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Molecular methods for the specific detection of *Colletotrichum sansevieriae*[*]

Métodos moleculares para la detección específica de *Colletotrichum sansevieriae*

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Abstract

Introduction. *Sansevieria* anthracnose, caused by *Colletotrichum sansevieriae*, poses a significant risk to the cultivation and export of this ornamental plant. Effective and swift identification methods for this pathogen are crucial for implementing control measures to prevent its spread to uninfected areas. **Objective.** To implement and optimize molecular methods for the rapid and reliable identification of *C. sansevieriae*. **Materials and methods.** During 2016, a β-tubulin-2 (β-tub2) gene fragment of *C. sansevieria* isolated from a local farm in Alajuela, Costa Rica was analyzed. PCR-RFLP of the partial β-tubulin-2 (β-tub2) gene fragment was implemented with the enzyme MseI (Tru1I). In addition, species-specific primers for *C. sansevieriae* detection and PCR-RFLP analysis of the amplified fragment were applied. **Results.** The digestion consistently produced a two-band restriction pattern specific to *C. sansevieriae*. The designed primers successfully amplified a 383 bp fragment of the β-tub2 from all *C. sansevieriae* strains tested. No amplification was observed from other *Colletotrichum* species within the *C. gloesporioides* and *C. acutatum* complexes, as well as from *C. truncatum* and *Fusarium oxysporum* isolates. Moreover, this restriction site, located within the amplicon generated by the species-specific primers for *C.* sansevieriae, enabled successful validation of the species through digestion. **Conclusions.** Both PCR based methods proved sensitive enough to detect *C. sansevieriae* in naturally and artificially infected *Sansevieria* leaves without requiring isolation of the pathogen in pure cultures, making the diagnostic process more efficient and accessible.

Keywords: RFLP, anthracnose, plant disease, β-tubulin gene, plant pathogen, fungal diagnostics.

Resumen

Introducción. La antracnosis de *Sansevieria*, causada por *Colletotrichum sansevieriae*, representa un riesgo significativo para el cultivo y la exportación de esta planta ornamental. Los métodos efectivos y rápidos de identificación de este patógeno son cruciales para implementar medidas de control que prevengan su propagación a

áreas no infectadas. **Objetivo.** Implementar y optimizar métodos moleculares para la identificación rápida y confiable de *C. sansevieriae*. **Materiales y métodos.** Durante 2016, se analizó un fragmento del gen β-tubulina-2 (β-tub2) de *C. sansevieriae* aislado de una finca local en Alajuela, Costa Rica. Se implementó PCR-RFLP del fragmento parcial del gen β-tubulina-2 (β-tub2) con la enzima MseI (Tru1I). Además, se aplicaron cebadores específicos para la detección de *C. sansevieriae* y análisis de PCR-RFLP del fragmento amplificado. **Resultados.** La digestión produjo de manera consistente un patrón de restricción de dos bandas específico para *C. sansevieriae*. Los cebadores diseñados amplificaron con éxito un fragmento de 383 pb del β-tub2 de todas las cepas de *C. sansevieriae* probadas. No se observó amplificación de otras especies de *Colletotrichum* dentro de los complejos *C. gloesporioides* y *C. acutatum*, ni de aislamientos de *C. truncatum* y *Fusarium oxysporum*. Además, este sitio de restricción, ubicado dentro del amplicón generado por los cebadores específicos para C. sansevieriae, permitió la validación exitosa de la especie mediante digestión. **Conclusiones.** Ambos métodos basados en PCR demostraron ser lo suficientemente sensibles como para detectar C. sansevieriae en hojas de Sansevieria infectadas de manera natural y artificial sin necesidad de aislar el patógeno en cultivos puros, lo que hace que el proceso diagnóstico sea más eficiente y accesible.

Palabras clave: RFLP, antracnosis, enfermedad vegetal, gen β-tubulina, patógeno vegetal, diagnóstico fúngico.

Introduction

The group *Sansevieria*, which is now included in the genus *Dracaena*, comprises plants native to Africa and Asia and includes about 80 plant species (van Kleinwee et al., 2022). For purposes of this research, the genus *Sansevieria* is retained (Brand & Wichura, 2023). Plants from this group are commonly known as bowstring hemp, snake plant and mother-in-law's tongue (Khalumba et al., 2005; Takawira & Nordal, 2001). *Sansevieria* plants serve a major role in the ornamental foliage industry due to their aesthetic appeal and adaptability, making them a significant subject of interest in horticultural research and commercial cultivation (Rêgo et al., 2020). These plants are widely used for interior and exterior decoration as well as a landscape foliage plants (Khalumba et al., 2005; Takawira & Nordal, 2001). *Sansevieria* has been a profitable ornamental crop due to low input costs for crop maintenance and minimal incidence of pests and diseases (Campoverde & Palmateer, 2012). In addition to their use as ornamental plants, *Sansevieria* species are also valuable for the fiber content and medicinal properties as well as for soil conservation (Khalumba et al., 2005; Takawira & Stedje, 2011). Moreover, these plants are renowned for their air purification qualities as they remove human health harmful volatile organic compounds (VOCs) such formaldehyde, benzene, and carbon dioxide from the atmosphere (Dela Cruz et al., 2014).

Sansevieria plants are affected by different fungal diseases, which can compromise their ornamental and economic value. One of the most damaging diseases is anthracnose, caused by the fungus *Colletotrichum sansevieriae* (Kee et al., 2020a). In 2006, this pathogen was first identified as the cause of *Sansevieria* anthracnose in Japan (Nakamura et al., 2006). Since this first report, *Sansevieria* anthracnose associated with *C. sansevieriae* has been reported in different countries worldwide, including Australia (Aldaoud et al., 2011), the United States (Campoverde & Palmateer, 2012; Palmateer et al., 2012, Grskovich et al., 2024), India (Gautam et al., 2012), Costa Rica (Pérez-León et al., 2013), Korea (Park et al., 2013), Malaysia (Kee et al., 2020a), China (Li et al., 2023), and Germany (Brand & Wichura, 2023).

The disease was first identified on *Sanseviera trifasciata* (actually *Dracaena trifasciata*, van Kleinwee et al., 2022) Prain var. Laurentii, but also affects other *S. trifasciata* varieties (Black Gold, Coral, Hahnii, Moonshine & Zeylanica), as well as other *Sanseviera* species (Campoverde & Palmateer, 2012; Nakamura et al., 2006; Pérez-León et al., 2013). The pathogen causes water-soaked lesions which enlarge and ultimately coalesce resulting in

severe leaf blight and plant destruction if left unattended (Kee et al, 2020b). While the fungus is highly specific to *Sanseviera* spp and does not affect other plants, its high host specificity results in a significant impact particularly for the varieties use in commercial production (Nakamura et al., 2006; Pérez-León et al., 2013). While some *Sansevieria* species and cultivars are resistant, the most famous and traditionally used varieties are susceptible (Campoverde & Palmateer, 2012; Pérez-León et al., 2013).

This susceptibility has significant economic implications. However, there are currently no scientific reports available that quantify the yield damage caused by this disease. In South Florida, for instance, when the disease spread in local nurseries, the *Sansevieria* production was stopped (Campoverde & Palmateer, 2012). The disease also affects bare-rooted plants produced in Central America, which are exported to North America. Although *C. sansevieriae* can be managed with sanitary measures and weekly applications of fungicides that include different modes of action in a preventive approach (Campoverde & Palmateer, 2012; Pérez-León et al., 2015), producers struggle with its management, even when they use recommended guidelines. In addition, the practices to manage the disease add cost to the production, making the crop unprofitable, further threatening *Sansevieria* production (Campoverde & Palmateer, 2012).

Management strategies as quarantine, exclusion, and containment are necessary to prevent the introduction and establishment of the pathogen into disease-free areas. Accurate and rapid identification methods of plant pathogens are pivotal to implementing these strategies. Traditionally, morphological and molecular identification techniques are part of the diagnostic routines to identify *Colletotrichum* species with recent advancements of a multilocus approach (Cao et al., 2024). However, morphological identification of *Colletotrichum* species is challenging because of overlapping characteristics among species and intraspecific variability (Ferrucho et al., 2024). Moreover, some *Colletotrichum* identification methods require considerable time, resources, and effort (Cao et al., 2024). Therefore, developing PCR endpoint or qPCR-specific primers for identifying *Colletotrichum* species has become an alternative (Ferrucho et al., 2024; McHenry & Aćimović, 2024).

Different genomic regions, such as the β-tubulin, the Internal Transcribed Spacer (ITS), actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chitin synthase, and calmodulin have been used to identify *Colletotrichum* species (Huang et al., 2021; Silva et al., 2017). These genomic regions have proven effective in differentiating among *Colletotrichum* species causing anthracnose in several economically important crops (Ferrucho et al., 2024; Gang et al., 2015; Martinez-Culebras et al., 2000; 2003; Rampersad, 2011; Tapia-Tussell et al., 2008; Torres-Calzada et al., 2011).

The ITS and β-tub2 are the most used DNA markers for the identification and characterization of *Colletotrichum* species (Kee et al., 2020a; Lee et al., 2007; Ramdial & Rampersad, 2015; Schena et al., 2014; Torres-Calzada et al., 2011; Watanabe et al., 2016; Yamagishi et al., 2016). Species-specific primers and PCR-RFLP methods targeting the β-tub2 gene can be more informative than ITS region sequences, as evidenced by the comparisons of DNA polymorphism indices, showing very little or no intraspecific variation (Rampersad et al., 2016; Silva et al., 2012). Moreover, these methods are among the most effective at distinguishing single *Colletotrichum* species (Damm et al., 2012; Weir et al., 2012), including *C. sansevieriae* (Kee et al., 2020b). In addition, some species-specific molecular diagnostic methods based on ITS sequences have demonstrated to be taxon-specific rather than species-specific, pointing out the low-resolution power of ITS for closely related species (Afanador-Kafuri et al., 2014; Álvarez et al., 2014; Silva et al., 2012). Building on these successful molecular approaches, this study aimed to implement and optimize molecular methods for the rapid and reliable identification of *C. sansevieriae*.

Materials and methods

Fungal isolation and identification of *C. sansevieria* isolates

Open field-grown leaves of *Sanseviera trifasciata* 'Laurentii' and 'Hahnii' with typical symptoms of anthracnose were collected during 2016 from a local farm in Alajuela, Costa Rica (Figure 1). The leaves were transported to Plant Biotechnology Laboratory of the Agronomic Research Center (CIA), at the University of Costa Rica.

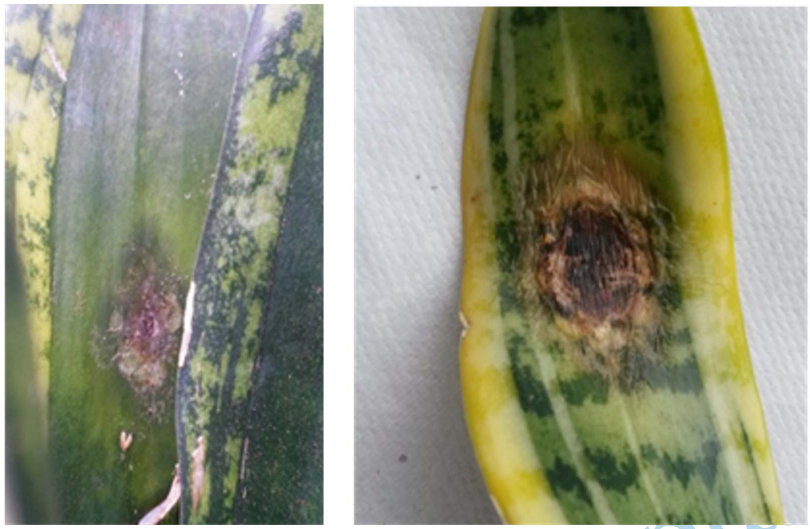

Figure 1. Symptoms caused by *Colletotrichum sansevieriae* on *Sansevieria trifasciata*. Plant Biotechnology Laboratory, Agronomic Research Center (CIA), Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2016.

Figura 1. Síntomas causados por *Colletotrichum sansevieriae* en *Sansevieria trifasciata*. Laboratorio de Biotecnología de Plantas, Centro de Investigaciones Agronómicas (CIA), Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2016.

The leaves were cut into small fragments (1 cm x 1 cm), whose surface was sterilized by dipping in 1.5 % sodium hypochlorite (NaOCl) for 2 min, rinsed three times with sterilized water, and dried on sterilized tissue paper under a laminar flow chamber (High Ten, China). The sterilized leaf samples were aseptically cut into pieces of 0.2 cm x 0.3 cm from the advancing edge of each lesion and placed on Acidified Potato Dextrose Agar (APDA) medium and incubated at room temperature (25 ± 2 °C) in the dark. The growing edges of any fungal hyphae emerging from the plated segments were then transferred aseptically into APDA. Pure cultures were obtained by single spore isolation and maintained on PDA. Reference isolates of other *Colletotrichum* species, previously identified and characterized in our laboratory by multilocus sequence analysis (Ruiz-Campos et al., 2017; 2022), were included in this study (Table 1).

Colonies showing a grayish-white, felted, with cottony-white aerial mycelium on a gray to olivaceous-gray background on PDA, consistent with the description of *C. sansevieriae* (Nakamura et al., 2006; Park et al., 2013). Additionally, *Fusarium oxysporum*, a pathogen commonly found in *Sansevieria* leaves, was used to validate the specificity of the methods used in this research to identify *C. sansevieriae* as part of a standard procedure in this type of experiments. To achieve this, colonies resembling *Fusarium* spp. were obtained and subsequently identified

Table 1. Isolates, *Colletotrichum* species and *Fusarium oxysporum*, used in this study. Plant Biotechnology Laboratory, Agronomic Research Center (CIA), Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2016.

Cuadro 1. Aislamientos de las especies de *Colletotrichum* y *Fusarium oxysporum*, utilizados en este estudio. Centro de Investigaciones Agronómicas (CIA), Laboratorio de Biotecnología de Plantas, Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2016.

Species	Isolate	Source
Colletotrichum. fructicola	TE 3.1	Ruiz-Campos et al. (2017; 2022)
	EB 11.1	Ruiz-Campos et al. (2017; 2022)
C. magnum	C.m	Ruiz-Campos et al. (2017; 2022)
C. sansevieriae	Cs1	This study
	Cs2	This study
	Cs3	This study
	Cs4	This study
	Cs5	This study
	Cs6	This study
C. simmondsii	Lp 9.2	Ruiz-Campos et al. (2017; 2022)
C. theobromicola	EH1.1	Ruiz-Campos et al. (2017; 2022)
C. tropicale	BN5.1	Ruiz-Campos et al. (2017; 2022)
C. truncatum (C. capsici)	EB11.1	Ruiz-Campos et al. (2017; 2022)
	TE9.1	Ruiz-Campos et al. (2017; 2022)
Fusarium oxysporum	F1	This study

and characterized by nucleotide sequence analysis of two independent gene regions, the ITS1–5.8S–ITS2 region of rDNA (ITS) and partial β-tubulin-2 gene.

Genomic DNA was extracted from mycelium scraped from 7-10-days old colonies grown on PDA using the CTAB method (Doyle & Doyle, 1987). Amplification of ITS region, β-tubulin-2, TEF1-α, was conducted using primer pairs ITS1/ITS4 (White et al., 1990), T1/Bt2b (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997), and EF1/EF2 (O'Donnell et al., 1998), respectively. PCR reactions were performed in a total volume of 25 μL which contained 1X 10X PCR Buffer, 1.7 mM $MgCl_2$, 0.2 mM dNTP, 0.8 mg/mL Bovine Serum Albumin (BS), 0.8 μM of each primer, 0.05 U/ μL Dream Taq Polymerase, 3.0 μL of crude DNA. The final volume was adjusted with sterile Nanopure water to 25 μL. All reagents were from Thermo Fisher Scientific, Waltham, MA, USA.

PCR reactions with no DNA template were used as a negative control. DNA amplification was carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The cycling conditions for the ITS region were as follows: 93 °C for 3 min, 35 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 0.45 s and final extension at 72 °C for 5 min. The thermal conditions for the β-tubulin and TEF1-α genes were 94 °C for 5 min, 35 cycles at 94 °C for 45 s, 65 °C for 45 s, 72 °C for 1 min and final extension at 72 °C for 5 min. The PCR products were visualized by electrophoresis in 1.6 % agarose gel (1.6 g of agarose in 100 mL of TRIS-Borate-EDTA 0.5X buffer). Before loading 5 μL of the PCR products in the gels, 2 μL of 6X Loading Buffer containing GelRed (Biotium, Fremont, CA, USA) (100X dilution) were added. Fragments sizes were estimated using a 100 bp GeneRuler DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). Electrophoresis was run at 110V for 1 h and observed in a transilluminator, BioDoc-It2® 315 Imaging System LMS-26 (UVP, Upland, CA). All PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea) in both directions using the same

PCR primers. Sequences were edited using BioEdit Sequence Alignment Editor Software (Hall, 1999). The identity of the isolates was established by comparison with those available in the GenBank by BLAST search.

PCR-RFLP analysis of β-tubulin partial gene for detection of *C. sansevieriae*

The β-tubulin partial gene sequences of *C. sansevieriae* were imported into BioEdit (Hall, 1999). A restriction map was generated with all restriction enzymes included in the software. Enzyme MseI, which generated a clear and discriminatory two-band pattern (320 and 480 bp), was selected. Amplicons (800 bp) of β-tubulin gene were obtained by PCR using primer set T1/Bt2b as described above and digested with MseI (Fermentas, Ontario, Canada).

The digest reactions were performed in volumes of 10 μL containing 8 μL PCR product and 2 μL of enzyme mix (2 μL of Buffer R (Fermentas, Ontario, Canada), 2 U/μL MseI restriction enzyme, and 6 μL of sterile distilled water). The digested reactions were incubated at 65 °C for 16 h (overnight digest to ensure complete digestion of DNA). Fragments were separated on 2.5% agarose gels and visualized under UV light as described above. Fragments sizes were estimated using a 100 bp GeneRuler DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). The MseI restriction site was also within the amplicon obtained with the *C. sansevieriae* species-specific primers CsTubF2/CsTubR3, therefore it was also subjected to restriction digest according to the procedure previously described.

Species-specific PCR primer design and identification of *C. sansevieriae*

β-tubulin partial gene sequences (800 bp) of *C. sansevieriae* obtained in this study and those from other *Colletotrichum* species retrieved from the GenBank, including the accession LC180128 of the isolate Sa 1-2 from Japan, used by Nakamura et al. (2006) to describe *C. sansevieriae*, were edited with BioEdit (Hall, 1999) and aligned using CLUSTAL as implemented in MEGA 5 (Kumar et al., 2018). Several sets of forward and reverse primers specific to *C. sansevieriae* were designed using the primer designing tools Primer3Plus program (Untergasser et al., 2007) and Primer-Blast (Ye et al., 2012). The degree of specificity of these primers to other potential target sequences was determined using Primer-Blast algorithm (Ye et al., 2012), limiting the search to i) Non-redundant (nr) and ii) Organism-specific database (*Colletotrichum*) in the GenBank. The primers were synthesized by Macrogen Inc. (Seoul, South Korea). A primer set, CsTubF2 (5'-TTCCACCACGTCGACACTTA-3') and CsTubR3 (5'-TATTGGGAGGATCAGCGGTC-3'), which amplify a 383 bp fragment and provided the most consistent and specific DNA amplification following PCR optimization, was selected for further analysis.

PCR reactions were performed in a total volume of 25 μL containing 2.5 μL 10X PCR Buffer, 1.7 mM $MgCl_2$ (25 mM), 0.2 mM dNTP mix (2 mM), 0.8 μM of each primer, 1.0 mg/mL BSA (20 mg/mL), 0.05 U/μL Dream Taq DNA, 2.0 μL of crude DNA. Sterile Nanopure water was used to adjust the final volume to 25 μL. All reagents were from Thermo Fisher Scientific, Waltham, MA, USA. PCR reactions with no DNA template were used as negative control. DNA amplification was carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: 30 s at 95 °C, followed by 25 cycles with 10 s at 95 °C, 15 s at 61 °C, and 45 s at 72 °C. The reaction was completed by a final extension temperature of 72 °C for 5 minutes. The PCR products were visualized by electrophoresis in 1.6 % agarose gel (1.6 g of agarose in 100 mL of TRIS-Borate-EDTA 0.5X buffer). Before loading 5 μL of the PCR products in the gels, 2 μL of 6X Loading Buffer containing GelRed (Biotium, Fremont, CA, USA) (100X dilution) were added. Fragment sizes were estimated using a 100 bp GeneRuler DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). Electrophoresis was run at 110V for 1 h and observed in a transilluminator, BioDoc-It2® 315 Imaging System LMS-26 (UVP, Upland, CA).

PCR products obtained with specific-primers CsTubF2/CsTubR3 were purified and sequenced, in both directions by Macrogen Inc. (Seoul, South Korea), using the same primer pair. Sequences were aligned and compared using BioEdit Sequence Alignment Editor Software (Hall, 1999). The identity of the samples was established by comparison with those available in the GenBank using BLAST search.

Detection of *C. sansevieriae* from naturally and artificially infected *Sansevieria*

DNA was extracted directly from leaves of five infected field-grown *S. trifasciata* var. Laurentii plants showing characteristic symptoms of anthracnose. Approximately 100 mg of fresh tissue from infected lesion was processed according to the method described by Saghai-Maroof et al. (1984). Extracted DNA was both amplified by PCR using *C. sansevieriae*-specific primers CsTubF2/CsTubR3 and subjected to PCR-RFLP with MseI restriction enzyme, under the reaction conditions previously described. DNA from pure culture of *C. sansevieriae*, characterized by sequencing of the ITS region and β-tubulin partial gene sequences in this study, sterile distilled water and DNA from healthy plants (*in vitro*-grown plants) served as controls.

PCR products obtained with specific primers CsTubF2/CsTubR3 were purified and sequenced by Macrogen Inc. (Seoul, South Korea), in both directions using the same primer pair. Sequences were aligned and compared using BioEdit Sequence Alignment Editor Software (Hall, 1999). The identity of the samples was established by comparison with those available in the GenBank using BLAST search.

Results

Identification of *C. sansevieriae* and *Fusarium oxysporum* isolates

Ten single-spore isolates of *C. sansevieriae* and one single-spore isolate of *Fusarium oxysporum* were successfully identified using ITS and partial β-tubulin-2 gene sequence analysis. The results showed that *C. sansevieria* isolates had 99 % and 100 % similarity, with sequences of strain Sa 1-2 (LC179806 and LC180128, respectively) from Japan, used to describe *C. sansevieriae*. ITS sequences also shared 99 % identity with other *C. sansevieriae* sequences available in the GenBank from Australia (HQ433226), USA (JF911350), Iran (KP835682) and South Korea (KC847065). No β-tubulin sequences were available for these strains. The β-tubulin-2 gene nucleotide sequence of two isolates (Sa3.1 and Sa18.1) were deposited into GenBank (OP713761 and OP713762, respectively). *F. oxysporum* shared more than 99 % similarity with sequences of several *F. oxysporum* strains for ITS (MT530269, MT529531) and TEF1-α (MN386726, MN386739, KF575346) sequences.

Analysis of β-tubulin partial gene and PCR-RFLP for detection of *C. sansevieriae*

PCR products obtained with T1/Bt2b primers for isolates belonging to the seven *Colletotrichum* species generated an amplicon of approximately 800 bp, and 600 to 700 bp for the *F. oxysporum* isolate (Figura 2A).

MseI digestion from the PCR product from the amplification with the T1 and βt2b consistently produced a two-fragment pattern (320 and 480 bp) for all *C. sansevieriae* isolates (Figure 2B). A two-band pattern with different band sizes was also observed for *F. oxysporum* (~200 and 350 bp approx.), *C. capsici* (200 and 350 bp approx.) and *C. simmondsii* (200 and 600 bp). PCR products (800 bp) for *C. fructicola*, *C. tropicale*, *C. theobromicola*, and *C. magnum* isolates were not digested (Figure 2B). The negative control of undigested PCR amplicon resulted in only one fragment of 800 pb for *Colletotrichum* isolates (Figure 2B).

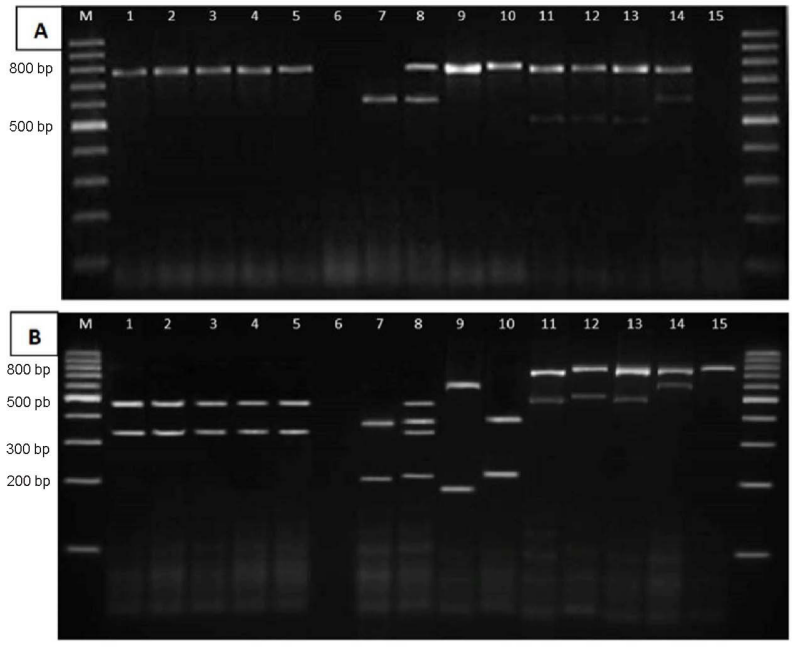

Figure 2. A) PCR amplification of β-tubulin 2 with T1 and βt2b primer pair, and B) Restriction digestion pattern of amplicons using Mse1 (Tru1): 100 bp DNA ladder (Lane M), *C. sansevieriae* DNA extracted from mycelium (Lanes 1, 2 and 3), *C. sansevieriae* DNA extracted from *S. trifasciata* diseased leaf (Lanes 4 and 5), DNA extracted from *S. trifasciata* without diseased leaf (Lane 6), *F. oxysporum* (Lane 7), *F. oxysporum* and *C. sansevieriae* mixture (Lane 8), *C. simmondsii* (Lane 9), *C. truncatum (capsici)* (Lane 10), C. *fructicola* (Lane 11), *C. tropicale* (Lane 12), *C. theobromicola* (Lane 13), *C. magnum* (Lane 14), negative control (Lane 15, panel A) and undigested PCR amplicon from *C. sansevieriae* (Lane 15, panel B). Plant Biotechnology Laboratory, Agronomic Research Center (CIA), Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2017.

Figura 2. A) Amplificación por PCR de *β*-tubulina 2 con el par de cebadores T1 y *β*t2b, y B) Patrón de digestión por restricción de amplicones usando Mse1 (Tru1): escalera de ADN de 100 bp (carril M), ADN de *C. sansevieriae* extraído del micelio (carriles 1, 2 y 3), ADN de *C. sansevieriae* extraído de hoja enferma de *S. trifasciata* (carriles 4 y 5), ADN extraído de hoja sin enfermedad de *S. trifasciata* (carril 6), *F. oxysporum* (carril 7), *F. oxysporum* y mezcla de *C. sansevieriae* (carril 8), *C. simmondsii* (carril 9), *C. truncatum* (*capsici*) (carril 10), *C. fructicola* (carril 11), *C. tropicale* (carril 12), *C. theobromicola* (carril 13), *C. magnum* (carril 14), control negativo (carril 15, panel A) y amplicón de PCR no digerido de *C. sansevieriae* (carril 15, panel B). Laboratorio de Biotecnología de Plantas, Centro de Investigaciones Agronómicas (CIA), Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2017.

Species-specific PCR primers for *C. sansevieriae* detection and PCR-RFLP analysis of the amplified fragment

The primer pair, CsTubF2/CsTubR3 designed in this research for the detection of *C. sansevieriae* showed a perfect match to the partial β-tubulin sequence from *C. sansevieriae* strain Sa 1-2, with no off-target amplification found in the *Colletotrichum genus* or *F. oxysporum*. The primers amplified a 383 bp fragment from all *C. sansevieriae* isolates tested, but did not amplify DNA from other *Colletotrichum* species (*C. simmondsii*, *C. truncatum*, *C. fructicola*, *C. tropicale*, *C. theobromicola*, *C. magnum*) or *F. oxysporum* (Figure 3A). A positive PCR reaction was observed for DNA samples from all isolates tested using the universal primer pair ITS4/ITS5, showing that the extracted DNA was PCR amplifiable (data not shown).

The research showed no false positives when tested against DNA from other *Colletotrichum* species and *F. oxysporum*. Similarly, there were no false negatives observed in PCR reactions for all *C. sansevieriae* isolates tested.

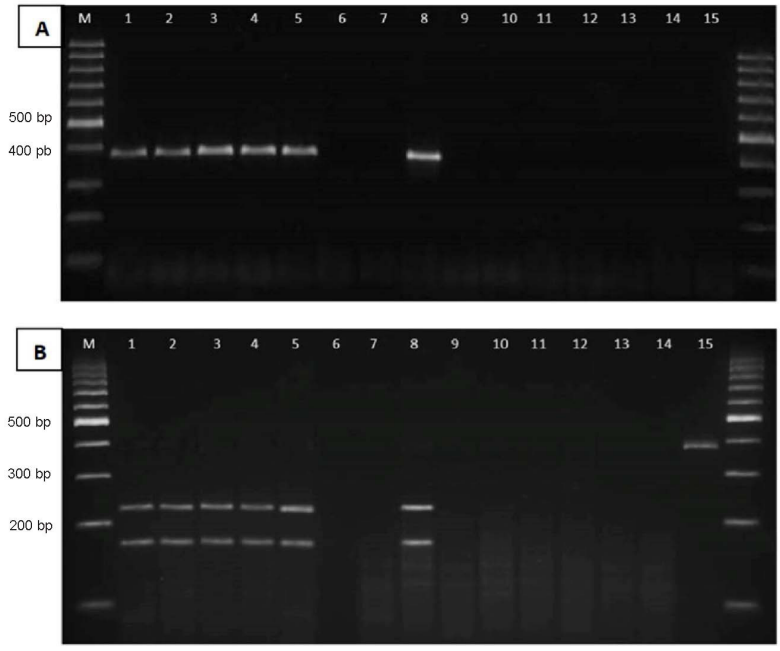

Figure 3. A) Specific PCR amplification for *C. sansevieriae* with primers CstubF2/CstubR3 (383 bp), and B) Restriction digestion pattern of β-tubulin 2 amplicons obtained with CstubF2/CstubR3 primer pair using MseI: 100 bp DNA ladder (Lane M), *C. sansevieriae* DNA extracted from mycelium (Lanes 1, 2 and 3), *C. sansevieriae* DNA extracted from *S. trifasciata* diseased leaf (Lanes 4 and 5), DNA extracted from *S. trifasciata* without diseased leaf (Lane 6), *F. oxysporum* (Lane 7), *F. oxysporum* and *C. sansevieriae* mixture (Lane 8), *C. simmondsii* (Lane 9), *C. (truncatum) capsici* (Lane 10), C. *fructicola* (Lane 11), *C. tropicale* (Lane 12), *C. theobromicola* (Lane 13), *C. magnum* (Lane 14), negative control (Lane 15, panel A) and undigested PCR amplicon from *C. sansevieriae* (Lane 15, panel B). Plant Biotechnology Laboratory, Agronomic Research Center (CIA), Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2017.

Figura 3. A) Amplificación PCR específica para *C. sansevieriae* con los imprimadores CstubF2/CstubR3 (383 bp), y B) Patrón de digestión por restricción de los amplicones de β-tubulina 2 obtenidos con el par de imprimadores CstubF2/CstubR3 utilizando MseI: escalera de ADN de 100 bp (carril M), ADN de *C. sansevieriae* extraído del micelio (carriles 1, 2 y 3), ADN de *C. sansevieriae* extraído de una hoja enferma de *S. trifasciata* (carriles 4 y 5), ADN extraído de *S. trifasciata* sin hoja enferma (carril 6), *F. oxysporum* (carril 7), mezcla de *F. oxysporum* y *C. sansevieriae* (carril 8), *C. simmondsii* (carril 9), C. *truncatum* (*capsici*) (carril 10), *C. fructicola* (carril 11), *C. tropicale* (carril 12), *C. theobromicola* (carril 13), *C. magnum* (carril 14), control negativo (carril 15, panel A) y amplicón PCR no digerido de *C. sansevieriae* (carril 15, panel B). Laboratorio de Biotecnología de Plantas, Centro de Investigaciones Agronómicas (CIA), Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2017.

The sequencing of the PCR products obtained with primers CsTubF2/CsTubR3 confirmed that the amplified sequences were identical (100% sequence identity) to the expected partial β-tubulin gene sequence of *C. sansevieriae* strain Sa 1-2 (GenBank accession no. LC180128).

The restriction enzyme, MseI (Tru1I) was selected for the PCR-RFLP analysis. For *C. sansevieria* specific primers (CsTubF2/CsTubR3), digestion with the MseI consistently produced two fragments of 170 and 210 bp (Figure 3B).

Detection of *C. sansevieriae* from naturally and artificially infected *Sanseviera*

Total DNA was extracted from naturally and artificially infected *Sanseviera* leaves and subjected to PCR amplification using species-specific CsTubF2/CsTubR3 primers and PCR-RFLP assay. The species-specific

primers successfully amplified a band of 380 bp from DNA samples extracted from both naturally and artificially infected leaves. No visible PCR products were obtained from healthy leaves and the water control (Figure 3A).

Sequencing of the amplified PCR products obtained with the specific primers confirmed the identity of the pathogen as *C. sansevieriae*. These sequences shared 100% identity with the partial β-tubulin gene sequence of *C. sansevieriae* strain Sa 1-2 (GenBank accession no. LC180128), confirming the identity of the pathogen.

DNA from infected leaves (artificially and naturally) was successfully amplified by PCR using either the β-tubulin T1/Bt2b or CsTubF2/CsTubR3 primers and generated the PCR fragments of the expected size, 800 and 380 bp, respectively (Figures 2A and 3A). In some samples from naturally infected leaves additional fragments of different sizes were observed when T1/Bt2b primers were used. No visible PCR products were obtained from healthy leaves and the water control (Figures 2B and 3B). MseI digestion generated fragments around 320 and 480 bp when the T1/Bt2b were used, and 170 and 210 bp when CsTubF2/CsTubR3 were used, specifically for *C. sansevieriae* (Figures 2B and 3B). The presence of additional fragments did not interfere with the scoreability of the two-band *C. sansevieriae* specific restriction pattern (Figure 3B).

Discussion

Sansevieria anthracnose caused by *C. sansevieriae* is an emerging disease threatening *Dracaena* production (Brand & Wichura, 2023). This study used End-point PCR and PCR-RFLP for the direct detection of *C. sansevieriae* from infected plant tissue. This combined approach allowed the discrimination of *C. sansevieria* from other *Colletotrichum* species.

The *C. sansevieria-specific* primer pair CsTubF2/CsTubR3 was tested *In silico* (Ye et al., 2012) and showed no significant matches to other *Colletotrichum* species or *F. oxysporum*, a pathogen commonly found in *Sansevieria* leave infections (Kee at al., 2020b). This confirms the high specificity of the designed primers to *C. sansevieriae*. The β-tub2 gene sequences have been previously used for primer design for rapid and reliable diagnostic PCR of other *Colletotrichum* species as *C. truncatum* (Rampersad, 2011), *C. acutatum* (Talhinhas et al., 2005) and *C. gloeosporioides* (Chung et al., 2010; Talhinhas et al., 2005) as well as for phylogenetic analysis (Damm et al., 2012; Weir et al., 2012). In other research, species-specific primers have been used to successfully identify other *Colletotrichum* species, including *C. gloesporiodes* and *C. acutatum* (Prakoso et al., 2019). Similarly, in this research, the use of species-specific primers allowed the accurate identification of *Colletotrichum* species without amplifying F. *oxysporum* (Pinzón Gutiérrez et al., 2013).

The design of species-specific primers for the identification of *C. sansevieria* could offer a less complex and time-consuming alternative than other molecular and morphological approaches, as reported in other *Colletotrichum* species (Pinzón Gutiérrez et al., 2013; Tapia-Tussell et al., 2008). It is, however, necessary to examine additional isolates of *Colletotrichum* spp. and other fungi causing leaf spots on *Sansevieria* (Bhunjun et al., 2021; Li et al., 2013), to confirm the efficiency and specificity of the CsTubF2/CsTubR3 primers, as the possibility that the primers could amplify other targets cannot be excluded due to the lack of sequence data for some *Colletotrichum* species in the database, hence non-target species could be amplified even if they have a few mismatches to the primers (Ye et al., 2012). Therefore, further analysis including other species and pathogens is suggested.

The PCR-RFLP based on the β-tub2 gene allowed a clear identification of *C. sansevieriae* strains and their discrimination from other *Colletotrichum* species. PCR-RFLP of several genome regions, including β-tub2 gene, has been used to differentiate among *Colletotrichum* species (Gang et al., 2015; Maharaj & Rampersad, 2012; Martinez-Culebras et al., 2000; Ramdeen & Rampersad, 2013; Tapia-Tussell et al., 2008). β-tubulin gene has also been used in PCR-RFLP for differentiating benzimidazole-resistant isolates among a *C. gloeosporioides* population (Chung et al., 2010).

Consistent with previous research, in this study the PCR product from the β-tubulin gene from species belonging to the *C. gloeosporioides* species complex were (Weir et al., 2012) not digested the by the MseI enzyme. Other research has also demonstrated that β-tubulin gene sequences of isolates belonging to *C. gloeosporioides* species complex do not contain recognition sites for enzyme digestion by MseI, reducing the number of possible targets and preventing the detection of species within the *C. gloeosporioides* species complex (Ramdeen & Rampersad, 2013). However, the use of this enzyme allowed for the differentiation of *C. sansevieria* from these species.

In general, the *Colletotrichum* species tested in this research that produced two amplicons for β-tub2 gene with the T1 and βt2b primers did not interfere with the scorability of the two-band *C. sansevieriae* specific restriction pattern. Consistently, in a previous research two amplicons for β-tub2 gene from *C. gloeosporioides* and *C. nymphaeae* isolates with primer pair T1/T2 for these species, however, this precluded recovery and sequencing of β-tub2 gene for these strains (Eaton el al., 2021).

Colletotrichum neosansevieriae, a described species from South Africa, also causes a leaf spot disease of *Sansevieria*. This species is phylogenetically clearly distinct from *C. sansevieriae* (Kee et al., 2020a). DNA samples from *C. neosansevieriae* were not available in this study. Therefore, both molecular-based methods, PCR-species specific primers and PCR-RFLP, were not tested on this species. However, sequence comparison using BioEdit showed no priming sites, and the MseI restriction site was also absent within the β-tub2 gene region of *C. neosansevieriae*. These results indicate that both species can be differentiated using the two diagnostic methods presented here. Further analysis is suggested.

One aspect that requires clarification is that in this study, the sensitivity of the method was not determined, specifically the minimum DNA concentration required to detect the pathogen. However, it was demonstrated that *C. sansevieria* could be detected from infected tissue without the need for isolation on culture media. The PCR-RFLP and PCR-specific primers used in this research enabled the detection of *C. sansevieria* directly from diseased leaves of *S. trifasciata* cv. Laurentii naturally and artificially inoculated with *C. sansevieriae*. Although *Colletotrichum* isolation from leaves is a typical method to identify the pathogen (Syafitri et al., 2023), all the diseased samples directly extracted from the leaves were positive in this study, rendering isolation of the pathogen unnecessary. Since pathogen isolation is time-consuming and labor-intensive, the techniques used in this research provide a faster alternative for identifying of *C. sansevieriae* directly from field collected leaf tissues, a key issue for the quick implementation of adequate disease control measurements to limit spread of the pathogen, including containment and eradication procedures (Ma & Michailides, 2007).

Although the PCR-RFLP method presented here was able to detect *C. sansevieriae* and *F. oxysporum* simultaneously, *F. oxysporum* was frequently recovered from *Sansevieria* diseased leaves. Several *Fusarium* species, including *F. oxysporum*, cause foliar disease on *S. trifasciata* (Kee et al., 2020a). Therefore, attention should be paid to these new causal agent pathogens.

While the combination of endpoint-PCR with specific primers and RFLP techniques provides a more comprehensive and accurate diagnosis remains to be defined whether these techniques can be sufficiently reliable and implemented for routine diagnosis of the disease in the diagnostic laboratories from the country. The results presented in this study suggest that both techniques promising diagnostic potential, but further studies are needed to fully assess its reliability and feasibility for widespread, routine use. This could help determine whether it could serve as a viable alternative to the more resource-intensive combination of these and other techniques.

Conclusions

The Polymerase Chain Reaction (PCR) species-specific primers and/or the combination PCR-RFLP (Restriction Fragment Length Polymorphism) described in this research could be integrated into a specific diagnostic protocol

for *C. sansevieriae*. This would contribute to reduce the pathogen dissemination into new areas, locally and internationally (quarantine decisions), as well as to take informed disease management strategies. While the methods presented in this study offer a promising approach for the rapid and accurate detection of *C. sansevieriae* in *Sansevieria* plants, additional research is needed to confirm their practical application, assess sensitivity, and ensure their reliability for widespread use in plant pathology diagnostics.

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Interests conflict

The authors have no conflicts of interest to declare.

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