ALINE MYUKI OMORI

FUSARIUM VERTICILLIOIDES: IDENTIFICAÇÃO DE ANTÍGENO ESPÉCIE-ESPECÍFICO E DESENVOLVIMENTO DE PCR-ELISA PARA DETECÇÃO DO FUNGO

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RESUMO

Fusarium verticillioides é o principal patógeno de milho e pode causar desde uma infecção assintomática até o apodrecimento de todas as partes da planta. Além disso, esse fungo é o principal produtor de fumonisinas, que estão relacionadas a doenças em humanos e animais. Os métodos tradicionais utilizados para detectar fungos baseado em suas características morfológicas são demorados e apresentam baixa especificidade e sensibilidade. A identificação e a caracterização de possíveis antígenos espécie-específicos com potencial para serem utilizados em métodos imunológicos bem como o desenvolvimento de métodos rápidos, sensíveis e específicos para detecção desse fungo são importantes para o controle de qualidade do milho destinado à alimentação humana e animal. Portanto, os objetivos deste trabalho foram identificar e caracterizar um antígeno de 67 kDa possivelmente espécie-específico de *F. verticillioides* e desenvolver uma PCR-ELISA baseada no gene *FUM21*. A proteína de 67 kDa (p67) foi analisada por espectrometria de massa e sua atividade enzimática foi determinada em gel de poliacrilamida copolimerizado com amido, gelatina e soro albumina bovina. A PCR-ELISA foi desenvolvida utilizando *primers* e sonda específicos para o gene *FUM21*. A especificidade da PCR-ELISA e da PCR convencional foi determinada analisando DNA de isolados de *F. verticillioides*, *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *Aspergillus niger*, *A. ochraceus*, *A. carbonarius* e *Penicillium variabile* e a sensibilidade dos métodos foi avaliada utilizando concentrações de 250 fg a 25 ng de DNA de *F. verticillioides* 103G. O efeito de matriz e a sensibilidade da PCR-ELISA e da PCR convencional em milho também foram analisados inoculando de 10 a 10^7 conídeos de *F. verticillioides* 103G em milho triturado. Duas possíveis proteínas resultaram da análise da p67 por espectrometria de massa: a proteína relacionada ao precursor da aminopeptidase Y de *F. fujikuroi* e a provável glicoamilase GMY2 de *F. verticillioides*. A análise da atividade enzimática da p67 revelou que a proteína apresenta atividade de amilase, sugerindo, portanto, que a p67 é a provável glicoamilase GMY2 de *F. verticillioides*. A PCR-ELISA não apresentou positividade com DNA de *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *A. niger*, *A. ochraceus*, *A. carbonarius* e *P. variabile*, e foi capaz de detectar cinco isolados de *F. verticillioides* apresentando sensibilidade de 2,5 pg e 10^4 conídeos/g para DNA de cultura pura e para DNA de milho inoculado com suspensão de conídeos de *F. verticillioides*, respectivamente, sendo 100 vezes mais sensível do que a PCR convencional. Portanto, a p67 foi identificada como provável glicoamilase GMY2 de *F. verticillioides* o que permitirá a produção dessa proteína em sua forma recombinante em larga escala visando o desenvolvimento de método imunológico espécie-específico. A PCR-ELISA foi específica para *F. verticillioides*, foi mais sensível que a PCR convencional e apresentou potencial para ser utilizada na detecção de *F. verticillioides* em amostras de milho.

Palavras-chaves: *Fusarium verticillioides*. Fumonisinas. Glicoamilase. PCR-ELISA. *FUM21*.

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ABSTRACT

Fusarium verticillioides is the main pathogen of maize and could cause since an asymptomatic infection until rotting of all parts of the plant. Besides that, this fungus is the main producer of fumonisins, which are related to diseases in humans and animals. The traditional methods used to detect fungi based on their morphological characteristics are time-consuming and present low sensitivity and specificity. The identification and characterization of specie-specific antigens with potential to be used in immunological methods as well as the development of rapid, sensitive and specific methods for detection of this fungus are important for the quality control of the maize intended for human and animal consumption. Therefore, the objectives of this study were to identify and to characterize a possible specie-specific antigen of *F. verticillioides* and to develop a PCR-ELISA based on *FUM21* gene of this fungus. The 67 kDa protein (p67) was analyzed by mass spectrometry and its enzymatic activity was determined in polyacrylamide gel copolymerized with starch, gelatine and bovine serum albumine. PCR-ELISA was developed using specific primers and probe for the *FUM21* gene. The specificity of PCR-ELISA and conventional PCR was determined by analyzing DNA from *F. verticillioides*, *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *Aspergillus niger*, *A. ochraceus*, *A. carbonarius* and *Penicillium variabile* isolates and the sensitivity of the methods was evaluated using amounts from 250 fg to 25 ng of *F. verticillioides* 103G DNA. The matrix effect and the sensitivity of PCR-ELISA and conventional PCR in maize were analyzed by inoculating from 10 to 10^7 conidia of *F. verticillioides* 103G in ground corn. Two possible proteins resulted from the p67 analysis by mass spectrometry: protein related to aminopeptidase Y precursor of *F. fujikuroi* and putative glucoamylase GMY2 of *F. verticillioides*. The analysis of p67 enzymatic activity showed that the protein presents amylase activity, suggesting, therefore, that the p67 is the putative glucoamylase GMY2 of *F. verticillioides*. PCR-ELISA showed no positivity with DNA of *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *A. niger*, *A. ochraceus*, *A. carbonarius*, *P. variabile* and was able to detect five isolates of *F. verticillioides* and presented detection limits of 2.5 pg and 10^4 conidia/g for DNA of pure culture and for DNA of maize inoculated with conidia suspension of *F. verticillioides*, respectively, and was 100 folds more sensitive than conventional PCR. Therefore, the p67 was identified as putative glucoamylase GMY2 of *F. verticillioides* which will allow the production of this protein in its recombinant form on a large scale aiming the development of specie-specific immunological method. Moreover, the developed PCR-ELISA was specific, more sensitive when compared to the conventional PCR and showed potential to be used in the detection of *F. verticillioides* isolates in maize samples.

Key-words: *Fusarium verticillioides*. Fumonisins. Glucoamylase. PCR-ELISA. *FUM21*.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
ACN	Acetonitrile
Akt	Proteína quinase B
ANVISA	Agência Nacional de Vigilância Sanitária
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumine
CFU	Colony foming units
CoA	Coenzima A
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAO	Food and Agriculture Organization of the United Nations
FB_1	Fumonisina B_1
GSK-3β	Glicogênio sintase quinase 3β
IARC	International Agency for Research on Cancer
ic-ELISA	Indirect competitive enzyme-linked immunosorbent assay
ITS	Internal transcribed spacer
MDA	Malondialdeído
mPCR	Reação em cadeia da polymerase multiplex/Multiplex polymerase chain reaction
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCBI	National Center for Biotechnology Information
p67	Proteína de 67 kDa/67 kDa protein

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Reação em cadeia da polimerase/Polymerase chain reaction
PCR-ELISA	Polymerase chain reaction – Enzyme-linked immunosorbent assay
q-TOF	Quadrupole time-of flight
SDS	Sodium dodecyl sulfate
TFA	Trifluoroacetic acid
UPLC	Ultra-performance liquid chromatography

SUMÁRIO

1 INTRODUÇÃO

1.1 MILHO

O milho (*Zea mays*) é o cereal mais cultivado no mundo e apresenta um amplo espectro de aplicações sendo utilizado desde a alimentação animal até a produção de combustível (USDA, 2016). A Divisão de Estatísticas da Organização das Nações Unidas para Agricultura e Alimentação (FAO) (2015) estima que a produção mundial de milho em 2014 foi de 1 bilhão e 38 milhões, sendo os Estados Unidos, a China e o Brasil os maiores produtores, colhendo 35%, 21% e 8% da produção total de milho, respectivamente.

O milho apresenta propriedades nutricionais muito ricas contendo aproximadamente 72% de amido, 10% de proteínas e 4% de lipídeos, além de vitaminas do complexo B e minerais, sendo um dos principais componentes da ração animal e a base alimentar em muitos países (INGLETT, 1970; NUSS; TANUMIHARDJO, 2010; RANUM et al., 2014). Esse alto teor nutricional também o torna suscetível à contaminação por microrganismos que comprometem a produtividade e a qualidade dos grãos e das sementes (DALCERO et al., 1998; KPODO; THRANE; HALD, 2000; GHIASIAN et al., 2004; MEIRELLES et al., 2007; COVARELLI; BECCARI; SALVI, 2011).

Os principais patógenos do milho são os fungos do gênero *Fusarium*. Cerca de 90% das amostras de milho estão contaminadas por *Fusarium* spp., sendo *F. verticillioides* a espécie prevalente, com frequência de aproximadamente 70% (KEDERA; PLATTNER; DESJARDINS, 1999; ALMEIDA et al., 2000; MEIRELLES et al., 2007; NERBASS, 2008; COVARELLI; BECCARI; SALVI, 2011; LANZA, 2013).

1.2 *FUSARIUM VERTICILLIOIDES*

F. verticillioides é um fungo filamentoso pertencente ao filo Ascomycota, classe Sordariomycetes, ordem Hypocreales e família Nectriaceae (MYCOBANK, 2016). Em meio ágar batata dextrose, as culturas apresentam inicialmente micélios brancos que podem desenvolver pigmentos violetas com o tempo. Os macroconídeos são relativamente longos e delgados, ligeiramente curvados ou retos, de paredes finas com a célula apical curvada, a célula basal em forma de pé e o número de septos variando de 3 a 5 (**Figura 1** A e B). Os microconídeos são encontrados em grande quantidade nos micélios aéreos, são ovais ou em

forma de disco com a base achatada e normalmente não apresentam septos (**Figura 1** C e D). Os micélios aéreos são comumente formados por cadeias longas de microconídeos, mas agregados pequenos podem ocorrer ocasionalmente (**Figura 1** E e F) (LESLIE; SUMMERELL, 2006).

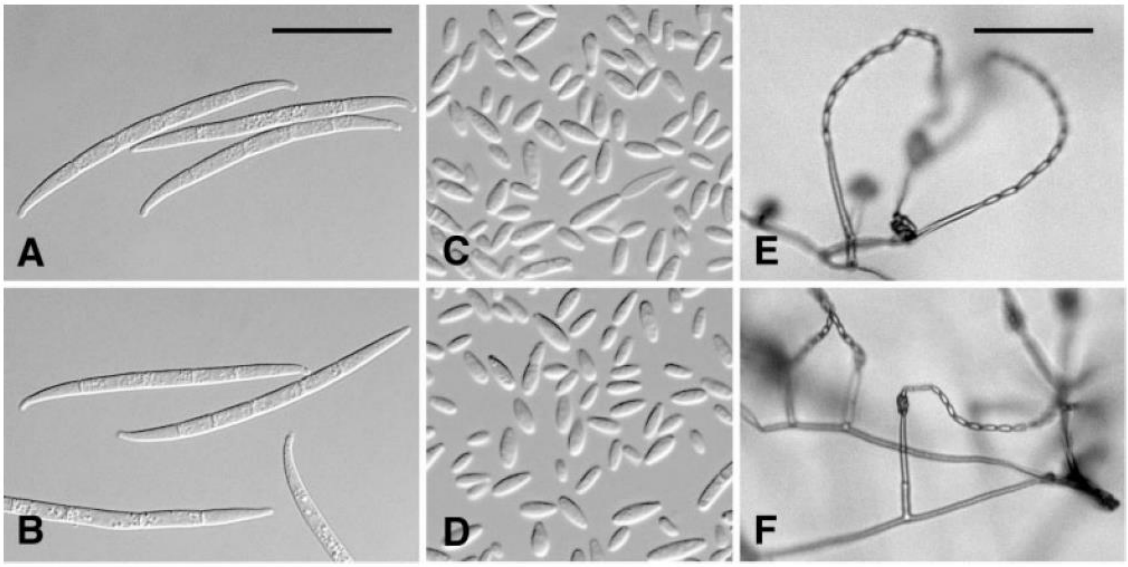


Figura 1 – Morfologia de *F. verticillioides* em microscopia óptica. A – B: Macroconídeos; C – D: Microconídeos; E – F: Microconídeos *in situ* em ágar folha de cravo. A – D, barra de escala = 25 µm; E – F, barra de escala = 50 µm.
Fonte: Leslie e Summerell (2006)

A contaminação de milho por *F. verticillioides* pode ocorrer por diversas vias. A forma mais comum é através de conídeos transportados pelo ar que invadem o milho através do estigma ou de lesões causadas por insetos (OOKA; KOMMEDAHL, 1977; HEADRICK; PATAKY, 1991; MUNKVOLD; CARLTON, 1997; MUNKVOLD; HELMICH; SHOWERS, 1997; MUNKVOLD; McGEE; CARLTON, 1997). Outra via de infecção é a sistêmica, que é causada por micélios ou conídeos presentes no interior ou na superfície da semente (FOLEY, 1962). Nessa via, o fungo se desenvolve dentro da plântula, se desloca das raízes para o caule e atinge a espiga e os grãos (KEDERA; LESLIE; CLAFLIN, 1992; MUNKVOLD; HELMICH; SHOWERS, 1997; MUNKVOLD; McGEE; CARLTON, 1997). A infecção sistêmica também pode ocorrer através de inóculos que sobrevivem em resíduos de colheita no solo e contaminam as sementes (LESLIE et al., 1990; RHEEDER; MARASAS, 1998).

F. verticillioides pode causar danos em todas as fases do desenvolvimento do milho, podendo levar a podridão de raiz, caule, espiga e deterioração de grãos (**Figura 2**). Geralmente,

o aparecimento de tecidos visivelmente danificados está associado à grande produção de micélios e a secreção de enzimas hidrolíticas (OREN et al., 2003).

Esse fungo também pode causar uma infecção assintomática, que é caracterizada por um desenvolvimento organizado do fungo restrito a tecidos específicos como as raízes laterais, onde ocorre a reprodução do fungo dentro de um número pequeno de células sem causar danos a células circundantes (OREN et al., 2003).

Além de causar prejuízos econômicos para a agricultura por afetar a qualidade do milho, *F. verticillioides* também é responsável por produzir diversas micotoxinas como fumonisinas, fusarina C e ácido fusárico e seus derivados (LESLIE; SUMMERELL, 2006).

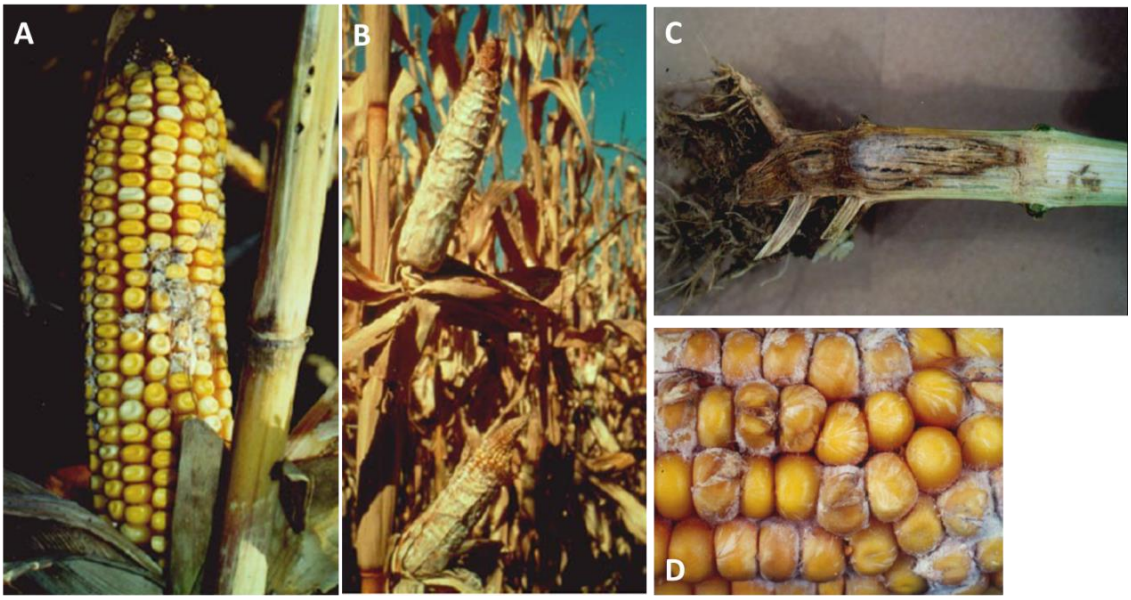


Figura 2 – Danos causados por *F. verticillioides* em milho. A: Podridão moderada na espiga; B: Podridão severa na espiga; C: Podridão no caule; D: Deterioração dos grãos.
Fonte: Munkvold e Desjardins (1997)

1.3 MICOTOXINAS

Micotoxinas são metabólitos secundários produzidos por fungos filamentosos que são capazes de causar efeitos tóxicos em animais e seres humanos (SMITH; MOSS, 1985). Constituem um grupo diversificado de compostos orgânicos de baixa massa molecular, que apresentam grande estabilidade térmica e química, permitindo sua persistência no alimento mesmo após a inativação dos fungos pelos processos usuais de industrialização e, ao serem metabolizadas pelos animais podem ocorrer em carnes, ovos e leite (NORRED, 2000; CAST, 2003).

A exposição às micotoxinas ocorre, predominantemente, por meio da ingestão de alimentos e rações contaminados (CHU, 1991). No entanto, a intoxicação por via dérmica e por inalação já foi relatada (CAST, 2003). As enfermidades causadas por micotoxinas são denominadas micotoxicoses e podem ser divididas em agudas e crônicas. As micotoxicoses agudas ocorrem quando há consumo de altas doses de micotoxinas e são caracterizadas por hepatite, hemorragia, nefrite, necrose das mucosas digestivas e podem levar a morte. As micotoxicoses crônicas são mais frequentes, ocorrem quando existe o consumo prolongado de baixas doses de micotoxinas e são caracterizadas por redução da eficiência reprodutiva, diminuição da conversão alimentar, redução da taxa de crescimento, diminuição no ganho de peso e podem atuar no desenvolvimento de câncer (SMITH; MOSS, 1985; IARC, 2002).

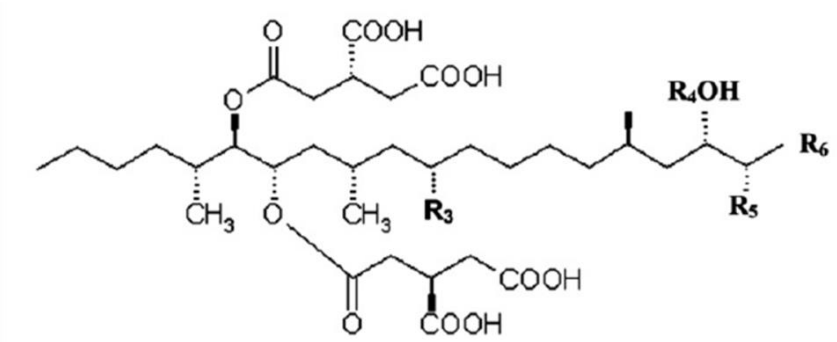
Embora animais e seres humanos sejam suscetíveis a esses efeitos, os animais apresentam maior probabilidade de contaminação devido à ingestão de grãos de baixa qualidade (D'MELLO; PLACINTA; MACDONALD, 1999), sendo o maior problema das micotoxicoses atribuído aos prejuízos relacionados à queda no desempenho produtivo de animais (SMITH; MOSS, 1985).

1.3.1 Fumonisinas

As fumonisinas foram isoladas pela primeira vez em 1988 de culturas de *F. verticillioides*. Atualmente, sabe-se que essas micotoxinas também são produzidas por *F. proliferatum*, *F. napiforme*, *F. oxysporum*, *F.dlamini* e *F. nygamai* (BEZUIDENHOUT et al., 1988; GELDERBLOM et al., 1988; KNAFLEWSKI et al., 2008).

As fumonisinas são divididas em quatro grupos de acordo com a sua estrutura química: A, B, C e P (**Figura 3**). As fumonisinas das séries B são as mais frequentemente encontradas em amostras de milho, sendo a fumonisina B_1 (FB_1) a mais abundante, representando cerca de 70 a 80% do total de fumonisinas em culturas de *F. verticillioides* e em alimentos naturalmente contaminados (WASKIEWICZ, BESZTERDA; GOLINSKI, 2012).

Estudos realizados em países da África, Ásia, Europa e América do Sul mostram que a incidência de fumonisinas em amostras de milho pode chegar a 100% e em alguns casos com nível médio acima do limite máximo recomendado pela União Européia (1000 μg/Kg) (DOKO et al., 1995; RAMIREZ et al., 1996; JINDAL; MAHIPAL; ROTTINGHAUS, 1999; MEDINA-MARTÍNEZ; MARTÍNEZ, 2000; KPODO; THRANE; HALD, 2000; FANDOHAN et al., 2005; GONG et al., 2009; COVARELLI; BECCARI; SALVI, 2011; OLIVEIRA et al., 2017).



Fumonisins	Empirical formula	Molecular weight	R_3	R_4	R_5	R_6
Fumonisin A_1	$C_{36}H_{61}NO_{16}$	763	OH	OH	$NHCOCH_3$	CH_3
Fumonisin A_2	$C_{36}H_{61}NO_{15}$	747	H	OH	$NHCOCH_3$	CH_3
Fumonisin A_3	$C_{36}H_{61}NO_{15}$	747	OH	H	$NHCOCH_3$	CH_3
Fumonisin B_1	$C_{34}H_{59}NO_{15}$	721	OH	OH	NH_2	CH_3
Fumonisin B_2	$C_{34}H_{59}NO_{14}$	705	H	OH	NH_2	CH_3
Fumonisin B_3	$C_{34}H_{59}NO_{14}$	705	OH	H	NH_2	CH_3
Fumonisin C_1	$C_{33}H_{57}NO_{15}$	707	OH	OH	NH_2	H
Fumonisin P_1	$C_{39}H_{62}NO_{16}$	800	OH	OH	3HP	CH_3
Fumonisin P_2	$C_{39}H_{62}NO_{15}$	784	H	OH	3HP	CH_3
Fumonisin P_3	$C_{39}H_{62}NO_{15}$	784	OH	H	3HP	CH_3

Figura 3 – Estrutura química das fumonisinas.
Fonte: Waskiewicz, Beszterda e Golinski (2012)

A ingestão de milho e derivados contaminados com fumonisinas está associada a diversas doenças em humanos e animais. As fumonisinas causam leucoencefalomalácia em equinos (MARASAS et al., 1988), edema pulmonar em suínos (ROSS et al. 1990), redução no desenvolvimento e imunossupressão em aves (WEIBKING et al., 1993; LI et al., 1999) e em ratos foi comprovada a ação hepatotóxica e hepatocarcinogênica (GELDERBLOM et al., 1988). Em seres humanos, estudos epidemiológicos indicam a sua associação com defeitos no tubo neural de fetos (MISSMER et al., 2006) e com o câncer esofágico e hepático primário (YOSHIZAWA; YAMASHITA; LUO, 1994; UENO et al., 1997). Essas micotoxinas são classificadas pela Agência Internacional de Pesquisa sobre Câncer (IARC) (2002) como carcinógeno do grupo 2B, ou seja, provavelmente carcinogênico.

A atividade tóxica das fumonisinas se deve à sua ação no metabolismo dos esfingolipídeos, que são lipídeos importantes na estruturação da membrana plasmática e que podem atuar como sítios de reconhecimento nas superfícies de algumas células. Devido à semelhança estrutural entre as fumonisinas e as bases esfingóides (esfinganina e esfingosina), metabólitos importantes na biossíntese de esfingolipídeos, ocorre uma competição pela enzima ceramida sintase, responsável por converter a esfinganina em dihidroceramida e a esfingosina

em ceramida. As fumonisinas, ao se ligarem à ceramida sintase, promovem sua inibição, diminuindo a biossíntese de ceramidas, aumentando esfinganina e esfingosina livres e reduzindo a degradação dos esfingolipídeos provenientes da dieta (MERRILL et al., 1993; NORRED et al., 1997). O balanço entre esses compostos está intimamente relacionado à apoptose, uma vez que a diminuição nos níveis de ceramida e aumento nos níveis de esfingosina inibem a apoptose e o aumento nos níveis de ceramida, esfinganina e ácidos graxos induzem a apoptose (YOO et al., 1992; SCHROEDER et al., 1994; YOO et al., 1996; SCHMELZ e al., 1998; RILEY et al., 2001). Assim, as fumonisinas podem tanto induzir como inibir a apoptose, apresentando resultados controversos (RILEY et al., 2001). Além disso, a diminuição dos complexos esfingóides afeta a função de proteínas de membrana como a proteína de ligação ao folato (receptor α de folato) o que pode estar associado a defeitos no tubo neural de fetos (STEVENS; TANG, 1997; ZHANG et al., 2008).

Essas micotoxinas também podem induzir a peroxidação lipídica que está relacionada a diversos processos patológicos (SEVANIAN; HOCHSTEIN, 1985). Yin et al. (1998) demonstraram que as fumonisinas aumentam a permeabilidade e o transporte de oxigênio na membrana e observaram que essas toxinas podem promover a produção de radicais livres intermediários e acelerar a reação em cadeia associada à peroxidação lipídica. Abado-Becognee et al. (1998) observaram que a FB_1 é um potente indutor de malondialdeído (MDA), um dos produtos secundários da peroxidação lipídica.

As fumonisinas também causam alterações na atividade de proteínas quinases envolvidas no ciclo celular como a ciclina D1, a glicogênio sintase quinase 3β (GSK-3β) e a proteína quinase B (Akt), que podem levar a ruptura no ponto de restrição do ciclo celular, sendo um possível mecanismo pelo qual essas micotoxinas atuam no desenvolvimento do câncer (RAMLJAK et al., 2000).

Diversos países estabeleceram limites máximos tolerados para fumonisinas em alimentos e rações. A União Europeia recomenda um limite máximo de fumonisinas ($B_1 + B_2$) em milho e produtos derivados para humanos de 1.000 µg/Kg e para animais de 60.000 µg/Kg (COMISSÃO EUROPEIA, 2006, 2007). No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) estabeleceu em 2012 um limite máximo de fumonisinas de 2.000 µg/Kg para produtos à base de milho e de 2.500 µg/Kg para derivados de milho destinados ao consumo humano. Os limites máximos tolerados de fumonisinas em ração animal no Brasil não foram determinados pela legislação, no entanto o Grupo de Trabalho sobre Micotoxinas (2006) criado

pelo Ministério da Agricultura recomenda um limite máximo de 10.000 µg/Kg em milho destinado à alimentação animal.

1.4 GENES *FUM*

A biossíntese de fumonisinas é coordenada por diversas proteínas codificadas por genes conhecidos como genes *FUM* que se encontram agrupados no genoma do fungo (PROCTOR et al., 2003). O agrupamento de genes envolvidos na biossíntese de metabólitos secundários em ascomicetos filamentosos é comum (BROWN et al., 1996; TUDZYNSKI; HOLTER, 1998).

Atualmente, são conhecidos 17 genes *FUM* (*FUM1*, *FUM2*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM10*, *FUM11*, *FUM13*, *FUM14*, *FUM15*, *FUM16*, *FUM17*, *FUM18*, *FUM19*, *FUM20* e *FUM21*) que abrangem uma região de 46 kb do genoma de *F. verticillioides* (PROCTOR et al., 2003; PROCTOR et al., 2013) (**Figura 4**).

Figura 4 – Organização dos genes *FUM*. Há evidências de que um pequeno gene (*FUM20*) está localizado entre os genes *FUM2* e *FUM13* (BROWN et al., 2005).
Fonte: Proctor et al. (2013)

As funções dos genes *FUM* na biossíntese de fumonisinas em *F. verticillioides* têm sido preditas baseadas na estrutura química das fumonisinas e na similaridade dos genes *FUM* com genes cujas funções já são conhecidas ou têm sido determinadas geneticamente pela ruptura do gene e complementação do mutante (PROCTOR et al., 1999; SEO; PROCTOR; PLATTNER, 2001; BUTCHKO; PLATTNER; PROCTOR et al., 2003a, 2003b; BOJJA et al., 2004; BROWN et al., 2005; BUTCHKO; PLATTNER; PROCTOR, 2006; ZALETA-RIVERA et al., 2006; BROWN et al., 2007). O provável produto dos genes *FUM* e as suas funções são mostrados no Quadro 1.

Quadro 1 – Prováveis produtos dos genes *FUM* e suas funções.

Gene	Provável produto	Provável função	Referência

FUM1	Policetídeo Sintase	Catalisar a síntese do policetídeo linear de 18 carbonos formando a estrutura da fumonisina do carbono 3 (C-3) ao carbono 20 (C-20)	Proctor et al., 1999 Bojja et al., 2004
FUM2	Citocromo P-450 monoxigenase	Catalisar a hidroxilação do carbono 10 (C-10) da estrutura da fumonisina	Proctor et al., 2003
FUM3	Dioxigenase	Catalisar a hidroxilação do carbono 5 (C-5) da estrutura da fumonisina	Seo; Proctor; Plattner, 2001 Butchko; Plattner; Proctor, 2003b
FUM6	Citocromo P-450 monoxigenase fusionado a P450 redutase dependente de NADPH	Catalisar a adição de oxigênio no carbono 14 (C-14) e/ou carbono 15 (C-15) na estrutura da fumonisina	Seo; Proctor; Plattner, 2001
FUM7	Desidrogenase	Catalisar a redução da dupla ligação do grupo alceno presente nos ésteres tricarbalílicos ligados ao C-14 e ao C-15.	Seo; Proctor; Plattner, 2001 Butchko; Plattner; Proctor, 2006 Zaleta-Rivera et al., 2006
FUM8	Oxoamino sintase	Catalisar a liberação da cadeia policetídeo de 18 carbonos da enzima Fum1p e a condensação de alanina ao policetídeo formando a estrutura da fumonisina do carbono 1 (C-1) ao C-20 com o grupo amino no carbono 2 (C-2)	Seo; Proctor; Plattner, 2001
FUM10	Acil-Coenzima A Sintase	Ativar o policetídeo da fumonisina e/ou o precursor do éster tricarboxílico através de CoA	Proctor et al., 2003 Butchko; Plattner; Proctor, 2006 Zaleta-Rivera et al., 2006
FUM11	Transportador mitocondrial de tricarboxilatos	Transportar ácidos tricarboxílicos precursores de ésteres tricarboxílicos do lumen mitocondrial para o citoplasma	Proctor et al., 2003 Butchko; Plattner; Proctor, 2006
FUM13	Desidrogenase de cadeia curta ou Redutase	Catalisar a redução do grupo carbonila no C-3 da fumonisina para uma hidroxila	Butchko; Plattner; Proctor, 2003a Proctor et al., 2003
FUM14	Domínios de condensação de peptídeo sintase não ribossomal	Catalisar a esterificação dos ácidos tricarboxílicos ativados por CoA no carbono C-14 e C-15 da estrutura da fumonisina	Butchko; Plattner; Proctor, 2006 Zaleta-Rivera et al., 2006
FUM15	Citocromo P-450 monoxigenase	Catalisar a hidroxilação da estrutura da fumonisina	Proctor et al., 2003
FUM16	Acil-Coenzima A Sintase	Ativar o policetídeo da fumonisina e/ou o precursor do éster tricarboxílico através de CoA	Proctor et al., 2003 Butchko; Plattner; Proctor, 2006
FUM17	Fator de garantia de Longevidade	Proteger contra a ação das fumonisinas	Proctor et al., 2003
FUM18	Fator de garantia de Longevidade	Proteger contra a ação das fumonisinas	Proctor et al., 2003
FUM19	Transportador ABC	Transportar compostos do interior das células para o meio externo	Proctor et al., 2003
FUM21	Fator de transcrição	Regular positivamente a expressão dos genes *FUM*	Brown et al., 2007

Diversos pesquisadores utilizaram a PCR, do inglês *Polymerase Chain Reaction*, baseado em genes *FUM* com o objetivo de identificar isolados produtores de fumonisinas. O

gene *FUM1* foi utilizado como alvo nos trabalhos de Bluhm et al. (2002) e Sreenivasa et al. (2006) e foi capaz de detectar isolados de *F. anthophilum*, *F. proliferatum* e *F. verticillioides*, porém alguns isolados dessas espécies foram negativos no teste, sugerindo que não são produtores de fumonisinas ou que o método apresenta baixa sensibilidade.

Ramana et al. (2011) e Rashmi et al. (2012) desenvolveram uma PCR baseada nos genes *FUM1* e *FUM13* que detectou tanto isolados produtores como não produtores de fumonisinas provavelmente porque as condições do experimento não foram apropriadas para a expressão do gene ou devido à baixa especificidade do método. Em amostras de arroz e milheto naturalmente contaminadas, a PCR apresentou resultados negativos para amostras positivas para fumonisinas possivelmente devido à degradação de DNA, baixa sensibilidade ou extração ineficiente de DNA a partir dos grãos (RAMANA et al., 2011).

Mudili et al. (2014) utilizaram os *primers* para o gene *FUM13* desenhados por Ramana et al. (2011) para detectar isolados produtores de fumonisinas e observaram que o método foi capaz de detectar todos os isolados de *F. proliferatum* e *F. verticillioides* testados, porém não verificaram se eram produtores de fumonisinas.

Os genes *FUM6* e *FUM8* foram utilizados como alvos na PCR desenvolvida por Dawidiziuk et al. (2014). O método foi testado em 96 isolados fúngicos, dos quais 72 eram isolados de *Fusarium* sp. e 24 eram isolados de fungos filamentosos não-*Fusarium*, e apresentou sensibilidade e especificidade de 89%. Em amostras de grãos de trigo, a PCR apresentou resultados positivos para amostras contaminadas, porém, não foi capaz de detectar amostras contaminadas de palha de trigo provavelmente devido à baixa qualidade do DNA extraído da palha que pode conter inibidores da PCR, como polissacarídeos (DEMEKE; JENKINS, 2010).

Divakara et al. (2014) desenvolveram uma PCR multiplex (mPCR) baseada nos genes *FUM1*, *FUM8* e *FUM21* para diferenciar *Fusarium* spp. produtores e não produtores de fumonisinas provenientes de sementes de soja. Os isolados de *F. verticillioides* produtores de fumonisinas foram positivos para os três genes, enquanto que o isolado não produtor de fumonisinas foi positivo apenas para *FUM1* e *FUM8*, indicando que o gene *FUM21* apresenta maior potencial para diferenciar isolados de *F. verticillioides* produtores de não produtores de fumonisinas.

1.4.1 Gene *FUM21*

O gene *FUM21* está localizado adjacente ao gene *FUM1* e possivelmente codifica um fator de transcrição que contém um motivo de ligação ao DNA Zn(II)2Cys6 e regula positivamente a produção de fumonisinas. A deleção desse gene em isolados de *F. verticillioides* resulta na ausência ou baixa produção de fumonisinas e a complementação dos mutantes deficientes com gene *FUM21* restaura a produção de fumonisinas, indicando sua participação na biossíntese de fumonisinas (BROWN et al., 2007).

Assim como as fumonisinas, a expressão do gene *FUM21* é afetada por diversos fatores como temperatura, atividade de água, salinidade e pH. Fanelli et al. (2013) observaram que a expressão do gene *FUM21* reflete o perfil de produção de fumonisinas em diferentes condições com exceção da temperatura, uma vez que o gene foi expresso em maior quantidade a 15°C e a produção máxima de fumonisinas ocorreu a 30°C. Sabe-se que a regulação da expressão do gene *FUM21* envolve fatores epigenéticos. Visenstin et al. (2012) observaram que um estado de hiperacetilação das histonas próximo à região promotora está associado a um aumento na expressão desse gene.

1.5 EXOANTÍGENOS

Exoantígenos são macromoléculas imunogênicas produzidas por microrganismos durante o seu cultivo, sendo detectados em filtrados de culturas. Esses antígenos são importantes na identificação imunológica de patógenos fúngicos e na resolução de problemas taxonômicos, uma vez que os fungos produzem antígenos únicos que permitem a identificação específica (KAUFMAN; STANDARD, 1987).

Meirelles et al. (2006) produziram e caracterizaram exoantígenos de 8 isolados de *F. verticillioides*. Os exoantígenos apresentaram concentração média de proteína de 296,6 µg/mL e concentração média de carboidratos de 152,3 µg/mL. O perfil eletroforético mostrou uma grande variedade de bandas com massas moleculares variando de 17 a 170 kDa. Esses exoantígenos foram utilizados para imunizar coelhos, resultando em alto título de anticorpos policlonais que apresentaram potencial para serem utilizados na imunodetecção desse fungo.

Diversos trabalhos desenvolveram ELISAs (*Enzyme-linked Immunosorbent Assays*), baseados em exoantígenos capazes de detectar *Fusarium* spp. a nível de gênero em grãos (ABRAMSON et al., 1998; IYER; COUSIN, 2003; ROHDE; RABENSTEIN, 2005; MEIRELLES et al., 2007). Abramson et al. (1998) utilizaram exoantígenos de *F. graminearum* e *F. sporotrichioides* para produzir anticorpos em galinhas e desenvolveram um ELISA indireto

para estimar o nível de exoantígenos de espécies de *Fusarium* em amostras de trigo. Meirelles et al. (2007) desenvolveram um ELISA competitivo indireto baseado em anticorpos policlonais contra exoantígeno de *F. verticillioides* capaz de detectar *Fusarium* sp. em amostras de milho.

A produção de um método imunológico espécie-específico capaz de identificar isolados de *F. verticillioides*, principais contaminantes do milho e produtores de fumonisinas, depende da identificação de proteínas espécie-específicas. Biazon et al. (2006) observaram a presença de duas proteínas de massas moleculares aparentes de 67 kDa e 114 kDa no exoantígeno de *F. verticillioides* reconhecidas apenas em isolados desta espécie e que portanto apresentam potencial para serem espécie-específicas. A identificação e caracterização dessas proteínas seriam de grande importância para o desenvolvimento de métodos imunológicos específicos para *F. verticillioides*.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Identificar a proteína possivelmente espécie-específica de 67 kDa do exoantígeno de *F. verticillioides* e desenvolver uma PCR-ELISA baseada no gene *FUM21* para detecção de *F. verticillioides*.

2.2 OBJETIVOS ESPECÍFICOS

- Produzir exoantígeno de *F. verticillioides* 97K;
- Proceder a extração in-gel de peptídeos da proteína de 67 kDa (p67) do exoantígeno de *F. verticillioides* 97K;
- Analisar os peptídeos da p67 por espectrometria de massa;
- Analisar a atividade enzimática da p67;
- Padronizar a extração de DNA de culturas puras e de amostras de milho;
- Padronizar uma PCR convencional utilizando *primers* para o gene *FUM21*;
- Padronizar uma PCR-ELISA utilizando *primers* e sonda para o gene *FUM21*;
- Determinar a especificidade da PCR convencional e da PCR-ELISA;
- Determinar a sensibilidade da PCR convencional e da PCR-ELISA;
- Comparar a PCR-ELISA com a PCR convencional.

ARTIGOS

ARTIGO 1:

IDENTIFICATION OF A POSSIBLE SPECIE-SPECIFIC PROTEIN OF *FUSARIUM VERTICILLIOIDES* EXOANTIGEN

Identification of a possible specie-specific protein of *F. verticillioides* exoantigen

Abstract

Fusarium verticillioides is an important maize pathogen and produces mycotoxins such as fumonisins that can cause damage to human and animal health. The development of a rapid, sensitive and specific method to detect this fungus is necessary and immunological detection is a promising alternative. The discovery, identification and characterization of specie-specific antigens are important in the production process of a specific immunological technique. Therefore, the objective of this study was to identify the 67 kDa protein (p67) of *F. verticillioides* exoantigen which presents high potential to be a specie-specific antigen. The identification of p67 was performed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) and by in-gel analysis of the enzymatic activity. The analysis of in-gel trypsin digested p67 by mass spectrometry revealed two possible proteins: the protein related to aminopeptidase Y precursor of *F. fujikuroi* and the putative glucoamylase GMY2 of *F. verticillioides*. To confirm which of these proteins the p67 is, the enzymatic activity was explored and showed that this protein presents an amylase activity indicating that p67 is the putative glucoamylase GMY2 of *F. verticillioides*.

Keywords: Toxigenic fungi; p67; immunological method; fumonisin; mycotoxin.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg (syn. *F. moniliforme* J. Sheld) is an important maize pathogen [1-3] and causes since asymptomatic infection until severe rotting of all parts of the plant.[4] Besides damaging the plants, this fungus produces mycotoxins, such as fumonisins which are associated with neural tube defects [5] and esophageal and liver cancer in humans [6,7] being classified as Group 2B (possibly carcinogenic) by International Agency for Research on Cancer (IARC).[8] In animals, this mycotoxin is associated with leukoencephalomalacia in equines,[9] pulmonary edema in swines,[10] growth reduction and immunosuppression in birds[11,12] and have hepatotoxic and hepatocarcinogenic effect in rats.[13]

Studies carried out in countries of the Africa, Asia, Europe and South America show that the incidence of fumonisins in corn samples can reach 100% and in some cases with

average level above the maximum limit recommended by the European Union (1000 μg/Kg).[3,14-21]

Due to the high contamination of maize by *F. verticillioides* and fumonisins [2,3,18] and the health problems caused by this mycotoxin, it is necessary to develop rapid, sensitive and specific methods for quality control of corn for feeding. Culture in several media, microscopic examination and chemical analyzes of chitin, ergosterol and secondary metabolites are the methods traditionally used for detection of fungus and mycotoxins contamination.[22-25] These methods present low specificity and sensitivity and are time-consuming, except for the identification of secondary metabolites by chromatography and mass spectrometry that although present high sensitivity and specificity are laborious, use toxic reagents and require extensive cleaning process of the sample.

Enzyme-linked immunosorbent assay (ELISA) is an alternative method for detection of *F. verticillioides* because this method allows analysis of several samples in a single test, presents simple sample processing and high sensitivity and specificity and can detect the presence of fungi in food even after heat treatment which enables the evaluation of contamination in processed foods. ELISA based in exoantigens, that are immunogenic macromolecules produced and released to the culture medium throughout the growth of the fungus, is broadly employed in the pathogenic fungi identification and detection because most fungi produce specie-specific exoantigens.[2,26,27]

The ELISAs developed until now for detection of *Fusarium* species in food are genus-specific detecting both fumonisin non-producing and fumonisin producing species of *Fusarium*.[2,28] Taking into account the high contamination of maize with *F. verticillioides* and that fumonisins are harmful to humans and animals' health, it is necessary to develop specific methods to detect this fungus to improve the quality control of the corn. In this way, it is very important the identification and characterization of specie-specific proteins which present potential to differentiate *F. verticillioides*, the main fumonisins producer, from other species of *Fusarium*.

Biazon et al. (2006)[29] described a possible specie-specific protein of *F. verticillioides* isolates with apparent molecular weight of 67 kDa. According to studies carried by our group, the indirect competitive ELISA using antibodies specific to 67 kDa protein (p67) was able to quantify *F. verticillioides* exoantigen in poultry feed sample. The concentration of *F. verticillioides* exoantigen determined by this method showed high positive correlation with the

fumonisin level, with Pearson's correlation coefficient (r) of 0.76, which reinforces the idea that p67 is specific for *F. verticillioides* since this is the main producer of fumonisin.

Although the use of p67 could increase the specificity of the method, the purification of the protein from exoantigen is laborious and presents low yield. Therefore, it is important to identify this protein to determine the gene responsible for its synthesis which would allow the production of a recombinant p67. The mass spectrometry (MS) has been widely used in the identification of several compounds of different organisms.[30-34]

Taking into account the need of specific and sensitive methods for detection of *F. verticillioides* and the probable specie-specificity of p67, the objective of this study was to identify this protein for future applications in the development of a specific immunological method to detect *F. verticillioides* in foods.

Material and Methods

Fusarium verticillioides culture and exoantigen production

F. verticillioides 97K isolate was kindly provided by Prof. Dr. Elisa Yoko Hirooka, Department of Food and Drug Technology, State University of Londrina, Paraná, Brazil and was routinely grown on potato dextrose agar at room temperature. The exoantigen of *F. verticillioides* 97K was obtained according to Meirelles et al. (2006).[35] Briefly, suspension of 10^7 conidia/mL from *F. verticillioides* 97K grown on potato dextrose agar for 7 days was prepared in sterile 0.15 M phosphate buffer saline (PBS) containing 0.1% Tween 80 and aliquots of 1 mL were transferred to 250 mL of brain heart infusion broth. The cultures were incubated at 28°C under agitation for 14 days and were inactivated with 0.02% thimerosal for 24 hours at 4°C, followed by vacuum filtration and centrifugation at 4,500 x *g* at 4°C for 20 minutes. The supernatant containing the exoantigen was dialyzed for 24 hours at 4°C with distilled water and PBS in dialysis tubes with exclusion limit of 12 – 16 kDa, was lyophilized and was resuspended in distilled water. The protein concentration was determined by Bradford method using bovine serum albumin (BSA) (Sigma, Saint Louis, USA) as standard.[36]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The separation of p67 from the other components of the *F. verticillioides* 97K exoantigen was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970).[37] Briefly, *F. verticillioides* 97K exoantigen was mixed with sample buffer (80 mM Tris; 6% v/v SDS; 46% v/v glycerol; 7.7% v/v β-mercaptoethanol; 0.0125% v/v bromophenol blue), boiled for 5 min and 15 µL of the mixture and 10 µL of the molecular weight standard from 10 to 200 kDa (Invitrogen, Carlsbad, USA) were applied in the channels of the gel (5% stacking gel and 10% resolving gel). The electrophoresis was carried out at 100 V until the dye reaches the end of the gel. The gel was then stained with Coomassie Brilliant Blue (Biorad, Hercules, USA).[38] BSA was submitted to the same process to be used as control in MS.

The concentration of p67 was determined by the software of public domain Image J (Research Service Branch, National Institute of Health, USA).

In-gel digestion and extraction of peptides

The digestion of p67 and the extraction of its peptides from the gel were performed according to Shevichenko et al (2006).[39] Briefly, after electrophoresis and gel staining with Coomasie, the bands of p67 were highlighted from the gel, cut into small cubes, transferred to a microtube and destained with 100 mM ammonium bicarbonate (Sigma, Saint Louis, USA) in 50% acetonitrile (ACN) (Sigma, Saint Louis, USA). The gel containing the target protein was then dehydrated with pure ACN, vacuum-dried and treated with trypsin solution (Sigma, Saint Louis, USA) at 20 ng/µL overnight. The extraction buffer containing 5% v/v trifluoroacetic acid (TFA) (Sigma, Saint Louis, USA) in 67% v/v ACN was then added to the microtube followed by incubation for 1 h at 37°C under gentle agitation. The supernatant containing the peptides was then transferred to a new microtube, was vacuum-dried and stored at -20°C until the analysis by UPLC-MS/MS.

The BSA and a peace of the gel without any protein were submitted to the same procedures and were used as controls in MS.

Analysis by Mass Spectrometry

The electrospray ionization tandem mass spectrometry (ESI-MS/MS) using a quadrupole time-of-flight (Q-TOF) linked to ultra-performance liquid chromatography (UPLC) (Bruker Corporation, Massachusetts, USA) was used to analysis the peptides generated from the trypsin digestion in gel. The dry extracts of p67, BSA and gel without any protein were ressuspended in 0.1% TFA. The system was equipped with a 150 x 2.0 mm C18, 3µm column (Phenomenex, California, USA) and the solvents used were as follows: 0.03% v/v TFA (solvent A); 90% ACN v/v with 0.03% v/v TFA (solvent B). The gradient elution used was 0–30 min, 2–60% B; 30–36 min, 60 – 90% B; 36–45 min, 90–2% B at 40°C with a flow rate of 200 µL/min and injection volume of 10 µL. ESI voltage of 1.3 kV in positive ion mode, nebulizing pressure of 3 bar and drying gas flow of 9 L/min were applied for ionization using nitrogen.

The data processing was performed using DataAnalysis (Bruker, California, USA) and the proteins identification was made using the commercially available search software Mascot (Matrix Science, London, England) with the following parameters: National Center for Biotechnology Information (NCBI) for database, all entries for taxonomy, trypsin for enzyme, one missed cleavage allowed, peptide mass tolerance of ± 1.2 Da, fragment mass tolerance of ± 0.6 Da, none fixed modification, none variable modification, ions score cut off of 20 and $p < 0.05$ for significance threshold.

In-gel analysis of the enzymatic activity of the p67

The amylase activity of the *F. verticillioides* exoantigen proteins was determined according to Martínez et al. (2000)[40] with some modifications. Briefly, the *F. verticillioides* 97K exoantigen was mixed with sample buffer (0.125 M Tris; 20% v/v glycerol; 0.04% v/v bromophenol blue) and was submitted to the SDS-PAGE[37] in a 10% resolving gel containing 0.25% v/v copolymerized starch. The gel was subjected to a voltage of 100 V at 4°C until the dye reaches the end of the gel. After electrophoresis, the gel was washed for 1 h with 2.5% Triton X-100 at room temperature and then was incubated for 3 h in 0.1 M Tris-HCl pH 7.6 containing 2 mM $CaCl_2$ at 39°C. After this period, the gel was washed with distillated water, the bands were fixed with 12% w/v Trichloroacetic acid and the gel was stained with Lugol solution (6.7 mg/mL KI and 3.3 mg/mL I_2).

The protease activity of the *F. verticillioides* exoantigen proteins was analyzed according to the Heussen and Dowdle (1980)[41] with some modifications. Briefly, the *F.*

verticillioides 97K exoantigen was mixed with sample buffer (7.5% v/v SDS; 3% v/v sucrose (Sigma, Saint Louis, USA); 12 µg/mL bromophenol blue) and was submitted to the SDS-PAGE[37] in a 10% resolving gel containing 0.1% v/v gelatin and in a 10% resolving gel containing 0.1% v/v BSA. The electrophoresis was carried out at 100 V at 4°C until the dye reaches the end of the gel. The gel was then washed with 2.5% Triton X-100 for 1 h at room temperature and was incubated with 0.1 M Glycine pH 8.3 for 4 h at 37°C. After this period, the gel was stained with Coomasie Brilliant Blue.[38] Trypsin was used as a positive control.

Results and Discussion

The exoantigen of *F. verticillioides* 97K showed a concentration of 1.5 mg/mL. After SDS-PAGE and Coomassie staining, it was possible to distinguish p67 band (Figure 1) from the other bands. The BSA presented some extra bands probably due to its degradation or contamination however it remained the most abundant protein (**Figure 1**). The Image J estimated that p67 represents 30% of the total proteins in the *F. verticillioides* 97K exoantigen. Approximately 1 mmol of p67 were highlighted from the gel and submitted to in-gel trypsin digestion.

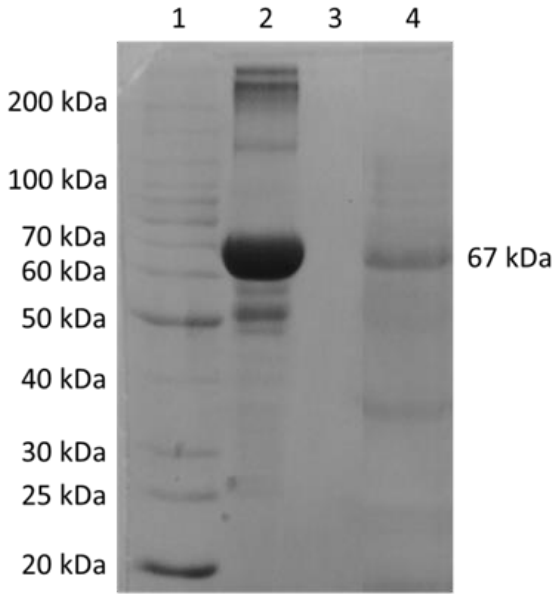


Figure 1. Electrophoretic pattern of *F. verticillioides* 97K exoantigen and BSA in polyacrylamide gel stained by Coomassie Brilliant Blue. 1. Molecular weight standard; 2. BSA; 3. Empty; 4. *F. verticillioides* 97K exoantigen.

The analysis of the in-gel trypsin digested p67 by UPLC-ESI-qTOF MS/MS and the subsequent research in NCBI database using Mascot resulted in two possible proteins, the

protein related to aminopeptidase Y precursor of *F. fujikuroi* (Acession number: CCT71037)[42] with score of 1502 and the putative glucoamylase GMY2 of *F. verticillioides* (Acession number: ABY89281)[43] with score of 989. Nineteen peptides generated by the digestion of p67 matches with the peptides present in the protein related to aminopeptidase Y precursor covering 50% of its amino acid sequence. On the other hand, the putative glucoamylase GMY2 presented 34% of its amino acid sequence covered by sixteen peptides generated by the digestion of p67.

In the negative control that consisted by gel digested with trypsin without any protein only cationic trypsin of *Bos taurus* (Acession number: P00769)[44] was identified with score of 2167. Five peptides generated by negative control digestion covered 27% of the total amino acid sequence of identified protein. And as expected, the in-gel trypsin digested BSA was identified as serum albumin of *Bos taurus* (Acession number: P02769)[45] with score of 3669. Nineteen peptides generated by the digestion of BSA in gel covered 29% of the amino acid sequence of the identified protein. These results indicate that there was no contamination during the sample processing and that the data obtained are reliable.

The protein related to aminopeptidase Y precursor of *F. fujikuroi* is composed by 491 amino acids and contains region similar to protease-associated domain and polypeptide binding site suggesting that this protein presents protease activity.[42] This protein has an identity of 99% with aminopeptidase of *F. verticillioides* (Acession number: XP_018752314)[46] which is also composed by 491 amino acids suggesting that p67 could be the aminopeptidase of *F. verticillioides*.

The putative glucoamylase GMY2 of *F. verticillioides* contains 583 amino acids and includes starch-binding domain and two starch-binding sites which indicate that this protein presents amylase activity.[43]

For a reliable identification of the p67, the amylase and protease activity of this protein was analyzed using polyacrylamide gels copolymerized with starch, gelatin and BSA. The p67 presented amylase activity and did not show protease activity confirming that this protein is the putative glucoamylase GMY2 of *F. verticillioides* (**Figure 2**). The protease activity was observed in the 44 kDa protein of the *F. verticillioides* exoantigen in polyacrylamide gel copolymerized with gelatin, in the 83 kDa protein of the *F. verticillioides* exoantigen in polyacrylamide gel copolymerized with BSA and in the trypsin used as a positive control (**Figure 3** and **4**).

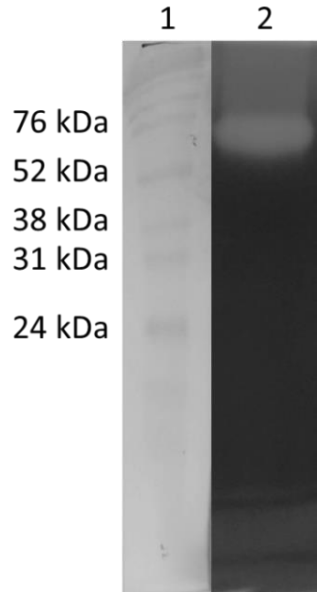


Figure 2. Amylase activity analysis of the proteins of the *F. verticillioides* 97K exoantigen in polyacrylamide gel copolymerized with starch. 1. Molecular weight standard; 2. *F. verticillioides* 97K exoantigen

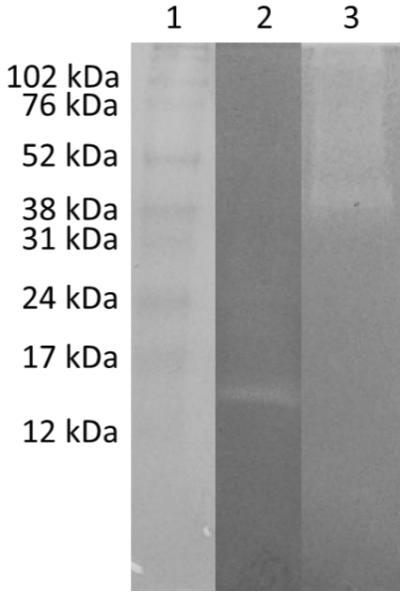


Figure 3. Protease activity analysis of the proteins of the *F. verticillioides* 97K exoantigen in polyacrylamide gel copolymerized with gelatin. 1. Molecular weight standard; 2. Trypsin; 3. *F. verticillioides* 97K exoantigen.

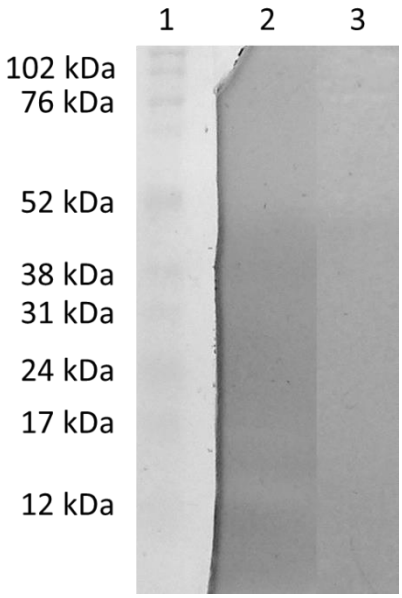


Figure 4. Protease activity analysis of the proteins of the *F. verticillioides* 97K exoantigen in polyacrylamide gel copolymerized with BSA. 1. Molecular weight standard; 2. Trypsin; 3. *F. verticillioides* 97K exoantigen.

The glucoamylase together with the amylase represent the two major classes of starch-degrading enzymes identified in the fungi. The glucoamylase cleaves single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner, while the α-amylase randomly hydrolyses the 1,4-α-D-glucosidic links between adjacent glucose residues in linear amylose chains.[47]

Bluhm and Woloshuk (2005)[48] observed that the kernels lacking starch due to physiological immaturity did not accumulate fumonisin B_1 and demonstrated the importance of the starch degradation by amylase activity in the fumonisin B_1 synthesis working with a mutant strain of *F. verticillioides* with a disrupted α-amylase gene. They showed that the amylopectin, a component of starch, induces fumonisin B_1 production in *F. verticillioides*. Thus the high correlation between the fumonisin and p67 levels ($r = 0.76$) (now known to be the putative glucoamylase GMY2 of *F. verticillioides*) observed in the study carried out in our laboratory could be explained by the influence of the amylase activity on the pathway of the fumonisin synthesis.

The complete coding sequence of the putative glucoamylase GMY2 gene of *F. verticillioides* (Acession number: EU247509.1)[49] is already available which facilitates the production of the recombinant protein aiming the development of an immunological method specific to *F. verticillioides* able to estimate the level of fumonisin in food samples.

Conclusion

The p67 of *F. verticillioides* exoantigen was identified as putative glucoamylase GMY2 by UPLC-ESI-qTOF-MS/MS and enzymatic study in polyacrylamide gel. This identification will allow the production of the recombinant protein on a large scale and the development of an immunological method specific to *F. verticillioides* isolates.

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ARTIGO 2:

DETECTION OF *FUSARIUM VERTICILLIOIDES* BY PCR-ELISA BASED ON *FUM21* GENE

Detection of *Fusarium verticillioides* by PCR-ELISA based on *FUM21* gene

Abstract

Fusarium verticillioides is a primary corn pathogen and fumonisin producer which is associated with toxic effects in humans and animals. The traditional methods for detection of fungal contamination based on morphological characteristics are time-consuming and show low sensitivity and specificity. Therefore, the objective of this study was to develop a PCR-ELISA based on the *FUM21* gene for *F. verticillioides* detection. The DNA of the *F. verticillioides*, *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. isolates was analyzed by conventional PCR and PCR-ELISA to determine the specificity. The sensitivity was evaluated by analyzing *F. verticillioides* DNA ranging from 250 fg to 25 ng. The matrix effect and the sensitivity in corn were analyzed by inoculating from 10 to 10^7 conidia of *F. verticillioides* in ground corn. PCR-ELISA was specific to *F. verticillioides* isolates. The method showed a 2.5 pg detection limit and was 100-fold more sensitive than conventional PCR. In corn samples inoculated with *F. verticillioides* conidia, the detection limit of the PCR-ELISA was 1 x 10^4 conidia/g and was also 100-fold more sensitive than conventional PCR. PCR-ELISA based on the *FUM21* gene is more sensitive than conventional PCR and shows potential to be used for *F. verticillioides* detection in corn samples.

Keywords: Toxigenic fungi; food-borne fungi; PCR-ELISA; fumonisin; corn; mycotoxin.

1. Introduction

Corn (*Zea mays* L.) is the most cultivated cereal in the world and presents a broad spectrum of applications ranging from animal feed to fuel production (USDA, 2017). The total world production of corn was 1 billion and 38 million in 2014 (FAO, 2015). Brazil is the third

largest corn producer in the world and accounted for 88 million tons, and exported 35 million tons in 2016 (CONAB, 2017).

Corn is one of the major components of the animal feed and is the staple food in many countries (Nuss and Tanumihardjo, 2010). Nevertheless, it is susceptible to fungal contamination which affects grain and seed productivity and quality. *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* (J.) Sheldon) is a primary corn pathogen and can cause from asymptomatic infection to severe rotting of all parts of the plant (CAST, 2003). Furthermore, this fungus produces fumonisins, a group of secondary metabolites which can cause toxic effects to human and animal health (Bezuidenhout et al., 1988).

Fumonisins are mainly produced by *F. verticillioides* and *F. proliferatum* (T. Matsushima) Nirenberg (CAST, 2003) and are associated with leukoencephalomalacia in equines (Marasas et al., 1988), pulmonary edema in pigs (Ross et al., 1990), growth reduction and immunosuppression in birds (Weibking et al., 1993) and have hepatotoxic and hepatocarcinogenic effect in rats (Gelderblom et al., 1988). In humans, epidemiological studies indicate the association of fumonisins with neural tube defects (Missmer et al., 2006), and with esophageal and liver cancer (Ueno et al., 1997). The International Agency for Research on Cancer (IARC, 2002) classified fumonisins as Group 2B, i. e. possibly carcinogenic to humans.

Studies carried out in African, Asian, European and South American countries have shown that the incidence of fumonisins in corn samples can reach 100% and in some cases with mean levels above the maximum limit recommended by the European Union (1000 μg/kg) (Covarelli et al., 2011; Fandohan et al., 2005; Gong et al., 2009; Medina-Martínez and Martínez, 2000; Oliveira et al., 2017).

Because of high contamination of corn by *F. verticillioides* and fumonisins and the health problems caused by these mycotoxins, it is essential to develop sensitive and specific methods to detect *F. verticillioides* contamination. Conventional methods for identification and

detection of fungal contamination include culture in several media, microscopic examination and chemical analyzes of chitin, ergosterol and secondary metabolites (Thrane, 1989). These methods have low specificity and sensitivity and are time-consuming, except for the identification of secondary metabolites by chromatography and mass spectrometry. In spite of the high sensitivity and specificity, they are laborious, use toxic reagents and require an extensive sample clean-up.

Polymerase Chain Reaction (PCR) based on genes involved in fumonisin production can be used to detect *F. verticillioides* in corn. The amplified PCR product is commonly detected by electrophoresis and is qualitative (Sue et al., 2014). An attractive approach is the detection of DNA using an enzyme linked immunosorbent assay (ELISA) that allows semi-quantitative analysis of several samples in a single test with high sensitivity and specificity (Grimm and Geisen, 1998).

The genes directly involved in the fumonisin biosynthesis are organized into a gene cluster, known as the fumonisin biosynthetic (*FUM*) gene cluster (Proctor et al., 2003). In *F. verticillioides* the cluster consists of 17 coordinately regulated genes, designated *FUM1*, *FUM2*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM10*, *FUM11*, *FUM13*, *FUM14*, *FUM15*, *FUM16*, *FUM17*, *FUM18*, *FUM19*, *FUM20* and *FUM21*. Functions of the *FUM* genes in fumonisin biosynthesis in *F. verticillioides* have been predicted based on fumonisin chemical structures and the similarity of the *FUM* genes to genes whose functions are already known or have been determined genetically by gene disruption and mutant complementation (Brown et al., 2005; Brown et al., 2007; Butchko et al., 2003, 2006; Proctor et al., 2003).

The *FUM21* gene probably codes a transcription factor that positively regulates the *FUM* genes expression and was the last *FUM* gene to be described so far (Brown et al., 2007). The presence of a Zn (II) 2Cys6 DNA-binding domain in the predicted protein suggests that this gene is involved in transcriptional regulation. *FUM21* deletion mutants produced little to

no fumonisins and the complementation of these mutants with a wild-type copy of the *FUM21* gene restored fumonisin production (Brown et al., 2007).

Divakara et al. (2014) developed a multiplex PCR (mPCR) to differentiate toxigenic and non-toxigenic *Fusarium* sp. using primer for the *FUM21* gene along with the *FUM1* and *FUM8* genes. The researchers analyzed 27 *Fusarium* sp. isolates associated to sorghum seeds and the positivity to the *FUM21* gene was observed only in fumonisin producing *F. verticillioides* isolates, while positivity for the *FUM1* and *FUM8* genes was observed both in fumonisin producer and in fumonisin non-producing *F. verticillioides* isolates, indicating that the *FUM21* gene shows greater potential to differentiate fumonisin producers from fumonisin non-producer isolates.

Taking into account the high frequency of *F. verticillioides* and fumonisins in corn, the health problems associated with these mycotoxins, the relation of *FUM21* with fumonisin production and the presence of this gene only in fumonisin producing *F. verticillioides*, the objective of this study was to develop a PCR-ELISA based on the *FUM21* gene to detect *F. verticillioides* in corn.

2. Material and Methods

2.1. Fungal isolates and growth conditions

F. verticillioides isolates (97K, 103F, 103G, 113F and 119Br) and *Penicillium variabile* belongs to the Mycological Culture Collection of the Department of Food Science and Technology at the State University of Londrina.; *F. graminearum* FSP27 and FRS26 were provided by the Mycological Culture Collection of Laboratory of Toxigenic Fungi and Mycotoxins of the Department of Microbiology of Biomedical Sciences Institute, University of

São Paulo (São Paulo-Brazil); isolates of *F. graminearum* 102, *F. proliferatum* 559, *F. subglutinans* 332, *Aspergillus niger* (911, 104CF, 219CF, 444CF, 642AN), *A. carbonarius* (168, 180) and *A. ochraceus* (4363, 4368) were provided by the Mycological Culture Collection of Department of General Biology, State University of Londrina, Paraná, Brazil. All isolates were routinely grown in potato dextrose agar at 25°C.

2.2. DNA extraction from fungal isolates

The DNA of fungal isolates was extracted according to the method described by Van Burik et al. (1998) with some modifications. Briefly, whole mycelium of the isolates grown on Petri dishes was inoculated in potato dextrose broth using an inoculating loop and the culture was incubated for 3 days at 28°C. Then, the mycelium was harvested by filtration, transferred to a mortar and lysis buffer (10 mmol/l Tris HCl pH 7.5; 20 ml/l Triton X-100; 10 mmol/l sodium acetate; 1 mmol/l EDTA pH 8.0; 10 g/l SDS) was added. The mycelium was then macerated with a pestle, transferred to a microtube containing glass beads and phenol and the mixture was submitted to agitation in the TissueLyser (Qiagen, Hilden, Germany) and centrifuged for 10 min at 9,000 x *g*. The supernatant was treated with phenol:chloroform:isoamyl alcohol (25:24:1), followed by further agitation and centrifugation. The supernatant was treated with chloroform:isoamyl alcohol (24:1) and the mixture was agitated and centrifuged. This last process was repeated until the supernatant was clear. The supernatant was then treated with RNAse (Invitrogen, Carlsbad, USA) at a final concentration of 100 mg/l at 37°C for 30 min. Then the DNA was precipitated by adding absolute ethanol and the pellet was washed with 70% ethanol and dried at room temperature. The DNA was then re-suspended in ultrapure water and stored at -20°C until use.

The DNA concentration was measured at 260 nm and the DNA quality was determined by calculating the ratio between A260 nm and A280 nm using a NanoDrop Lite spectrophotometer (Thermo Scientific, Massachusetts, USA).

2.3. Conventional PCR

The PCR was carried out in a final reaction volume of 25 µl. The amplification mixture consisted of 2.5 µl DNA template, 1.5 mmol/l $MgCl_2$, 1x PCR buffer (Invitrogen, Carlsbad, USA), 200 µmol/l dNTP mix (Invitrogen, Carlsbad, USA), 1.25 U of Taq polymerase (Invitrogen, Carlsbad, USA) and primers at a concentration of 0.2 µmol/l (Invitrogen, Carlsbad, USA). The set of primers designed by Divakara et al. (2014) from the *FUM21* gene (*FUM21F*: 5' GCAACATACAAGGGGGAGTT 3', *FUM21R*: 5' GGGTGGGAATAGGTCAGTT 3') was used and PCR products of 598 bp were expected. PCR was performed by initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR products were submitted to electrophoresis on 10% polyacrylamide gel with 100 bp ladder (Invitrogen, Carlsbad, USA) and visualized by silver staining.

2.4. DNA Sequencing

DNA sequences of the PCR products of *F. verticillioides* isolates was determined using the primers *FUM21F* and *FUM21R* and the BigDye Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions in the ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence information was assembled and edited using the Bio Edit Sequence Alignment Editor (Ibis Biosciences,

Carlsbad, CA). The Nucleotide Basic Local Alignment Search Tool (BLAST) was used to compare the DNA sequences obtained with the sequences in the National Center for Biotechnology Information (NCBI) database. They were aligned using the program Clustal Omega (European Molecular Biology Laboratory, Heidelberg, Germany).

2.5. PCR-ELISA

For the PCR-ELISA, an oligonucleotide based on the sequence amplified by the *FUM21* primers was designed to be used as internal capture probe. This oligonucleotide was biotin labelled at the 5' end and had the following sequence: 5' TGTAATGGATCAGCGGAAGAGTCT 3' (*FUM21S*) (Invitrogen, Carlsbad, USA).

A commercial kit (Roche, Applied Science, Mannheim, Germany) was used for the PCR-ELISA. The PCR-ELISA amplification mixture was similar to conventional PCR except for the dNTP that was replaced by the PCR DIG Labeling mix (Roche Applied Science, Mannheim, Germany) which is a mixture of dATP, dCTP, dGTP, dTTP and digoxigenin -11-dUTP and is used to incorporate DIG into the PCR product. The ELISA reaction was performed according to the manufacturer. Briefly, the labeled PCR products were denatured with NaOH solution and incubated with *FUM21S* probe at 37°C for 3 h on a microplate previously coated with streptavidin. After washing, anti-DIG antibody peroxidase conjugate was added, followed by incubation at 37°C for 30 min. The wells were then washed and incubated with the substrate of the peroxidase ABTS® (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt). The absorbance was measured by Multiskan Ex Reader (Labsystems, Helsinki, Finland) at 405 nm. The cut off was determined by the following equation (Frey et al., 1998):

$$\text{Cutoff} = \text{X} + \text{SD}\ t\sqrt{1 + \frac{1}{n}}$$

Where X is the mean of independent negative control absorbance, SD is the standard deviation, n is the number of independent controls, *t* is the (1 – α)th percentile of the one-tailed *t*-distribution with (n-1) degrees of freedom and confidence level of 95%.

2.6. Specificity of conventional PCR and PCR-ELISA

Specificity analysis of conventional PCR and PCR-ELISA was performed using DNA of *F. graminearum* FRS26, FSP27 and 102, *F. proliferatum* 559, *F. subglutinans* 332, *A. niger* 104CF, 219CF, 444CF, 642AN and 911, *A. ochraceus* 4363 and 4368, *A. carbonarius* 168 and 180 and *P. variabile* at 10 ng/μl.

The similarity between the *FUM21* genes of fumonisin producer isolates was analyzed by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

2.7. Sensitivity of conventional PCR and PCR-ELISA

The sensitivity of conventional PCR and PCR-ELISA was determined by testing *F. verticillioides* 103G DNA in amounts from 250 fg to 25 ng.

2.8. Artificial corn contamination

A ground corn sample was previously evaluated for presence of the *FUM21* gene and presented negative result. A suspension of 10^7 conidia/ ml of *F. verticillioides* 103G was prepared in 10 ml of sterile 0.15 mol/l phosphate saline buffer containing 1 ml/l Tween 80 (v/v) and serial dilutions using 10 factor dilution were made transferring 1ml of the suspension to a tube containing 9 ml of the same diluent until the concentration of 10 conidia/ ml. One milliliter of each concentration was then inoculated in 250 mg ground corn. Corn inoculated with 1 ml of diluent was used as negative control.

2.9. DNA extraction from artificially contaminated corn

DNA was extracted from corn samples according to the method described by Cenis (1992) with some modifications. Two hundred and fifty milligrams of ground corn artificially contaminated with *F. verticillioides* (as previously described) were mixed with lysis buffer (200 mmol/l Tris-HCl pH 8.5; 250 mmol/l NaCl; 25 mmol/l EDTA; 5 g/l SDS) and glass beads in the TissueLyser. The suspension was centrifuged for 10 min at 9,000 x *g* and the supernatant was treated with chloroform: isoamyl alcohol (24:1). The mixture was centrifuged and the last process was repeated. The supernatant was mixed with 3 mol/l sodium acetate pH 5.2, incubated for 10 min at -20°C and centrifuged. From the next step, which consisted of adding RNAse, the procedures applied for DNA extraction and to determine DNA concentration and quality were the same as described for DNA extraction from fungal isolates.

3. Results and Discussion

The PCR based on the *FUM21* gene was positive for the five *F. verticillioides* isolates and was negative for isolates of *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *A. niger*, *A. ochraceus*, *A. carbonarius* and *P. variabile*, suggesting that the set of primers is specific to *F. verticillioides* (**Fig. 1**). This result is in agreement with the study by Divakara et al. (2014) who also observed the specificity of this set of primers to fumonisin- producing *F. verticillioides* isolates in a mPCR.

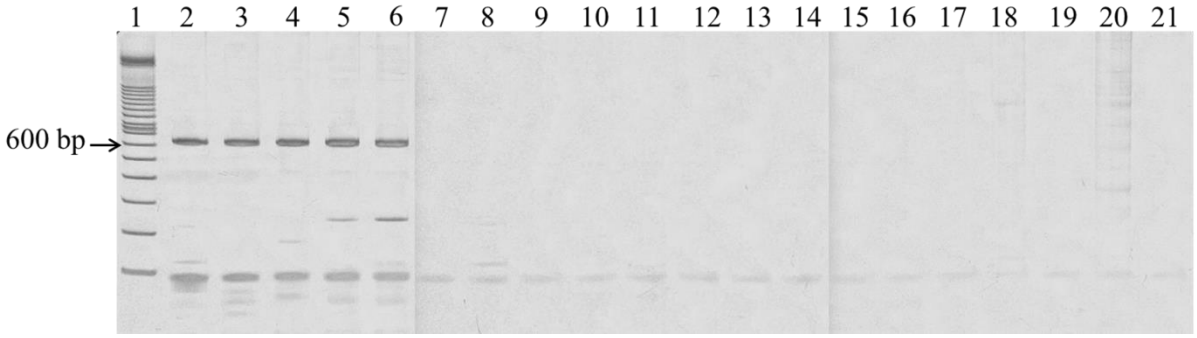

Fig. 1. Specificity of the conventional PCR based on *FUM21* gene. Lane 1: 100 bp ladder; lane 2: *F. verticillioides* 97K; lane 3: *F. verticillioides* 103F; lane 4: *F. verticillioides* 103G; lane 5: *F. verticillioides* 113F; lane 6: *F. verticillioides* 119Br; lane 7: *F. graminearum* FRS26; lane 8: *F. graminearum* FSP27; lane 9: *F. graminearum* 102; lane 10: *F. proliferatum* 559; lane 11: *F. subglutinans* 332; lane 12: *A. niger* 104CF; lane 13: *A. niger* 219CF; lane 14: *A. niger* 444CF; lane 15: *A. niger* 642AN; lane 16: *A. niger* 911; lane 17: *A. ochraceus* 4363; lane 18: *A. ochraceus* 4368; lane 19: *A. carbonarius* 168; lane 20: *A. carbonarius* 180; lane 21: *P. variabile*.

Several researchers developed PCR based on *FUM* genes (Bluhm et al., 2002; Dawidziuk et al., 2014; Mudili et al., 2014; Ramana et al., 2011; Rashmi et al., 2012; Sreenivasa et al., 2006). The *FUM1* gene was already used as target in PCR and was group-specific, detecting *F. anthophilum*, *F. proliferatum* and *F. verticillioides*, however some isolates of these

species were negative for the gene suggesting that they are fumonisin non-producing isolates or that the method showed low sensitivity (Bluhm et al., 2002; Sreenivasa et al., 2006).

The PCR based on *FUM13* gene showed positive results for fumonisin producing isolates and for some fumonisin non-producing isolates probably because the environmental conditions were not appropriate for the expression of the gene or due to the low specificity of the method. In naturally contaminated rice and finger millet samples, the PCR showed contradictory results giving negative results in samples positive for fumonisin, probably due to DNA degradation, low sensitivity or inefficient DNA extraction from the samples (Ramana et al., 2011; Rashmi et al., 2012). Mudili et al. (2014) used the primers for *FUM13* gene designed by Ramana et al. (2011) in a PCR for detection of fumonisin producing isolates and demonstrated that the method was able to detect all isolates of *F. proliferatum* and *F. verticillioides* evaluated in the study.

A PCR based on *FUM6* and *FUM8* genes was used to evaluate 72 *Fusarium* sp. isolates and 24 non-*Fusarium* filamentous fungal isolates and sensitivity and specificity of 89% was obtained. In wheat kernel samples, the method showed positive results for contaminated samples, however, was not successful in samples of wheat chaff because the DNA extracted from chaffs was not of sufficient quality to provide reliable results probably due to the presence of PCR inhibitors, such as polysaccharides (Dawidziuk et al., 2014; Demeke and Jenkins, 2010).

F. proliferatum is a potential fumonisin producer (CAST, 2003), but it has been detected at low frequency (Desjardins et al., 2000; Lanza et al., 2014; Piecková and Jesenska, 2001; Rocha et al., 2011). *F. proliferatum* was negative for the *FUM21* gene in the PCR based on *FUM21* gene developed in the present study. The sequence of the *F. proliferatum FUM21* gene is available in the NCBI database (Access number: KF482467). Despite the identity of 75% between *FUM21* gene sequences of *F. proliferatum* and *F. verticillioides*, the annealing

sequences for the primers used in the present study are not present in the *F. proliferatum FUM21* gene.

Frisvad et al. (2007) reported fumonisin B_2 production by *A. niger* isolates and the presence of a fumonisin-like biosynthetic gene cluster in *A. niger* genome has already been observed, but in the present study, *A. niger* was negative for the *FUM21* gene. *A. niger* has 11 genes similar to the *F. verticillioides FUM* genes (Access number: KJ934797) described so far, among them the *FUM21* gene, however only partial sequences are available in the NCBI database. Comparison of *A. niger* and *F. verticillioides FUM21* gene sequences by BLAST showed no significant similarity probably due to the absence of the *A. niger FUM21* gene complete sequence. The analysis of the *A. niger FUM21* gene partial sequence also revealed the absence of the annealing sequence for the primers used in the present study.

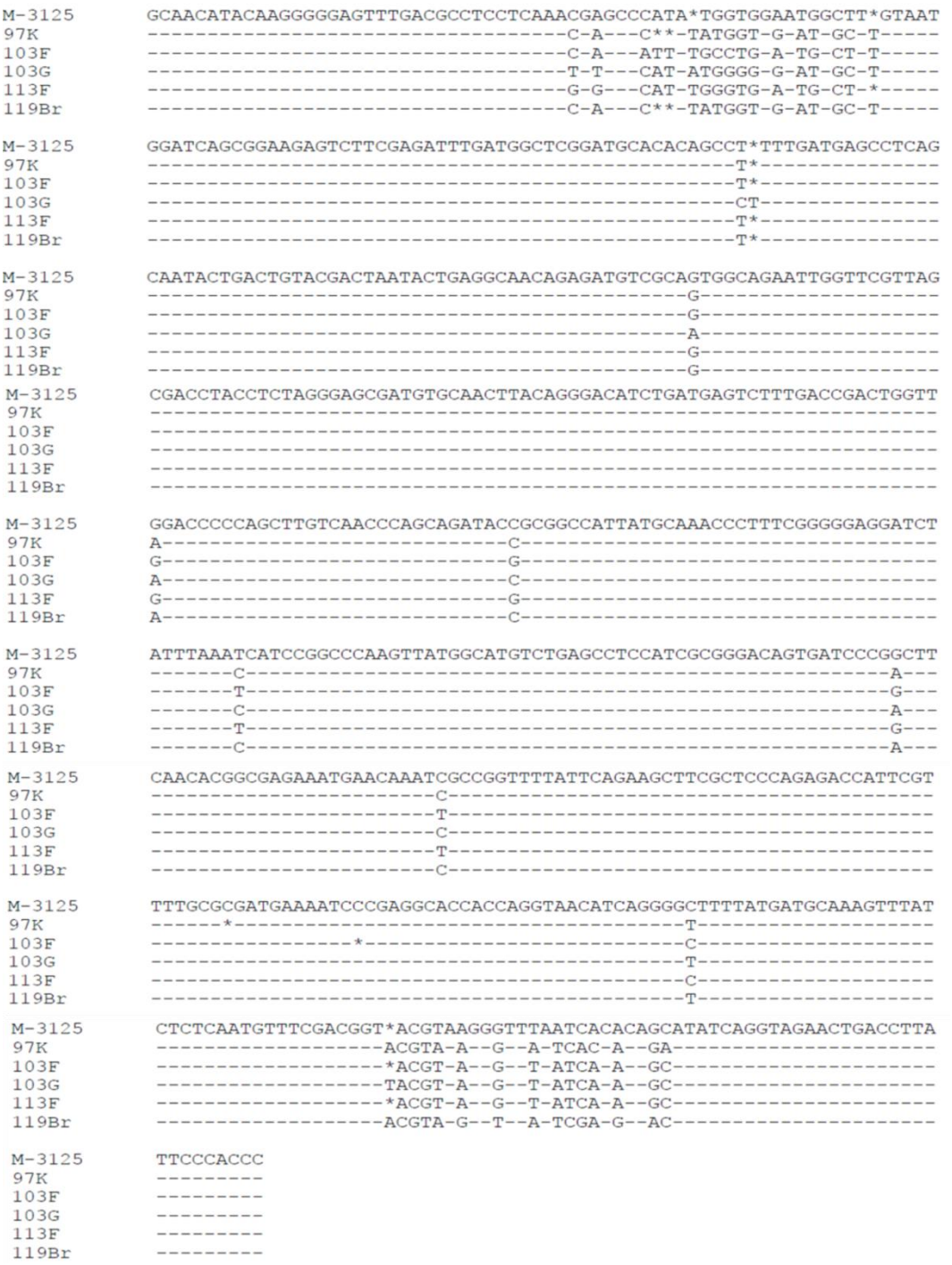

Fig. 2. Alignment of amplicons of the *F. verticillioides* isolates with the sequence of *F. verticillioides* M-3125 available in the NCBI database (Acession number: AF155773.5). - symbols equal nucleotides and * symbols gaps.

In the present study, the specific detection of *F. verticillioides* was desired because this species is the predominant one in corn samples (Covarelli et al., 2011; Fandohan et al., 2005;

Medina-Martínez and Martínez, 2000; Ono et al., 2008). The sequencing followed by analysis in BLAST confirmed that the fragments corresponded to the *FUM21* gene fragment. The fragments generated by the conventional PCR of the *F. verticillioides* isolates showed a 99% identity with the *FUM21* gene (Acession number: AF155773.5). The amplicon alignment showed the presence of some mutations which resulted in a slight variation in the size of the PCR products with the 97K, 103F, 103G, 113F and 119Br isolates showing a size of 597 bp, 599 bp, 603 bp, 599 bp and 599 bp, respectively (**Fig. 2**).

The DNA of different fungal isolates was also analyzed by PCR-ELISA which showed a cut off of 0.347. Only *F. verticillioides* isolates were positive indicating the specificity of the set of primers and *FUM21S* probe to *F. verticillioides* isolates (**Fig. 3**).

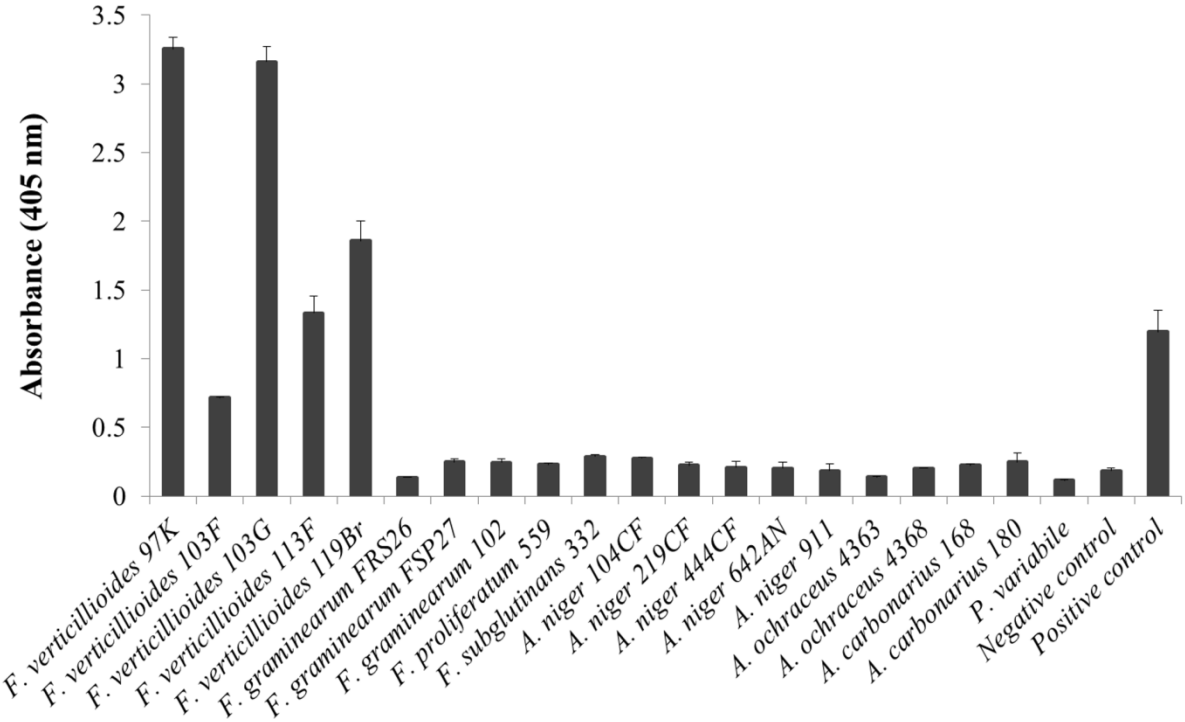

Fig. 3. Specificity of the PCR-ELISA based on *FUM21* gene. Positive control is a DIG labelled control DNA provided by the PCR-ELISA kit and negative control is the PCR product obtained by replacing DNA with ultrapure water.

Grimm and Geisen (1998) developed a PCR-ELISA for detection of potential fumonisin producing *Fusarium* species using the ribosomal internal transcribed spacer 1 (ITS1) sequence

as target. The PCR-ELISA was positive only for *F. napiforme*, *F. nygamai*, *F. proliferatum* and *F. verticillioides* isolates and was more specific than the conventional PCR which was also positive for *F. poae*, *F. solani*, *F. italicum*, *P. digitalum* and *A. flavus* isolates. However, *F. napiforme*, *F. nygamai* and *F. proliferatum* isolates were not fumonisin producers under the assayed conditions.

The conventional PCR showed a 250 pg detection limit for *F. verticillioides* 103G DNA (**Fig. 4**). Bluhm et al. (2002) developed a mPCR based on the *TRI6* gene and *FUM1* gene to detect trichothecene and fumonisin producing species of *Fusarium* in cornmeal with a 100 pg detection limit.

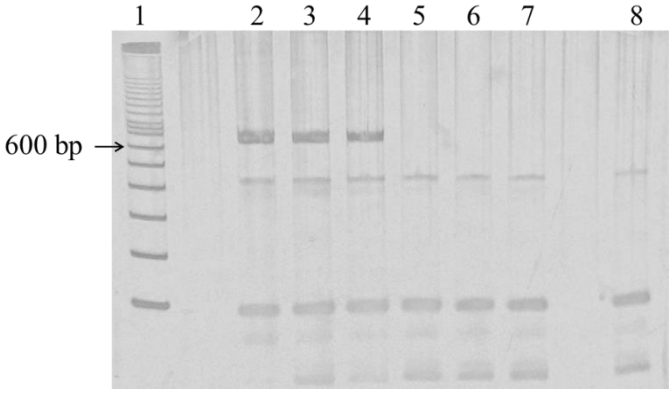

Fig. 4. Sensitivity of the conventional PCR based on *FUM21* gene using different amounts of *F. verticillioides* 103G DNA. Lane 1: 100 bp ladder; lane 2: 25000 pg; lane 3: 2500 pg; lane 4: 250 pg; lane 5: 25 pg; lane 6: 2.5 pg; lane 7: 0.25 pg; lane 8: negative control.

The PCR-ELISA was 100-fold more sensitive than conventional PCR with a 2.5 pg detection limit (**Fig. 5**). Although there are some studies on PCR-ELISA for human fungal pathogens, only few reports on PCR-ELISA for food-borne toxigenic fungi (Grimm and Geisen, 1998) were found to discuss the current data. Di Pinto et al. (2012) also reported a 100-fold increase in sensitivity with PCR-ELISA compared to the agarose gel-based detection method for *Vibrio parahaemolyticus* contamination in shellfish. The higher sensitivity observed in the

PCR-ELISA is probably related to the increase in the positive signal of biotin-labeled probe-bound PCR products due to the specific hybridization and enzymatic signal amplification.

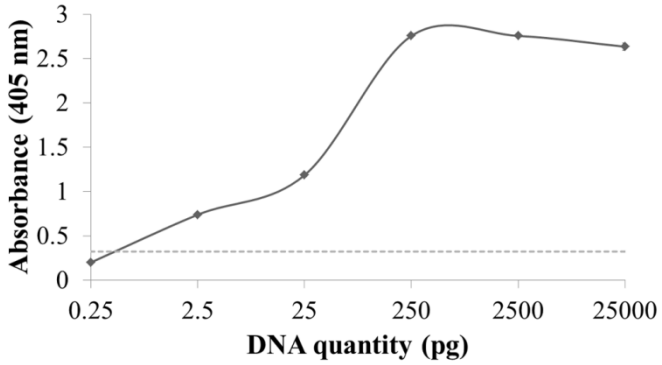

Fig. 5. Sensitivity of the PCR-ELISA based on *FUM21* gene using different amounts of *F. verticillioides* 103G DNA. The dotted line represents the cut off of 0.323.

When different amounts of conidia were inoculated on corn to evaluate the matrix effect and the sensitivity of the methods in corn samples, detection limit of conventional PCR was $1x10^6$ conidia/g for DNA of *F. verticillioides* 103G (**Fig. 6**). Bluhm et al. (2002) and Ramana et al. (2011) developed a mPCR to detect toxigenic *Fusarium* species using primers for *FUM1* and *FUM13* genes, respectively, and obtained a 10^5 CFU/g detection limit.

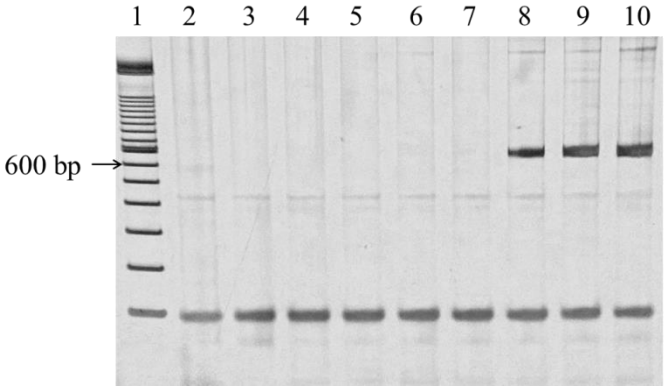

Fig. 6. Sensitivity of the conventional PCR based on *FUM21* gene in maize samples inoculated with *F. verticillioides* 103G conidia. Lane 1: 100bp ladder; lane 2: 0 conidium/g; lane 3: 1x10 conidia/g; lane 4: $1x10^2$ conidia/g; lane 5: $1x10^3$ conidia/g; lane 6: $1x10^4$ conidia/g; lane 7: $1x10^5$ conidia/g; lane 8: $1x10^6$ conidia/g; lane 9: $1x10^7$ conidia/g; lane 10: positive control.

The analysis of corn samples artificially contaminated with *F. verticillioides* 103G conidia by PCR-ELISA showed that this method is 100-fold more sensitive compared to conventional PCR with a 1 x 10^4 conidia/g detection limit (**Fig. 7**).

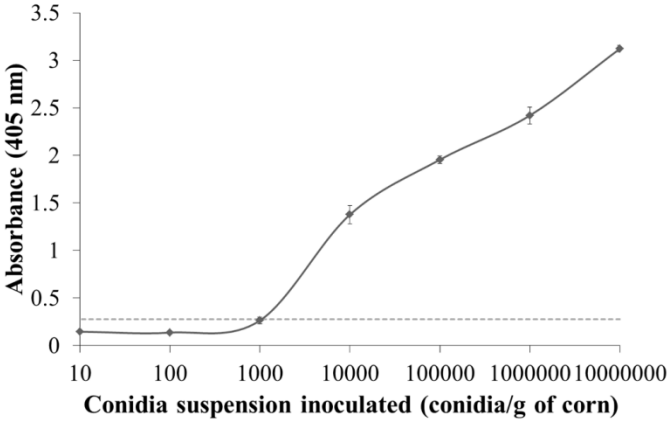

Fig. 7. Sensitivity of the PCR-ELISA based on *FUM21* gene in maize samples inoculated with *F. verticillioides* 103G conidia. The dotted line represents the cut off of 0.275.

Traditionally, the identification of *Fusarium* species has been performed by morphological characteristics such as presence or absence of microconidia, macroconidia shape and size, colony morphology and pigmentation and growth rates. However, this analysis is time consuming and requires considerable expertise (Leslie and Summerell, 2006). The PCR-ELISA based on the *FUM21* gene developed in the present study shows potential to be an alternative method to detect contamination by toxigenic *F. verticillioides* with high sensitivity. This method was able to detect *F. verticillioides* in artificially contaminated corn indicating that it is promising to detect *F. verticillioides* in corn samples.

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Conflicts of interest

The authors declare no conflict of interest.

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3 CONCLUSÕES GERAIS

A p67 foi identificada como provável glicoamilase GMY2 de *F. verticillioides* o que permitirá a produção dessa proteína em sua forma recombinante e o desenvolvimento de um método imunológico específico para isolados de *F. verticillioides*. A PCR-ELISA baseada no gene *FUM21* foi específica para isolados de *F. verticillioides*, apresentou sensibilidade 100 vezes maior do que a PCR convencional e foi capaz de detectar gene *FUM21* em amostra de milho contaminado artificialmente com até 10^4 conídeos de *F. verticillioides*/g de milho sugerindo que este método apresenta potencial para ser utilizado na detecção desse fungo em amostras de milho.

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