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TIAGO DE MELO NAZARETH

FUMIGAÇÃO DO ISOTIOCIANATO DE ALILA CONTRA O CRESCIMENTO DE FUNGOS PRODUTORES DE MICOTOXINAS EM MILHO ESTOCADO

(Fumigation of allyl isothiocyanate against the growth of mycotoxigenic fungi in stored corn)

CURITIBA
2017

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Saúde, Tecnologia e Produção Animal, da Escola de Ciências da Vida da Pontifícia Universidade Católica do Paraná, para obtenção do título de Mestre em Ciência Animal.

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ATA Nº 0102 E PARECER FINAL DA DEFESA DE DISSERTAÇÃO DE MESTRADO EM CIÊNCIA ANIMAL DO ALUNO TIAGO DE MELO NAZARETH

Aos vinte e um dias do mês de fevereiro do ano de dois mil e dezessete, às 13:30 horas, realizou-se na sala de vídeo 1, térreo, Bloco Verde, Escola de Ciências da Vida, da Pontifícia Universidade Católica do Paraná, localizada no Campus de Curitiba, Rua Imaculada Conceição, nº 1155, Prado Velho – Curitiba – PR, a sessão pública de defesa da Dissertação do mestrando Tiago de Melo Nazareth, intitulada: **"FUMIGAÇÃO DO ISOTIOCIANATO DE ALILA CONTRA O CRESCIMENTO DE FUNGOS PRODUTORES DE MICOTOXINAS EM MILHO ESTOCADO"**. O mestrando concluiu os créditos exigidos para obtenção do título de Mestre em Ciência Animal, segundo os registros constantes na secretaria do Programa. Os trabalhos foram conduzidos pelo Professor orientador e Presidente da banca, Dr. Fernando Bittencourt Luciano (PUCPR), auxiliado pelos Professores Doutores Leandro Batista Costa (PUCPR), Luciano Aparecido Panagio (UEL) e pela Doutora Keliani Bordin. Procedeu-se à exposição da Dissertação, seguida de sua argüição pública e defesa. Encerrada a fase, os examinadores expediram o parecer final sobre a Dissertação, que nos termos do Artigo 53 do Regulamento deste Programa de Pós-Graduação, foi considerada aprovado.

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Proclamado o resultado, ao Presidente da Banca Examinadora encerrou os trabalhos, e para que tudo conste, eu Caroline Nocera Bertton, confiro e assino a presente ata juntamente com os membros da Banca Examinadora.

Curitiba, 21 de fevereiro de 2017.

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ABREVIAÇÕES / ABREVIATIONS

ITCs: Isotiocianatos / isothiocyanates

AITC: Isotiocianato de alila / allyl isothiocyanate

PDA: Potato dextrose agar

FB_1: Fumonisin B_1

FB_2: Fumonisin B_2

AFs: Aflatoxins

AFB_1: Aflatoxin B_1

AFB_2: Aflatoxin B_2

AFG_1: Aflatoxin G_1

AFG_2: Aflatoxin G_2

HPLC-DAD: High performance Liquid Chromatography - Diode Array Detector

HPLC-FLD: High performance Liquid Chromatography - Fluorescence Detector

“Trabalhe com sinceridade de coração. E tudo quanto fizerdes, fazei-o de todo o coração, dê o seu melhor como para o Senhor, e não para o homem”.

Colossenses 3:22-23

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FORMATO DA DISSERTAÇÃO

A presente dissertação é composta por capítulos. O *Capítulo 1* apresenta uma introdução geral, a contextualização do tema, o estado da arte e os objetivos da pesquisa. O *Capítulo 2* trata-se do artigo científico completo redigido a partir dos resultados obtidos no estudo, formatado nas normas da revista para o qual o artigo será submetido. O *Capítulo 3* finaliza esta dissertação com conclusões gerais e considerações finais deste trabalho e sugestões para estudos futuros. As normas da revista estão disponíveis no Anexo I.

RESUMO GERAL

A busca por novas substâncias a partir de fontes naturais tem ganhado importância no cenário mundial como alternativa ao uso de fungicidas sintéticos. Dentre esses compostos naturais, o isotiocianato de alila (AITC) tem se destacado pelo seu potencial antifúngico. O objetivo do presente trabalho foi avaliar a eficiência do AITC na forma gasosa para a redução do crescimento de *Aspergillus parasiticus* e *Fusarium verticillioides* visando a redução da produção de micotoxinas (aflatoxinas AFB_1, AFB_2, AFG_1 e AFG_2; fumonisinas FB_1 e FB_2) em milho estocado por180 dias. Amostras de 300 g de grãos de milho foram tratados com AITC (0,5; 10 e 50 µL/L em volume do frasco) em frascos de vidro (830 mL), sendo então selados e armazenados por 2 dias sob condições ambientais de umidade e temperatura. Em seguida, os frascos foram abertos por 1 h para eliminar o excesso de AITC e inoculados respectivamente com 10^4 e 10^5 conídios/g de *A. parasiticus* CECT 2947 e *F. verticillioides* CECT 2983. Os grupos controle não receberam tratamento com AITC. Alíquotas de 10 g foram usadas para determinar a população fúngica após 0, 2, 7, 14, 30, 60, 90, 120, 150 e 180 dias. Similarmente, os níveis de aflatoxinas (B_1, B_2, G_1 e G_2), fumonisinas (FB_1 e FB_2) e AITC foram quantificados por HPLC nesses mesmos tempos. O AITC a 50 µL/L resultou na redução significativa ($p<0,05$) da população em 180 dias de estocagem, reduzindo 3,17 log UFC/g e 3,9 log UFC/g de *A. parasiticus* e *F. verticillioides*, respectivamente. Além disso, tratamentos com 10 e 50 µL/L evitaram a produção de FB_1. Os níveis de AITC residual no milho tratado com 10 e 50 µL/L foram respectivamente, 6,5 e 29,1% após 14 dias, enquanto que somente o tratamento de 50 µL/L apresentava AITC residual (25,9%) nos grãos após 30 dias. O AITC residual não foi detectado no grupo tratado com 0,5 µL/L já após 2 dias. O tratamento profilático com AITC foi eficaz na redução da população fúngica e inibiu a produção de FB_1. Por ser um composto volátil, o AITC mostrou-se capaz de penetrar no milho, sendo que doses de 50 µL/L apresentaram maior efeito residual. Todos esses resultados sugerem que o AITC pode ser usado como agente fumigante em silos ou até mesmo em embalagens, a fim de evitar a deterioração fúngica e, consequentemente, a produção de micotoxinas em grãos de milho.

Palavras-chave: Antimicrobiano natural; Deterioração fúngica; Redução de micotoxinas; Fungos toxigênicos; Armazenamento de grãos.

ABSTRACT

The search for new substances from natural sources has gained importance worldwide as an alternative to synthetic fungicides. Among these natural compounds, allyl isothiocyanate (AITC) has been evaluated for its antifungal potential. The objective of the present study was to evaluate the efficiency of gaseous AITC to inhibit the growth of *Aspergillus parasiticus* and *Fusarium verticillioides*, and to reduce mycotoxin production (aflatoxins B_1, B_2, G_1 and G_2, fumonisins B_1 and B_2) in corn during 180 days of storage. Samples (300 g) of corn kernels were treated with AITC (0.5, 10 and 50 μL/L) in glass jars (830 mL), then hermetically closed and stored for 2 d under normal environmental conditions. The flasks were opened for 1 h to let the excess of AITC to escape and inoculated with 10^4 and 10^5 spores/g of *A. parasiticus* CECT 2947 and *F. verticillioides* CECT 2983, respectively. Control groups were not treated with AITC. Aliquots of 10 g were used to determine the fungal population after 0, 2, 7, 14, 30, 60, 90, 120, 150 and 180 d. Similarly, levels of aflatoxins (B_1, B_2, G_1 and G_2), fumonisins (B_1 and B_2) and AITC were quantified by HPLC in these same time points using 5 g samples. AITC at 50 μL/L resulted in a significant reduction of the fungal population ($p < 0.05$) after 180 d of storage, decresing 3.17 log CFU/g and 3.9 log CFU/g of *A. parasiticus* and *F verticillioides*, respectively. In addition, treatments with 10 and 50 μL/L prevented the production of FB1. Residual levels of AITC in corn treated with 10 and 50 μL/L were 6.5 and 29.1% after 14 d; <LOD (Limit of Detection) and 25.9% after 30 d, respectively. Residual AITC was not detected in corn treated with 0.5 μL/L. Prophylactic treatment with AITC was effective in reducing the fungal population and inhibited FB_1 production. All these results suggest that AITC may be used as a fumigant agent in silos or packages to avoid fungal deterioration and, consequently, to reduce mycotoxins production in corn kernels.

Keywords: Natural antimicrobial, fungal spoilage, mycotoxins reduction, toxigenic fungi, stored grains.

CAPÍTULO 1

INTRODUÇÃO E CONTEXTUALIZAÇÃO

O milho é um dos cereais mais importantes cultivados e consumidos no mundo. Segundo a USDA (2016), a produção mundial chegou a 968,86 milhões de toneladas (t) em 2015. O Brasil é o terceiro maior produtor mundial com uma produção aproximada de 63,3 milhões de t no ano de 2016, estando atrás apenas dos EUA (384,8 milhões t) e China (219,6 milhões t) (GCEA/IBGE, 2017; USDA, 2017). Do total produzido no Brasil, 52 milhões de t foram destinadas a alimentação humana e animal (Abimilho, 2016).

Dentre as perdas que ocorrem durante o processamento e estocagem de grãos, os fungos são os agentes deteriorantes mais frequentes, principalmente em países de clima tropical, como o Brasil, onde o armazenamento inapropriado dos grãos, associado ao clima quente e úmido favorecem a deterioração fúngica (Astoreca et al., 2012). Como consequência, ocorrem alterações físico-químicas dos grãos e o decréscimo do seu valor nutricional, contribuindo para a redução do valor econômico e muitas vezes impossibilitando sua comercialização (Astoreca et al., 2012; Degraeve et al., 2016).

Além das perdas econômicas provocadas pela deterioração de grãos, muitos fungos são produtores de micotoxinas. As micotoxinas são metabólitos secundários tóxicos produzidos por fungos em diversos alimentos sob determinadas condições de temperatura e umidade (Nielsen et al., 2004). Esses compostos podem ser formados por fungos que se desenvolvem nos grãos quando ainda estão no campo ou durante a estocagem (Degraeve et al., 2016). Dentre os produtores de micotoxinas, destacam-se principalmente os fungos dos gêneros *Aspergillus, Fusarium* e *Penicillium,* que produzem aflatoxinas, fumonisinas, tricotecenos, zearalenona e ocratoxina (Sweeney e Dobson, 1998).

Incidência de micotoxinas em alimentos

A *Food and Agriculture Organization* (FAO, 2004) estima que cerca de 25% dos grãos no mundo possam estar impróprios para o consumo devido a contaminação por micotoxinas. Para evitar essa alta incidência, métodos preventivos são tomados, tais

como, a implementação de boas práticas de produção, estocagem e distribuição. Porém essas toxinas continuam sendo um problema mundial (ONU/FAO, 2016). Como exemplo, nos Estados Unidos, a *Food and Drug Administration* (FDA) estimou que as perdas provocadas pelas micotoxinas na indústria animal podem custar centenas de milhões de dólares ao ano (CAST, 2003).

A presença dos fungos micotoxigênicos raramente é visualmente perceptível em alimentos, o que não significa a ausência de micotoxinas nos mesmos. Da mesma forma, a presença de fungos não significa produção de micotoxinas, pois as condições de temperatura e atividade de água para a produção de micotoxinas são distintas das condições ótimas de crescimento, e geralmente são mais restritas (Binder et al., 2007). Dessa forma, as condições ambientais podem permitir o crescimento do fungo, porém a produção de micotoxinas pode ser limitada ou até mesmo evitada por fatores de estresse durante o crescimento do mofo (Astoreca et al., 2012).

A exposição humana às micotoxinas pode acontecer de forma direta ou indireta. A direta ocorre pela ingestão de grãos ou produtos alimentícios contaminados; e de maneira indireta, se dá quando o indivíduo ingere alimentos como o leite e a carne de animais contaminados (Rocha et al., 2014). A elevada exposição animal e humana às micotoxinas pode resultar em efeitos adversos agudos e crônicos, dependendo da espécie e da susceptibilidade do animal. Estes efeitos adversos estão geralmente associados ao potencial teratogênico, carcinogênico, estrogênico e efeitos imunossupressores destas toxinas (Binder et al., 2007).

Legislação

Atualmente, mais de 100 países possuem legislação para determinar o limite máximo tolerável de micotoxinas em alimentos e ração animal (Wu e Guclu, 2012). Essas legislações foram criadas com o intuito de regular a qualidade dos grãos comercializados, evitando a entrada de alimentos que possam causar doenças para a população. No Brasil, a RDC nº 7 de 18 de fevereiro de 2011 da ANVISA determina o limite máximo tolerado (LMT) de micotoxinas em alimentos. Na Tabela 1, estão apresentados os LMT para micotoxinas em milho e produtos derivados do milho. Da mesma maneira, a legislações da FDA (2011) nos Estados Unidos e da Comissão Europeia (EC Nº 1881/2006) determinam os valores máximos toleráveis de micotoxinas em milho e seus produtos

derivados. Os níveis máximos toleráveis no Brasil são geralmente maiores, contudo a cada nova normativa, a legislação se torna mais restrita.

Tabela 1. Limites máximos toleráveis (LMT) de micotoxinas encontradas em milho segundo a RDC nº 7 de 18 de Fevereiro de 2011 da ANVISA.

Micotoxina	Alimentos	LMT (µg/kg)
Aflatoxinas (B_1+B_2+G_1+G_2)	Milho, milho em grão (inteiro, partido, amassado, moído), farinhas ou sêmolas de milho	20
	Alimentos à base de cereais para crianças	1
Fumonisinas (B_1+B_2)	Milho de pipoca	2000
	Alimentos à base de milho para alimentação infantil	200
	Milho grão para processamento	5000
	Farinha de milho, creme de milho, fubá, flocos, canjica, canjiquinha	1500
	Amido de milho e outros produtos à base de milho	1000
Zearalenona	Milho grão para processamento	400
	Milho de pipoca, canjiquinha, canjica, produtos e subprodutos à base de milho	150

Métodos de controle

A segurança alimentar tornou-se uma preocupação mundial, e estratégias eficientes de controle da contaminação fúngica devem ser adotadas. Segundo Leibetseder (2006), prevenir a contaminação pelo microrganismo e estabelecer pontos críticos de controle são as principais maneiras de se reduzir a deterioração fúngica e consequentemente, reduzir a contaminação por micotoxinas.

O controle da produção das micotoxinas pode ser realizado por métodos químicos, físicos e/ou biológicos. Contudo, apesar dessa variedade de métodos, ao serem produzidas no alimento, a mitigação das micotoxinas se torna muito difícil (Varga et al., 2010). A aplicação de fungicidas é o recurso mais tradicional. Entretanto, têm se sugerido alternativas mais racionais para a redução da contaminação por micotoxinas (Nathanail et al., 2016; Serrano et al., 2016; Zhu et al., 2016). Os métodos são normalmente preventivos, pois é a maneira mais barata e eficiente de evitar a produção de micotoxinas,

além de reduzir os efeitos sobre a saúde animal, bem como as perdas econômicas (Smith e Girish, 2012). Infelizmente, é impossível evitar a contaminação fúngica completamente, portanto, algumas medidas de desintoxicação vêm sendo propostas, com aplicação durante as fases de pré-colheita, pós-colheita, ou até mesmo no produto final (Zhu et al., 2016). Dentre esses métodos, os adsorventes de micotoxinas (argilas e bentonitas) são os mais comuns, sendo disponível comercialmente para a utilização na alimentação animal, os quais se ligam às toxinas no trato gastrintestinal impedindo a sua absorção sistêmica (Avantaggiato et al., 2004).

O uso de microrganismos (*Saccharomyces pastorianus, Saccharomyces cerevisiae* e bactérias ácido láticas) capazes de biotransformar as micotoxinas também vem sendo proposto e avaliado (Corassin et al., 2013; Meca et al., 2013; Nathanail et al., 2016). Entretanto, até o momento não há um método disponível capaz de eliminar totalmente as micotoxinas sem alterar a qualidade dos produtos.

A busca por novas substâncias a partir de fontes naturais tem ganhado importância no cenário mundial como alternativa ao uso de aditivos sintéticos (Ramos, et al., 2012). Os óleos essenciais são misturas complexas de substâncias voláteis e lipofílicas obtidos de plantas principalmente por destilação com arraste a vapor d'água (Santos et al., 2013). Esses compostos possuem capacidade de inibir diversos patógenos em alimentos e, devido a aceitação e segurança para os consumidores, tendem a ser cada vez mais utilizados (Luciano e Holley, 2009).

Entre os antimicrobianos naturais, os isotiocianatos (ITCs) se destacam pelo potencial antibacteriano e antifúngico. Estes compostos são produtos da hidrólise enzimática dos glucosinolatos, sendo encontrados em plantas da família *Brassicaceae* (Azaiez et al., 2013). São conhecidos mais de 120 glucosinolatos com diferentes estruturas químicas (Fahey et al., 2001). Dentre eles, a sinigrina encontrada na mostarda marrom (*Brassica juncea*), quando hidrolisada pela enzima mirosinase, é convertida principalmente em isotiocianato de alila (AITC) (Luciano e Holley, 2009).

O AITC é um composto volátil que está relacionado com diversos efeitos benéficos à saúde humana incluindo potencial antiangiogênico, anti-inflamatório, neuroprotetor e anticarcinogênico (Bhattacharya et al., 2013). O AITC é o mais potente antimicrobiano entre os ITCs, devido a sua ação antimicrobiana em doses menores (Olaimat e Holley, 2016). Nesse contexto, alguns autores demonstraram a capacidade do AITC em volatilizar e inibir o crescimento de entidades micotoxigênicas, como *Fusarium graminearum, Aspergillus parasiticus, Penicillium expansum e Fusarium poae,*

demonstrando um efeito dose dependente e com efeitos de mitigação da produção de micotoxinas a partir de 10 µL/L em fase gasosa (Azaiez et al., 2013; Manyes et al., 2015; Nazareth et al., 2016). Além disso, o AITC tem a capacidade de reagir diretamente com micotoxinas formando novos compostos e reduzindo sua presença em soluções e matrizes alimentares (Luciano et al., 2014; Meca et al., 2012).

Por ser um composto volátil, o AITC pode ser utilizado como fumigante, podendo reduzir os níveis de micotoxinas em milho pela inibição do crescimento de fungos toxigênicos ou pela reação direta com micotoxinas. Dessa forma, o objetivo do presente trabalho foi avaliar a eficiência do AITC na forma gasosa para a redução do crescimento de *Aspergillus parasiticus* CECT 2947 e *Fusarium verticillioides* CECT 2983, visando a redução da produção de micotoxinas (aflatoxinas B_1, B_2, G_1 e G_2; fumonisinas B_1 e B_2) em milho durante 180 dias de estocagem.

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CAPÍTULO 2

Fumigation of allyl isothiocyanate against the growth of mycotoxigenic fungi in stored corn

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ABSTRACT

The objective of this study was to evaluate the efficacy of gaseous AITC to inhibit the growth of *A. parasiticus* and *F. verticillioides*, and mycotoxin production (aflatoxins B_1, B_2, G_1 and G_2, fumonisins B_1 and B_2) in corn during 180 d. Samples of corn were treated with AITC (0.5, 10 and 50 µL/L) in glass jars. The samples were inoculated with either 10^4 or 10^5 spores/g of *A. parasiticus* CECT 2947 and *F. verticillioides* CECT 2983, respectively. Aliquots of 10 g were used to determine the fungal population after 0, 2, 7, 14, 30, 60, 90, 120, 150 and 180 d. Similarly, levels of aflatoxins, fumonisins and AITC were quantified by HPLC during the same period. AITC at 50 µL/L resulted in a significant reduction of the fungal population ($p < 0.05$) after 180 d, decreasing 3.17 log CFU/g and 3.9 log CFU/g of *A. parasiticus* and *F verticillioides*, respectively. In addition, treatments with 10 and 50 µL/L prevented the production of FB1. Residual levels of AITC in corn treated with 10 and 50 µL/L were detected after 14 and 30 d, respectively. Therefore, prophylactic treatment with AITC reduced the fungal population and inhibited FB_1 production in stored corn.

Keywords

Natural antimicrobial, fungal spoilage, mycotoxins reduction, toxigenic fungi, stored grains.

1. Introduction

Corn is one of the most important food grains cultivated around the world. Brazil is the third largest producer worldwide with approximately 63.3 million tons produced in 2016 (GCEA/IBGE, 2017). Fungal spoilage and mycotoxin contamination are frequent problems that reduce the quality of corn kernels and cause significant economic loses. Toxigenic fungi are capable to grow under a wide range of conditions, but food and feedborne mycotoxins have a more significant importance in tropical countries, where the weather is warm and humid (Astoreca et al., 2012; Degraeve et al., 2016). The Food and Agricultural Organization estimated that approximately 25% of cereals produced worldwide are contaminated with dangerous levels of mycotoxins (Duarte et al., 2010; Iqbal et al., 2013).

Aflatoxins (AFs) are polyketide secondary metabolites produced by toxigenic species of *Aspergillus*, such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* and others (Khayoon et al., 2010; McKean et al., 2006). The most common aflatoxins in food products are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). These toxins are mainly found in grains and nuts, whereas aflatoxin M_1, which is derived from the metabolism of AFB_1 in mammals, is found in milk and dairy products (Giray et al., 2007). Among AFs, AFB_1 is the most potent carcinogenic compound to mammals, classified as group 1 by the International Agency for Research on Cancer (IARC, 1993). The consumption of feed contaminated with aflatoxins by different species is associated with teratogenic, mutagenic and carcinogenic effects (Binder, 2007; Manyes et al., 2015). Moreover, acute hepatitis, hemorrhage and edema have been also reported in animals that ingested high levels of

AFs (Iqbal et al., 2014). *Aspergillus* species are commonly associated with the storage of grains and, consequently, AFs are produced after harvest.

Fumonisins are secondary metabolites of *Fusarium* species, mainly *Fusarium verticillioides* and *Fusarium proliferatum*. These fungi are present in the environment and contaminate the grains in the field, where corn the most affected product (Piacentini et al., 2015). Once contaminated, fumonisins can be produced during pre or post-harvest. Exposure to fumonisins may cause harmful responses to human and animal. In humans, high exposure to FB_1 is associated with increased risk of developing esophageal cancer (Ling et al., 2015). In animals, they cause various diseases including equine leukoencephalomalacia and pulmonary edema in swine (Queiroz et al, 2012; Latorre et al, 2015).

Many countries have established maximum tolerable levels of mycotoxins in corn kernels and corn kernels products. In Brazil, the tolerable limit of aflatoxins and fumonisins in corn kernels range from one to 20 ng/g and 200 to 5000 ng/g, respectively (ANVISA, 2011). In 2006, the Commission of the European Community set the levels for aflatoxins ranging from five to 10 ng/g for AFs and 200 to 2000 ng/g for FMs in corn (EC nº. 1881/2006). On the other hand, the US allows a limit of 20 ng/g for AFB_1, and a limit ranging from 2000 to 4000 ng/g for FMs in corn (FDA, 2011).

There has been an increasing interest in the application of natural antimicrobial compounds in foods due to their safety status and higher consumer acceptance (Luciano and Holley, 2009). Some vegetables such as broccoli, cauliflower, cabbage, brussels sprout and various mustard species are known to produce high concentrations of isothiocyanates (ITCs). These compounds are naturally formed after hydrolysis of glucosinolates, and act as a defense mechanism against fungal diseases, insects and herbivores (Mansour et al., 2012; Neoh et al., 2012). ITCs have an electrophilic carbon

that enables them to bind to thiol and amino groups of amino acids, peptides and proteins, forming conjugates (Cejpek et al., 2000). Thus, these compounds can inhibit microbial enzymes and vital metabolic pathways of different pathogens at low concentration, including insects, bacteria, nematodes and fungi.

Allyl isothiocyanate (AITC), derived from brown and black mustard, is a volatile compound that has presented the most potent antimicrobial activity among ITCs. Its small molecule can penetrate the matrix of stored foods through fumigation (Olaimat and Holley, 2016; Siahaan et al., 2014), presenting potent activity against mold growth and mycotoxin production in different foods, including bread (Azaiez et al., 2013), pizza crust (Quiles et al., 2015), nuts (Hontanaya et al., 2015), wheat tortillas (Saladino et al., 2016), wheat flour (Nazareth et al., 2016), strawberries (Ugolini et al., 2014) and corn kernels (Tracz et al., 2016).

The objective of this study was to evaluate the effects of AITC as fumigant agent to inhibit the growth of *Aspergillus parasiticus* CECT 2947 and *Fusarium verticillioides* CECT 2983 and reduce mycotoxin production in corn kernels during 180 d of storage. As a secondary objective, we evaluated the residual concentrations of AITC during the same period to verify when reapplications of the fumigant would be necessary.

2. Materials and Methods

2.1. Materials

Aflatoxins (B_1, B_2, G_1, G_2), fumonisins (FB_1, FB_2) and allyl isothiocyanate (>95% purity) analytical standards were obtained from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile, methanol, o-phthalaldehyde and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO) and acetic acid from LabMaster (Biotec, Pinhais,

Brazil). Deionized water (<18 MU/cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Darmstadt, Germany).

2.2. Fungal strains and inoculum

Fusarium verticillioides CECT 2983 and *Aspergillus parasiticus* CECT 2947 were obtained from Spanish Type Culture Collection (CECT Valencia, Spain). The strains were grown in potato dextrose agar (PDA, Himedia, Mumbai, India) for 7 d at 25 ºC. Then, 5 mL of 0.1% peptone water (Oxoid, Basingstoke, England) were added on top of the grown colonies, which were scraped to form a suspension. The mixture was transferred to a lab tube and adjusted to 10^6 conidia/mL for *Aspergillus parasiticus* or 10^7 conidia/mL for *Fusarium verticillioides*. These solutions were used to contaminate corn samples.

2.3. AITC fumigation treatment

Whole corn kernels (300 g) were placed in glass jars (830 mL) and autoclaved for 20 min at 121°C. Jars were cooled to room temperature (23ºC) and treated with AITC (0.5 µL/L, 10 µL/L and 50 µL/L). Control samples did not receive any treatment. The jars were hermetically closed with metal caps, homogenized and incubated for 48 h at 25°C. Thereafter, bottles were opened for one hour in sterilized air to allow ventilation of the grains and evaporation of AITC excess, as described and adapted by Tracz et al. (2016). Finally, each sample was inoculated with 20 mL of sterilized water to increase environmental humidity and 3 mL of either fungal suspension. Amounts of 20 g of corn kernels were taken at 10 different time-points (0, 2, 7, 14, 30, 60, 90, 120, 150, 180 d), where 10 g were used for microbial counting and 10 g were used for mycotoxin analysis. Experiments were repeated twice in triplicates for each treatment (n = 6).

2.4. Determination of fungal population

Corn samples (10 g) were added to 90 mL of sterile 0.1% peptone water. The mixture was poured inside sterile mortars (500 mL) and the kernels were subsequently crushed with a pestle for 5 min in a level 2 biosafety cabinet. Then, mixtures were serially diluted in tubes containing 0.1% peptone water and 0.1 mL of each dilution were plated on PDA in duplicates. The plates were incubated for 7 d at 25 ºC in the dark. Results are presented in log CFU/g.

2.5. Fumonisins analysis

Fumonisin extraction and quantification were performed according to Shephard et al. (1990) with some modifications. Corn samples (12.5 g) were extracted with 25 mL of methanol: water (75:25, v/v). The mixture was stirred for 30 min in water bath (40 ^{o}C) and filtered (Whatman N° 1 filter). Clean-up of extracts was performed using solid-phase extraction (SPE) columns (Strata SAX, Phenomenex, Torrance, USA), which were conditioned with 8 mL of methanol followed by 8 mL methanol:water (75:25, v/v). A 2 mL aliquot of the extract was passed through the column and washed with 5 mL of methanol:water (75:25, v/v) followed by 3 mL of methanol. Fumonisins B_1 and B_2 were eluted with 15 mL of methanol:acetic acid (99.5:0.5, v/v). Extract was separated in triplicate and evaporated using airflow. Dried extracts were suspended in 2 mL of methanol:water (50:50, v/v), evaporated and diluted in 0.2 mL acetonitrile:water (50:50, v/v). Prior to derivatization. O-phthalaldehyde (OPA) reagent was prepared dissolving OPA (0,02 g) in methanol (0.5 ml) and adding 2.5 ml of 0.1 M sodium borate and 25 µL of 2-mercaptoethanol. Derivatization was carried out manually prior to the HPLC analysis by suspending the extract (100 µL) with OPA reagent (200 µL). Then, 20 µL was injected

in the HPLC system (1220 infinity, Agilent, Santa Clara, USA) with fluorescence detector (1260 infinity, Agilent) at excitation and emission wavelengths of 335 and 440 nm, respectively. Agilent OpenLAB CDS ChemStation Editor was used for data analysis. A C18 column (Phenomenex, Torrance, USA) 4.6 x 150mm, 3μm particle size was used as stationary phase. Gradient mobile phase was acetonitrile (B) and water + acetic acid 1% (A) at a flow rate of 1 mL/min. Mobile phase was 30% B at 0 - 8.5 min, increasing to 70% B at 18 min, hold for 4 min and increasing to 95% B at 23 min, which was kept until 24 min, decreasing to 30% B at 25 min.

2.6. Determination of residual AITC in corn

Corn samples were treated with different doses of AITC (0.5, 10 and 50 μL/L). AITC residual levels were evaluated at 2, 14, 30 and 60 d of storage. The experiment was conducted in triplicate and the analyses were carried out in duplicates (n = 6). The extraction was performed as described by Tracz et al. (2016). Samples (5 g) were placed in a 15 mL falcon tube containing 10 mL of methanol. The mixture was mixed for 30 min in water bath at 40 ºC. Thereafter, tubes were placed inside an ultrasonic bath for 10 min, followed by centrifugation at 4000 x *g* for 5 min at 20 ºC. The supernatant was recovered and filtered through 0.22 μm nylon membrane. An aliquot of 20 μL was injected in HPLC with diode array detector (LC-DAD) 1220 infinity (Agilent, Santa Clara, USA) at 236 nm, with Agilent OpenLAB CDS ChemStation Editor for data analysis. A Gemini C_{18} column (Phenomenex, Torrance, USA) 4.6 x 150mm, 3 μm particle size was used as stationary phase. The isocratic mobile phase was water:acetonitrile (60:40, v/v) and flow rate of 0.8 mL/min.

2.7. Validation procedures

Validation of the analytical method was performed according to guidelines established by ICH (ICH, Harmonised Tripartite Guideline, 2005). Recovery and standard deviations (RSDs) were measured in spiked samples (100, 200 and 300 ng/g for FB_1 and FB_2; 2, 5 and 10 ng/g for AFB_1, AFB_2, AFG_1, AFG_2; 62.5, 125 and 250 μL/L for AITC) submitted to same treatment conditions, in four different days, using three replicates in each day. The samples were previously evaluated for endogenous mycotoxins. The limits of detection (LOD) and quantification (LOQ) were calculated based on signal: noise ratio of 3:1 and 10:1, respectively. Linearity was evaluated by calibration curve using six concentrations in triplicate. Matrix effects were investigated by comparing the slopes of standards diluted in solvent with the slopes of matrix extract spiked with standards.

2.8. Statistical analysis

*S*oftware Prism version 3.00 (GraphPad, La Jolla, CA, USA) for Windows was used for statistical analysis. ANOVA was performed followed by Tukey HSD post-hoc test for multiple comparisons. The level of significance considered was $p \leq 0.05$.

3. Results and Discussion

3.1. Effects of allyl isothiocyanate on fungal growth

Despite the increasing interest in the use of natural compounds to prevent the growth of microorganisms in foods, there are no studies reporting the use of AITC to inhibit the growth of micotoxigenic fungi in corn kernels during a long period of storage. Table 2 shows the effect of a prophylactic treatment with AITC (0.5, 10 and 50 μL/L) in corn to inhibit the growth of *A. parasiticus* CECT 2947 for 180 d. At day 0, all treatment

had significant difference due residual AITC. The treatments of 0.5 and 10 µL/L had no effect in the fungal growth in comparison with the control group after day 0. However, 50 µL/L could decrease the population of *A. parasiticus* to levels below our detection limit (0.92 CFU/g) after 150 d. Our results corroborated with Manyes et al. (2015), which reported the inhibition effect of AITC in mycelial growth of *Aspergillus parasiticus* CECT 2947. The authors demonstrated that doses lower than 25 mg of AITC per disc (disc diffusion method) could not inhibit the mycelial growth in petri dishes incubated at room temperature during 20 d. Such results combined show that AITC can inhibit the growth of *A. Parasiticus* CECT 2947.

Okano et al. (2015) determined the effectiveness of AITC as vapor treatment with mustard seed extract to control aflatoxin producing fungi on stored corn. AITC at 54.6 ng/mL inhibited visible growth of aflatoxigenic molds in unsterilized corn and in sterilized corn inoculated with various aflatoxigenic fungi after 14 d. However, f *A. glaucus*, *A. penicillioides* and *A. restrictus* were detected when corn was homogenized in peptone water and cultured in agar. Similarly, our study demonstrated that treatment with 10 µL/L inhibit the visual growth after 15 d (data not shown) but strain of *A. parasiticus* could be detected in PDA medium.

Table 2

Effect of prophylactic fumigation of allyl isotiocyanate (0.5, 10 and 50 µL/L) in corn kernels for 48 h stored in hermetically closed glass jars. Jars were opened for 1 h (Day 0) to allow evaporation of excess of AITC and *Aspergillus parasiticus* CECT 2947 was inoculated at ~ 4 log UFC/g. Fungal growth was followed during 180 d.

	A. parasiticus CECT 2947			
	Fungal population (Log CFU/g) (Mean± SE)			
Days	Control	0.5 µL/L	10 µL/L	50 µL/L
0	4.31±0.02^{a}	4.16±0.04^{b}	4.09±0.03^{b}	4.09±0.02^{b}
2	4.03±0.06^{a}	4.03±0.06^{a}	3.78±0.09^{a}	3.90±0.07^{a}
7	2.89±0.07^{a}	2.77±0.08^{ab}	2.76±0.08^{ab}	2.45±0.13^{b}
14	2.94±0.08^{a}	2.96±0.04^{a}	2.85±0.04^{a}	2.13±0.17^{b}
30	3.67±0.05^{a}	3.68±0.05^{a}	3.74±0.07^{a}	2.37±0.18^{b}
60	3.91±0.04^{a}	3.73±0.09^{a}	4.10±0.24^{a}	1.85±0.17^{b}
90	3.59±0.05^{a}	3.97±0.08^{a}	3.73±0.17^{a}	1.43±0.14^{b}
120	3.39±0.09^{a}	3.70±0.04^{a}	3.37±0.06^{a}	1.22±0.15^{b}
150	3.36±0.09^{a}	3.63±0.04^{a}	3.64±0.19^{a}	<0.92±0.00^{b}
180	3.30±0.06^{a}	3.66±0.12^{a}	3.07±0.30^{a}	<0.92±0.00^{b}

Different letters show significant difference among treatments in the same row ($p \leq 0.05$)

Quiles et al. (2015) evaluated the use of antimicrobial devices containing allyl isothiocyanate to inhibit the growth of *A. parasiticus* in pizza crust. None of the treatments tested was able to completely inhibit the fungal growth. However, treatments containing 10 µL/L could reduce fungal population > 5 log CFU/g in comparison to control group after 30 d. These different results can be explained by differences in both methods. The pizza crusts were contaminated and incubated at 4 ºC. This temperature is not ideal for the growth of *Aspergillus parasiticus*. Furthermore, this species is commonly found in corn kernels, where the microorganism is more adapted. *A. parasiticus* depend

of O_2 to grow, and in our study the headspace in jars was around 60%. The hermetic storage in bags can reduce the headspace and consequently reduce the free O_2 (Donahaye et al., 1991), compromising the regular growth.

The effects of AITC against *F. verticillioides* growth are presented in table 3. The fumigation of 10 and 50 µL/L of AITC avoided the fungal growth after day 7, although, only the treatment of 50 µL/L reduced the fungal population to levels below the detection limit (0.92 CFU/g) after 150 d. Azaiez et al. (2013) evaluated the inhibitory effect of AITC, PITC and BITC in mycelial growth of *F. moniliforme* CECT 2987 in doses of 10, 25 and 50 mg/disc using disc diffusion method. The authors demonstrated that AITC was able to reduce the mycelial growth ranging from 64.1 to 88.7%. Our study corroborates with these results, since the doses of AITC ≥10 µL/L could avoid *Fusarium* growth.

In this study, the AITC showed a dose-dependent fungicide ability, with faster reduction in the fungal population at higher doses. Thus, the AITC may be used as natural fumigant to avoid fungal spoilage by *F. verticillioides* and *A. parasiticus* in corn kernerls.

Table 3

Effect of prophylactic fumigation of allyl isotiocyanate (0.5, 10 and 50 μL/L) in corn kernels for 48 h stored in hermetically closed glass jars. Jars were opened for 1 h (Day 0) to allow evaporation of excess of AITC and *Fusarium verticillioides* was inoculated at ~ 5 log UFC/g. Fungal growth was followed during 180 d.

	Fusarium verticillioides CECT 2987			
	Fungi population (Log CFU/g) (Mean± SE)			
Days	Control	0.5 μL/L	10 μL/L	50 μL/L
0	4.94±0.05[a]	4.86±0.04[a]	4.91±0.03[a]	4.79±0.06[a]
2	4.67±0.16[a]	4.81±0.10[a]	4.48±0.08[a]	2.88±0.30[b]
7	4.84±0.06[a]	5.02±0.08[a]	3.71±0.24[b]	1.77±0.12[c]
14	5.04±0.15[a]	5.14±0.18[a]	2.77±0.43[b]	0.92±0.00[c]
30	4.29±0.18[a]	4.90±0.33[a]	2.53±0.36[b]	1.31±0.14[c]
60	3.95±0.23[a]	4.82±0.34[a]	2.45±0.35[b]	<0.92±0.00[c]
90	3.81±0.16[a]	4.49±0.38[a]	1.83±0.21[b]	<0.92±0.00[b]
120	4.62±0.18[a]	4.38±0.37[a]	2.10±0.24[b]	1.10±0.14[b]
150	3.36±0.09[a]	3.63±0.04[a]	1.64±0.19[b]	<0.92±0.00[b]
180	3.73±0.55[a]	4.40±0.23[a]	1.98±0.39[b]	<0.92±0.00[b]

Different letters show significant difference among treatments in the same row ($p \leq 0.05$).

3.2. Effects of allyl isothiocyanate on mycotoxin production

There is an increase in FB_1 concentration in the control and 0.5 μL/L groups during 180 d (Table 4), reaching higher concentrations than the maximum established by European Community (2006). On the other hand, treatments of 10 μL/L and 50 μL/L avoided FB_1 production during 180 d. It is possible that *F. verticillioides* CECT 2987 does not produce FB_2 during storage (data not shown). Tracz et al. (2016) evaluated the effect of gaseous AITC on fumonisin production in corn kernels treated with 50, 100 and 500 μL/L during 30 d. The authors demonstrated that all treatments could avoid

mycotoxin production and suggested application of lower doses of AITC should be tested. Comparing the data from such study with ours, we conclude that AITC is effective to avoid FB1 if corn kernels are stored to up 180 d. It becomes important for long-term storages needs.

Table 4

Production of fumonisin B1 by *Fusarium verticillioides* in corn kernels pre-treated with 0.5, 10 or 50 µg/L of gaseous allyl isothiocyanate (AITC). Kernels were stored hermetically for 180 d.

Days	Treatment (µL/L)			
	Control	0.5 µL/L	10 µL/L	50 µL/L
	Concentration of FB_1 by *Fusarium verticillioides* CECT 2987 (µg/g)			
0	1.19^{ABa} ±0.10	1.05^{ABa} ±0.20	1.04^{Aa} ±0.25	0.93^{Aa} ±0.09
30	0.83^{Aa} ±0.22	0.96^{ABa} ±0.20	0.8^{Aa} ±0.16	1.26^{Aa} ±0.32
60	0.76^{Aa} ±0.15	0.92^{ABa} ±0.25	0.9^{Aa} ±0.07	0.78^{Aa} ±0.11
90	1.81^{BCa} ±0.14	0.89^{Ab} ±0.21	0.92^{Ab} ±0.18	0.94^{Ab} ±0.19
120	1.39^{ABa} ±0.10	1.86^{ABb} ±0.28	0.83^{Ac} ±0.17	1.1^{Ac} ±0.09
150	2.32^{Ca} ±0.33	1.86^{ABab} ±0.30	1.1^{Ab} ±0.20	1.05^{Ab} ±0.10
180	2.53^{Ca} ±0.29	1.92^{Ba} ±0.20	0.81^{Ab} ±0.10	0.82^{Ab} ±0.10

Different capital letters show significant difference among days ($p \leq 0.05$). Lower case letters show significant difference among treatments ($p \leq 0.05$).

3.3. Residual AITC results

Figure 1 shows the residual concentration of AITC after fumigation for 48 h, ventilation for 1 h and storage during 180 d. Dose of 0.5 µL/L of AITC was not detected in the first day of analysis (day 2). However, doses of 10 and 50 µL/L showed

respectively, 75% and 68% of residual concentration at day 2, 6.5 and 29.1% after 14 d; <LOD (Limit of Detection) and 25.9% after 30 d. Therefore, AITC could penetrate corn kernels and was gradually released to levels below LOD at day 14 and 60 for treatments of 10 and 50 µL/L, respectively. These results suggest that further protection of corn kernels from fungal spoilage could be reached with new applications of 10 µL/L of AITC in 14 d intervals and 50 µL/L after 60 d of fumigation.

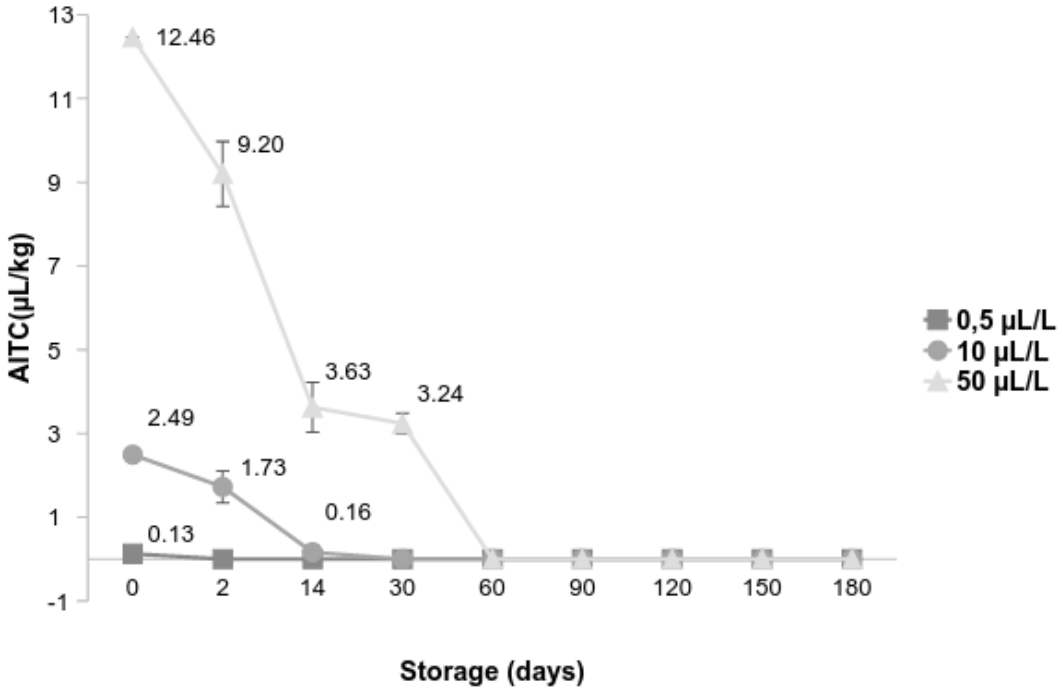

Figure 1

Residual allyl isothiocyanate in corn after 48 h of fumigation (0.5, 10 and 50 µL/L) (Day 0) in hermetic glass jars, 1 h of ventilation (Day 2) and 180 d of storage at 23 ºC.

The validation parameters results are show in Table 5. The doses used to recovery test were 62.5 µL/L, 125 µL/L and 250 µL of AITC/L and the mean of recovery were, respectively, 66.8% (RSD 8.8%), 75.7% (RSD 16.4%) and 72.0% (RSD 11.5%). The values of LOD and LOQ were 0.2 and 0.75 µL AITC/kg, respectively. The coefficient of

linearity was 0.9995 and the matrix effect indicated low interference at the results (110.4%).

AFs recovery test were performed out with doses of 2, 10 and 20 ng/g. The means of recovery ranged from 70 to 102.7 % with lower recovery standard deviation (RSD). These results suggest that the method can be used to mycotoxin recovery with higher reproducibility. The coefficient of linearity was next to 1 in both aflatoxins calibration curve, however the matrix effect of AFB_1, AFB_2, and AFG_2 were higher than 120 %, therefore the quantification of samples was carried out with a matrix effect calibration curve.

AITC is a GRAS (generally recognized as safe) compound permitted for use as a food preservative in US, but its use is limited due to its strong odor. For this reason, researchers have investigated the application of microencapsulation associated to low concentrations of AITC (Ko et al., 2012). Corn is generally processed before consumption, what might reduce AITC to undetectable levels. Anyway, AITC was below LOD (0.2 µL/kg of corn) after 60 days when the most concentrated treatment (50 µL/L) was used, and usually levels below 2 µL/kg are not perceived by the human palate (Nielsen and Rios, 2000).

Overall, AITC residual concentration was proportional to fungicide effect. When higher was the residual concentration, higher was the impact on the fungal population. Consequently, 50 µL/L showed the lower FB_1 production.

Table 5

Results of validation method parameters for detection and quantification of allyl isothiocyanate, AFB_1, AFB_2, AFG_1, AFG_2, $FB_1$and FB_2 in corn kernels.

Standard	Conc (ng/g) [a] (µL/L) [b]	Lin	Matrix Effect (%)	LOD (ng g)	LOQ	Recovery (%)	RSD (%)
	62.5[b]					66.8	8.8
AITC	125 [b]	0.9995	110.4	0.2[c]	0.75[c]	75.7	16.4
	250 [b]					72	11.5
	2 [a]					74	3.4
AFB_1	10 [a]	0.9993	140.3			70	2.1
	50 [a]					89.1	8.1
	2 [a]					77.6	5.3
AFB_2	10 [a]	0.9998	200.4			78.9	3.3
	50 [a]					81.1	12.5
	2 [a]					78.5	3.5
AFG_1	10 [a]	0.9986	117.8			72.3	2.3
	50 [a]					75.9	5.4
	2 [a]					102.7	8.6
AFG_2	10 [a]	0.9997	169.7			96.9	2.1
	50 [a]					95.2	3.6
	100						
FB_1	200	0.9932	94.5				
	300						
	100						
FB_2	200	0.9938	92.9				
	300						

Conc: concentration spiked in corn kernels; Lin : liniarity; M.E : matrix effect; LOD : limit of detection; LOQ : limit of quantification; M.R: % mean of recovery; RSD: recovery standard deviation; a : ng of mycotoxin/g; b : µL of AITC/L; c : µL of AITC/kg

4. Conclusions

The use of gaseous AITC at 10 and 50 µL/L inhibited the *F. verticillioides* and FB_1 production. Moreover, 50 µL/L reduced the population of *A. parasiticus* to undetectable levels in corn kernels after 150 d. AITC was absorbed by corn and gradually released. Residual levels of AITC could extend product shelf-life and improve its safety. Reapplication of 10 and 50 µL/L could be necessary after 14 d and 60 d, respectively, to confer further protection to grains against mold contamination. These results suggest that AITC is a potential fumigant agent that can be used in silos or packages to avoid fungal deterioration and mycotoxins production in corn kernels. Future studies should apply fungal contaminations at different time points of storage to verify the effectiveness of residual concentration of AITC.

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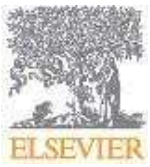
CAPÍTULO 3

CONSIDERAÇÕES FINAIS

O uso de AITC gasoso a 10 e 50 μL/L inibiu o crescimento de *F. verticillioides* e a produção de FB_1. Além disso, 50 μL/L evitou o crescimento de *A. parasiticus* em grãos de milho após 180 dias. O efeito residual da AITC demonstrou que a reaplicação de 10 e 50 μL/L após 14 e 60 dias, respectivamente, pode ser necessária para evitar novas contaminações fúngica e estender a vida de prateleira do milho. Esses resultados sugerem que o AITC pode ser utilizado como agente fumigante em silos ou embalagens, para evitar a deterioração fúngica e a produção de micotoxinas em grãos de milho.

Para estudos futuros, sugere-se a contaminação fúngica em diferentes tempos de armazenamento para verificar a eficácia da concentração residual de AITC. Da mesma forma, sugere-se avaliar a reaplicação de 10 e 50 μL/L de AITC em 14 e 60 dias de intervalos, respectivamente, para evitar contaminações por *A. parasiticus* e *F. verticillioides.* Além disso, o experimento pode ser realizado com diferentes grãos, como o trigo, o feijão, o arroz e a soja, os quais permanecem estocados por longos períodos de tempo, permitindo a deterioração por fungos micotoxigênicos.

ANEXO I



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