Journal of Seed Science
ISSN 2317-1545
www.abrates.org.br/revista

Journal of Seed Science, v.40, n.4, p.370-377, 2018
http://dx.doi.org/10.1590/2317-1545v40n4192761

Molecular detection of *Colletotrichum lindemuthianum* in bean seed samples[1]

Stélio Jorge Castro Gadaga[2], Carolina da Silva Siqueira[2*], José da Cruz Machado[2]

ABSTRACT - *Colletotrichum lindemuthianum* is the causal agent of anthracnose in common bean, and infected seeds are the most typical propagation form of the disease. Thus, using common bean seeds free of *C. lindemuthianum* is crucial to managing this pest, as well as employing fast and accurate detection techniques to ensure high seed quality. In this study, both conventional and quantitative PCR techniques (cPCR and qPCR) were used for the detection and quantification of *C. lindemuthianum* in samples of common bean seeds. For that, seeds were inoculated by exposing them to fungal colonies for different periods of time, 0 h, 36 h, 72 h, 108 h and 144 h, each period corresponding to an inoculum potential. Then, they were mixed with healthy seeds, so incidences of 0.25%, 0.50%, 1%, 10%, and 100% of seeds with different inoculum potentials were obtained, in samples of 400 seeds. Both cPRC and qPCR techniques were effective in detecting the fungus. With the cPCR method, the highest sensitivity was recorded in those samples with 10% inoculated seeds with inoculum potential P36. On the other hand, with the qPCR technique, the highest sensitivity in detecting the fungus was observed in samples with 0.25% inoculated seeds with inoculum potential P36.

Index terms: *Phaseolus vulgaris*, anthracnose, PCR.

Detecção molecular de *Colletotrichum lindemuthianum* em amostras de sementes de feijão

RESUMO - *Colletotrichum lindemuthianum* é o agente causal da antracnose do feijoeiro, tendo a semente como uma forma de disseminação. O uso de sementes de feijão livres do patógeno é uma das estratégias de manejo, e o uso de técnicas de detecção precisas e rápidas torna-se indispensável para a análise sanitária prévia de sementes. Neste estudo, as técnicas de PCR convencional (cPCR) e PCR em tempo real (qPCR) foram utilizadas para a detecção e quantificação de *C. lindemuthianum* em amostras de sementes de feijão. Para isso, sementes foram inoculadas por meio de sua exposição às colônias do fungo por tempos variados, 0, 36, 72, 108, e 144 horas, cada período correspondendo a um potencial de inóculo, P0, P36, P72, P108 e P144. As sementes inoculadas foram misturadas com sementes sadias, obtendo-se incidências de 0,25% 0,50%, 1%, 10% e 100% de sementes para cada potencial de inóculo em amostras de 400 sementes. Tanto a cPRC como a qPCR foram eficazes na detecção do fungo. A partir das amostras de sementes com 10% de incidência e com P36, foi possível detectar o fungo por cPCR. Pela técnica de qPCR o patógeno foi detectado em amostras a partir da incidência de 0,25% no menor potencial de inóculo P36.

Termos para indexação: *Phaseolus vulgaris*, antracnose, PCR.

Introduction

Widely spread in Brazil, the common bean crop is susceptible to several diseases, such as anthracnose (*Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi and Cav.), fusarium wilt (*Fusarium oxysporum* f. sp. *phaseoli*), rust (*Uromyces phaseoli*), and bean blight (*Xanthomonas axonopodis* pv. *phaseoli*). Among those caused by fungi, anthracnose is a great concern nowadays, not only because of its economic importance, but also due to the capacity of devastating crops within a short time, under favorable environmental conditions. Yield losses usually oscillate between 20 and 50%; but cases in which entire crops were compromised have also been reported (Santana and Mahuku, 2002).

[1]Submitted on 03/12/2018. Accepted for publication on 08/26/2018.
[2] Departamento de Fitopatologia, Universidade Federal de Lavras, 37200-000 – Lavras, MG, Brasil.
*Corresponding author <kerolpet@gmail.com>

The seed is one of the most important forms of survival and long-distance dissemination used by the fungus. This fact increases the importance of the quality analyses of seeds, once it can indicate the presence of *C. lindemuthianum* in samples. Damasceno Silva et al. (2007) report that approximately 50 physiological races of *C. lindemuthianum* have been identified in Brazil, among which 65, 73, and 81 stand out as the most frequent ones, considering the virulence.

According to the literature, the occurrence of *C. lindemuthianum* in bean seeds was first described in Brazil by incubating seeds in an agar medium containing sodium 2,4-dichlorophenoxyacetate at a 50-ppm concentration. The paper roll method was proposed by Anselme and Champion (1981) and made official by ISTA (1985). This procedure has been successfully used in many countries, and it is part of the *Manual de Análise Sanitária de Sementes no Brasil* (Brasil, 2009b). In that case the assessment is based on symptoms produced on the cotyledons of seeds carrying the pathogen. Nevertheless, despite being specific and sensitive as a biologic method, the paper roll technique presents some difficulties of evaluation.

Due to the necessity of fast obtaining sensitive and reliable results, molecular methods have arisen as promising alternatives for detecting *C. lindemuthianum* in bean seed lots, especially for routine analyses. The conventional PCR technique has already been used to diagnose anthracnose in bean plant tissues (Coêlho et al., 2016; Chen et al., 2007; Chen et al., 2013; Barcelos et al., 2014; García- Serrano et al., 2008; Wang et al., 2008).

The present work aimed to evaluate the viability of detecting *C. lindemuthianum* in common bean seed samples through the conventional PCR and real-time PCR, emphasizing the sensitivity of these techniques.

Material and Methods

The experiment was carried out in the Laboratory of Seed Pathology of the Department of Plant Pathology, at the *Universidade Federal de Lavras* (UFLA).

Source and multiplication of the fungus and characterization of the seeds

For this study, the isolate LV238 of *C. lindemuthianum*, race 65, was used. It was provided by the Laboratory of Plant Resistance of the Department of Biology, at UFLA. The isolate was cultivated in 15-cm diameter Petri dishes containing M3 medium (10 g of sucrose, 20 g of agar, 2 g of $KH_2(PO_4)$, 1 g of $MgSO_4.7H_2O$, 6 g of neopeptone/peptone, 4 mL of panvit, and 1 mL of chloramphenicol; all diluted in one liter of distilled water) (Damasceno Silva et al., 2007). They were maintained in a BOD incubator at 20±2 °C, with a 12-h photoperiod for seven days. Other isolates obtained from bean seeds, namely *Sclerotinia sclerotiorum*, *Fusarium oxysporum* f. sp. *phaseoli* and *Rhizoctonia solani*, from the mycological collection of the Laboratory of Seed Pathology at UFLA, were also multiplied in similar conditions to those previously described, but using PDA (potato, dextrose, agar) as culture medium instead.

The experiment used seeds of the cultivar *Pérola*, from the group *Carioca*, which are susceptible to *C. lindemuthianum*. The physiological and pathological analyses as specified by Rules for Seed Testing (Brasil, 2009a) showed a germination rate of 95.5%, and an incidence of *Aspergillus flavus* and *Penicillium* sp. below 0.5%, assessed by the health test, described in the *Manual de Análise Sanitária de Sementes* (Brasil, 2009b).

Seed inoculation and preparation of the samples

Bean seeds inoculated with *C. lindemuthianum* were obtained through the osmotic conditioning methodology proposed by Machado et al. (2012). First, they were disinfested with 1% sodium hypochlorite, for 1 minute, and then rinsed three times with distilled water. After that, they were dried over two germitest paper sheets, placed inside plastic trays and let under laboratory environment for 48 h.

Fungus colonies aged seven days were cultivated in Petri dishes containing M3 medium added of mannitol, with the osmotic potential adjusted to -1.0 MPa (Michel and Radcliffe, 1995). The seeds were evenly distributed in a single layer and kept in a BOD incubator at 20 ± 2 °C, with a 12-h photoperiod. They were exposed to the fungus colonies for periods of 0, 36, 72, 108, and 144 h, which corresponded to the inoculum potentials P0, P36, P72, P108, and P144, respectively, as described by Machado et al. (2004). After each period of contact between fungus and seeds, the latter were transferred from the Petri dishes to plastic trays containing two dry germitest paper sheets, where they stayed for 48 h at room temperature.

The inoculated seeds were then mixed with healthy ones at specified quantities (Table 1) to obtain various incidence levels (0.25%, 0.50%, 1%, 10%, and 100%) of inoculated/infected seeds, with different inoculum potentials (P0, P36, P72, P108, and P144), within a sample of 400 seeds.

DNA extraction

First, in order to extract the DNA from pure *C. lindemuthianum* cultures, the fungus was cultivated in 15-cm diameter Petri dishes with the M3 medium for seven days. The mycelium was carefully scraped off with a scalpel blade (Solidor) and then ground with liquid nitrogen in a mortar, until it formed a powder. An amount of 40 mg of this

material was transferred to 1.5-mL microtubes. Samples of other fungi that naturally occur in bean seeds, to be specific *S. sclerotiorum*, *F. oxysporum* f. sp. *phaseoli*, and *R. solani*, were also prepared following the same procedures.

To extract the fungal DNA from the bean seeds mixed as described in Table 1, an A11 Basic IKA mill was employed to ground each sample individually with liquid nitrogen. The maceration of samples proceeded in crescent order of incidence level to prevent contamination and, after each round, the mill was cleaned and disinfested with 70% alcohol. At the end of the procedure, three subsamples of 40 g were transferred to 1.5-mL microtubes. Samples of healthy seeds were also macerated.

All DNA extractions, either from pure cultures or bean seed samples, were performed with the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), following the manufacturer's guidelines.

DNA quality was checked in 1% agarose gel and 1xTBE buffer solution (40 mM tris-borate, 1 mM EDTA, pH 8.0). The samples were stained with GelRed® (Biotum) and visualized with an L-PIX UV transilluminator (Loccus Biotecnologia, Brazil). The DNA was quantified with a NanoDrop 3300 spectrophotometer (Thermo Scientific).

Conventional PCR and real-time PCR

Specific primers, previously described in the literature, were used for the detection of *C. lindemuthianum*, both by conventional PCR (cPCR) and real-time PCR (qPCR) (Table 2). The amplification of the 638 base pair fragments, by the cPCR technique, and their quantification, by qPCR, followed the reaction procedures and cycle conditions described by Wang et al. (2008) and Chen et al. (2013), respectively.

The amplifications by cPCR were performed with a Multigene thermocycler (Labnet, NJ, USA). Then, each product was evaluated in 1% agarose gel, using GelRed® (Biotium) as a stain for observation in an L-PIX UV transilluminator (Loccus Biotecnologia, Brazil). For the qPCR, a Rotor-Gene 6500 thermocycler (Corbett Research, Mortlake, Australia) and the software Rotor-Gene version 1.7.75 (Corbett) were used.

Evaluation of specificity and sensitivity of the conventional PCR and real-time PCR in detecting C. lindemuthianum race 65

After the DNA extraction from *C. lindemuthianum* pure cultures and other bean-contaminant fungi (*S. sclerotiorum*, *F. oxysporum* f. sp. *phaseoli*, and *R. solani*), tests were conducted to confirm the specificity of the primers CD1 and CD2, used in cPCR for detecting the isolate of *C. lindemuthianum* race 65. These primers were also employed to verify the responsiveness in identifying the pathogen in samples of bean seeds with different incidence levels (Table 1). Ultrapure sterile water and DNA from healthy seeds were used as a

Table 1. Incidence levels obtained from mixing healthy bean seeds (cultivar Pérola) with seeds inoculated with the isolate LV238 of *Colletotrichum lindemuthianum* race 65, for different seed-fungus contact times, corresponding to the inoculum potentials P0 - 0 h, P36 - 36 h, P72 - 72 h, P108 - 108 h, and P144 - 144 h.

Inoculum potential*	Incidence levels (%)	Nº of infected seeds	Nº of healthy seeds	Total of seeds
P36	0.25	1	399	400
	0.5	2	398	400
	1	4	396	400
	10	40	360	400
	100	400	0	400
P72	0.25	1	399	400
	0.5	2	398	400
	1	4	396	400
	10	40	360	400
	100	400	0	400
P108	0.25	1	399	400
	0.5	2	398	400
	1	4	396	400
	10	40	360	400
	100	400	0	400
P144	0.25	1	399	400
	0.5	2	398	400
	1	4	396	400
	10	40	360	400
	100	400	0	400

*For each inoculum potential, all incidence levels were tested separately

Table 2. Primer sequences used in the conventional PCR (cPCR) and real-time PCR (qPCR) reactions for detecting *Colletotrichum lindemuthianum* in bean seeds artificially inoculated.

Technique	Primers	Sequence	Reference
cPCR	CD1	5'-ACC TGG ACA CAT AAG TCA AAG-3'	Wang et al. (2008)
	CD2	5'-CAA CAA TGC CAG TAT CAG AG-3'	
qPCR	ClF432	5'-GGA GCC TCC TTT GCG TAG TAAC-3'	Chen et al. (2013)
	ClR533	5'-ACC TGA TCC GAG GTC AAC CTT GTT-3'	

negative control. All tests were performed in triplicates.

For the evaluation of the specificity and sensitivity of the qPCR technique, when using the pair of primers CIF432 and CIF533, standard calibration curves were generated through serial fungal DNA dilutions, varying from 200 ng/µL to 0,0002 ng/µL. The negative control was ultrapure sterile water, and the tests were carried out in two replications.

Detection of C. lindemuthianum by real-time PCR in dissected seeds

The quantification of *C. lindemuthianum*, race 65, in whole and dissected bean seeds that had been artificially inoculated with different inoculum potentials (P36, P72, P108, and P144 h) was carried out by the qPCR technique.

For the dissection, forty bean seeds were placed for 18 h in 15-cm Petri dishes, containing filter paper moistened with sterile distilled water. Then, they had the tegument and embryo carefully separated. Right after the dissection, these fractions were individually ground in a mortar with liquid nitrogen, until obtaining a powder. The DNA was extracted and used for the qPCR technique, according to the procedures previously detailed.

Results and Discussion

Specificity, sensitivity, and limit of detection of the conventional PCR and real-time PCR for C. lindemuthianum in pure cultures

This work showed that the pair of primers used for the cPCR process was specific for detecting the isolate of *C. lindemuthianum*, race 65, but not for other pathogens and saprophytes present on bean seeds. The cPCR products generated single bands of 638 base pairs for the positive diagnose of the pathogen studied (Figure 1).

Based on this outcome, the cPCR technique can be applied for routine laboratory analyses, because it is easily reproducible and takes little time to provide reliable results. Similar findings of other authors endorse this recommendation (Chen et al., 2007; Gutiérrez et al., 2014; Mesquita et al., 1998; Pacheco et al., 2014).

As for the limit of DNA detection of *C. lindemuthianum,* race 65, in a pure culture, the pair of primers CD1/ CD2 was able to identify very low concentrations, sensing the minimum of 0.02 ng/µL, from dilutions of an initial concentration of 200 ng/µL (Figure 2).

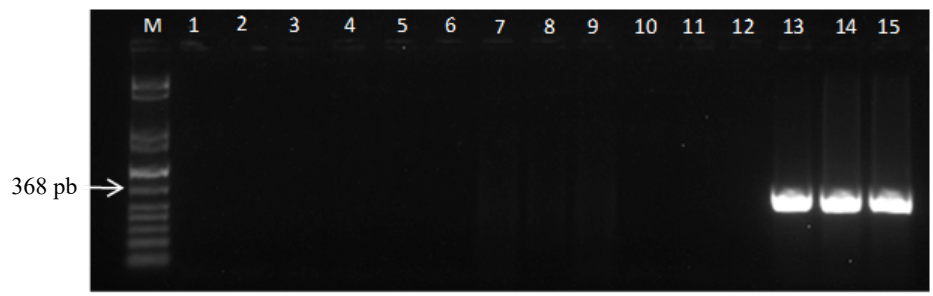

Figure 1. Specificity of the pair of primers CD1/ CD2 in detecting *Colletotrichum lindemunthianum* by conventional PCR. M: 1 kb marker (Qiagen GelPilot®); Columns 1-3: *Sclerotinia sclerotiorum*; 4-6: *Fusarium oxysporum* f. sp. *phaseoli*; 7-9: *Rhizoctonia solani*; 10-12: negative control; and 13-15: positive control.

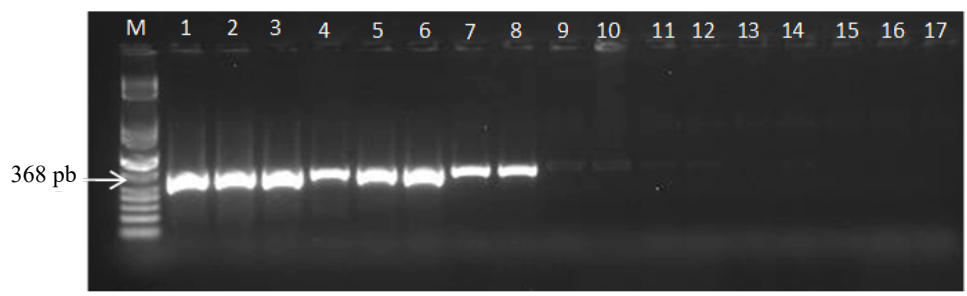

Figure 2. Limit of detection of the pair of primers CD1/ CD2 for *Colletotrichum lindemuthianum*, by the conventional PCR technique. M: 1 Kb marker (Qiagen GelPilot®). Columns 1-2: 200 ng/µL; 3-4: 20 ng/µL; 5-6: 2 ng/µL; 7-8: 0.2 ng/µL; 9-10: 0.02 ng/µL; 11-12: 0.002 ng/µL; 13-14: 0.0002 ng/µL; 15-16: 0.00002 ng/µL; and 17: negative control.

Regarding the sensitivity results of the pair of primers CD1/ CD2, used in the cPCR technique to test bean seeds with different incidence levels, from mixtures of inoculated and healthy seeds, the fungus was spotted in those samples with incidence values above 10%, with inoculum potential P36. However, the pathogen could also be detected from levels up from 0.25% incidence within the higher potentials P72, P108, and P144 (Table 3). These results were consistent in all three replications, which validates the verification analyses.

The pair of primers CD1/ CD2 used in cPCR was efficient and sensitive at diagnosing *C. lindemuthianum*, race 65, in samples of bean seeds, once they could detect the presence of the fungus in all inoculum potentials assessed. Other authors who studied *Stenocarpella* sp. and *Corynespora cassiicola* remarked that others specifics primers were efficient for the detection of pathogens in soybean and corn seeds inoculated with different inoculum potentials, respectively (Barrocas et al., 2012; Sousa et al., 2016).

With regards to the qPCR method, the primers ClF432 and ClR533 were specific and sensitive for the detection of *C. lindemuthianum,* race 65, in bean seed samples with different levels of pathogen incidence. The outcomes of the calibration curve and Ct values (threshold cycle) pointed to the linearity of the curve as a function of the dilution of fungal DNA (Figures 3A and 3B). The efficiency of the qPCR was of 1.03, as determined by the linear regression equation, with the mean values of the corresponding amplifications (r^2 = 0.99). The technique sensitivity was checked through the dissociation curve, by the equation Y = -3.25 + 12.58.

The results in Figure 3 show that it was possible to quantify the genetic material in the DNA diluted samples of *C. lindemuthianum,* race 65, by using the standard curve and the Ct values. Thus, they confirm the sensitivity of the pair of primers in tracing even low concentrations. The highest value of Ct was observed at the lowest dilution of 0.002 ng/µL.

This study demonstrated that the qPCR is a useful, viable, responsive technique for day-to-day health seed analyses aiming at diagnosing *C. lindemuthianum*. Its advantages are numerous, including reliability and reduced time taken to produce results on the presence/ absence of the fungus in bean samples or other pathosystems (Chen et al., 2013; Wang et al., 2008; Botelho et al., 2015; Vanegas-Berrouet et al., 2014).

Comparatively, it became clear that the qPCR technique was more sensitive than the cPCR one. For that reason, employing that type of analysis in national certification programs could be recommended as a way of detecting *C. lindemuthianum* in samples of bean seeds to be commercialized.

As for the DNA quantification of *C. lindemuthianum* in bean samples with different fungal incidence levels and variable inoculum potentials, it was noticed that even at the lowest values of incidence and inoculum potential, 0.161 ng/µL of fungus DNA was quantified. The highest inoculum concentration, however, was found in samples with an incidence of 100% among seeds and P144. In this case, an amount of 842 ng/µL was detected (Table 4).

Thus, as the inoculum potential and the incidence among the artificially inoculated and healthy seeds increased, the amount of DNA detected by the qPCR reactions also increased. Such result reveals that the longer the exposure time of the seeds to the pathogen the higher the fungal capacity to penetrate and colonize tissues of the seeds. Authors who worked with molecular detection of other fungi in different hosts also observed the same correlation, when using the qPCR technique (Botelho et al., 2015; Guimarães et al., 2017; Vanegas-Berrouet et al., 2014; Sousa et al., 2015).

Table 3. Amplification of the genomic DNA of *Colletotrichum lindemuthianum* by conventional PCR (cPCR), using the pair of primers CD1/ CD2, in samples of 400 bean seeds with different levels of incidence and treated with different inoculum potentials P0 - 0 h, P36 - 36 h, P72 - 72 h, P108 - 108 h, and P144 - 144 h.

Inoculum potential*	Incidence of seeds inoculated with *Colletotrichum lindemuthianum* (%)	cPCR detection in seeds (positive results/ replications)
Negative control	-	0/3
Positive control	+	3/3
P36	0.25	0/3
	0.5	0/3
	1	0/3
	10	3/3
	100	3/3
P72	0.25	3/3
	0.5	3/3
	1	3/3
	10	3/3
	100	3/3
P108	0.25	3/3
	0.5	3/3
	1	3/3
	10	3/3
	100	3/3
P144	0.25	3/3
	0.5	3/3
	1	3/3
	10	3/3
	100	3/3

*Regarding the different seed-fungus contact time.

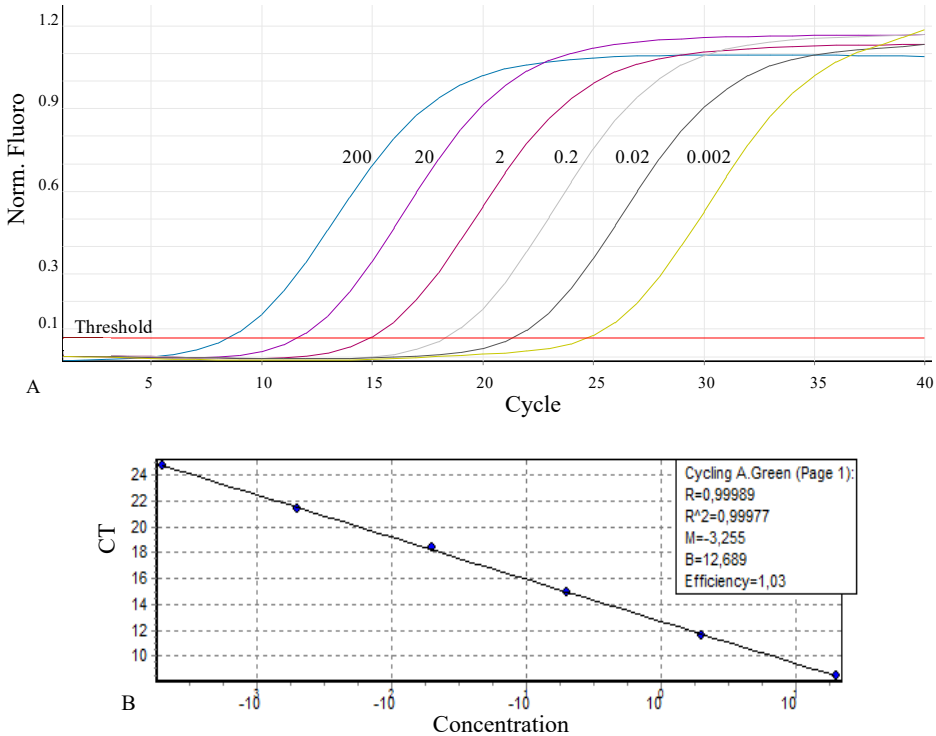

Figure 3. Real-time PCR for the detection and quantification of *Colletotrichum lindemuthianum*. A – Amplification curve with serial dilutions of the target DNA, from 200 ng/µL to 0.002 ng/µL, and negative control (NC). B – Standard curve; Threshold cycle (Ct), graphically represented as a function of the logarithm values of dilution and fluorescence emitted during the reaction.

Table 4. Quantification of *Colletotrichum lindemuthianum* (ng/µL*1000), by real-time PCR, in bean samples with different incidence levels of inoculated seeds (0.25%, 0.5%, 1%, 10%, 100%), treated with different seed-fungus contact time, corresponding to the inoculum potentials P0 - 0 h, P36 - 36 h, P72 - 72 h, P108 - 108 h, and P144 - 144 h.

Incidence levels * (%)	Quantification of *Colletotrichum lindemuthianum* (ng/µL)			
	P36	P72	P108	P144
0.25	0.161	3.24	5.25	8.71
0.5	0.354	6.22	11.44	19.1
1	0.443	12.33	19.6	27.4
10	14.9	41.8	99.3	131
100	120	204	443	842

*Incidence levels in lots of 400 seeds.

Quantification of C. lindemuthianum in seed fractions by real-time PCR

When whole seeds and dissected fractions (tegument and embryo) were analyzed as for the concentration of *C. lindemuthianum* DNA in all inoculum potentials, the highest value (60.3 ng/µL) was verified in whole seeds, at P144. Lower concentration of the fungus DNA, 20.4 ng/µL, was detected in tegument, followed by the lowest concentration, 0.15 ng/µL, quantified in the single embryo (Table 5).

The findings from the dissection of inoculated seeds showed that, at the highest inoculum potentials, the fungus could colonize the inner part of the seed reaching vital tissues. This fact leads to the deterioration of internal membranes and consequently reduction of the vigor and quality of the seed. For that reason,

Table 5. Concentration of *Colletotrichum lindemuthianum* DNA (ng/µL*100), quantified by real-time PCR, in bean samples (whole seeds, tegument, and embryo) treated with different seed-fungus contact time, corresponding to the inoculum potentials P0 - 0 h, P36 - 36 h, P72 - 72 h, P108 - 108 h, and P144 - 144 h.

Structure	P36	P72	P108	P144
Whole seed	0.987	5.835	48.35	60.3
Tegument	0.513	0.792	6.497	20.433
Embryo	0.021	0.017	0.081	0.145

the inoculum potential of pathogens can be accounted as a determinant factor for seed quality. The studies with soybean and *Aspergillus ochraceus* of Rocha et al. (2014) corroborate the results of this work. They also noticed that, when the inoculum potential was high, seed quality and vigor were committed.

Conclusions

The cPCR technique can detect *C. lindemuthianum* in bean seed samples at the minimum incidence level of 10% and seeds with lowest inoculum potential.

The qPCR technique can detect *C. lindemuthianum* in bean seed samples at the incidence level of 0.25% and seeds with lowest inoculum potential reaching the tegument and embryo.

The qPCR technique is able to quantify 0.161 ng/µL of *C. lindemuthianum,* race 65, in common bean seeds with the lowest incidence of seed and minimum inoculum potential.

Based on the qPCR evaluations, *C. lindemuthianum* colonizes the inner part of seeds.

Acknowledgments

The authors express their acknowledgment to the institutions *Universidade Federal de Lavras* and *Universidade Pedagógica de Quelimane*. Also, to CAPES, CNPq, and FAPEMIG, for the financial support; to Seprotec company, for providing the seeds used in this study; to the Laboratory of Plants Resistance and Professor Elaine Souza, for all the assistance.

References

ANSELME, C.; CHAMPION, R. *Bean anthracnose – Phaseolus vulgaris, Colletotrichum lindemuthianum.* In: ISTA Handbook in seed health testing. Zurich: International Seed Testing Association (ISTA), 1981/1982 (Woking sheet Nº 45).

BARCELOS, Q.L.; PINTO, J.M.A.; VAILLANCOURT, L.J.; SOUZA, E.A. Characterization of *glomerella* strains recovered from anthracnose lesions on common bean plants in Brazil. *Plos One*, v.9, n.3, p.900-919, 2014. http://journal.ashspublications.org/content/123/6/1038.abstract

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 with maize seeds. *Revista Brasileira de Sementes*, v.34, n.2, p.218-224, 2012. http://www.scielo.br/pdf/rbs/v34n2/05.pdf

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.055-062, 2015. http://www.scielo.br/scielo.php?script=sci_arttext&pid=S2317-15372015000100055

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, 2009a. 395p. http://www.agricultura.gov.br/arq_editor/file/2946_regras_analise__sementes.pdf

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. 1 ed., 2009b. 200p. http://www.bs.cca.ufsc.br/publicacoes/manual_analises_sanitarias.pdf

CHEN, Y.Y; CONNER, R.L.; GILLARD, C.L.; BOLAND, G.J.; BABCOCK, C.; CHANG, K.F.; HWANG, S.F.; BALASUBRAMANIAN, P.M. A specific and sensitive method for the detection of *Colletotrichum lindemuthianum* in dry bean tissue. *Plant Disease*, v.91, n.10, p.271-276, 2007. http://www.apsnet.org/publications/plantdisease/2007/October/Pages/91_10_1271.aspx

CHEN, Y.Y.; CONNER, R. L.; GILLARD, C.L.; McLAREND, D.L.; BOLANDE, G.J.; BALASUBRAMANIANF, P.M.; STASOLLAG, C.; ZHOUH, Q.X.; HWANGH, S.F.; CHANGH, K.F.; BABCOCK, C. A quantitative real-time PCR assay for detection of *Colletotrichum lindemuthianum* in navy bean seeds. *Plant Pathology*, v.62, p.900–907, 2013. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3059.2012.02692.x/epdf

COÊLHO, M.; GONÇALVES-VIDIGAL, M.C.; SOUSA, L.L.; NUNES, M.P.B.A.; AZEVEDO, R.F.; GALVÁN, M.Z. Characterization of race 65 of *Colletotrichum lindemuthianum* by sequencing ITS regions. *Acta Scientiarum. Agronomy*, v.38, n.4, p.429-438, 2016. http://www.scielo.br/pdf/asagr/v38n4/1807-8621-asagr-38-04-00429.pdf

DAMASCENO SILVA, K.J.; SOUZA, E.A.; ISHIKAWA, F.H. Characterization of *Colletotrichum lindemuthianum* isolates from the State of Minas Gerais, Brazil. *Journal of Phytopathology*, v.155, n.4, p.241-247, 2007. http://onlinelibrary.wiley.com/doi/10.1111/j.1439-0434.2007.01226.x/epdf

GARCÍA-SERRANO, M.; RODRÍGUEZ-GUERRA, E.A.L.R.; SIMPSON, J. Analysis of the MAT1-2-1 gene of *Colletotrichum lindemuthianum*. *Mycoscience*, v.49, p.312–317, 2008. https://link.springer.com/article/10.1007/s10267-009-0484-2

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

GUTIÉRREZ, P.; YEPES, M.S.; RESTREPO, J.F.A.; BERROUET, K.V.; MONTOYA, M.M. The CD1/CD2 marker for specific detection of *Colletotrichum lindemuthianum* is an iron transporter pseudogene. *Tropical Plant Pathology*, v.39, n.4, p.275-283, 2014. http://www.scielo.br/pdf/tpp/v39n4/v39n4a02.pdf

ISTA. International Seed Testing Association. *Handbook on Seed Health Testing*. Switzerland. Second edition. 1985. 45 sheets.

MACHADO, J.C.; OLIVEIRA, J.A.; VIEIRA, M.G.G.C.; ALVES, M.C. Uso da restrição hídrica na inoculação de fungos em sementes de algodoeiro (*Gossypium hirsutum*). *Revista Brasileira de Sementes*, v.26, n.1, p.62-67, 2004. http://www.scielo.br/pdf/rbs/v26n1/a10v26n1.pdf

MACHADO, J.C.; BARROCAS, E.N.; COSTA, M.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* (Impresso), v.20, p.37-63, 2012.

MESQUITA, A.G.G.; PAULA JUNIOR, T.J.; MOREIRA, M.A.; BARROS, E.G. Identification of races of *Colletotrichum lindemuthianum* with the aid of PCR-Based molecular markers. *Plant Disease*, v.82, n.10, p.1084-1087, 1998. http://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS.1998.82.10.1084

MICHEL, B.E.; RADCLIFFE, D.A. Computer program relating solute potencial to solution composition for five solutes. *Agronomy Journal*, v.87, n.1, p.131-136, 1995.

PACHECO, L.M.; BERROUET, K.V.; YEPES, M.S.; ÁNCHEZ, P.G.; MONTOYA, M.M. Detección por PCR de *Colletotrichum lindemuthianum* em cultivos y semillas de frijol en Antioquia, Colombia. *Acta Agronómica*, v.63, n.4, p.377-387, 2014. http://www.scielo.org.co/pdf/acag/v63n4/v63n4a10.pdf

ROCHA, F.S.; CATÃO, H.C.R.M.; BRANDÃO, A.A.; GOMES, L.A.A. Danos causados por diferentes potenciais de inóculo de *Aspergillus ochraceus* no vigor de sementes de soja. *Semina: Ciências Agrárias*, v.35, n.6, p.2895-2904, 2014. http://dx.doi.org/10.5433/1679-0359.2014v35n6p2895

SANTANA, E.; MAHUKU, G. Diversidad de razas de *Colletotrichum lindemuthianum* em Antioquia y evaluación de germoplasma de frijol crema-rojo por resistencia a antracnosis. *Agronomía Mesoamericana*, v.13, p.95-103, 2002. http://www.mag.go.cr/rev_meso/v13n02_095.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. http://onlinelibrary.wiley.com/doi/10.1111/ppa.12257/epdf

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.085-091, 2016. http://www.scielo.br/pdf/jss/v38n2/2317-1545-jss-v38n2152049.pdf

VANEGAS-BERROUET, K.; MARTÍNEZ-PACHECO, L.; SALAZAR-YEPES, M.; GUTIÉRREZ-SÁNCHEZ, P.; MARÍN-MONTOYA, M. Detección y cuantificación por qPCR de *Colletotrichum lindemuthianum* en tejidos y semillas de frijol en Antioquia, Colombia. *Bioagro*, v.26, n.1, p.13-20, 2014. http://www.redalyc.org/pdf/857/85730396002.pdf

WANG, W.; TANG, J.H.; WANG, Y.C. Molecular detection of *Colletotrichum lindemuthianum* by duplex PCR. *J. Phytopathology*, v.156, p.431-437, 2008. http://onlinelibrary.wiley.com/doi/10.1111/j.1439-0434.2007.01386.x/epdf