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Morphological characterization and molecular identification of *Colletotrichum* spp. associated with *Schlumbergera gaertneri*

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ABSTRACT: The Whitsun cactus (*Schlumbergera gaertneri*), native to Brazil, is used as ornamental plant worldwide. Symptoms of anthracnose were identified in plants cultivated in southern Brazil, causing the loss of ornamental value of the affected plants. Thus, the objective of this work was to isolate, characterize and identify the causal agent of anthracnose in *S. gaertneri*, through morphological and molecular characters. For the pathogenicity test, seedlings were injured with a toothpick and inoculated with a conidia suspension, while the morphological characterization of the causal agent was performed in Mathur culture medium. The Internal Transcribed Spacer (ITS), Actin, β-tubulin, Glyceraldehyde-3-phosphate dehydrogenase and Calmodulin regions were used to molecularly identify the species. The isolates obtained were pathogenic to *S. gaertneri*, presenting morphological characteristics compatible with species of the genus *Colletotrichum*. By sequencing the genomic regions, Glyceraldehyde-3-phosphate dehydrogenase, Calmodulin and β-tubulin we identified *Colletotrichum karsti* and *C. siamensis* as causal agents of anthracnose in *S. gaertneri*.

Key words: anthracnose; calmodulin; Whitsun cactus

Caracterização morfológica e identificação molecular de *Colletotrichum* spp. associado a *Schlumbergera gaertneri*

RESUMO: O cacto de Whitsun (*Schlumbergera gaertneri*), planta nativa do Brasil, é utilizada como ornamental no mundo inteiro. Sintomas de antracnose foram identificados em plantas cultivadas no sul do Brasil, provocando a perda do valor ornamental dos indivíduos acometidos. Desse modo, o objetivo do trabalho foi isolar, caracterizar e identificar o agente causal da antracnose em *S. gaertneri*, por meio de caracteres morfológicos e moleculares. Para o teste de patogenicidade, mudas foram feridas com palito de dente e inoculadas com uma suspensão de conídios, enquanto que a caracterização morfológica do agente causal foi realizada em meio de cultura Mathur. As regiões do Espaçador Interno Transcrito (ITS), Actina, β-tubulina, Gliceraldeído-3-fosfato desidrogenase e Calmodulina foram utilizadas para identificar molecularmente as espécies. Os isolados obtidos foram patogênicos à *S. gaertneri*, apresentando características morfológicas compatíveis com espécies do gênero *Colletotrichum*. Ao sequenciar as regiões genômicas, Gliceraldeído-3-fosfato desidrogenase, Calmodulina e β-tubulina identificamos *Colletotrichum karsti* e *C. siamensis* como agentes causais da antracnose em *S. gaertneri*.

Palavras-chave: antracnose; calmodulina; cacto de Whitsun

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Introduction

Schlumbergera spp. comprises a genus of epiphytic cacti native to the Atlantic Forest in Brazil (Soller et al., 2014). The species of the genus, despite not being so well studied, have great ornamental and economic value, being widely cultivated worldwide for the production of flowers, present between autumn and late spring. Within the genus, the species *Schlumbergera gaertneri* (Regel) Britton & Rose (syn. *Hatiora gaertneri*), popularly known as the Whitsun cactus, is one of the most widespread, being common in homes in Southern Brazil.

Colletotrichum spp. the causal agents of anthracnose, comprise a group of fungal pathogens known to cause severe losses in many crops. Damm et al. (2012) point out that species of the genus are among the most scientifically and also economically important, due to their wide worldwide distribution and occurrence on a wide range of hosts.

Brown lesions, strongly depressed in the center, which after progression, in some cases presented the formation of orange-colored masses, were found in individuals of *S. gaertneri* grown in Southern Brazil. The disease also affected the aesthetic aspect and the following flowering, as the affected plants emitted fewer flowers. The visual symptoms were compatible with those observed for species of the genus *Colletotrichum*, although to date, there was no scientific evidence of such a pathosystem. Given the above, the objective of this study was to isolate, characterize, and identify the causal agent of anthracnose in *S. gaertneri* by means of morphological and molecular characters.

Materials and Methods

Material collection and experimental site

In March 2021, arthicles (segment of the articulated stem system) of *S. gaertneri* were observed with anthracnose symptoms, showing irregular and strongly depressed dark brown spots (6 to 17 mm in diameter), on individuals grown in the municipality of Santa Maria, Rio Grande do Sul, Brazil (29° 41' 49" S, 53° 48' 49" W). The disease affected approximately 15% of the arthicles and resulted in some of these falling off, which significantly compromised the emission of floral buds in the following flowering period. Symptomatic arthicles were taken to the Phytopathology Laboratory "Dr. Elocy Minussi", at the Department of Plant Protection of the Universidade Federal de Santa Maria, where the analyses were performed.

Obtaining fungal isolates

The arthicles were disinfected for 1 minute in 70% alcohol solution, followed by 1 minute in 1% sodium hypochlorite solution, and then washed in sterile water twice. Subsequently, they were placed in a humid chamber and kept at 25 ± 2 °C and a 12 hours photoperiod for four days. Isolation was performed by transferring fungal structures grown on necrotic areas to 70 mm diameter Petri plates containing synthetic Merck KGaA® Batata-Dextrose-Ágar (BDA) culture medium. After filling the surface of the culture medium (seven days, 25 ± 2 °C, 12 hours photoperiod), the isolates were purified using the monospore culture technique as described by Fernandez (1993), where a single spore was used to obtain a pure fungal culture. At five days of growth (25 ± 2 °C, 12 hours photoperiod) after purification, two distinct colonies (H3 and I2), but similar to the genus *Colletotrichum*, were selected, and part of the material (mycelium + spores) was removed, transferred to tubes containing BDA culture medium and stored in the laboratory fungus collection for further use.

Pathogenicity test

The isolates stored in the tubes were reactivated by removing fragments of culture medium containing fungal structures and repotted onto Petri plates containing BDA. After growth (fifteen days, 25 ± 2 °C, 12 hours photoperiod), 10 mL of sterile distilled water was added to each plate. The surface of the culture medium was scraped off with the aid of a Drigalski loop and the suspension was filtered through a double layer of gauze into a beaker. All processes were performed in a laminar flow chamber and the materials used were previously sterilized in an autoclave (25 minutes, 121 °C, 1 atm).

Next, to keep the spores in suspension, two drops of Tween® were added and the solution was placed on a magnetic stirrer to homogenize the sample. The conidia concentration was counted in a Neubauer chamber and the suspension adjusted to 2×10^6 conidia mL^{-1}.

The inoculation methodology followed the study of Zhao et al. (2018), with adaptations, where healthy *S. gaertneri* seedlings were taken to the Phytopathology Laboratory and deposited in transparent boxes.

Using a sterile toothpick, eight wounds (approximately 1 mm deep × 1 mm in diameter) were made in each plant and 100 µL of the previously adjusted conidia suspension was divided and deposited over the wounds. In the control treatment, the conidia suspension was replaced with sterile distilled water. Six replicates were used for each treatment, each consisting of one plant. A portion of absorbent cotton dampened with water was placed in the boxes to maintain humidity, and then the boxes were closed. Cotton was kept moist throughout the pathogenicity test. The boxes were kept in an air-conditioned room (25 ± 2 °C, 12 hours photoperiod). At the end of the test, the incidence of the disease was evaluated for each plant for the presence of symptoms in each of the locations where the suspension was deposited. If, in at least one of the eight locations there was the manifestation of symptoms, the incidence was considered positive for the plant.

Morphological characterization

To morphologically characterize the isolates, 7 mm diameter discs of culture medium containing fungal structures were transferred to the center of Petri plates (90 mm) filled with Mathur culture medium (Mathur et al., 1950). The plates were kept in the growth chamber (25 ± 2 °C, 12 hours

photoperiod) until the entire surface of the culture medium was filled in at least one repetition. The colonies were measured daily with a digital caliper, obtaining the average growth of two dimensions, for each repetition. Six replicates were used for each isolate, each consisting of one plate. At the end of the test, the final colony diameter and the mycelial growth velocity index (MGVI) of the isolates were obtained, the latter calculated using Equation 1, proposed by Maguire (1962), and adapted by Oliveira (1991):

$$MGVI = \sum\left(\frac{D - Da}{N}\right) \quad (1)$$

where: D - current average colony diameter; Da - average colony diameter on the previous day; and, N - number of days after inoculation.

The sporulation of each isolate was determined as described in the pathogenicity test by adding to each pure culture plate, 10 mL of sterile water, followed by scraping, filtering in double layer of gauze, adding Tween® and determining the number of conidia by Neubauer chamber. A 100 µL aliquot of the suspension was used to prepare and characterize 50 conidia units of each isolate. Each of the conidia was also evaluated for format, as proposed by Tozze Júnior (2008) (Table 1). These, were randomly chosen and evaluated using an OSM Olympus® eyepiece, attached to the microscope, at 40×.

The methodology used by Silva (2016) and adapted by Savian et al. (2020), was used to analyze the form of fifty apressoria produced by each isolate. The production of fruiting bodies was also evaluated according to Savian et al. (2020). Figure 1 illustrates the evaluation of the format of each apressoria (Silva, 2016).

Table 1. Morphology of conidia of *Colletotrichum* spp. evaluated.

Numeric code	Description
1	Straight, spindle-shaped, with tapered apices
2	Straight, oblong, with rounded apices
3	Straight, clavate, tapered at one end and round at the other
4	Straight, with constriction
5	Falcate, with tapered apices

Adapted from Tozze Júnior (2008).

Figure 1. Formats of the apressoria: 1. Lobbed; 2. Slightly lobbed; 3. Rounded.

Molecular identification

DNA from the isolates was extracted using the CTAB method (Doyle, 1990), and then subjected to Polymerase Chain Reaction (PCR) to amplify and sequence the Internal Transcribed Spacer (ITS) regions, Actin (ACT), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Calmodulin (CAL) and β-tubulin (βTUB), using primers SR6R/LR1, ACT512F/ACT783R, GDF/GDR, CL1C/CL2C, respectively (Weir et al., 2012), and TUB2F/TUB4F (Copes & Ojiambo, 2021). The analysis of the sequenced fragments was performed by the BioEdit software, where the sequences of nitrogenous bases were compared to the GenBank deposits, and those with higher coverage and similarity values were selected and aligned with the sequences obtained in this study. For this process, we used the software ClustalW algorithm.

Phylogenetic analysis was conducted with the "Neighbor-joining" method (1,000 replicates), by MEGA software (version 7). The nucleotide sequence similarity between the isolates was calculated with the Basic Local Alignment Search Tool - BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The base pair sequences obtained were deposited in GenBank, while samples of the lyophilized isolates were deposited in the SMDB Herbarium (Herbarium of the Biology Department of the Universidade Federal de Santa Maria).

Statistical analysis

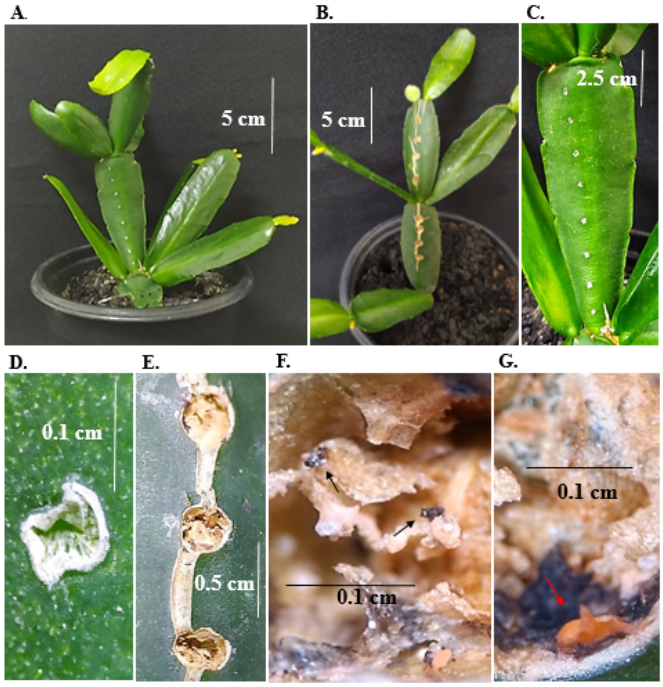
To compare the size of the lesions caused by the isolates, initially the arithmetic mean of the lesions on each plant was taken, and each plant was considered a repetition (n = 6). In the control treatment, the average diameter of the wounds caused by the toothpick was used. Subsequently, the treatments (isolates H3, I2, and control) were compared using Dunnet statistical test, with $p < 0.05$, using the Sisvar software (Ferreira, 2014).

Results and Discussion

Pathogenicity test

Both isolates were shown to be pathogenic for *S. gaertneri* (Figure 2). Lesions on the arthicles appeared around 15 days after inoculation (DAI), with formation of a small depressed area over the site of spore suspension deposition in some inoculated plants. At the end of the test, which occurred at 45 DAI, all plants inoculated with spore suspension showed symptoms, while plants inoculated with sterile distilled water did not. The symptoms presented at the end of the test, for both isolates, consisted of strongly depressed, brown spots, some with the presence of dark spots that resembled achevuli. Koch postulates were closed with the re-isolation of the pathogen from infected tissues, thus constituting the first report of anthracnose for this plant in the world.

In the family Cactaceae, in which *S. gaertneri* is included, species of the *C. boninense* and *C. gloeosporioides* complexes are most frequently reported (Conforto et al., 2017; Zhao et al., 2018; Abirami et al., 2019; Nascimento et al., 2019; Reyes-Fornet et al., 2019), causing anthracnose, although species from distinct complexes are also pathogenic (Custodio et al., 2021). In addition, these reports include the description of symptoms, color, and form of the lesion varying depending on the host and identity of the pathogen.



Where: A, B, and C: general appearance of plants at 45 days after inoculation (DAI). D and E: wound site 45 DAI with water and spore suspension, respectively. F. presence of acerbicles at the pathogen inoculation site. G: formation of a conidial mass (conidiomata) on a plant inoculated with the pathogen. Bars indicate the scale.

Figure 2. Appearance of *Schlumbergera gaertneri* seedlings during pathogenicity test with conidial suspension of *Colletotrichum* spp.

The incidence of the disease was positive for all plants inoculated with the spore suspensions of both isolates (Table 2). In all areas where the suspensions were deposited, similar patches of varying size were created, and thus the severity was considered to be 100%, with each lesion equivalent to 12.50% severity. In the control treatment there was no formation of these brown lesions, with only the injury site caused by the toothpick being apparent.

In our study, the isolate I2 proved to be more aggressive than the isolate H3, since the size of the lesion caused by the former was statistically higher, at 4.4 mm (Table 3). The isolate H3, despite presenting a smaller lesion, of 3.5 mm, also differed statistically from the control treatment, whose wound remained at approximately 1 mm, demonstrating the action of the pathogens on the tissue of inoculated plants.

In evaluating the diversity and pathogenicity of *Colletotrichum* species obtained from strawberry (*Fragaria x ananassa*), among them *C. siamense* and *C. karsti*, Chung et al. (2020) observed initial symptoms about two days after inoculation of spore suspension on wounded leaves. In this study, the authors also found that the size of the lesions caused by *C. siamense* was statistically higher than those obtained by inoculation with *C. karsti*, corroborating our study, where the isolate I2 (later identified as *C. siamense*) caused the largest lesions on the tissue, previously injured, which may indicate a greater aggressiveness of this species when compared to *C. karsti*.

Table 2. Incidence, number of lesions per plant, and disease severity in *Schlumbergera gaertneri* seedlings inoculated with *Colletotrichum* spp.

Isolated	Pathogenicity	Average number of dark lesions per plant	Disease severity (%)
H3	+	8	100
I2	+	8	100
Control	-	0	0

Where: (+):pathogenic; (-): non pathogenic; 1 lesion corresponds to 12.50% severity.

Table 3. Average lesion size caused by *Colletotrichum* spp. on *Schlumbergera gaertneri* seedlings at 45 days after inoculation.

Isolated	Lesion size (mm)
H3	3.5 b*
I2	4.4 a
Control**	1.0 c
CV (%)	19.4

* Means followed by the same letter in the column do not differ statistically by the Dunett test at 5% probability of error. ** Without inoculation of the pathogen, only with wounding. CV: Coefficient of variation.

Morphological characterization

The isolates showed variation in the characteristics analyzed on Mathur culture medium. Regarding the variable final colony diameter (FCD), the isolate I2 showed the maximum value, that is, 90.0 mm at 8 DAI, while for the isolate H3 the value was 69.8 mm (Table 4) in the same period of time, demonstrating slower mycelial growth. As for the MGVI variable, isolate I2 presented 51.4% higher index than isolate H3 (25.8 × 17.1). The sporulation presented by the isolates was similar, with 1.6×10^6 for I2 and 1.5×10^6 for isolate H3.

Chung et al. (2020), also found differences between the growth of *Colletotrichum* isolates obtained from the same host. In the study developed by the authors, four species of the genus were found to be responsible for causing anthracnose on strawberry (*Fragaria × ananassa*) in Taiwan, reinforcing the initial hypothesis of our study that more than one pathogen could be the causal agent of the disease, even though they were collected from the same host as *S. gaertneri*. In contrast, Soares et al. (2021), found differences between the mycelial growth of *C. siamense* isolates obtained from avocado fruits with anthracnose symptoms in Southern Brazil, thus indicating that growth variation cannot be exclusively taken into account for the distinction of species of the genus.

Regarding the general appearance of the colonies, it was possible to see variation among the isolates. The isolate H3 showed low aerial mycelium with an irregular colony border, while for isolate I2, the mycelium, although low, was

Table 4. Colony diameter, mean mycelial growth velocity index (MGVI) and sporulation of *Colletotrichum* spp. on Mathur culture medium.

Isolated	FCD (mm)[1]	MGVI	Sporulation ($\times 10^6$)[3]
H3	69.8	17.1[2]	1.5
I2	90.0	25.8[2]	1.6

[1] Final colony diameter at eight days of growth. [2] MGVI: considering eight days of growth. [3] Sporulation at 15 days of incubation, in conidia mL^{-1}.

interposed by tufts of protruding hyphae (Figure 3). There was abundant conidia mass production (conidiomata) for both isolates and neither showed the formation of arrows. The reverse side of the plates for isolate H3 had orange coloration, while for I2 the coloration was more opaque.

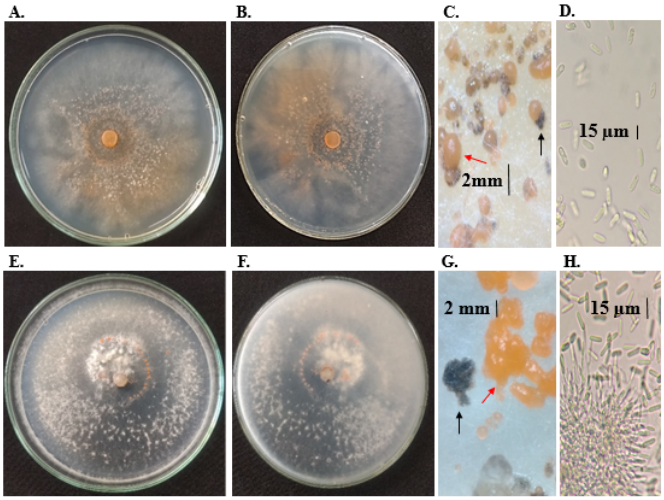
The isolate H3 produced conidia with average dimensions of 14.3 × 5.0 µm, while for I2 the dimensions were 14.6 × 3.9 µm (Table 5). For both isolates, the highest percentage of conidia consisted of format 2, characterized as straight oblong with rounded apices. The length-to-width ratio was highest for I2 with 3.7 while H3 reached a ratio of 2.9. Such features are compatible with species of the *C. boninense* complexes and also *C. gloeosporioides* (Damm et al., 2012; Weir et al., 2012). For both, there was no formation of conidia classified as "straight, with constriction", and, the lowest percentage was considered "falcate, with tapered apices".

Both isolates showed the formation of apressoria (Table 6). For H3, most of the apressoria produced were classified as rounded (87%), while 10 and 3% were classified as lobbed and slightly lobbed, respectively. For I2, most of the apressoria were also classified as rounded, while none of those observed were considered slightly lobbed.

In an extensive review on species of the *C. gloeosporioides* complex, Weir et al. (2012) observed variations among the apressoria produced by different isolates of this group, and the same was found by Damm et al. (2012) when reviewing species of the *C. acutatum* complex. In our study it was also possible to visualize a great variation in the aspects of the apressoria presented by isolates H3 and I2 (Figure 4).

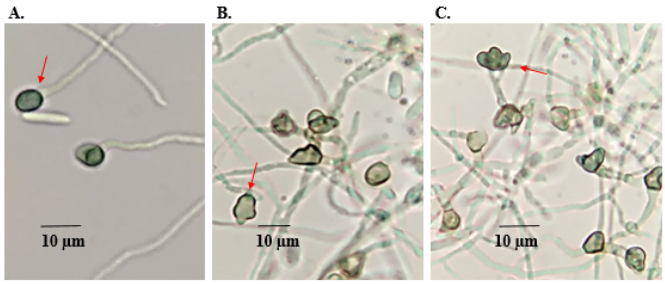
Differentiation of *Colletotrichum* spp. is typically based on morphological features such as mycelial growth, conidia size, colony staining, format of apressoria, and presence of arrows. However, it should be noted that many species within the genus may share one or more of these characteristics in common. Furthermore, these characteristics are plastic, being influenced by environmental factors, culture media, and light, which can cause the same pathogen to produce them or not (Cannon et al., 2012).

In our study, the synapomorphic characteristics of the isolates did not allow us to identify to which species or species H3 and I2 belong. For this reason, molecular identification was employed aiming at identification at the species level.



Where: A, B, C, and D: isolated H3. E, F, G, and H: isolated I2. A and E: top view of the colony. B and F: bottom view of the colony. C and G: black arrow indicates acerule production, red arrow indicates conidiomata production. D and H: general appearance of the conidia. Bars indicate the scale.

Figure 3. General appearance of colonies and conidia of *Colletotrichum* spp. grown on Mathur culture medium at 15 days of incubation.



Where: arrows indicate in: A: Rounded; B: Slightly lobbed; C: Lobbed. Arrows indicate each of the formats. Bars indicate the scale.

Figure 4. Format of apressoria produced by *Colletotrichum* spp.

Molecular identification

Sequencing of the Internal Transcribed Spacer (ITS) and Actin regions were not conclusive in identifying the species level of the isolates, so they were not described in the present study. The phylogenetic tree (Figure 5) results from aligning isolates H3 and I2 with sequences of the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) region of *Colletotrichum*

Table 5. Length, width, length-width ratio, and conidial types of *Colletotrichum* spp. isolated from *Schlumbergera gaertneri*.

Isolated	Length (µm)	Width (µm)	Length-to-width ratio (mean)	Conidia type (%)				
				1	2	3	4	5
H3	14.3	5.0	2.9	8	76	12	0	4
I2	14.6	3.9	3.7	6	75	16	0	3

Where: 1. Straight, spindle-shaped, with tapered apices. 2. Straight, oblong, with rounded apices. 3. Straight, clavate, tapered at one end and round at the other. 4. Straight, with constriction. 5. Falcate, with tapered apices.

Table 6. Characteristics of apressoria of *Colletotrichum* spp. isolated from *Schlumbergera gaertneri*.

Isolated	Length (µm)	Width (µm)	Length-to-width ratio (mean)	Percentage (%)		
				1	2	3
H3	7.5	8.4	0.9	10	3	87
I2	9.6	7.6	1.3	40	0	60

Where: Formats of the apressoria: 1. Lobbed; 2. Slightly lobbed; 3. Rounded.

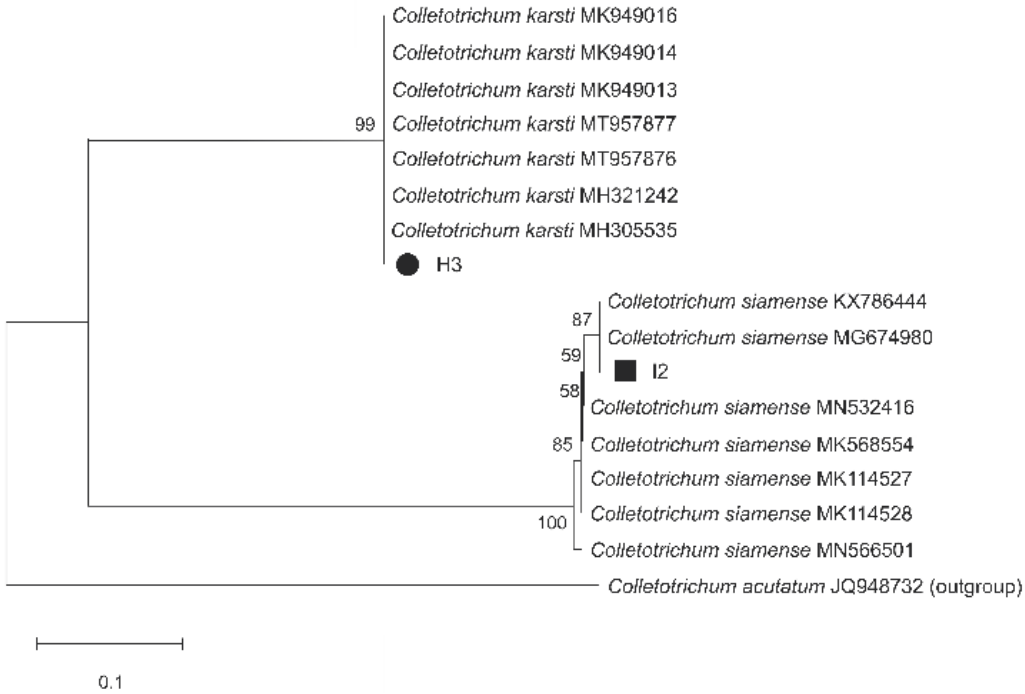


Figure 5. Phylogenetic tree inferred by Neighbour-Joining method from DNA sequences of the Glyceraldehyde-3-phosphate dehydrogenase region, based on 1,000 bootstrap replicates. The number in the branches represents the bootstrap number. A sequence from *Colletotrichum acutatum* was used as an outgroup.

isolates from the GenBank database. The isolate H3 showed a high genetic similarity value with a bootstrap value of 99, to several sequences (MK949016, MK949014, MK949013, MT957877, MT957876, MH321242, and MH305535) of *C. karsti* as can be seen. The isolate encoded as I2 in turn was positioned in a distinct clade, containing sequences belonging to the species *C. siamense*. The bootstrap support value in this case was 87.

The phylogenetic tree generated from the Calmodulin region alignment (Figure 6) was efficient in identifying the species, corroborating the results obtained by sequencing the GAPDH region. The support value of 99 and 100, respectively, for *C. karsti* and *C. siamense* confirmed the genetic identity of the isolates pathogenic to *S. gaertneri*.

With the sequencing of the β-tubulin region it was possible to obtain the phylogenetic tree of the corresponding region, presented in Figure 7. This region kept the isolates in distinct clusters, each with bootstrap support values of 100, confirming the identity of isolates I2 and H3 as being *C. siamense* and *C. karsti*, respectively.

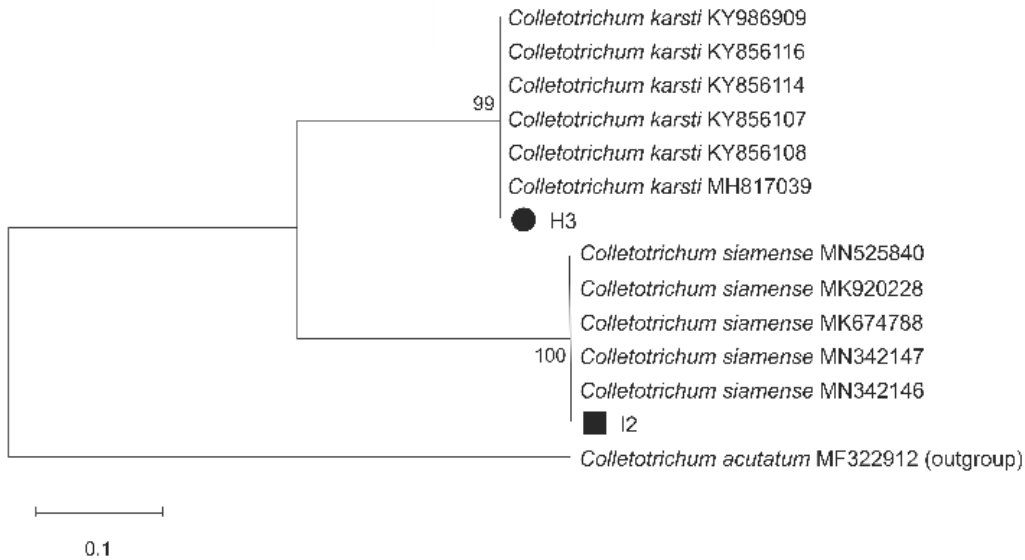


Figure 6. Phylogenetic tree inferred by Neighbour-Joining method from DNA sequences of the Calmodulin region, based on 1,000 bootstrap replicates. The number in the branches represents the bootstrap number. A sequence from *Colletotrichum acutatum* was used as an outgroup.

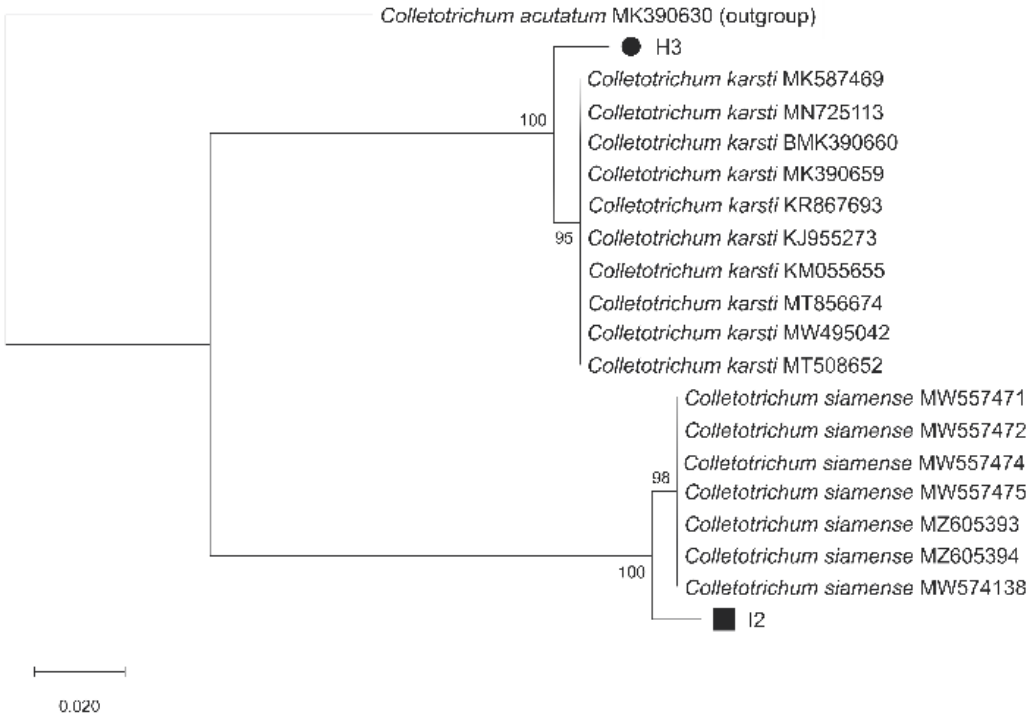


Figure 7. Phylogenetic tree based on the Neighbour-Joining method from DNA sequences of the β-tubulin region, based on 1,000 bootstrap replicates. The number in the branches represents the bootstrap number. A sequence from *Colletotrichum acutatum* was used as an outgroup.

Table 7. Isolates, SMDB access, and sequence code deposited in GenBank.

Isolated	Código GenBank				
	DNA regions				
	ITS	ACT	GAPDH	CAL	β-TUB
H3	OL826793	OL744075	OL770244	OL692093	OL779249
I2	OL826823	OL800701	OL801319	OL742650	OL830406

Where: ITS: Internal Transcribed Spacer; ACT: Actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; CAL: Calmodulin; β-TUB: β-tubulin.

After the molecular identification of the isolates of the present study, the sequences of the ITS, CAL, ACT, GAPDH and β-TUB regions of each isolate could be deposited in the GenBank database, obtaining the codes presented in Table 7.

From the identification of the species to which H3 and I2 belonged, it was possible to obtain a registration code with the Brazilian National System for Genetic Heritage Management (Sistema Nacional de Gestão do Patrimônio Genético - SisGen) (code A77AFA0). A pure culture of each fungal isolate was deposited in the Santa Maria Herbarium-Biology Department (SMDB) and given a registration number (20649 for H3 and 20648 for I2).

Conclusions

Colletotrichum karsti and *C. siamensis* are the causal agents of anthracnose on *Schlumbergera gaertneri*, which differ in the size of the lesion caused.

In the morphological characterization, the colonies presented, differences in the general aspect, such as the type of mycelium and coloration of the conidiomata. The isolates showed formation of apressoria and absence of arrows.

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Compliance with Ethical Standards

Author contributions: Conceptualization: LGS, JSS; Data curation: LGS; Formal analysis: LGS, JSS, MAS, LSM; Methodology: LGS, JSS, JRTK, MAS, LSM, MFBM; Supervision: MFBM; Validation: MFBM; Writing - original draft: LGS; JSS; JRTK; MAS; LSM; Writing - review & editing: MFBM.

Conflict of interest: The authors declare that they have no conflict of interest.

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