ABRATES

Journal of Seed Science

ISSN 2317-1545

www.abrates.org.br/revista

NOTE

Journal of Seed Science, v.42, e202042012, 2020

http://dx.doi.org/10.1590/2317-1545v42229530

Specificity and sensibility of primer pair in the detection of *Colletotrichum gossypii* var. *cephalosporioides* in cotton seeds by PCR technique

Mirella Figueiró de Almeida[1*], Sarah da Silva Costa[1], Iara Eleutéria Dias[1], Carolina da Silva Siqueira[1], José da Cruz Machado[1]

ABSTRACT: Cotton Ramulosis (*Gossypium hirsutum*) is an important disease affecting cotton plantations in Brazil, and its causal agent, *Colletotrichum gossypii* var. *cephalosporioides* (Cgc), according to the Brazilian phytosanitary authority, was considered a regulated non quarantine pest. It makes this microorganism subject to standardization in seed certification programs. The current seed health testing for detecting that pathogen in seed samples does not provide reliable results for routine analysis. On this paper, attempts were made to design specific primers for detection of Cgc associated with cotton seed. Two primer sets were selected based on the analysis of a multiple alignment of gene's sequence encoding the glyceraldehyde 3-phosphate dehydrogenase from Cgc, *C. gossypii* and reference strains of the *C. gloeosporioides* species complex. The conserved sites unique to Cgc strains were used to design specific fragment of 140 bp. The primer specificity was confirmed by using other fungi. The primers produced a detectable band of target DNA of Cgc in all inoculum potentials of the pathogen artificially inoculated by the water restriction technique. The developed primer pair represents, therefore, a reliable and rapid mean to diagnose the Ramulosis agent in cotton seed.

Index terms: *Colletotrichum gossypii*, *Colletotrichum gloeosporioides*, Ramulosis, water restriction.

Especificidade e sensibilidade de um par de primer na detecção de *Colletotrichum gossypii* var. *cephalosporioides* em sementes de algodão pela técnica de PCR

RESUMO: A ramulose do algodão (*Gossypium hirsutum*), causada por *Colletotrichum gossypii* var. *cephalosporioides* (Cgc), é uma doença importante que afeta as plantações de algodão no Brasil. De acordo com as autoridades fitossanitárias brasileiras, esse organismo tem sido considerado uma praga quarentenária não regulamentada, o que faz com que ela seja objeto de padronização em programas de certificação de sementes. Neste trabalho, um par de primer foi selecionado com base na análise de um alinhamento múltiplo de sequências do gene que codifica a gliceraldeído-3-fosfato desidrogenase a partir de Cgc, *C. gossypii* e isolados de referência representantes de outras espécies do complexo *C. gloeosporioides*. Uma única região conservada de Cgc foi utilizada para desenhar um par de primer específico de 140 pb. A especificidade dos primers foi confirmada pela utilização de outros fungos isolados de semente algodão. Os primers produziram uma banda detectável de DNA de Cgc em todos os potenciais de inóculo artificialmente inoculados pela técnica de restrição hídrica. Os primers desenvolvidos representam, portanto, um meio confiável e rápido para diagnosticar Cgc em amostras de sementes de algodão.

Termos para indexação: *Colletotrichum gossypii*, *Colletotrichum gloeosporioides*, ramulose, restrição hídrica.

***Corresponding author**
E-mail: mirellafa@yahoo.com.br

Received: 10/3/2019.
Accepted: 2/5/2020.

[1]Departamento de Fitopatologia, Universidade Federal de Lavras, Caixa Postal 3037, 37200-000 – Lavras, MG, Brasil.

INTRODUCTION

Ramulosis is one of the most prominent diseases in cotton (*Gossypium hirsutum)* in Brazil, and it is caused by *Colletotrichum gossypii* var. *cephalosporioides* A. S. Costa. This organism belongs to the *Colletotrichum gloeosporioides* species complex, as well as *Colletotrichum gossypii* South. (Cg), which causes Anthracnose in cotton (Salustiano et al., 2014).

These fungi (*C. gossypii* var. *cephalosporioides* and *C. gossypii*) belong to the *Ascomycota* phylum, having as main feature the production of conidial mass with orange color in acervuli and conidia morphologically similar (Bailey et al., 1996). Both pathogens are transmitted by seeds and cause damages in cotton plants (Silva-Mann et al., 2005; Mehta and Mehta, 2010).

Colletotrichum taxonomy was subject of extensive discussion by the variability of species classified in this genus; so, there are difficulties in the identification and separation of these organisms. Traditionally, the identification of that genus' members was based on some morphological characteristics, with emphasis on morphometry of conidia, colony color, mycelial growth rate and pathogenicity (Bailey et al., 1996; Tozze-Júnior et al., 2006). Specifically for the *Colletotrichum* complex associated with cotton, it is not always possible to differ what are the pathogens involved in the symptomatology of Ramulosis and Anthracnose, as well as the different degrees of aggressiveness and symptoms (Carvalho et al., 2015).

Within the seed pathology, the detection and differentiation between *C. gossypii* var. *cephalosporioides* and *C. gossypii* were carried out by using the "blotter" method, in which the assessment is based on mycelial growth habit of fungi developed in seeds after an incubation period (Tanaka et al., 1996). In this case, the high morphological similarities and isolate variability of these fungi make the results of such analysis questionable and not always consistent (Silva-Mann et al., 2002; Mehta and Mehta, 2010), determining the need to develop more accurate and reliable methods for this task.

Accuracy in identification of *C. gossypii* var. *cephalosporioides* and *C. gossypii* is, thus, necessary and indispensable to diagnose and control the involved diseases, as well as demand for detection methods of these fungi in seed samples on laboratory routine activities (Carvalho et al., 2015).

Molecular techniques and DNA sequence analysis were important to distinguish and identify populations of organisms at different levels. Currently, the PCR technique is used for direct detection of fungi and other organisms in association with seeds (Lee et al., 2002; Munkvold, 2009; Barrocas et al., 2012). This technology was successful in detecting, for example, *Stenocarpella* complex (*S. maydis* and *S. macrospora*) in maize (Romero and Wise, 2015), *Fusarium oxysporum* f.sp. *phaseoli* in bean seeds (Sousa et al., 2015), *Sclerotinia sclerotiorum* in soybean seeds (Botelho et al., 2015) and *Corynespora cassiicola* in soybean seeds (Sousa et al., 2016).

This study aimed to design specific primer pair to detect *Colletotrichum gossypii* var. *cephalosporioides* in cotton seeds and establish a protocol for safer and more sensitive sanitary analysis in the detection of this pathogen by PCR, ensuring to the cotton producers a safer quality control and providing better protection for agricultural production environments in the country.

MATERIAL AND METHODS

Isolates obtention: Colletotrichum gossypii var. *cephalosporioides* isolates and other fungi species were obtained from the mycological collection of the Mycology Laboratory and of the Seed Pathology Laboratory of the *Universidade Federal de Lavras* (UFLA), in Lavras, MG, Brazil (Table 1).

DNA extraction: genomic DNA was extracted from monosporic cultures of isolates grown on potato dextrose agar (PDA) for five days. The mycelium was scraped and homogenized in liquid nitrogen, and the extraction was performed using the Wizard®Genomic DNA purification kit (Promega, Madison, WI), according to the DNA extraction protocol recommended by the manufacturer. DNA concentrations were estimated using the NanoDrop 2000 instrument and visually in 1.2% agarose gel, by comparison of band intensity with a fragment size marker of 1 kb (Invitrogen).

Table 1. Isolates of *Colletotrichum gossypii* var. *cephalosporioides* and others fungal species associated with cotton and others hosts used in the specificity test.

Species	CML[1]	Other code[2]	Geographic origine[3]	Host	Specific Primer[4]
C. gossypii var. *cephalosporioides*		LAPS 22	Maracaju, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 23	Maracaju, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 24	Tangará da Serra, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 32	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2371	LAPS 259	Santa Helena de Goiás, GO	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2372	LAPS 260	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2373	LAPS 261	Pedra Preta, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2374	LAPS 263	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2375	LAPS 264	Mineiros, GO	Gossypium hirsutum	+
C. gossypii var. *cephalosporioides*	2376	LAPS 265	Campo Verde, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2377	LAPS 266	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2378	LAPS 267	Mineiros, GO	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2379	LAPS 268	Mineiros, GO	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2380	LAPS 269	Nova São Joaquim, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2381	LAPS 270	Nova São Joaquim, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2382	LAPS 271	Pedra Preta, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2383	LAPS 272	Itiquira, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2384	LAPS 273	Itiquira, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2386	LAPS 275	Itiquira, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*	2387	LAPS 276	Itiquira, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 277	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 392	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 393	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 396	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 397	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 398	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		LAPS 400	Primavera do Leste, MT	*Gossypium hirsutum*	+
C. gossypii var. *cephalosporioides*		CGCUber	Uberlândia, MG	*Gossypium hirsutum*	+
Colletotrichum gossypii	2327	CG3LEM	Luís Eduardo Magalhães, BA	*Gossypium hirsutum*	-
Colletotrichum siamense sensu lato	2884	CCJ73	Campo Grande, PB	*Anacardium occidentale*	-
Colletotrichum tropicale	2888	CCJ105	Fortaleza, CE	*Anacardium occidentale*	-
Colletotrichum asianum	2893	CCJ204	São Luís, MA	*Anacardium occidentale*	-
Colletotrichum theobromicola	2931	MT68	Pacajus, CE	*Anacardium occidentale*	-
Colletotrichum truncatum		LAPS133	Rio Verde, GO	*Phaseolus vulgaris*	-
Colletotrichum gloeosporioides		CAA115/1	Acari, MG	*Annona reticulata*	-
Colletotrichum fructicola		CAA137	Acari, MG	*Annona crassiflora*	-

Continue...

Table 1. Continuation.

Species	CML[1]	Other code[2]	Geographic origine[3]	Host	Specific Primer[4]
Colletotrichum karstii		CAA81	Umbuzeiro, MG	*Annona crassiflora*	-
Colletotrichum gigasporum	3316	LabioMMi3311	Brazil	*Piper aducum*	-
Aspergillus flavus	2708		Montividiu, GO	Solo (*Gossypium hirsutum*)	-
Aspergillus clavatus	2734		Ibiá, MG	*Seed (Glycine max)*	-
Aspergillus chevalieri	2737		Lavras, MG	*Seed (Phaseolus vulgaris)*	-
Bipolaris sorokiniana	3315	LabioMMi285	São Carlos, SP	*Piper aducum*	-
Curvularia sp.		CTC15	Pará		-
Alternaria alternata	3314	LabioMMi06	Brazil		-
Diaphorte sp.		LAPS559	São Paulo, SP	*Glycine max*	-
Phoma tarda	716		Campanha, MG	*Coffea arabica*	-
Phoma exígua	940		Coromandel, MG	*Coffea arabica*	-
Penicillium citrunum	3310	LabioMMi249	Teresina, PI		-
Penicillium terrigenum	1226		Montividiu, GO	*Gossypium hirsutum*	-
Fusarium oxysporum f. sp. *vasinfectum*	1119		Mato Grosso	*Gossypium hirsutum*	-
Ascochyta sp.	361		Lavras, MG	*Baccharis* sp.	-
Phomopsis sp.		FEL89	Brazil		-
Clonostachys roseum		CSO36	Brazil		-
Cercospora sp.		LAPS255	Campo Verde, MT	*Glycine max*	-
Fusarium paranaense	1830		Brazil	*Glycine max*	-
Didymella sp.	193		Machado, MG	*Coffea arabica*	-
Macrophomina sp.		MA01	Primavera do Leste, MT	*Glycine max*	-
Corynespora cassiicola		LAPS467	São Paulo, SP	*Glycine max*	-
Sclerotinia sp.		LAPS242	Uberlândia, MG	*Glycine max*	-

[1]CML = mycological collection of the Plant Pathology Department, *Universidade Federal de Lavras*, Lavras, MG, Brazil.
[2]LAPS = mycological collection of the Seed Pathology Laboratory, *Universidade Federal de Lavras*, Lavras, MG, Brazil.
[2]LaBioMMi = Microorganisms Micromolecular Biochemistry Laboratory, Chemistry Department, *Universidade Federal de São Carlos*, São Carlos, SP, Brazil.
[3]States of Brazil: BA = Bahia; CE = Ceará; GO = Goiás; MG = Minas Gerais; MA = Maranhão; MT = Mato Grosso; PB = Paraíba; PI = Piauí; SP = São Paulo.
[4]Specific primer; (+) PCR amplification; (-) no PCR amplification.

Development of specific primers for detection and identification of C. gossypii var. *cephalosporioides:* alignments generated from the sequences of the work of Salustiano et al. (2014), using ClustalW implemented by MEGA5 (Tamura et al., 2011), were obtained for the partial DNA of glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) of Cgc isolates and other species from the *C. gloesporioides* species complex. Unique sites in the sequences of the Ramulosis' etiologic agent were identified and used to design species-specific primers. The primer sequences were compared using the BLAST program in order to verify its homology with sequences previously deposited in GenBank (https://www.ncbi.nlm.nih.gov/) (Table 2). The developed primer pair was analyzed for performance characteristics such as hairpin structure, potential self-dimer formation and stability of 3 termini, using OligoAnalyzer 3.1 integrated platform (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). The primers' synthesis was performed by Sigma-

Aldrich Brazil LTD. The genomic material isolated from *C. gossypii* var. *cephalosporioides* was subjected to PCR analysis.

Determining primer specificity: the specificity of the primer pair was tested by PCR amplification of genomic DNA of 28 Cgc's isolates, ten isolates of *Colletotrichum's* other species and 21 isolates of other fungal species, which were reported in cotton seed and other host (Table 1). PCR was performed using 25 µL mix for PCR OneTaq (BioLabs), containing 10 pmol of forward and reverse primers and DNA 10 ng. The DNA amplification was performed under the following cycle conditions: 94 °C for four minutes (initial denaturation), 94 °C for 45 seconds (denaturation), 65 °C for 45 seconds (annealing), 72 °C for one minute (extension), and 34 cycles of 72 °C for ten minutes (final extension). To separate PCR products, an aliquot of 10 µL was used on 1.2% agarose gel, stained with GelRed® (Biotium®, Hayward, 95 CA, USA). The PCR products were observed in UV transilluminator, L-Pix HE equipament (Loccus Biotechnology, Brazil). Before using the specific primers, a PCR reaction was performed using universal GDF primers GDF (5´- GCCGTCAACGACCCCTTCATTGA-3') and universal GDR primers GDR (5´- GGGTGGAGTCGTACTTGAGCATGT- 3') (Templeton et al., 1992), with the genomic DNA of all species used in this study to test if the genomic DNA was adequate for PCR amplification. The experiments were repeated at least two times.

Sensivity evaluation of primers developed in seed samples: to evaluate the sensitivity of PCR reaction using primer pair, cotton seed with different infestation level inoculated with *C. gossypii* var. *cephalosporioides* was used, and a four-hundred-seed sample were prepared by mixing the artificially inoculated seeds with healthy seeds generating three infestation level (100%, 10% and 1%) per inoculum. For each infestation level of seeds, the test was performed in four replicates, and the experiment was repeated twice.

Table 2. GenBank accession numbers of *Colletotrichum gossypii* var. *cephalosporioides* and other species from the *C. gloesporioides* species complex used to obtain specific primer pair to Cgc.

Species	CML[1]	Other code[2]	Host	Origin[3]	GenBank number
C. gossypii var. cephalosporioides	2373	LAPS 261	*Gossypium hirsutum*	Pedra Preta, MT	JX847009
C. gossypii var. cephalosporioides	2379	LAPS 268	*Gossypium hirsutum*	Mineiros, GO	JX847010
C. gossypii var. cephalosporioides	2384	LAPS 273	*Gossypium hirsutum*	Itiquira, MT	JX847011
C. gossypii var. cephalosporioides	2388	IAC 13350	*Gossypium hirsutum*	Piracicaba, SP	JX847012
C. gossypii var. cephalosporioides	2389	IAC 12405	*Gossypium hirsutum*	Ituverava, SP	JX847013
Colletotrichum gossypii	2324	IAC 1025	*Gossypium hirsutum*	Campinas, SP	JX847014
Colletotrichum gossypii	2325	CG 1 LEM	*Gossypium hirsutum*	Luis Eduardo Magalhães, BA	JX847015
Colletotrichum gossypii	2327	CG 3 LEM	*Gossypium hirsutum*	Luis Eduardo Magalhães, BA	JX847016
C. kahawae subsp. kahawae		ICMP 17905	*Coffea arabica*	Kenya	JX010012
Colletotrichum gloeosporioides		IMI 356878	*Citrus sinensis*	Italy	JX010056
Colletotrichum fructicola		ICMP 18581	*Coffea arabica*	Thailand	JX010033
Colletotrichum siamense		ICMP 18578	*Coffea arabica*	Thailand	JX009924
Colletotrichum asianum		ICMP 18580	*Coffea arabica*	Thailand	JX010053
Colletotrichum theobromicola		ICMP 17958	*Stylosanthes guianensis*	Australia	JX009948
Colletotrichum boninense		CBS 112115	*Leucospermum* sp.	Australia	JQ005247

[1]CML = mycological collection of the Plant Pathology Department, *Universidade Federal de Lavras*, Lavras, MG, Brazil.
[2]LAPS = mycological collection of the Seed Pathology Laboratory, *Universidade Federal de Lavras*, Lavras, MG, Brazil.
IAC = Campinas Agronomic Institute, Campinas, SP, Brazil.
ICMP = International Collection of Microorganisms from Plants, New Zeland.
CBS = Centralalbureau voor Schimmelcultures, Utrecht, The Netherlands.
[3]States of Brazil: BA = Bahia; GO = Goiás; MT = Mato Grosso; SP = São Paulo.

Seed inoculation: cotton seeds CV delta opal susceptible to the Ramulosis' etiologic agent were disinfected in 70% alcohol for one minute, followed by 1% of sodium hypochlorite solution for two minutes, then washed four times with autoclaved distilled water. The sterilized seeds were arranged in trays where they remained for 24 hours at room temperature to complete drying. After drying, it was used physiological conditioning method or water restriction for seed inoculation (Machado et al., 2012; Barrocas et al., 2014).

Then, the seeds were artificially inoculated with the *C. gossypii* var. *cephalosporioides* strain CML2374 that growed in petri dishes with fifteen cm diameter containing PDA medium, modified by the addition of manitol adjusted with water potential of -1.0 MPa, as SPPM Software (computer program that relates solute potencial to solution composition). A sequence of data was generated over temperature, concentration, or potential ranges by specifying an initial value (Michel and Radcliffe, 1995), remaining seven days in BOD at 25 °C with a photoperiod of twelve hours. The seeds were placed in a single layer on the fungus colony, where they remained for 24 and 48 hours, being removed and placed in sterilized trays and dried in a laminar flow chamber for 24 hours. As controls, seeds were used without the fungus and with incubation in substrate with water restriction.

DNA extraction of seed samples: the inoculated seed samples were macerated in mill (IKA® A11 analytical basic mill) with liquid nitrogen to obtain a thin powder. Samples with 0.04 g of this powder were placed in 1.5 mL microtubes in four replicates. The extraction was carried out with the use of Wizard®Genomic DNA purification kit (Promega, Madison, WI), according to the DNA extraction protocol recommended by the manufacturer. The PCR reaction and the cycle conditions were the same described for the specificity of the primer pair.

RESULTS AND DISCUSSION

Colletotrichum gossypii var. *cephalosporioides* specific primers designed from the GAPDH gene had the following sequences: CGC1F (5'- CAG ACT ACA AGG CCA ACG C- 3') and CGC1R (5'- GAG TCG TAC TTG AGC ATG TAG- 3'). This primer pair amplifies a fragment of 140bp. This primers' pair specifically amplified DNA of only its respective target, Cgc*,* in all reactions (Figure 1A). The primers did not cross-react with DNA of any other *Colletotrichum* species or other fungal species tested (Figure 1B and Table 1).

The sensitivity of the primers' pair may be considered high due to their capacity of detecting the pathogen in seed samples with minimal incidence of 1%, which was the limit used in this study. In the controls, there was no amplification of the genomic DNA from the causative agent of cotton Ramulosis (Figure 2).

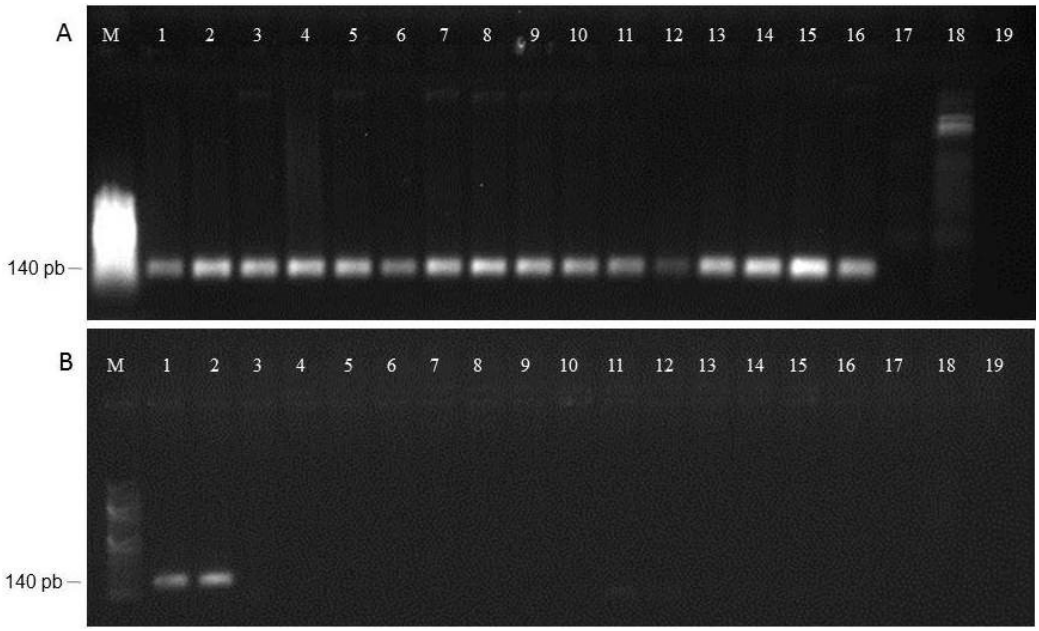
A PCR-based diagnostic assay using specific primers derived from the gene encoding the glyceraldehyde 3-phosphate dehydrogenase was developed for the Ramulosis' causal agent from cotton, *C. gossypii* var. *cephalosporioides.* Furthermore, the primers were able to detect the pathogen in artificially infested cotton seeds.

The PCR products obtained from the seeds showed characteristic bands, as observed in the pathogen's DNA amplification in pure cultures. Thus, it was evident that the primer pair was effective in detecting the Ramulosis' etiological agent in artificially infested cotton seeds, indicating no false positive result for contamination. These primer pair allowed the amplification of the genomic DNA samples from the *C. gossypii* var. *cephalosporioides* tested, being effective in detection of fungal incidences from 1 to 100% at different inoculum potential tested.

In a study conducted by Guimarães et al. (2017), the pair of primers designed and described was used to quantify *C. gossypii* var. *cephalosporioides* in artificially inoculated cotton seeds by cPCR and qPCR techniques. The results showed that the primers used were reliable. Primers showed linearity in the standard curve generated by qPCR technique at each dilution level of Cgc DNA extracted from pure culture. The quantification of the inoculum potential by qPCR was 1.44 pg/ µL DNA at P24, which increases to 6.89 pg/ µL at P48 and 24.5 pg/ µL at P96. The authors concluded that there was proportionality between fungal DNA, inoculum potential, effects on germination and seed vigor.

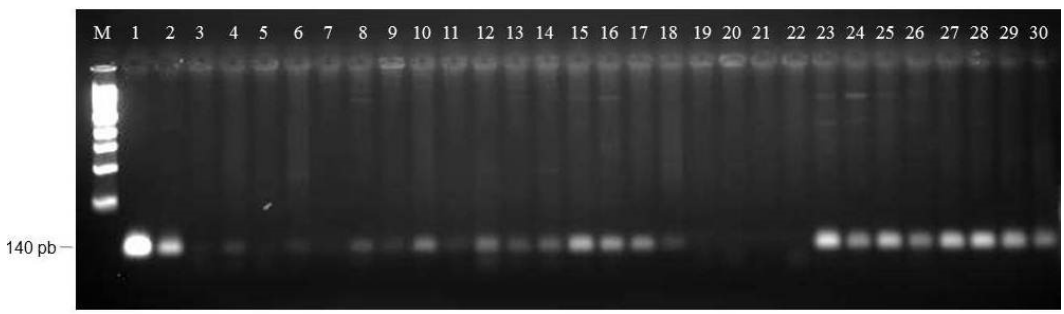
For other pathosystems, the sensitivity in detecting seeds' pathogens is variable. For example, in a study conducted by Barrocas et al. (2012), *Sternocarpella* was detected in maize seeds infected with minimal incidence of 2% in the studied samples. In a study conducted by Sousa et al. (2015), *Fusarium oxysporum* f. sp. *phaseoli* fungus was detected

in lower levels of infection, and 0.25% incidence in beans seeds. One possibility of increasing the PCR sensitivity is prior incubation in favorable conditions for the development of fungi in seeds. Other example, cPCR and qPCR techniques were effective in detecting *Colletotrichum lindemuthianum* in beans seeds. It was possible using cPCR to detect the fungus in seed samples with 10% of incidence and with 0.25% incidence by qPCR technique (Gadaga et al., 2018).



A: lane M-100-bp marker (Axygen); lanes 1-16 (positive control): *Colletotrichum gossypii* var. *cephalosporioides* (LAPS22, LAPS23, LAPS32, CML2371, CML2372, CML2373, CML2374, CML2375, CML2376, CML2377, CML2378, CML2381, CML2382, CML2383, CML2384, CML2386); lanes 17-19 (negative control): lane 17: CML1119 (*Fusarium oxysporum* f. sp. *vasinfectum*); lane 18: MA01 (*Macrophomina* sp.); lane 19: water. B: lane M-100-bp marker (Axygen); lanes 1 and 2 (positive control): *C. gossypii* var. *cephalosporioides* (CML2374 e 2379); lanes 3-19 (negative control): lane 3: *C. gossypii* (CML2327); 4-12: other species of *Colletotrichum* (CML2884, CML2888, CML2893, CML2931, LAPS133, CAA115/1, CAA137, CAA81, CML3316); 13-19: other fungal species (CML2708, CML2734, CML2737, CML3315, CTC15, CML3314, LAPS559).

Figure 1. Specificity test of conventional PCR with primer pair CGC1F/ CGC1R.



Lane M: 50kb marker; lanes 1 and 2 - Cgc - CML2384 and 2374 isolates; lanes 3 to 6: 1% infection with seeds inoculated for 24 hours; lanes 7 to 10: 10% infection with seeds inoculated for 24 hours; lanes 11 to 14: 1% infection with seeds inoculated for 48 hours; lanes 15 to 18: 10% infection with seeds inoculated for 48 hours; lanes 19 to 22: control without fungus; lanes 23 to 26: 100% seeds infected with inoculation of 24 hours; lanes 27 to 30: 100% seeds infected with inoculation of 48 hours.

Figure 2. Sensitivity test of conventional PCR with primer pair CGC1F/ CGC1R in the detection of *Colletotrichum gossypii* var. *cephalosporioides* in samples of cotton seeds with different infection levels.

CONCLUSIONS

The results of this study, which complement previous work done by the pathologist group involved in this project in order to detect the causal agent of cotton Ramulosis in seed samples, meet a long-year demand from seed producers in Brazil. This technology enables a sanitary quality control of cotton seeds with greater accuracy and speed, making health analysis of seeds, which is viable and extremely important for the cotton producers.

It is also important to point out that, in practical terms, the health test protocol for the detection of *C. gossypii* var. *cephalosporioides* in cotton seed samples for quality certification programs can be made by implementing a health test by two methods, a molecular and a biological. In this case, samples would be initially subjected to PCR and subsequently applying the blotter test, as it was done by the current Rules for Seed Testing (Brasil, 2009a, b) for samples that had positive results in molecular testing. It is understood that combining these two methods makes the diagnosis of Ramulosis' agent in cotton seed samples safer and feasible from an operational point of view on health routine analytical laboratories.

ACKNOWLEDGEMENTS

The authors thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for providing the scholarship for the first author. To the CNPq, the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG) for funding and supporting for research.

REFERENCES

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Manual de análise sanitária de sementes*. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília: MAPA/ACS, 2009a. 200p. http://www.agricultura.gov.br/assuntos/insumos-agropecuarios/insumos-agricolas/sementes-e-mudas/publicacoes-sementes-e-mudas/manual-de-analise-sanitaria-de-sementes/view

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Regras para análise de sementes*. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília: MAPA/ACS, 2009b. 395p. http:// www.agricultura.gov.br/arq_editor/file/2946_regras_analise__sementes.pdf

BAILEY, J.A; NASH, C.; MORGAN, L.W.; O'CONNELL, R.J.; TEBEEST, D.O. Molecular taxonomy of *Colletotrichum* species causing Anthracnose on the Malvaceae. *Phytopathology*, v.86, p.1076-1083, 1996. https://www.apsnet.org/publications/phytopathology/backissues/Documents/1996Articles/Phyto86n10_1076.PDF

BARROCAS, E.N.; MACHADO, J.C.; ALMEIDA, M.F.; BOTELHO, L.S.; VON PINHO, E.V.R. Sensibility of the PCR technique in the detection of *Stenocarpella* sp. associated to maize seeds. *Revista Brasileira de Sementes*, v.34, n.2, p.218-224, 2012. http://www.scielo.br/pdf/rbs/v34n2/05.pdf

BARROCAS, E.N.; MACHADO, J.C.; ALVES, M.C.; CORREA, C.L. Desempenho de sementes de algodão submetidas à deficiência hídrica e presença de *Colletotrichum gossypii* var. *cephalosporioides*. *Bioscience Journal*, v.30, n.2, p.421-428, 2014. http://www.seer.ufu.br/index.php/biosciencejournal/article/view/17993/13757

BOTELHO, L.S.; BARROCAS, E.N.; MACHADO, J.C.; MARTINS, R.S. Detection of *Sclerotinia sclerotiorum* in soybean seeds by conventional and quantitative PCR techniques. *Journal of Seed Science,* v.37, n.1, p.55-62, 2015. http://dx.doi.org/10.1590/2317-1545v37n1141460

CARVALHO, E.M.; FIGUEIRA, A.R.; MACHADO, J.C.; ARAÚJO, D.V.; MACHADO, C.F. Variability of seed-borne *Colletotrichum* strains in cotton based on its1 and its2 ribossomal genes analysis. *Bioscience Journal*, v.31, n.3, p.691-700, 2015. https://doi.org/10.14393/BJ-v31n3a2015-23126

GADAGA, S.J.C.; SIQUEIRA, C.S.; MACHADO, J.C. Molecular detection of *Colletotrichum lindemuthianum* in bean seed samples. *Journal of Seed Science*, v.40, n.4, p.370-377, 2018. http://dx.doi.org/10.1590/2317-1545v40n4192761

GUIMARÃES, M.R.F.; SIQUEIRA, C.S.; MACHADO, J.C.; FRANÇA, S.K.S.; GUIMARÃES, G.C. Evaluation of inoculum potential of pathogens in seeds: relation to physiological quality and DNA quantification by qPCR. *Journal of Seed Science*, v.39, n.3, p.224-233, 2017. http://www.scielo.br/pdf/jss/v39n3/2317-1545-jss-39-03-00224.pdf

LEE, H.K.; TEWARI, J.P.; TURKINGTON, T.K. Quantification of seedborne infection by *Rhinchosporium secalis* in barley using competitive PCR. *Plant Pathology*, v.51, p.217-224, 2002. https://bsppjournals.onlinelibrary.wiley.com/doi/pdf/10.1046/j.1365-3059.2002.00685.x

MACHADO, J.C.; BARROCAS, E.N.; COSTA, L.N.; GUIMARÃES, R.M.; MACHADO, C. Uso da técnica de restrição hídrica ou condicionamento osmótico em patologia de sementes. *Revisão Anual de Patologia de Plantas*, v.20, p.37-63, 2012.

MEHTA, Y.R.; MEHTA, A. Variabilidade genética entre isolados de *Colletotrichum gossypii* do algodoeiro. *Summa Phytopathologica*, v.36, n.1, p.40-44, 2010. http://www.scielo.br/pdf/sp/v36n1/07.pdf

MICHEL, B.E.; RADCLIFFE, D.A. Computer program relating solute potencial to solution composition for five solutes. *Agronomy Journal*, v.87, p.131-136, 1995.

MUNKVOLD, G.P. Seed pathology progress in academia and industry. *Annual Review of Phytopathology,* v.47, p.285-311, 2009. https://doi.org/10.1146/annurev-phyto-080508-081916

ROMERO, M.P.; WISE, K.A. Development of molecular assays for detection of *Stenocarpella maydis* and *Stenocarpella macrospora* in corn. *Plant Disease*, v.99, p.761-769, 2015. http://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-09-14-0917-RE

SALUSTIANO, M.E.; RONDON, M.N.; ABREU, L.M.; COSTA, S.S.; MACHADO, J.C.; PFENNING, L.H. The etiological agent of cotton Ramulosis represents a single phylogenetic lineage within the *Colletotrichum gloeosporioides* species complex. *Tropical Plant Pathology*, v.39, n.5, p.357-367, 2014. http://www.scielo.br/pdf/tpp/v39n5/v39n5a02.pdf

SILVA-MANN, R.; SALGADO, K.C.C.; VIEIRA, M.G.G.C.; MACHADO, J.C. Variabilidade genética de isolados do complexo *Colletotrichum* associados a sementes de algodoeiro, por meio de técnicas moleculares e inoculação em plantas. *Fitopatologia Brasileira*, v.27, n.1, p.27-32, 2002. http://www.scielo.br/pdf/fb/v27n1/8464.pdf

SILVA-MANN, R.; VIEIRA, M.G.G.C.; MACHADO, J.C.; BERNARDINO-FILHO, J.R.; SALGADO, K.C.C.; STEVENS, M.R. AFLP markers differentiate *Colletotrichum gossypii* from *C. gossypii* var. *cephalosporioides*. *Fitopatologia Brasileira*, v.30, n.2, p.169-172, 2005. http://www.scielo.br/pdf/fb/v30n2/a11v30n2.pdf

SOUSA, M.V.; SIQUEIRA, C.S.; MACHADO, J.C. Conventional PCR for detection of *Corynespora cassiicola* in soybean seeds. *Journal of Seed Science*, v.38, n.2, p.85-91, 2016. http://www.scielo.br/pdf/jss/v38n2/2317-1545-jss-v38n2152049.pdf

SOUSA, M.V.; MACHADO, J.C.; SIMMONS, H.E.; MUNKVOLD, G.P. Real-time quantitative PCR assays for the rapid detection and quantification of *Fusarium oxysporum* f. sp. *phaseoli* in *Phaseolus vulgaris* (common bean) seeds. *Plant Pathology*, v.64, p.478-488, 2015. https://bsppjournals.onlinelibrary.wiley.com/doi/epdf/10.1111/ppa.12257

TAMURA, K.; PETERSON, D.; PETERSON, N.; STECHER, G.; NEI, M.; KUMAR, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution*, v.28, n.10, p.2731-2739, 2011. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3203626/pdf/msr121.pdf

TANAKA, M.A.S.; MENTEN, J.O.M.; MACHADO, J.C. Hábito de crescimento de *Colletotrichum gossypii* e *C. gossypii* var. *cephalosporioides* em sementes de algodoeiro. *Bragantia*, v.55, n.1, p.95-104, 1996. http://www.scielo.br/pdf/brag/v55n1/11.pdf

TEMPLETON, M.D.; RIKKERINK, E.H.A.; SOLON, S.L.; CROWHURT, R.N. Cloning and molecular characterization the glyceraldehyde-3-phosphate dehydrogenase-encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. *Gene*, v.122, n.1, p.225-230, 1992. https://doi.org/10.1016/0378-1119(92)90055-T

TOZZE-JÚNIOR, H.J.; BUENO, C.R.N.C.; MASSOLA-JÚNIOR, N.S. Caracterização morfológica e molecular de isolados de *Colletotrichum* spp. de hortaliças solanáceas. *Summa Phytopathologica,* v.32, n.1, p.71-79, 2006. http://www.scielo.br/pdf/sp/v32n1/v32n1a11.pdf