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

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**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.

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**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.
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 | Other code | Geographic origine | Host                   | Specific Primer |
|----------------------------------------------|-----|------------|--------------------|------------------------|-----------------|
| *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    | Tangarã 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 asiamum                      | 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                             | CML1 | Other code2 | Geographic origine3 | Host                                      | Specific Primer4 |
|-------------------------------------|------|-------------|---------------------|-------------------------------------------|-----------------|
| Colletotrichum karstii              |      | CAA81       | Umbuzeiro, MG       | Annona crassiflora                       | -               |
| Colletotrichum gigasporum           | 3316 | LabioMMi3311| Brazil              | Piper aduncum                            | -               |
| 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              |                                            | -               |
| Diaporthe sp.                     |      | LAP5559     | São Paulo, SP       | Glicyne max                              | -               |
| Phoma tardo                        | 716  | Campanha, MG| Coffea arabica      |                                            | -               |
| Phoma exigua                       | 940  | Coromandel, MG| Coffea arabica      |                                            | -               |
| Penicillium citruntum             | 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.                    |      | LAP255      | Campo Verde, MT     | Glicyne max                              | -               |
| Fusarium paranaense               | 1830 | Brazil      | Glicyne max         |                                            | -               |
| Didymella sp.                     | 193  | Machado, MG | Coffea arabica      |                                            | -               |
| Macrophomina sp.                  |      | MA01        | Primavera do Leste, MT| Glicyne max                           | -               |
| Corynespora cassiiicola           |      | LAP247      | São Paulo, SP       | Glicyne max                              | -               |
| Sclerotinia sp.                   |      | LAP242      | Uberlândia, MG      | Glicyne max                              | -               |

1CML = mycological collection of the Plant Pathology Department, Universidade Federal de Lavras, Lavras, MG, Brazil.
2LAPS = mycological collection of the Seed Pathology Laboratory, Universidade Federal de Lavras, Lavras, MG, Brazil.
3LaBioMMi = Microorganisms Micromolecular Biochemistry Laboratory, Chemistry Department, Universidade Federal de São Carlos, São Carlos, SP, Brazil.
4States of Brazil: BA = Bahia; CE = Ceará; GO = Goiás; MG = Minas Gerais; MA = Maranhão; MT = Mato Grosso; PB = Paraíba; PI = Piauí; SP = São Paulo.
5Specific 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 MEGAS (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-

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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.

**Sensitivity 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 | CML¹ | Other code² | Host | Origin³ | 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 |
| *Colletotrichum gossypii* | 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 asiaman* | ICMP 18580 | | *Coffea arabica* | Thailand | JX010053 |
| *Colletotrichum theobromicola* | ICMP 17958 | | *Stylosanthes guianensis* | Australia | JX009948 |
| *Colletotrichum boninense* | CBS 112115 | | *Leucospermum* sp. | Australia | JQ005247 |

¹CML = mycological collection of the Plant Pathology Department, Universidade Federal de Lavras, Lavras, MG, Brazil.

²LAPS = mycological collection of the Seed Pathology Laboratory, Universidade Federal de Lavras, Lavras, MG, Brazil.

³IAC = Campinas Agronomic Institute, Campinas, SP, Brazil.

ICMP = International Collection of Microorganisms from Plants, New Zeland.

CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

³States 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 grew 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 potential 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.
 Specific primers to detection of *Colletotrichum gossypii* var. *cephalosporioides*

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).

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

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.

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