

Putative pathogenicity genes of *Aspergillus niger* in sisal and their expression *in vitro*

Jorge Teodoro de Souza¹, Eliane Santos Jesus², Adailson Feitoza de Jesus Santos³

¹ Universidade Federal de Lavras, Departamento de Fitopatologia, Campus Universitário, CEP 37200-000, Lavras-MG, Brasil. E-mail: jorge.souza@dfp.ufla.br

² Universidade Federal do Recôncavo da Bahia, Rua Rui Barbosa, 710, Centro, CEP 44380-000, Cruz das Almas-BA, Brasil. E-mail: vera.00@hotmail.com

³ Universidade do Estado da Bahia, Departamento de Tecnologia e Ciências Sociais - Campus III. Rua Edgard Chastinet, s/n. São Geraldo. CEP:48905-680, Juazeiro-BA, Brasil. E-mail: adailsonmicrobiologia@gmail.com

ABSTRACT

Sisal (*Agave sisalana* Perrine ex Engelm) is cultivated in large areas of the Brazilian semi-arid Northeastern region. This crop allows thousands of people to obtain minimal wages. However, a disease known as bole rot, caused by *Aspergillus* species, is causing a decline in production. The objective of this study was to investigate the involvement of hydrolytic enzymes (cellulase, cutinase, protease and lipase) and ochratoxin A in an *in vitro* simulation of the interaction between *A. niger* Tiegh. and sisal to understand the mechanisms of fungal pathogenicity. Primers for cutinase lipase, protease, ochratoxin A, and elongation factor 1- α (EF), with this last one used as endogenous control were designed and optimized for real time quantitative PCR (qPCR) with the SYBR Green methodology. The genes potentially involved in the pathogenicity of *A. niger* showed higher expression in medium supplemented with sisal as compared to the minimal medium. The primers reported herein will allow detailed studies of the biological events leading to bole rot disease in sisal. These results represent the first data on genes putatively involved in the pathogenicity of *A. niger* to sisal.

Key words: *Agave sisalana*; bole rot disease; quantitative real time PCR

Prováveis genes de patogenicidade de *Aspergillus niger* em sisal e sua expressão *in vitro*

RESUMO

O sisal (*Agave sisalana* Perrine ex Engelm) é cultivado em larga escala na região semi-árida do Nordeste brasileiro. Esta planta proporciona a obtenção de renda mínima para milhares de famílias. No entanto, observa-se um declínio da cultura do sisal devido à podridão vermelha do caule do sisal, doença causada por espécies de *Aspergillus*. O objetivo desse trabalho foi estudar o envolvimento dos genes que codificam a micotoxina ocratoxina A e enzimas hidrolíticas (celulase, cutinase, protease e lipase) em uma simulação *in vitro* da interação entre *A. niger* Tiegh. e sisal para entender os mecanismos de patogenicidade do fungo. Primers para celulase, cutinase, lipase, protease, ocratoxina A, fator de alongação 1- α (EF), sendo o último utilizado como controle endógeno, foram desenhados e otimizados para amplificação por PCR em Tempo Real (qPCR) utilizando a metodologia SYBR Green. Os genes potencialmente envolvidos na patogenicidade de *A. niger* apresentaram maior expressão no meio suplementado com sisal, quando comparado com o meio mínimo. Os primers relatados nesse trabalho permitirão a realização de estudos detalhados dos eventos que levam a podridão vermelha do sisal. Os resultados apresentados representam as primeiras informações sobre os genes de *A. niger* potencialmente envolvidos na patogenicidade ao sisal.

Palavras-chave: *Agave sisalana*; podridão vermelha do sisal; PCR em tempo real

Introduction

Sisal (*Agave sisalana* Perrine ex Engelm) is cultivated in large areas of the American, Asian and African continents, but its highest concentration is found in Latin America. Brazil is the biggest producer of sisal fiber in the world and contributed with 53% of the production in 2013 (Faostat, 2015). In Brazil sisal cultivation is concentrated in the northeast region, where Bahia State is the biggest producer, with 94% of the national production, followed by the States of Paraíba and Rio Grande do Norte (Silva et al., 2008). Sisal is the source of the main hard fiber produced in the world. The commercialization of this fiber in the external and internal Brazilian markets generates revenues of the order of 80 million dollars per year. Additionally, the sisal production chain employs approximately 500 million people in activities that include maintaining the plantations, harvesting, fiber extraction and processing, industrialization and use in handicraft products (Silva et al., 2008).

Despite its importance to northeastern families, sisal cultivation has been declining in the last few years. The disease known as bole rot is one of the main factors leading to the sisal decadence. Although *A. niger* Thiegh, has been initially pointed out as the sole cause of the disease, other species in the section *Nigri*, such as *A. brasiliensis* and *A. tubingensis* are also pathogenic to sisal (Santos et al., 2014). This disease is considered to be the most important phytosanitary problem of sisal in Brazil by producers, extensionists and scientists due to its high destructive potential. Leaves of infected plants become improper for fiber extraction and plants die with the progress of the disease. Progressive darkening of internal infected tissues characterize the disease. The color of infected tissues varies from brown to red and normally extends from the base of the stem to the base of the leaves. All growth stages of sisal are susceptible to the pathogen (Bock, 1965; Silva et al., 2008). Although fatal, infected adult sisal plants survive for some time in the field due to the relatively slow colonization rates of the pathogen. Plants with advanced stages of the disease show yellow leaves and rotten stem or bole, which leads to plant death (Coutinho et al., 2006).

The process of penetration and colonization of *Aspergillus* spp. in sisal was not studied yet. However, it is known that necrotrophic pathogens in general depend on the production of hydrolytic enzymes to penetrate and toxins and hormones to colonize the host tissues (Agrios, 2005). Hydrolytic enzymes are required to degrade components of plant cell walls. Genes coding for enzymes that catalyze the hydrolysis of lipids and proteins, such as lipases, proteases, cutinases and cellulases are present in the genome of *A. niger* (Pel et al., 2007), but little is known on their expression and role on the pathogenicity to sisal. Species of black aspergilli, including isolates of *A. niger*, are able to produce toxins, such as ochratoxin A (OTA), a carcinogenic mycotoxin (Abarca et al., 1994). However, similar to the hydrolytic enzymes, no information is available on the role of OTA on the pathogenicity of *Aspergillus* spp. to sisal.

In this study, primers were designed and optimized to amplify fragments of genes that encode the synthesis of lipases, proteases, cutinases, cellulases and ochratoxin A in isolates *A.*

niger through quantitative real time PCR (qPCR). A simulation *in vitro* of the pathosystem *A. niger* x sisal was utilized.

Materials and Methods

Isolate AN18 of *A. niger* obtained by Santos et al. (2014) from a diseased sisal plant was used in all experiments. The cultivation of *A. niger* was done in two culture media: 1) one minimal medium (MM) composed of 10 g sucrose, 2 g NaNO₃, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, and 0.5 g KCl per litre plus 0.2 mL of a stock solution prepared with citric acid 5 g, ZnSO₄·6H₂O 5 g, Fe(NH₄)₂(SO₄)₂·6H₂O 1 g, CuSO₄·5H₂O 250 mg, MnSO₄ 50 mg, H₃BO₃ (Boric Acid) 50 mg, Na₂MoO₄·2H₂O 50 mg and distilled water 95 mL; and 2) the same medium amended with 1% of sisal extract (MS) before autoclaving at 120 °C for 20 min. The sisal extract was prepared by macerating 100 g of fresh sisal stem in 100 mL of distilled water. The *in vitro* experiments were installed in a complete random design with three replicates per treatment. For the analysis of gene expression seven sampling times were defined: 36, 42, 48, 54, 60, 66 and 72 h after the addition of 1 mL of a suspension containing 10⁸ conidia of *A. niger*/mL of sterile distilled water into the culture media and grown at 25 °C with 100 rpm of shaking. A total of 42 Erlenmeyer flasks containing 100 mL of MM or MS medium were the experimental units. Mycelial samples were collected by filtration and stored at -80 °C for RNA extraction. Total RNA was extracted from 75 mg of mycelium macerated in liquid nitrogen by using the RNAqueous® Kit (Ambion, West Leicestershire, England) following the manufacturer's recommendations. RNA samples were treated with Ambion® TURBO DNA-free TMDNase treatment and removal reagents to remove contaminating DNA. The RNA was quantified with the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, USA) and converted to cDNA by reverse transcription (RT) by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, São Paulo) according to the instructions of the manufacturer. The product of the reaction was stored at -20 °C.

For primer design the program ClustalW was used to align the sequences of each gene separately. The most conserved regions were selected for primer design in the on-line program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) adjusted with standard parameters, except length of the amplified fragments which was changed to 80 - 250 bp; primer length adjusted to 19-21 bp; annealing temperature was adjusted to 59 - 61 °C; and GC content from 40 - 60%. The accession numbers of the sequences used to design the primers were: ochratoxin A (XM 001397276.2), protease (XM 001396377.2), cellulase (XM 001389970.2), cutinase (XM 001399174.1), lipase (XM 001397840.2) α -tubulin (XM 003188558.1), translation and elongation factor 1- α (XM 001398394.2) and glyceraldehyde 3-phosphate dehydrogenase (XM 001397459.2).

The primers were optimized by conventional PCR. The cycling program utilized was: 94 °C for 2 min, followed by 9 cycles of denaturation at 94 °C for 45 s, annealing at 60°C for 45 s, and extension at 72 °C for 1 min and 29 cycles at 94 °C

for 30 s (denaturation), 48°C for 30 s (primer annealing), 72 °C for 1 min (extension) followed by a final extension of 72 °C for 10 min. Each reaction was composed of 5µL of 10X buffer (10 mM Tris and 50 mM KCl), 2 µL 50mM MgCl₂, 2 µL dNTPs (4x2.5 mM), 0.3 µL of Taq-polymerase (5 U.µL⁻¹), 0.75 µL of forward and reverse primers (10 mM) and 2 µL of genomic DNA and nuclease-free water in a total volume of 25 µL. Real time PCR reactions were conducted in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems). DNA amplification of target genes was done from total cDNA with the SYBR Green methodology. A cDNA serial dilution (1, 1:5, 1:50, 1:500 and 1:5000) was prepared in triplicate and used to test the efficiency of each primer. Amplification reactions were done in a total volume of 25 µL containing 12.5 µL of Power SYBR® Green Master Mix that contains SYBR® Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs, passive reference (ROX) and an optimized buffer, 0.75 µL of each primer (10 µM), 2 µL of cDNA sample and 9 µL of nuclease-free water. Control reactions were prepared with nuclease-free water instead of cDNA. The endogenous control adopted was the translation and elongation factor 1- α . The qPCR amplifications were run with incubations at 95 °C for 2 min, followed by 40 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min, and detection of the fluorescence signal at the end of each extension step. The melting curve was constructed for all samples. The relative quantification was done to determine the expression of the target genes by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The average expression of the target genes in the two culture media at different times was compared by the t test in the program Sisvar version 5.3 (Ferreira, 2011).

Results and Discussion

A total of 21 primers for the target genes were designed: 5 for ochratoxin A, 3 for cellulase, 3 for cutinase, 5 for protease and 5 for lipase. Additionally, 3 primer pairs were designed for the endogenous controls α -tubulin, translation and elongation factor and glyceraldehyde 3-phosphate dehydrogenase. All primer pairs, except 2 for ochratoxin A were optimized at 60 °C with conventional PCR (Data not shown). Among the

designed primers, five were randomly chosen to be used in qPCR (Table 1). The primers EF were chosen as endogenous controls because the efficiency of the PCR was close to 100%. The primers designed for lipase, although optimized in conventional PCR, did not show efficiency in qPCR and therefore the study of this enzyme was not further pursued.

In this study, qPCR analyses revealed that both hydrolytic enzymes and ochratoxin A were expressed at some moment during fungal growth in minimal medium (MM) and in the medium supplemented with sisal extract (MS). A comparison of the expression of each gene in MM and MS media with the t test showed significant differences for some sampling times (Figure 1).

With exception of cellulase at 48 h (Figure 1A), all the other enzymes presented higher expression rates in MS medium. Other studies showed that fungi such as *Cercospora zea-maydis* and *Heterobasidion parviporum* express genes

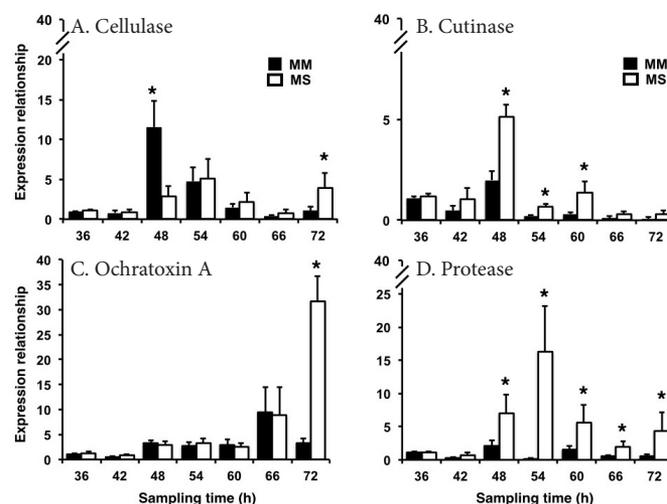


Figure 1. Expression analysis of target genes in minimal medium (black columns) and in minimal medium supplemented with sisal (white columns). The experiment was done *in vitro* by transferring *A. niger* into the media. Mycelium was collected at different sampling times, RNA was extracted and the expression of the target genes studied with real time PCR. The experiment was installed with three replicates and the error bars represent the standard deviation. Mean values marked with an asterisk are different when the media are compared in the same sampling time according to the t test ($p = 0.05$).

Table 1. Characteristics of the primers selected to be used in qPCR analyses.

Gene	Primer sequences ¹	Amplicon ²	Calibration function ($y = mx + b$) ³	Coefficient of correlation (R^2)	PCR efficiency (%) ⁴
Ocra4	F: ATGCGCAGTGTCCGACATGATTTG R: TCAAGAACCATGGATGCCTGGAGA	123	$Y = -3.121x + 25.441$	0.997	109
Pro3	F: TCCGGAAGTTGTGACATGGAGTGA R: AGCTGAGGAAACCCGTAAGTGGT	81	$Y = -2.985x + 33.457$	0.992	116
Cel1	F: AATGGTTGCCCTAGTGTGCATGCTG R: TTGATCGCCATCCAGACCACGTAT	132	$Y = -3.213x + 26.756$	0.999	104
Cut4	F: AGCAGCAAGGTGAAGGATTCCTGT R: CACTGCCATAAGTCAATGCGCCA	88	$Y = -3.229x + 28.278$	0.999	104
Tuba	F: TGGAAAGGGAGTTTACGGTTG R: ACTCACAACCCTCCCATCAG	201	$Y = -3.4x + 38.588$	0.898	96
EF	F: AAGATGGCCAAGCTCTACGA R: CTGTGGACAGGTGGATGTTG	203	$Y = -3.247x + 24.651$	0.998	103
Gli	F: CGAGTCCACTGGTGTCTTCA R: CAAGAAGCGTTGGAGAGGAC	168	$Y = -3.877x + 21.757$	0.996	81

¹Sequence of the forward (F) and reverse (R) primer

²Size of the fragment amplified by the primer pair (bp)

³ $y = mx + b$, where $b = y -$ intercepts the line of the standard curve and $m =$ slope of the line of the standard curve

⁴Efficiency of the PCR = $[(10^{(1/S)}) - 1] \times 100$, where S is the slope

coding for enzymes and mycotoxins during the process of pathogenesis to maize and *Picea abies*, respectively (Karlsson et al., 2007; Bluhm et al., 2008). Other studies of cellulase expression demonstrated the possible effect of this enzyme in the pathogenicity of some fungi. For example, both *Alternaria alternata* infecting leaves of *Morinda citrifolia* (Hubballi et al., 2011) and *Heterobasidion parvaporum* causing wood rot on Norway spruce (Karlsson et al., 2007) were able to produce cellulase, indicating that the enzyme plays a role in their pathogenicity.

The cutinase gene showed the lowest expression in both media when compared to the other genes studied, but its expression was generally higher in MS medium (Figure 1B). The role of cutinases in the pathogenicity of fungi is still unclear. Some studies point out to an essential role for cutinase in virulence and pathogenicity of *Fusarium solani* f. sp. *pisi* on pea (Rogers et al., 1994; Li et al., 2002) and *Botrytis cinerea* on tomato leaves (Commènil et al., 1998). Other authors, in studies employing deletion mutants of *Botrytis cinerea* (Reis et al., 2005) and *Fusarium oxysporum* (Rocha et al., 2008) have shown that this enzyme is dispensable for virulence and pathogenicity.

Ochratoxin A was never reported as a pathogenicity factor in fungi and was never studied under this aspect. In this study, ochratoxin A showed little expression, except at 66 in both media and at 72 h in MS medium (Figure 1C).

Our study shows a higher rate of expression of protease in the MS medium (Figure 1D), what may be one indication of its importance in pathogenicity. This enzyme is essential for the pathogenicity in other systems, such as *Sclerotinia sclerotiorum* on sunflower (Poussereau et al., 2001) and *Botrytis cinerea* on apple fruits (Rolland et al., 2009).

A temporal analysis of expression shows that cutinase was the first enzyme to be expressed by *A. niger*, followed by protease and cellulase. Ochratoxin A was expressed after all the hydrolytic enzymes.

Although we recognize that the definitive role of hydrolytic enzymes and ochratoxin A in the pathosystem *A. niger* x sisal was not completely unveiled in this study, we provided the first tools to study this interaction by qPCR. The study was done *in vitro* as a first step in the study of the interaction between sisal and the pathogen *A. niger*.

The data shown in this study, despite their preliminary nature, constitute the first information on genes of *A. niger* potentially involved in its pathogenicity to sisal. The primers reported here not only allow the study of the interaction *in vitro* but will also facilitate the analyses of the roles of cutinases, proteases, cellulases and ochratoxin A during the events of pre-penetration, penetration and colonization of sisal by *A. niger*.

Conclusions

Primers for cutinases, proteases, cellulases and ochratoxin A, genes putatively involved in the pathogenicity of *A. niger* to sisal were designed and optimized for qPCR.

The expression of these genes was higher in the medium supplemented with sisal than in the minimal medium.

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