomyl, low growth rate after 72 h of incubation, and orange color in the selective medium (Tab. 3). Eighty seven of the Tahiti lime isolates were found to be benomyl tolerant, and therefore classified as SGO (*C. acutatum*). The resting 13 ones were identified as *C. gloeosporioides* due to their benomyl susceptibility, high growth rate, and orange or grey color in the selective medium (Tab. 3).

TABLE 3. Classification and identification of the *Colletotrichum* isolates according to the fungicide sensitivity tests.

Original host	Benomyl	Classification in selective medium	Species
Tahiti lime	Tolerant	SGO	<i>C. acutatum</i>
	Susceptible	FGO	<i>C. gloeosporioides</i>
Tree tomato	Tolerant	SGO	<i>C. acutatum</i>
	Susceptible	FGG	
Mango	Susceptible	SGG	<i>C. gloeosporioides</i>
	Susceptible	FGS	

It can be seen how the mango isolates have a different growth pattern from the Tahiti lime and tree tomato ones, which were similar in either medium. This indicates the possibility that the population of *C. gloeosporioides* is inherently variable (Dodd *et al.*, 1991; Estrada *et al.*, 2000; Afanador *et al.*, 2003).

These results can also be used to infer isolate virulence. Abang (2003) found category SGG fungi to be the most aggressive ones, due to the degrading enzymes they possess, which are capable of destroying the cell membranes of young plant tissues. Notwithstanding, other studies point at *C. acutatum* as the most infectious species, because of its resistance to several fungicides (Peres *et al.*, 2002a).

Pathogenicity

Out of the 20 evaluated Tahiti lime isolates, those identified as *C. acutatum* provoked anthracnose symptoms in 188 out of 320 inoculated Tahiti lime petals (58.8%) 72 h after inoculation (Fig. 3). The pathogen was recovered and re-isolated from the infected tissue, thus proving that this species is the anthracnose infectious agent in this crop.

Regarding tree tomato, the first symptoms of the disease caused by this crop's isolates were seen on the 13th day after inoculation; on day 30th, the infection had reached medium incidence (Fig. 4). Again, the original pathogen was re-isolated from the infected fruits.

In the case of mango, out of the 30 isolates of the pathogen tested for original host infection, 19 were able to produce the first symptoms on 22 fruits on the fourth day after inoculation. By day 14th, the incidence of the disease had reached 100% of the fruit; on days 4th and 5th, the pathogen was grown again from infected fruit tissue samples in PDA medium. These results allowed determining that the evaluated isolates are highly infectious ($P \leq 0.05$).

Finally, the crossed infection tests gave negative results, as no symptoms of the disease were detected in organs inoculated with isolates taken from other crops (Tab. 4). Thus, the pathogens can be said to be host specific, which is confirmed by studies on the genetic diversity of the same collection analyzed in this work (Osorio *et al.*, unpublished results).

Molecular determination of the species

Out of the 293 isolates amplified in the present study, 182 were identified as *C. acutatum*, and 111 as *C. gloeosporioides* (Fig. 6). All the tree tomato isolates were identified as *C. acutatum*, and all the mango ones as *C. gloeosporioides*. Out



FIGURE 3. Pathogenicity test on Tahiti lime petals. A: 1 h after inoculation; B: 72 h after inoculation; C: colony of the pathogen re-isolated from the petals.



FIGURE 4. Fruits of tree tomato inoculated with *Colletotrichum* spp. isolates. A and B: fruits with anthracnose symptoms. C: control fruit inoculated with sterile agar discs.



FIGURE 5. Anthracnose infection process in fruits of mango. A: healthy fruits; B: initially infected fruits; C: fruits with 80 to 90% anthracnose incidence.

of the 93 Tahiti lime isolates, 83 corresponded to *C. acutatum*, and 10 to *C. gloeosporioides*. These results confirm those obtained with the morphological analysis and fungicide sensitivity tests. Similarly, Afanador *et al.* (2003) and Timmer and Brown (2000) characterized *Colletotrichum* spp. isolates obtained from different fruit crops, identifying *C. acutatum* as the anthracnose infectious agent in lime and tree tomato. Nevertheless, anthracnose affected plants of the latter crop infected by *C. gloeosporioides* have also been reported (Aranzazu and Rondón, 2001). Both species can be found in citrus, but only *C. acutatum* is responsible for premature fruit drop. The other species is just an associated saprophyte (Timmer and Brown, 2000).

Conclusions

1. The morphological, molecular, pathogenicity and fungicide sensitivity analyses conducted on the pathogens that determine anthracnose in the three studied crops allowed identifying them as *Colletotrichum* spp.
2. In Colombia, anthracnose in Tahiti lime and tree tomato is caused by *C. acutatum*, while in mango it is determined by *C. gloeosporioides*. The three infectious agents proved to be host specific, as far as no crossed infection among crops was seen.
3. Given that the therapeutic management tools and strategies currently in use for controlling the pathogens in

TABLE 4. Incidence of anthracnose symptoms in organs of the three studied fruit crops inoculated with isolates obtained from their original hosting crops, and from the other two crops.

Evaluated isolates [*]	Isolate origin	Anthracnose incidence (%)**		
		Tree tomato fruits	Mango fruits	Tahiti lime petals
C-189	Lime	31.0	0,0	100.0
C-595	Lime	0.0	0.0	0.0
C-774	Lime	0.0	0.0	100.0
C-799	Tree tomato	6.2	0.0	95.0
C-832	Tree tomato	31.0	0.0	100.0
C-869	Tree tomato	43.7	0.0	95.0
C-917	Mango	25.0	100.0	95.0
C-957	Mango	6.2	100.0	100.0
C-1011	Mango	0.0	100.0	95.0
Control		6.2	0.0	0.0

* National *Colletotrichum* collection code of the isolate.

** Symptom presence calculated as: (Number of infected organs / number of inoculated organs) x 100.

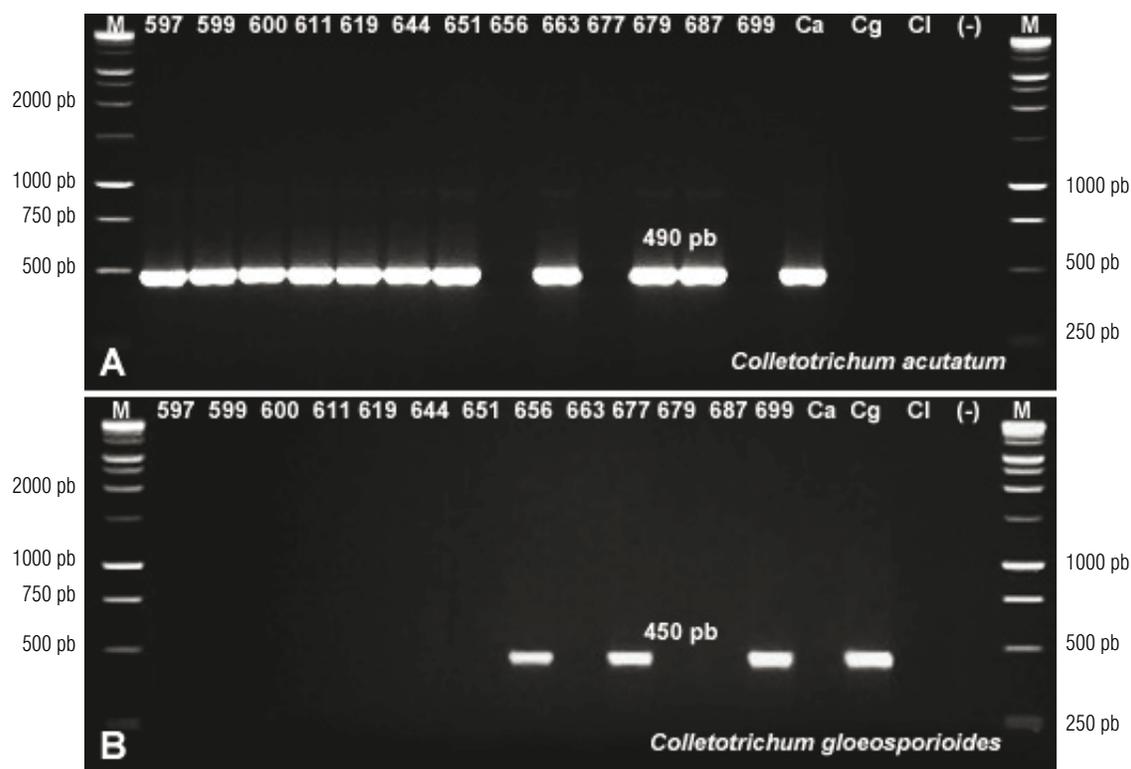


FIGURE 6. Identification of Tahiti lime *Colletotrichum* spp. isolates with species specific primers. A: *C. acutatum* (CaInt2/ITS4); B: *C. gloeosporioides* (CgInt/ITS4). (Ca): *C. acutatum* positive control; (Cg): *C. gloeosporioides* positive control; (Cl): *C. lindemuthianum* negative control; (-): negative control.

Colombia have not been sufficiently effective in reducing the impact of the disease, its affecting productivity or fungicide application levels, the newly acquired knowledge contributed by the present research study allows developing alternative disease management protocols. This is due to the fact that the two studied *Colletotrichum* species present different reactions to

control measures, among which we can count fungicides. The alternative scheme would focus on preventive control strategies, which, systematically applied to the plantations, would be in condition to reduce the incidence of the disease to more manageable levels, with less intense use of agrochemicals, and lower costs for the producer.

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