

## Sensibility of the PCR technique in the detection of *Stenocarpella* sp. associated with maize seeds<sup>1</sup>

Ellen Noly Barrocas<sup>2\*</sup>, José da Cruz Machado<sup>2</sup>, Mirella Figueiró de Almeida<sup>2</sup>,  
Luana Silva Botelho<sup>2</sup>, Édila Vilela de Resende Von Pinho<sup>3</sup>

**ABSTRACT** - Maize seeds, infected by *Stenocarpella* species, are important sources of inoculum for the introduction and dissemination of stalk and ear rot and macrospore leaf spot diseases. The use of healthy seeds is an important strategy for the preventive control of these diseases. However, one of the difficulties in the health quality control programs for maize seeds is the availability of a reliable and quick method for detecting these fungi during routine seed analyses. Therefore, the objective of the present study was to investigate the possibility of using the PCR technique as an alternative method for accurately detecting these pathogens in maize seed samples. Maize seeds were kept in contact with *S. maydis* colonie developed in PDA media containing mannitol at -1.4 MPa for 72 h. The seed samples used in this study were prepared with infected seeds at incidences of 100, 20, 10, 2, 1 and zero %. The primers used were able to detect *S. maydis* fungi in association with seeds with a maximum of 2% , however those primers were not able to differentiate between the two species.

Index terns: fungus, molecular detection, stalk and ear rot, macrospore leaf spot.

## Sensibilidade da técnica PCR na detecção de *Stenocarpella* sp. associados às sementes de milho

**RESUMO** - Sementes de milho infectadas por *Stenocarpella* são importantes fontes de inóculo para a introdução e disseminação da podridão do colmo e da espiga e mancha foliar de macrospora em campos de produção. O uso de sementes sadias constitui uma importante estratégia para o controle preventivo dessas doenças. A disponibilidade de métodos rápidos para a detecção desses fungos em análises de rotina é uma das dificuldades em programas de controle de qualidade de sementes de milho, já que o método utilizado demanda longo tempo e sua identificação é bastante subjetiva. O objetivo desse trabalho foi investigar o uso da técnica PCR para a detecção desses patógenos em sementes de milho. Para isto sementes de milho inoculadas com *S. maydis* foram misturadas a sementes sadias de modo que formassem lotes com incidências de 100, 20, 10, 2, 1 e zero%. O par de primers utilizado para detectar *S. maydis* foi capaz de detectar o máximo de 2% de incidência, todavia os primers não foram suficientes para diferenciar as duas espécies.

Termos para indexação: fungo, detecção molecular, podridão do colmo e da espiga, mancha foliar de macrospora.

### Introduction

The fungi *Stenocarpella maydis* (Berk.) Sacc. and *Stenocarpella macrospora*, Earle, are important pathogens

of maize, causing root, stem and ear rots in addition to seedling collapse and necrotic leaf spot (Casa et al., 2006). In Brazil, these diseases are present in all maize growing areas, where they may cause severe reduction in yields and

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<sup>2</sup>Departamento de Fitopatologia, Universidade Federal de Lavras, 37200-000 - Lavras, MG, Brasil

<sup>3</sup>Departamento de Agricultura, Universidade Federal de Lavras, 37200-000 - Lavras, MG, Brasil.

\*Corresponding author <ellenoly@gmail.com>

grain quality, besides producing mycotoxins, which are an important concern worldwide (Eddins, 1930; Dorrance et al., 1998).

The *Stenocarpella* species are necrotrophic, with a parasitic phase in the live tissues of the host and a saprophytic phase in the crop residue (Casa et al., 2003; Casa et al., 2006). As such, they can form pycnidia in the crop residue and may survive inside the seeds, which are important sources of primary inoculum (Casa et al., 1998), as a dormant mycelium. The infected seeds are the major source of introduction of the *Stenocarpella* complex in newly-planted areas.

In general, the use of healthy seeds is an important strategy in disease management and a reliable and fast diagnosis for diseases is necessary. The available methods to detect these species in routine seed analyses is based on morphological aspects of the fungal colonies, such as the color of colonies of infected seeds developed on a filter paper substrate (Mario and Reis, 2001), and other characteristics, as described in the methods recommended by the International Seed Testing Association-ISTA.

Nevertheless, the diagnosis of *Stenocarpella* sp. in maize seeds can be complicated by the presence of fast growing microorganisms, which are often associated with maize seeds (Phan et al., 2002). In addition, the period required for these fungi to produce pycnidia on the seeds, or in the substrate, may take too long thus making the method unsuitable for routine analysis purposes.

Due to its high importance for Brazilian agriculture, *Stenocarpella maydis* is now considered as a “non

quarantine regulated pest”, according to the Brazilian seed certification program and the pathogen is consequently subject to seed health standardization (Machado and Pozza, 2005).

The detection of fungi based on DNA analysis, by means of the polymerase chain reaction (PCR), has been proved to be a feasible alternative for a fast and sensitive diagnosis of several diseases and can be adapted for routine analysis (Lee et al., 2002). This method has been used for detection, identification and quantification of several plant pathogens. In the last decade, the PCR technique has been successfully employed, in combination with other techniques, for the detection of pathogens associated with seeds, including bacteria and other pathogenic agents.

The objective of this study was to evaluate the sensibility of the of PCR technique for detecting *Stenocarpella* sp. in maize seed samples, as required in seed health testing certification programs.

## Material and Methods

*Isolates origin and inoculum multiplication:* pure colonies of the two species of *Stenocarpella* and two different species of *Fusarium* were obtained from different sources (Table 1). They were initially transferred to potato dextrose agar medium (PDA) and incubated at a temperature of  $25 \pm 2$  °C with a photoperiod of 12 h for seven days. The mycelium of each fungal isolate, formed on the medium surface, was scraped, washed with sterile water, dried and stored in liquid nitrogen.

Table 1. Pathogenic species of fungi associated with maize seeds used in this study.

Fungi	Culture Code	Origin	Institution
<i>Stenocarpella maydis</i>	MY1	Sete Lagoas, Brazil	CNPMS <sup>b</sup>
<i>S. maydis</i>	MY2	Sete Lagoas, Brazil	CNPMS <sup>b</sup>
<i>S. maydis</i>	CML594	Brazil	CML <sup>a</sup>
<i>S. maydis</i>	CML 603	Brazil	CML <sup>a</sup>
<i>S. maydis</i>	CML 697	Brazil	CML <sup>a</sup>
<i>S. maydis</i>	CML 698	Brazil	CML <sup>a</sup>
<i>Stenocarpella macrospora</i>	MC1	Sete Lagoas, Brazil	CNPMS <sup>b</sup>
<i>S. macrospora</i>	MC2	Sete Lagoas, Brazil	CNPMS <sup>b</sup>
<i>S. macrospora</i>	MC-M	Monsanto, Brazil	
<i>Fusarium graminearum</i>	CML 780	Brazil	CML <sup>a</sup>
<i>F. verticillioides</i>	CML 323	Brazil	CML <sup>a</sup>

<sup>a</sup>Mycological Collection of the Federal University of Lavras-MG, Brazil.

<sup>b</sup>EMBRAPA- Maize and Sorghum Center, Sete Lagoas – MG, Brazil.

For the subsequent extraction of DNA, the mycelium of each species was macerated with a pestle and mortar in liquid nitrogen.

*Preparation and inoculation of seeds:* seeds of hybrid maize 3041 (Monsanto) were disinfested with 2% sodium hypochlorite for 1 min, shade dried and inoculated with isolate CML 697 of *S.maydis*, using the osmo-conditioning method (Machado et al., 2004). A conidial suspension of the pathogens was added to BDA medium in 15 cm Petri dishes, with the osmotic potential adjusted to -1.4 MPa, according to the SPPM software calculation (Michel and Radcliffe, 1995). The dishes were incubated at  $25 \pm 2$  °C with a photoperiod of 12 h, for seven days, and then seeds were placed on the fungal colonies, where they were incubated for 72 h, under the same conditions as previously described. The seeds were then shade dried and kept for future mixing with healthy seeds.

*DNA extraction:* the seed samples and the fungal sample were ground up in a refrigerated mill until a fine powder was produced. Approximately 1 g of each sample was macerated with liquid nitrogen and replicates of 0.04 g were weighed for the immediate extraction of DNA using a Wizard® Genomic DNA Purification kit (Promega). The primer pairs ITS1 / ITS4 (TCCGTAGGTGAACCTGCGG/TCCTCCGCTTATTGATATGC) and P1/2 (GTTGGGGGTTTAACGGCACG/GTTGCCTCGGCACAGGCCGG) were used for the amplifications of the target genomic regions for all fungal isolates. For the amplification of the fragments extracted from the inoculated seeds, only the primers P1 and P2 were used.

The quality of the DNA obtained was checked in a 0.8% agarose gel in TBE buffer stained with ethidium bromide ( $0.5 \text{ mgL}^{-1}$ ). The DNA was quantified in a Nano Drop ND 1000 Spectrophotometer.

*PCR Amplification:* the universal primers ITS1 and ITS4 were used to amplify ITS regions of the rDNA of both *Stenocarpella* species used in this study. The primers P1/P2 described by Xia and Achar (2001) were used to amplify the regions present only in the *S. maydis* isolate. PCR was carried out in 25  $\mu\text{L}$  of the reaction containing PCR buffer (supplied by the manufacturer), dNTPs (2.5 mM of each dNTP), primers (10  $\mu\text{M}$  of each forward or reverse primer) and 5  $\mu\text{L}$  units of Taq DNA polimerase (Phoneutria, Brazil), and using either 20 ng of DNA from fungal isolates or 2  $\mu\text{L}$  of concentrated DNA extracted from inoculated seeds. Initial denaturation of DNA was conducted at 95 °C for 3 min, followed by 30 cycles at 94 °C for 30 sec, 60 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 10 min. PCR fragments were visualized with ethidium bromide in 0.8% agarose gel in TBE buffer.

*Sensitivity and specificity of the PCR technique:* to determine the sensitivity and capacity of the specific primer pair (P1/P2) in amplifying the reduction in the amount of DNA, amplifications were performed with serial dilutions of isolated CML 697 of *S.maydis* at concentrations of  $20 \text{ ng.}\mu\text{L}^{-1}$  to  $2 \text{ pg.}\mu\text{L}^{-1}$  in two replications.

To determine the specificity of the primers P1/P2, genomic DNA were tested ( $20 \text{ ng.}\mu\text{L}^{-1}$ ) of 11 fungal isolates (Table 1) and compared with the universal pair of primers.

*Detection of **Stenocarpella** sp. in seed samples with different fungal incidence:* to determine the efficacy or sensibility of the PCR technique in the detection of *Stenocarpella* in maize seed samples, batches of 100 seeds were prepared by mixing artificially infected seeds and healthy seeds, in proportions to produce infection levels of: 100, 20, 10, 2, 1 and nil. The test was carried out with three replicates and the experiment was repeated three times for each level of seed infection.

## Results and Discussion

*Specificity of the P1/P2 primers for **Stenocarpella** sp. in pure cultures:* electrophoretic analysis in agarose gel at 0.8% of DNA fragments obtained by PCR amplification of fungi isolated from maize with the primers P1/P2, carried out by Xia and Achar (2001), generated bands of approximately 375 base pairs, showing specificity only to the genus *Stenocarpella* sp species. For *Fusarium verticillioides* and *F. graminearum* amplification of fragments of genomic DNA was observed only in relation to ITS1 and ITS4 primers, as occurred for all the DNA isolates of *Stenocarpella* analyzed, with bands of about 575 base pairs (Figure 1). The universal primers ITS1 and ITS4 were used as a positive control to ensure DNA quality and specificity of the primers target.

The P1/P2 primer pair was specific for differentiating species of *Stenocarpella* from other species, but was not specific enough to differentiate *S. maydis* from *S. macrospora*. Only the genomic DNA isolated MC 1 was not amplified with primers P1/P2 (Figure 1).

*Sensitivity of PCR to detect **Stenocarpella** sp. in pure culture:* the amplification reactions were conducted with decreasing amounts of genomic DNA of *S. maydis*, isolated to determine the minimum amount required to produce a product which can be detected by the pair P1/P2. This pair of primers was able to amplify the target DNA sequence with 200 pg when using isolated CML 697.

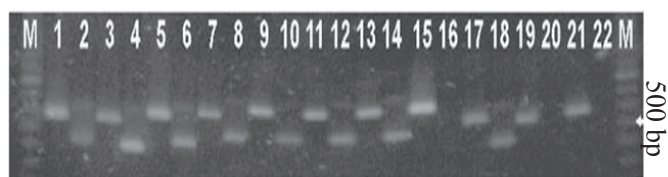


Figure 1. Amplification of genomic DNA of isolates of *Stenocarpella maydis*, *S. macrospora*, *Fusarium verticillioides* and *F. graminearum*. Columns 1,3,5,7,9,11,13,15,17,19,21 representing amplification of genomic DNA of pure cultures of the *Stenocarpella* isolates: MY1, MY2, CML697, CML698, CML594, CML603, MC-M, MC 1, MC 2, CML 780, CML 323, using the universal primers ITS1/ITS4. Lanes 2,4,6,8,10,12,14,16,18,20,22 representing the amplification of the genomic DNA of pure cultures of the same isolates using the primer P1/P2. Lane M – 100 bp ladder DNA.



Figure 2. Minimum level of the detection of genomic DNA of *Stenocarpella maydis* used with the pair of primers P1/P2. These pairs were used in the PCR with a serial dilution of the total genomic DNA of *S maydis* (CML 697). Lanes 1 to 7: 200ng, 20ng, 10ng, 200pg, 20pg, 2pg, 200fg of the total genomic DNA. Lane M contains a ladder with 100 bp.

*Detection of Stenocarpella sp. in infected maize seeds:* clear bands of 325 base pairs were observed from maize seed extracts, with the PCR technique, for seed samples with levels of incidence higher than 2% for both species of *Stenocarpella*.

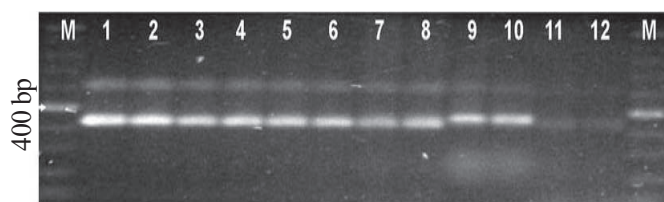


Figure 3. Amplification of genomic DNA of *Stenocarpella maydis* at different levels of infection in maize seeds. Lane M= 100 bp DNA ladder 1-2=100%, 3-4=20%, 5-6= 10%, 7-8=2%, 9-10=1%, 11-12=0%.

Maize seeds without infection by *Stenocarpella* sp. were tested in the present experiment as the control. For infection levels lower than 2%, the primers used in this investigation also proved to be efficient, although visible bands in DNA gel were not observed in some replicates at infection rates of 1% for both fungal species. The results of three replicates of each sample are summarized in Table 2.

Table 2. Results of the detection of *Stenocarpella* sp. in maize seeds infected artificially at different inoculum potentials using the PCR technique.

Infected seeds (%)	Detection of <i>Stenocarpella</i> sp by PCR <sup>a</sup>								
	Sample 1			Sample 2			Sample 3		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
100	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
1	+	+	-	-	+	-	+	+	-
0	-	-	-	-	-	-	-	-	-

<sup>a</sup>Presence(+) or absence(-) of amplified bands with the primer P1/P2 for the detection of *Stenocarpella* sp in maize seeds. R= replicates..

The internal transcribed spacer regions (ITS) of the rDNA have been widely used to differentiate and to detect closely related fungal species (Phan et al., 2002), as they are highly conserved parts of the genome within the same species and are variable among species (Lovic et al., 2002; Lee et al., 2002). Based on those regions, different primers have been designed and successful applications of them are reported for detection and identification of several target organisms (Lee et al., 2002; Iacomi-Vasilescu et al., 2009; Konstantinova et al., 2002; Rios et al., 2007; Ha et al., 2009; Consolo et al., 2009 and Ioos et al., 2007).

For some species with very similar morphological characteristics, designing specific primers is still a challenge. This mission may face difficulties for different reasons as pointed out by Iacomi-Vasilescu et al. (2002), in studies focused on designing primers to differentiate *Alternaria brassicae*, *A. brassicicola* and *A. japonica*. In this study the primers were only specific for distinguishing *A. brassicae* from *A. japonica*.

According to Xia and Achar (2001), the use of primers allows an accurate detection of *Stenocarpella maydis* associated with maize seeds. In the present study, the use of these primers was not efficient in differentiating

*Stenocarpella maydis* from *S.macrospora*. From the seed pathology point of view, both species are important pathogens, causing different symptoms and losses in the same plants and are able to significantly reduce maize yields.

It should be noted that both species are often found in the same seed sample of maize and their identification by conventional methods is unreliable and laborious. In practice, both species should be detected separately considering that they are different pathogens. The results of this study show that the development of specific primers to distinguish both species of *Stenocarpella* is necessary and should be investigated further. The use of sequences or genes related to a pathogenic trait may be an alternative to be explored, as mentioned by Guillemette et al. (2004) in the differentiation of *Alternaria brassicae* from other species associated with *Brassica* seeds.

Regarding sensitivity, the PCR technique used was able to reveal infection levels of both species of *Stenocarpella* at an incidence below 2% in the samples tested. Below that level of infection, bands were not seen in some replicates. As already mentioned, the molecular detection of fungi on seeds can be influenced by different factors, such as low concentration of target pathogens, the presence of other organisms and compounds like poly-phenols, tannins, polysaccharides and other natural components of the seeds. In other pathosystems, some compounds in the seed extraction have been demonstrated to interfere in the performance of Taq polymerase enzyme. In the literature, there are reports of the interference of polysaccharides from maize seeds in the DNA extraction. The increased viscosity of the extract makes pipetting difficult and interferes in the DNA concentration (Fang et al., 1992; Porebski et al., 1997).

An interesting point is the convenience of using commercial kits to extract DNA from plant tissues since it may prevent several factors interfering in the DNA extraction and its amplification. (Phan et al., 2002; Konstantinova et al., 2002; Guillemette et al., 2004; Ioos et al., 2007). In this study, the use of a commercial kit to extract DNA from maize seeds was shown to be efficient as no influence on the DNA extraction was observed. Because of this is important emphasize that this work the detection in pure culture and culture of fungus showed the same band (Figure 4).

According to a recent publications, the sensitivity of the detection of some pathogens by PCR can be considerably improved by using the real time PCR technique (qPCR), as has been demonstrated for some pathosystems. It is possible to quantify the presence of one single spore of pathogens associated with seeds

using such a technique (Chilvers et al., 2007). In work conducted on the detection of *Tilletia caries* in wheat seeds, McNeil et al., (2004) report that less than one spore of this pathogen was detected by that PCR technique at a 95% confidence level through calibration curves. The sensitivity of that technique in pure cultures was seen to be of 100 pg for *Alternaria brassicae* (Guillemette et al., 2004) and 10 fg for *Botrytis* spp. (Chilvers et al., 2007).



Figure 4. Electrophoresis gel of the PCR obtained with the universal primer of the ITS region (ITS1 e ITS4) and the specific primer (P1/P2) for the DNA of *Stenocarpella* sp. DNA obtained from pure cultures.(1-2) and from infected seeds (3-4). Lane M – 100 bp ladder DNA (Amersham, U.S.A).

Another strategy to be considered for improving the sensitivity of the PCR technique is to increase the biomass of the pathogens associated with seeds before the extraction of the DNA. This can be done by incubating the seeds for some period of time under favorable conditions for the development of the pathogens in association with seeds. After incubation, seeds are used for DNA extraction. Such a procedure has increased the sensitivity of the conventional PCR technique in detecting some pathosystems, as reported by Guillemette et al. (2004) working with *Alternaria radicina* in seed samples of cabbage and radish incubated for 48 hours. Through the conventional PCR technique, Vechiatto et al. (2006) detected *Diaphorte meridionalis* associated with a single soybean seed from a mixture of 399 healthy seeds, after an incubation period of 7 days by the conventional blotter method.

Early seed incubation may also be used to improve the sensitivity of the real time PCR technique. Chadha and Gopalakrishna (2006) were able to detect *Magnaporthe grisea* in rice seeds at levels of up to 0.2% incidence when seeds were incubated for 48 hours in PDA medium, whereas by the conventional PCR technique this level was 5%.

## Conclusions

The PCR technique as used in this study seems to be promising for detecting *Stenocarpella* sp in maize seeds

compared to the biological methods based on filter paper incubation (blotter test) or on acidified PDA. However, an investigation of new and more specific and sensitive primers, which are able to differentiate both species of *Stenocarpella* associated with maize seeds, should be a priority for the Brazilian seed certification program. In addition, the use of primers P1/P2 tested in this study can also be considered useful and may help in the initial screening for the presence of *Stenocarpella* in the seed lots commercialized.

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