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BIOCONTROLE DE *Aspergillus flavus* E *Fusarium verticillioides* POR *Bacillus* spp. ISOLADOS DE PLANTAS DE MILHO

Tiago Centeno Einloft
Biólogo, Mestre em Ciência de Alimentos

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TIAGO CENTENO EINLOFT
Bacharel em Ciências Biológicas - PUCRS
Mestre em Ciência e Tecnologia de Alimentos - UFRGS

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Por

RAFAEL GOMES DIONELLO
Orientador - PPG Fitotecnia

SIMONE MUNDSTOCK JAHNKE
Coordenadora do Programa de Pós-Graduação em Fitotecnia

RENAR JOÃO BENDER
PPG Fitotecnia/UFRGS

MICHELE HOELTZ
Universidade de Santa Cruz do Sul

LAURI LOURENÇO RADUNZ
Universidade Federal da Fronteira Sul

PEDRO ALBERTO SELBACH
Diretor da Faculdade de Agronomia

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RESUMO

Os fungos toxigênicos *Aspergillus flavus* e *Fusarium verticillioides* podem colonizar plantas de milho em todas as etapas do cultivo, através da contaminação fúngica presente no solo, levando a queda de produtividade e qualidade do grão. Em adição, estes fungos podem produzir micotoxinas que põem em grande risco a saúde de humanos e animais que consomem os grãos contaminados. O controle biológico é um método de controle fúngico alternativo ao uso de fungicidas químicos e o uso de bactérias provenientes do sistema radicular de plantas de milho é extremamente promissor em função da capacidade competitiva e de sobrevivência destes micro-organismos. O objetivo deste trabalho foi selecionar bactérias do sistema radicular de plantas de milho com potencial para o biocontrole de *Aspergillus flavus* e *Fusarium verticillioides* no agroecossistema de cultivo do milho. Foram isolados 1283 colônias bacterianas do sistema radicular de plantas de milho e destas 24 apresentaram atividade antifúngica e 3 foram selecionados para o restante do trabalho. Os três isolados selecionados foram identificados como *Bacillus safensis* RF69, *B. amyloliquefaciens* RP103 e *B. subtilis* RP242. Os isolados foram capazes de reduzir a taxa de crescimento micelial *in vitro* dos dois fungos, assim como foram capazes de reduzir a produção, germinação e sobrevivência de conídios. Foi detectada a produção de iturina A pelos isolados *B. amyloliquefaciens* RP103 e *B. subtilis* RP242, caracterizando este metabolito como um dos responsáveis pela atividade antifúngica. Os isolados *B. amyloliquefaciens* RP103 e *B. subtilis* RP242 demonstraram a manutenção da dominância frente a *A. flavus* e *F. verticillioides in vitro* em diferentes condições ambientais, caracterizando alta eficácia no controle fúngico em situações de stress hídrico e de térmico. A bacterização de sementes com os três isolados foi efetiva na proteção radicular frente aos dois fungos, assim como levou ao aumento do comprimento e peso das plântulas, da taxa de emergência de plântulas e na germinação de sementes. Os isolados foram capazes de reduzir drasticamente as contagens fúngicas em solo não-rizosférico e no grão de milho, reduzindo também a concentração de aflatoxina B1 e fumonisina B1 nos grãos. Os isolados demonstraram-se extremamente promissores para serem utilizados em um bioformulado objetivando o controle micotoxigênico em plantas de milho em diferentes etapas da produção de grãos.

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BIOCONTROL OF *Aspergillus flavus* AND *Fusarium verticillioides* BY *Bacillus* spp. ISOLATED FROM MAIZE ROOT SYSTEM

Author: Tiago Centeno Einloft
Adviser: Rafael Gomes Dionello

ABSTRACT

Toxigenic fungi *Aspergillus flavus* and *Fusarium verticillioides* may colonize maize grains on the field and during storage, leading to yield and quality losses, mainly by the production of aflatoxin B1 and fumonisin B1, carcinogenic and potentially carcinogenic mycotoxins, respectively. Biological control is a promising alternative control method and the usage of soil bacteria as biocontrol agents has great potential due to survival and competitive characteristics of those microorganisms. The objective of this study was to select bacteria from maize root system with potential to biocontrol *Aspergillus flavus* and *Fusarium verticillioides* on the agroecosystem of maize. We isolated 1283 bacterial colonies from maize root system, 24 of them presented antifungal activity and three were selected for the rest of the study. Selected isolates were identified as *Bacillus safensis* RP69, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242. The isolates were capable to reduce *in vitro* mycelial growth rate of both fungi and reduce conidial production, germination and survival. Iturin A production was detected by *B. amyloliquefaciens* RP103 and *B. subtilis* RP242, characterizing this metabolite as one of the responsible for the antifungal activity. Isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 maintained *in vitro* dominance towards *A. flavus* and *F. verticillioides* in different environmental conditions, characterizing high efficacy on fungal control even in water and temperature stress situations. Seed bacterization was effective on root protection towards both fungi, as well as increased seedling length and weight, emergency rate and seed germination percentage. The isolates were able to reduce fungal counts on non-rhizospheric soil and on grain, and were able to decrease aflatoxin B1 and fumonisin B1 final concentrations on maize grain. The selected isolates shown extremely promising to be used as biocontrol agents on a bioformulated product aiming mycotoxigenic control on maize plants during different steps of grain production.

[1] Doctoral thesis in Agronomy, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (147p.) February, 2016.

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1 INTRODUÇÃO

A cultura do milho é de extrema importância, pois além da alimentação humana e animal, seu principal destino no mundo, é utilizado nas áreas industrial e energética. No Brasil, o milho é um dos grãos mais produzidos e consumidos pela população, sendo uma das principais fontes de nutrientes para homens e animais. A demanda por grãos de alta qualidade vem crescendo a cada ano, sendo necessário garantir a alta produtividade para atender as necessidades dos consumidores.

Dentre os fatores que afetam negativamente a produtividade e qualidade da produção agrícola, as doenças de plantas assumem grande importância, especialmente aquelas causadas por fungos que afetam a qualidade dos grãos produzidos, tais como *Aspergillus flavus* e *Fusarium verticillioides*. Esses fungos são ainda potencialmente produtores de compostos que causam efeitos adversos nos animais e seres humanos, denominados micotoxinas.

Estima-se que cerca de 25% da produção anual de grãos esteja contaminada por micotoxinas, levando a perdas que chegam a 1 bilhão de toneladas de grãos anualmente. As principais micotoxinas produzidas por *A. flavus* e *F. verticillioides* são as aflatoxinas e fumonisinas, respectivamente, e os efeitos tóxicos desses compostos podem levar a sintomas leves, como a recusa alimentar e a perda da conversão nutricional, até doenças graves, como a leucoencefalomalácia equina, edema pulmonar, câncer hepático e renal e até a morte. Além do evidente risco a saúde de homens e animais, a ocorrência de

micotoxinas causa grandes prejuízos econômicos em função da morte de animais, decréscimo do ganho de peso e das barreiras comerciais impostas por países importadores de produtos alimentícios.

O controle preventivo de fungos toxigênicos é a maneira mais efetiva e segura de garantir a ausência destes compostos, que são de difícil remoção depois de produzidos no grão. Alguns autores têm preconizado o uso de fungicidas para o controle de fungos toxigênicos, porém, em função da toxicidade, eficácia limitada e possível estímulo da síntese de micotoxinas, tem se buscado alternativas que visam minimizar os efeitos causados pelos resíduos nos grãos e no ambiente.

O controle biológico, com utilização de micro-organismos antagonistas à patógenos tem se mostrado bastante promissor em função de sua eficácia, acessibilidade econômica e ausência de efeitos adversos à natureza, ao homem e animais. Embora eficazes, os bioformulados registrados representam apenas 1% da indústria de produtos antifúngicos.

Estudos recentes buscando o controle de fungos toxigênicos utilizando bactérias antagonistas evidenciaram o amplo espectro de utilização de bioformulados no controle fúngico. Estes têm potencial de utilização no campo, através da microbiolização de sementes, pulverização de espigas e infestação do solo. Em se tratando de armazenamento, a massa de grãos pode ser pulverizada com o bioformulado.

A busca de micro-organismos provenientes do sistema radicular de plantas de milho para o biocontrole de fitopatógenos é uma alternativa promissora, pois elimina o impacto ecológico e apresenta grande potencial no controle de fungos toxigênicos como o *A. flavus* e *F. verticillioides* em função da alta diversidade microbiana presente neste ambiente. Bactérias nativas do solo e do sistema radicular de culturas possuem características interessantes para serem utilizados como agentes de biocontrole em função

da pressão seletiva do ambiente em que foram retirados: excelente capacidade competitiva e de sobrevivência.

Dentre diversos gêneros de bactérias nativas do solo e colonizadoras do sistema radicular de grandes culturas, áquelas pertencentes ao gênero *Bacillus* se destacam em função do grande número de metabólitos bioativos com atividade antifúngica, que ressaltam o potencial biotecnológico deste gênero. Em adição, o potencial uso destas bactérias é amplificado em função de seus baixos requerimentos nutricionais, que facilitam a formulação de um bioproduto, e, por serem formadores de endósporos, estrutura que confere resistência em condições ambientais adversas, propiciando a sobrevivência dos agentes de biocontrole com as mudanças ambientais e garantindo a manutenção da efetividade antifúngica.

Com base nisto, os objetivos do presente trabalho foram isolar rizobactérias de plantas saudáveis de milho; testar seu potencial antagonista frente a cepas toxigênicas de *Aspergillus flavus* e *Fusarium verticillioides*; selecionar potenciais agentes de biocontrole para as duas espécies fúngicas; identificar em nível específico os isolados selecionados; avaliar o efeito dos agentes de biocontrole selecionados na taxa de crescimento micelial fúngico, na germinação, produção e sobrevivência de conídios; avaliar a capacidade dos isolados em influenciar no crescimento fúngico e produção de micotoxinas *in situ* no grão de milho; avaliar a capacidade dos agentes de biocontrole em influenciar nas contagens fúngicas em solo não-rizosférico e; avaliar o efeito da bacterização de sementes de milho com os agentes de biocontrole no crescimento fúngico nos tecidos vegetais radiculares e nos parâmetros agronômicos de crescimento vegetal de plântulas de milho.

2 REVISÃO BIBLIOGRÁFICA

2.1 A cultura do milho

O cultivo do milho (*Zea mays* L.) é uma atividade de extrema importância em nível global. Esta importância é dada em função de seu amplo uso, desde a alimentação até a indústria de alta tecnologia. A alimentação animal é para onde é destinada a maior parte da produção anual. Cerca de 70% da produção mundial é utilizada com este fim, enquanto no Brasil, os índices já chegaram a até 80% (ABIMILHO, 2007). Destes, cerca de 51% é destinada para a avicultura, 31% destinada à suinocultura e 11% para bovinos, assim sendo, tem um impacto indireto na alimentação humana (ABIMILHO, 2007; Duarte *et al*., 2011). Embora uma pequena parcela seja destinada diretamente para a alimentação humana (cerca de 3% da produção total), o milho (na forma de grão, farinha, óleo e outros derivados) representa um importante ingrediente, sendo considerado em algumas regiões, a principal fonte de energia e de nutrientes para a população consumidora (Cruz *et al*., 2000; Duarte *et al*., 2011).

Somado a isto, a cultura do milho exerce um impacto importante no âmbito social. No Brasil, cerca de 90% dos produtores de milho são considerados pequenos produtores, e destes, 25% dependem exclusivamente da cultura para a sua sobrevivência, para o seu consumo próprio (IBGE, 1996; Cruz *et al*., 2000). No que diz respeito a mão de obra, quase 15% dos funcionários em lavouras temporárias e 5% dos trabalhadores do

setor agrícola estão ligados diretamente a produção de milho (IBGE, 1996; Cruz *et al*., 2000; Duarte *et al.,* 2011).

Em função de sua ampla e diversa utilização e do grande impacto econômico e social, a cultura tem importante participação no contexto da produção mundial de cereais e atualmente é um dos cereais mais produzidos no mundo (USDA, 2015; FAO/WHO, 2015). A produção mundial de milho triplicou entre os anos de 1960 e 2001, aumento que basicamente acompanhou o crescimento da população de suínos e aves, pois é um ingrediente básico das rações destes animais (Cruz *et al*., 2000). Nos últimos quatro anos, a produção mundial média foi de 778,8 milhões de toneladas de milho enquanto na década de 1980 a média anual era de aproximadamente 450 milhões de toneladas, correspondendo um expressivo aumento na produção total deste grão no mundo (USDA, 2015; Demarchi, 2011).

Atualmente, os Estados Unidos é o principal produtor, tendo produzido mais de 361 milhões de toneladas produzidas na safra de 2014/2015 o que corresponde cerca de 35% da produção mundial. Em seguida, a China aparece com cerca de 205 milhões de toneladas, o que representa 22% da produção mundial (USDA, 2015; 2016). Juntos, os EUA e a China consomem mais de 54% do total de milho produzido no mundo. Este consumo aumentou em 8 e 24% nos últimos anos para os EUA e China, respectivamente. (Demarchi, 2011; ABIMILHO, 2015; USDA, 2015; 2016).

O Brasil é o terceiro maior produtor mundial de milho totalizando 84,6 milhões de toneladas na safra 2014/2015, com uma estimativa de colheita de aproximadamente 83 milhões de toneladas na safra 2015/2016. Foi o segundo grão mais produzido no Brasil na safra de 2014/2015, superado apenas pela soja, que é o grão mais produzido no país. Neste âmbito, o estado do Rio Grande do Sul produziu 6,17 milhões de toneladas de milho na safra 2014/15, um aumento em relação à safra 2013/2014, que atingiu 5,7 milhões de

toneladas. Por outro lado, o estado do Paraná produziu 15,8 milhões e o Mato Grosso 20,7 milhões de toneladas na safra de 2014/15. As regiões centro-sul do país são as principais produtoras desse grão, com produção de 75,86 milhões de toneladas de milho na safra de 2014/2015; com destaque para os estados do Paraná e Mato Grosso do Sul, que concentram 43% da produção nacional, enquanto o estado do Rio Grande do Sul representa cerca de 8% da produção (CONAB, 2015).

Em relação aos dados de produtividade, observa-se uma grande discrepância entre os países produtores. Enquanto os Estados Unidos, primeiro país em produtividade, obtiveram na safra de 2014/2015 uma produtividade de 10.700 kg ha^{-1}, o Brasil, considerado o quinto em produtividade, obteve na mesma safra 5396 kg ha^{-1} (ABIMILHO, 2015; NCGA, 2015; USDA, 2015). Esta grande discrepância entre os países pode ser atribuída a diversos fatores, entre eles o alto nível tecnológico aplicado nos sistemas e práticas de cultivo, bem como a alta organização política dos produtores americanos (ABRAMILHO, 2010).

Segundo o Ministério da Agricultura, Pecuária e Abastecimento do Brasil (BRASIL, 2011), para suprir a demanda, a produção de milho no Brasil está projetada para crescer em 2% ao ano até o ano 2021, com o aumento da área plantada em 0,3% ao ano no mesmo período. Este aumento de produtividade de 2%/ano intensifica a demanda pela maior qualidade de cultivo e consequentemente do grão, sendo necessárias medidas que minimizem a incidência de fitopatógenos que afetam a produção e a qualidade do produto final, como os fungos toxigênicos (BRASIL, 2011).

2.2 Fungos e micotoxinas em milho

A contaminação dos grãos de milho por diferentes grupos fúngicos pode causar um impacto muito grande na cadeia de cultivo do milho, resultando na queda da

produtividade e qualidade dos grãos. A importância desta contaminação ganha proporções ainda maiores quando os grupos fúngicos que atacam os grãos são potencialmente produtores de micotoxinas.

As micotoxinas são metabólitos secundários de estrutura química complexa e diversa produzidos por fungos filamentosos, que podem ser prejudiciais à saúde de animais ou seres humanos (Bennet & Klich, 2003). A ocorrência desses compostos existe de forma natural, podendo ou não ser produzida por um fungo potencialmente toxigênicos, dependendo de fatores intrínsecos e extrínsecos à população fúngica (Marin *et al*., 1998; Mogensen *et al*., 2009). A presença destes metabólitos tóxicos acarreta em um grande risco à saúde de animais e humanos consumidores dos produtos contaminados. A presença das micotoxinas leva consequentemente a perdas econômicas significantes, devido à redução conversão alimentar em animais, prejudicando o desenvolvimento de animais e a formação de barreiras econômicas entre países importadores e exportadores (Halasz *et al*., 2009).

Não existem métodos que eliminem completamente as micotoxinas de determinado alimento, principalmente em função da sua alta estabilidade molecular e a baixa reatividade com outras macromoléculas (Riley & Norred, 1999). Há relatos de significante redução das concentrações de algumas micotoxinas com tratamentos físicos (tratamento térmico, com microondas e degradação solar) e com a utilização de agentes químicos (bissulfito, amônia, peróxido de hidrogênio, bicarbonato de sódio, ozônio, aluminossilicatos hidratados de cálcio e sódio e hidrólises alcalinas) e biológicos (detoxificação por leveduras, diversos lactobacilos e rizobactérias) (Doyle *et al*., 1982; Samaraeva *et al*., 1990; Ahokas *et al*., 1998; Mayura *et al*., 1998; Katta *et al*., 1998; Riley & Norred, 1999; Cavaglieri *et al*., 2005; Bluma & Etcheverry, 2006; Halasz *et al*., 2009, Formenti *et al*., 2012).

Embora algumas técnicas tenham alcançado excelentes resultados, acabam esbarrando em algumas desvantagens, tais como: eficácia limitada, alto custo, a alteração da conformação química do alimento (levando a uma perda do valor nutricional e de palatabilidade) e até possíveis efeitos tóxicos dos resíduos de tratamento (Riley & Norred, 1999; Halasz *et al*., 2009). O controle e prevenção da incidência fúngica é a única maneira de garantir a ausência de micotoxinas, e por isso, o desenvolvimento de diferentes métodos de controle é de extrema relevância (Riley & Norred 1999; Magan & Ladred, 2007).

Atualmente, centenas de micotoxinas diferentes, produzidas por uma grande variedade de fungos filamentosos já foram descritas na literatura, mas algumas merecem maior destaque em função de sua alta toxicidade e ocorrência nos produtos alimentícios (Woloshuk & Shim, 2013). Na cultura do milho, as micotoxinas que merecem maior destaque, são as fumonisinas e as aflatoxinas, em função de sua capacidade toxigênica em humanos e animais, bem como a sua alta frequência e concentração encontradas nos grãos e em produtos derivados (Machinski & Soares, 2000; Camargos *et al*., 2001; IARC, 2002; Rocha *et al*., 2009; Reddy & Salleh, 2011; Alborch *et al*., 2012).

Os principais fungos produtores das aflatoxinas são àqueles pertencentes ao gênero *Aspergillus* seção *Flavi*, como as espécies *A. flavus* e *A. parasiticus*, sendo que apenas a primeira espécie é de relevância no contexto do milho. Os principais produtores das fumonisinas são os fungos do gênero *Fusarium* seção *Liseola* (*F. verticillioides* e *F. proliferatum*) e a espécie *Aspergillus niger*, destacando a espécie *F. verticillioides*, no contexto do cultivo do milho (Almeida *et al*., 2005; Frisvad *et al*., 2007; Magan & Ladred, 2007; Rocha *et al*., 2009; Alborch *et al*., 2012).

Os fungos *A. flavus* e *F. verticillioides* são fitopatógenos importantes do milho que causam podridões do grão e da espiga. A contaminação por estes fungos e suas

respectivas micotoxinas são alguns dos principais fatores que resultam no decréscimo da produtividade e da qualidade do grão (Almeida *et al*., 2005; Woloshuk & Shim, 2013).

2.2.1 *Aspergillus flavus* e Aflatoxina B1

2.2.1.1 Taxonomia e epidemiologia

O *A. flavus* (Teleomorfo: *Petromyces flavus*) é um fungo saprófita cosmopolita, isto é, encontrado em todo o mundo e em diferentes tipos de ambientes (Abbas *et al*., 2009; Horn *et al*., 2009). É uma espécie pertencente ao filo Ascomycota e em função da recente forma sexuada descoberta, foi inserido dentro do sub-filo Pezizomycotina (Horn *et al*., 2009; ÍNDEX FUNGORUM, 2016). Dentro do gênero *Aspergillus*, é um membro da seção *Flavi*, juntamente com o também aflatoxigênico *Aspergillus parasiticus* e *A. nomius*, com os quais divide diversas características em comum.

A espécie se caracteriza por colônias na coloração verde oliva (Figura 1a), em função de seus conídios fortemente pigmentados, produzidos em cabeças vesiculares cobertas por métulas e fiálidas, na grande maioria dos isolados (Figura 1 b-d) (Pitt & Hocking, 2009; IARC/WHO, 2012). Morfologicamente, a grande diferença entre o *A. flavus* e o *A. parasiticus* é a ornamentação conidial, que é lisa e com paredes finas em *A. flavus* e extremamente rugosa e com paredes grossas em *A. parasiticus* (IARC, 2012).

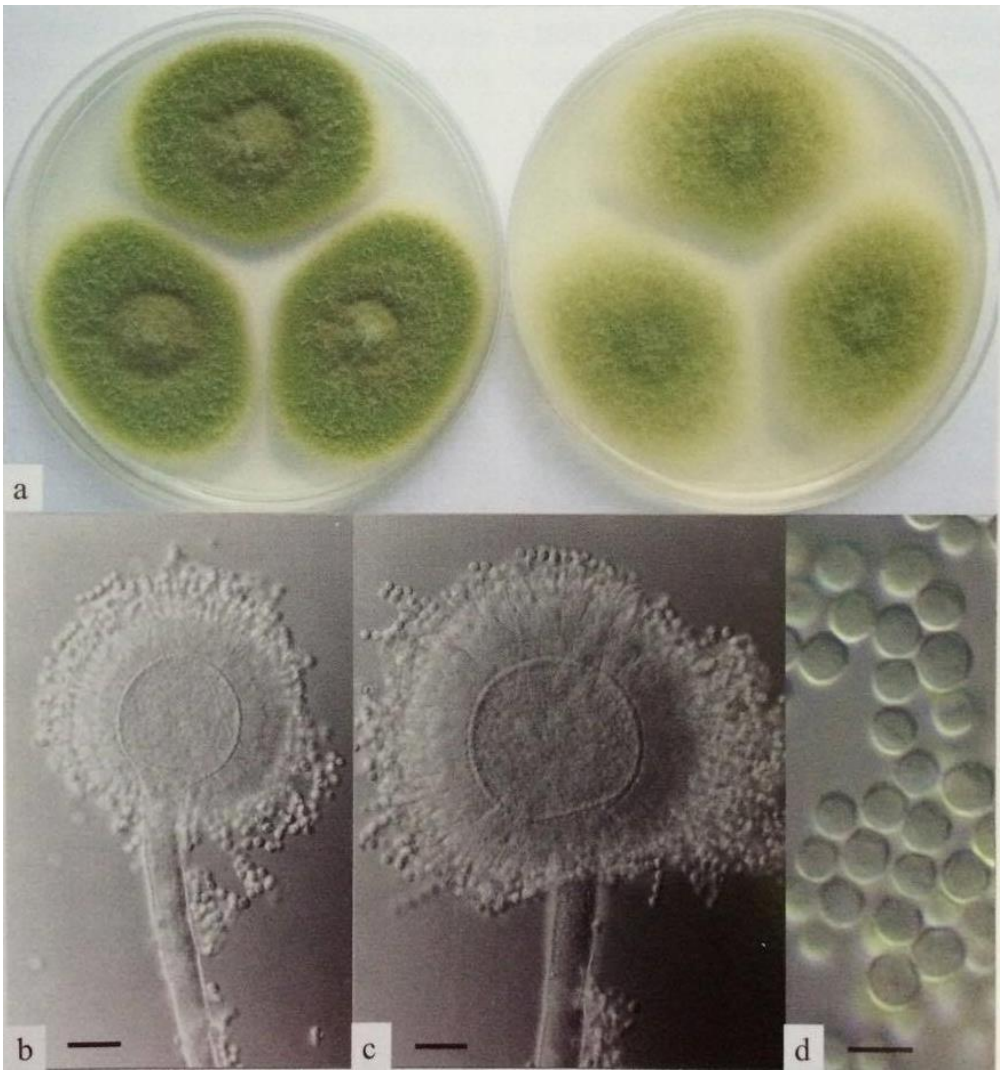

FIGURA 1. Macro e micromorfologia de *Aspergillus flavus*. Crescimento em meio CYA (a-esquerda) e MEA (a-direita), colônias de 7 dias a 25 ºC; (b-c) cabeças conidiais com vesículas, métulas e conídios (barra = 20 µm); (d) conídios ornamentação de conídios (barra = 5 µm). Fonte: Pitt & Hocking, 2009

Os *A. flavus* têm a capacidade de obter nutrientes de diferentes substratos e, por isso, é considerado um fungo extremamente ubíquo. Sua ocorrência é destacada principalmente em amendoim, algodão, soja, milho e outros cereais (Pitt & Hocking, 2009; Payne, 1998; Monge *et al*., 2012; IARC/WHO, 2012). Além da grande ubiquidade, é um fungo extremamente resistente, pois possui a habilidade de sobreviver no solo durante o período da entressafra na forma de micélio ou de estruturas de resistência, como

os escleródios (Wicklow *et al*., 1993; Payne, 1998; Jamie-Garcia & Cotty, 2004; Horn, 2007; Abbas *et al*., 2008; Abbas *et al*., 2009). Embora pouco se saiba sobre o ciclo de vida desta espécie no solo, seus propágulos latentes servem como inoculo inicial na infestação das partes radiculares das culturas posteriormente cultivadas, como pode ser observado na Figura 2 (Abbas *et al*., 2009).

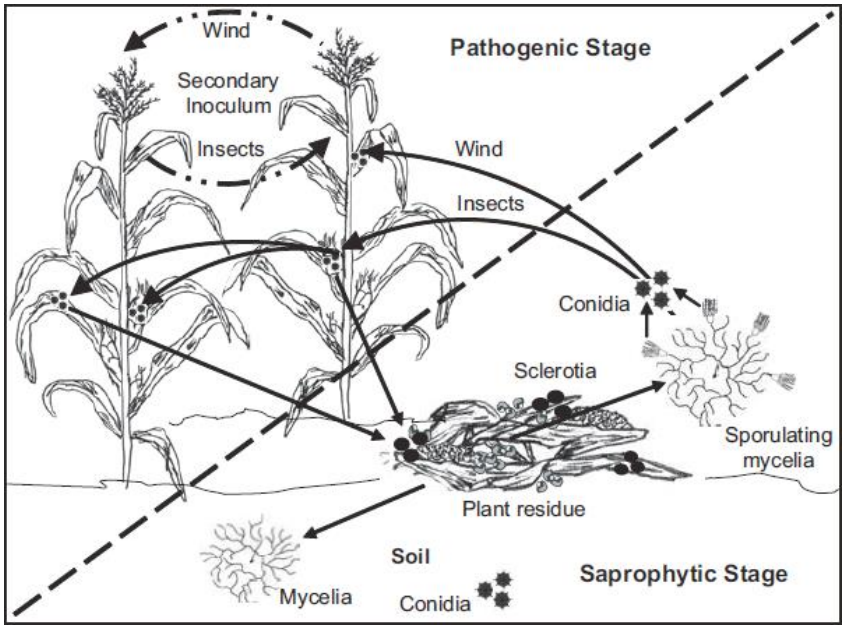

FIGURA 2. Ciclo de vida de *Aspergillus flavus* no agroecosisstema de cultivo do milho em estágios saprofíticos e patogênicos. Fonte: Abbas *et al*., 2009.

Por ser um micro-organismo extremamente difundido, é considerado tanto um patógeno de campo quanto de armazenamento, isto é, a contaminação por este fungo pode ocorrer tanto durante o período de pré-colheita quanto em pós-colheita. Na pré-colheita, a contaminação pelo solo, que serve de reservatório de micélio, esporos ou estruturas de resistência, ocorre através da penetração pelo sistema radicular e crescimento na planta como comensal, ou por ar, que carreia os esporos até a parte aérea da planta (Abbas *et al*., 2009; Chulze, 2010). Os esporos penetram no interior da planta de milho por aberturas feitas pelo ataque de insetos ou pela penetração direta do conídio germinado pelo canal

do estame, chegando na espiga onde ocorre a colonização dos grãos (Abbas *et al*., 2009). Este último ocorre principalmente durante a época de floração das plantas de milho, que estão susceptíveis tanto ao ataque de insetos quanto a penetração fúngica direta (Abbas *et al*., 2009). O *A. flavus* consegue crescer numa faixa de temperatura entre 18-35ºC, tendo como temperatura ótima de crescimento entre 30-35ºC, ou seja, desenvolve-se muito bem em países tropicais e subtropicais (IARC/WHO, 2012).

Já no período pós-colheita, a contaminação está relacionada ao transporte, processamento e principalmente durante o armazenamento do grão. Quando ocorrem atrasos na secagem ou até mesmo secagem incorreta e armazenamento com teor de agua acima de 12%, propágulos fúngicos trazidos do campo germinam e pode haver a proliferação fúngica na massa de grãos no interior de silos ou silo-bags de armazenamento (Magan & Aldred, 2007; Ahsan *et al*., 2010). Quando o armazenamento for incorreto, havendo o ataque de pragas e incremento de umidade, o desenvolvimento de *A. flavus* pode ocorrer de maneira explosiva (Magan & Aldred, 2007;).

2.2.1.2 Ocorrência de *A. flavus* em milho

Existem relatos da ocorrência de *A. flavus* em diferentes alimentos, mas com destaque para o amendoim e o milho como os principais substratos no qual o fungo pode ser encontrado, apresentando-se como um grande contaminante destas duas culturas (Taniwaki & Silva, 2001). Tipicamente, pode ser contabilizado até 10^6 conídios de *A. flavus* por grama de tecido vegetativo de milho após a colheita (Abbas *et al*., 2009).

A alta ocorrência de *A. flavus* em grãos de milho já foi descrita em diversos países. Alborch *et al*. (2012) relataram, na Espanha, a contaminação de 100 e 93% das amostras de grão de milho e derivados, respectivamente, com fungos do gênero *Aspergillus*, sendo que destes, em amostras de milho e em derivados, cerca de 95 e 50%

pertenciam a espécie *A. flavus*, respectivamente. Na Argentina, Astoreca *et al*. (2011) relataram a ocorrência de *Aspergillus* seção *Flavi* em altas concentrações em cerca de 50% das amostras de milho analisadas e, destes, 98% pertenciam a espécie *A. flavus*. Também na Argentina, Etcheverry *et al*. (1999) relataram a ocorrência do gênero *Aspergillus* em 60% das amostras de milho onde, destes, 78% foram identificados como pertencentes a espécie *A. flavus*. Recentemente no Brasil, Rocha *et al*. (2012) analisaram 200 amostras provenientes de quatro estados (Mato Grosso, São Paulo, Bahia e Rio Grande do Sul), dos quais aproximadamente 40% estavam contaminadas com *A. flavus*, sendo que entorno de 60% eram produtores de aflatoxina B1 e 50% eram produtores de aflatoxina B1 e B2.

Além da grande distribuição mundial, a ocorrência do *A. flavus* nos diferentes alimentos é agravada pela sua capacidade de produzir diferentes metabólitos potencialmente tóxicos para os animais e humanos. Esta espécie é potencialmente produtora das aflatoxinas B1 e B2, ácido aspergillico, ácido ciclopiazônico, ácido kójico, ácido helvulico, entre outros (Abbas *et al*., 2009).

Sua grande importância decorre da soma de diversos fatores como a sua alta ocorrência em diversos países com climas diferentes, da sua capacidade de sobrevivência e de contaminação de diferentes substratos alimentícios e, principalmente, da sua potencial habilidade de produzir diferentes metabólitos tóxicos para animais e seres humanos.

2.2.1.3 Aflatoxina B1: Toxicidade, ocorrência e legislação

Dos metabólitos tóxicos que o *A. flavus* é capaz de produzir, as aflatoxinas são consideradas as mais importantes. As aflatoxinas foram identificadas como compostos tóxicos depois da morte de cerca de 100.000 perus na Inglaterra em 1960, após a ingestão

de torta de amendoim contaminada com altas concentrações desta toxina (Blout, 1961). Após o isolamento deste composto, houve uma revolução no campo da micotoxicologia, com uma subsequente busca intensa de novos compostos toxigênicos em diferentes produtos alimentícios contaminados por diferentes grupos de fungos filamentosos (Abbas *et al*., 2009).

As aflatoxinas são quimicamente relacionadas com as bisfuranocumarinas (Figura 3) e afetam principalmente o fígado. A Aflatoxina B1 é considerada a mais tóxica, e, além da capacidade hepatotóxica, é altamente mutagênica, carcinogênica e provavelmente teratogênica para animais. A aflatoxina B1 causa a necrose hepatocelular, hiperplasia biliar, a elevação das enzimas séricas que produzem danos na membrana celular através do aumento da peroxidação lipídica (IARC, 2002; EFSA, 2009; Rocha *et al*., 2009). Em animais, há relatos da redução do ganho de peso, diminuição da ingestão alimentar, imunomodulação e até morte durante episódios de aflatoxicose aguda (EFSA, 2009). A Agência Internacional de Pesquisa sobre o Câncer (IARC) classifica a aflatoxina B1 como pertencente ao grupo 1, ou seja, comprovadamente carcinogênica para humanos e animais (IARC, 2002).

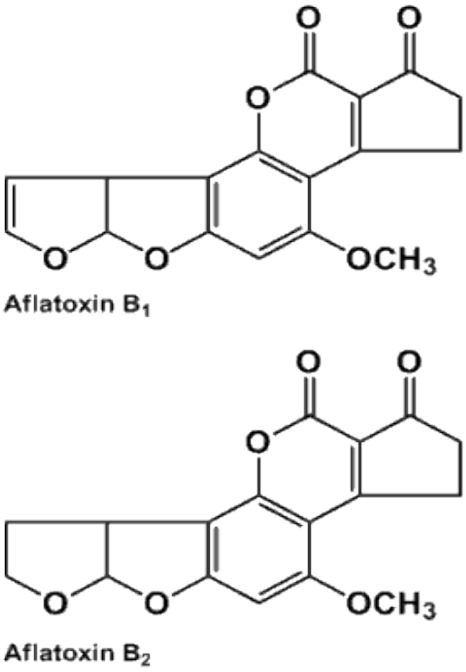

FIGURA 3. Estrutura química das aflatoxinas B1 e B2 Fonte: FDA, 2015

Assim como a alta ocorrência de *Aspergillus flavus*, foi observada a presença de aflatoxinas em um elevado número de amostras de milho e produtos derivados produzidos no Paquistão (AHSAN *et al*., 2010), Espanha (Alborch *et al*., 2012), Senegal (Diedhiou *et al*., 2011), Argentina (Etcheverry *et al*., 1999; Astoreca *et al*., 2011; Pereyra *et al*., 2011), Estados Unidos (Hawkins *et al*., 2008), China e Irã (Khosravi *et al*., 2007), México (Perez-Flores *et al*., 2011), entre outros países.

No Brasil, Hennigen & Dick (1995) relataram a presença de aflatoxina B1 em cerca de 40% das amostras analisadas, com concentrações chegando a até 906 µg kg^{-1} em grãos de milho. Já Sabino *et al*. (1989) relataram concentrações de até 900 µg kg^{-1} de aflatoxina B1 em grãos de milho produzidos nos estados do sul e sudeste do Brasil. Mais recentemente, em estatísticas geradas pelo Laboratório de Análises Micotoxicológicas da Universidade Federal de Santa Maria (LAMIC), foi relatado que cerca 60% de 2487 amostras de milho analisadas pelo laboratório apresentaram resultado positivo para a

presença de aflatoxina B1, com a concentração média de 20 µg kg^{-1} entre os meses de fevereiro e março de 2013 (LAMIC, 2013).

Devido à alta incidência e toxicidade desta micotoxina, as autoridades de diversos países fixaram limites máximos permitidos de aflatoxinas para determinados alimentos. Na União Européia, o limite máximo permitido para a aflatoxina B1 e para soma das aflatoxinas B1, B2, G1 e G2 é de 2,0 e 4,0 µg kg^{-1}, respectivamente, para todos os cereais e produtos derivados (EUROPEAN UNION, 2006). A União Européia ainda fixou um limite máximo de 5,0 e 10,0 µg kg^{-1} de aflatoxina B1 e para a soma das aflatoxinas, respectivamente, em milho que ainda será selecionado ou será submetido a algum outro tratamento físico (EUROPEAN UNION, 2006). Foi determinado também, um limite máximo de 0,10 µg kg^{-1} de aflatoxina B1 em alimentos para bebê e crianças com base em cereais, isto em função da maior suscetibilidade das crianças aos efeitos tóxicos desta micotoxina (EUROPEAN UNION, 2006). No Brasil, segundo a resolução RDC N° 7 de 18 de fevereiro de 2011, foi determinado o limite máximo permitido para a soma das aflatoxinas com aplicação imediata, conforme a Tabela 1 (ANVISA, 2011).

TABELA 1. Limites máximos permitidos para a soma das aflatoxinas no Brasil.

Alimento	Limite Máximo Permitido (µg kg^{-1})
Milho, milho em grão (inteiro, partido, amassado, moído), farinhas ou sêmolas de milho	20
Alimentos à base de cereais para alimentação infantil (lactentes e crianças de primeira infância)	1

Fonte: RDC nº 7, 18 de fevereiro de 2011, ANVISA.

A legislação brasileira permite concentrações maiores para a soma das aflatoxinas nos produtos à base de milho em relação à legislação europeia, provavelmente em função da precária realidade do país em relação ao problema das micotoxinas nos alimentos. Embora haja um limite máximo permitido, o ideal é a ausência completa deste composto,

levando em consideração o efeito altamente tóxico deste metabólito no corpo humano (IARC, 2002). Assim sendo, o controle fúngico é essencial para evitar a ocorrência de micotoxinas em alimentos. O *Aspergillus flavus* é considerado o principal produtor desta micotoxina em milho, então seu controle é imprescindível para assegurar a qualidade do grão em relação a esta toxina (Magan & Ladred, 2007).

2.2.2 *Fusarium verticillioides* e Fumonisina B1

2.2.2.1 Taxonomia e epidemiologia

A taxonomia de *F. verticillioides* foi assunto de muita controvérsia pelos taxonomistas. Este fungo já foi nomeado de *F. moniliforme*, nome específico o qual não é mais utilizado, pois hoje se sabe que a espécie *F. moniliforme* na realidade compreendia outras espécies, além de *F. verticillioides* (O'Donnell *et al*. 1998; Leslie *et al.*, 2005). O *F. verticillioides* tem como forma teleomórfica descrita a espécie *Giberella moniliformis*, mas também é pertencente ao complexo de espécies *Giberella fujikuroi*, porém, representando apenas isolados de *G. fujikuroi* com o *mating population* A, tendo os outros *mating populations* como representantes das espécie *F. subglutinans* e *F. proliferatum* (Leslie & Summerell, 2006).

Embora grande parte das espécies de *Fusarium* sejam produtoras de clamidosporos, o complexo *G. fujikuroi* tem como uma característica distintiva a ausência destas estruturas (Leslie & Summerell, 2006). Os conídios são produzidos em ramificações de hifa especializadas, chamadas de fiálides, as quais nesta espécie possuem a parede celular interna e externa contínua, permitindo a formação de cadeias longas de microconídios agregados, característica considerada distintiva desta espécie (Figura 4) (Tiedt & Jooste, 1992; Leslie & Summerell, 2006). A espécie regularmente produz abundande micelio aéreo com aspecto algodonoso e coloração esbranquiçada e o verso

com coloração que varia de creme ao rosa e violeta (Figura 5a) (IARC,2012). Os macroconídios, embora não produzidos em grandes números, possuem de 3 a 5 septos e cerca de 30-45 µm de comprimento (Figura 5b-d)

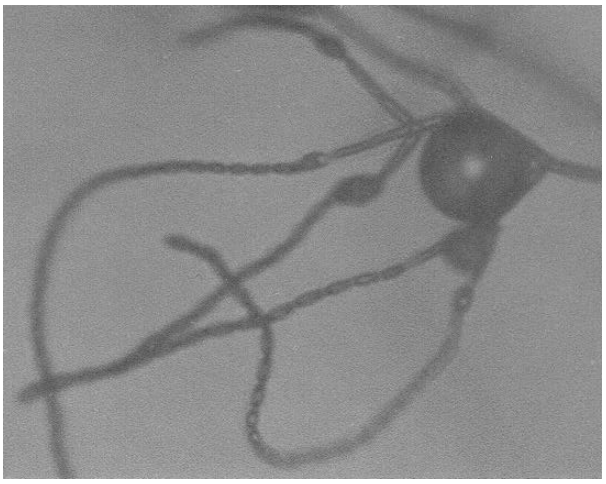

FIGURA 4. Fiálides produzindo cadeias longas de microconídios. Fonte: Glenn *et al*., 2004.

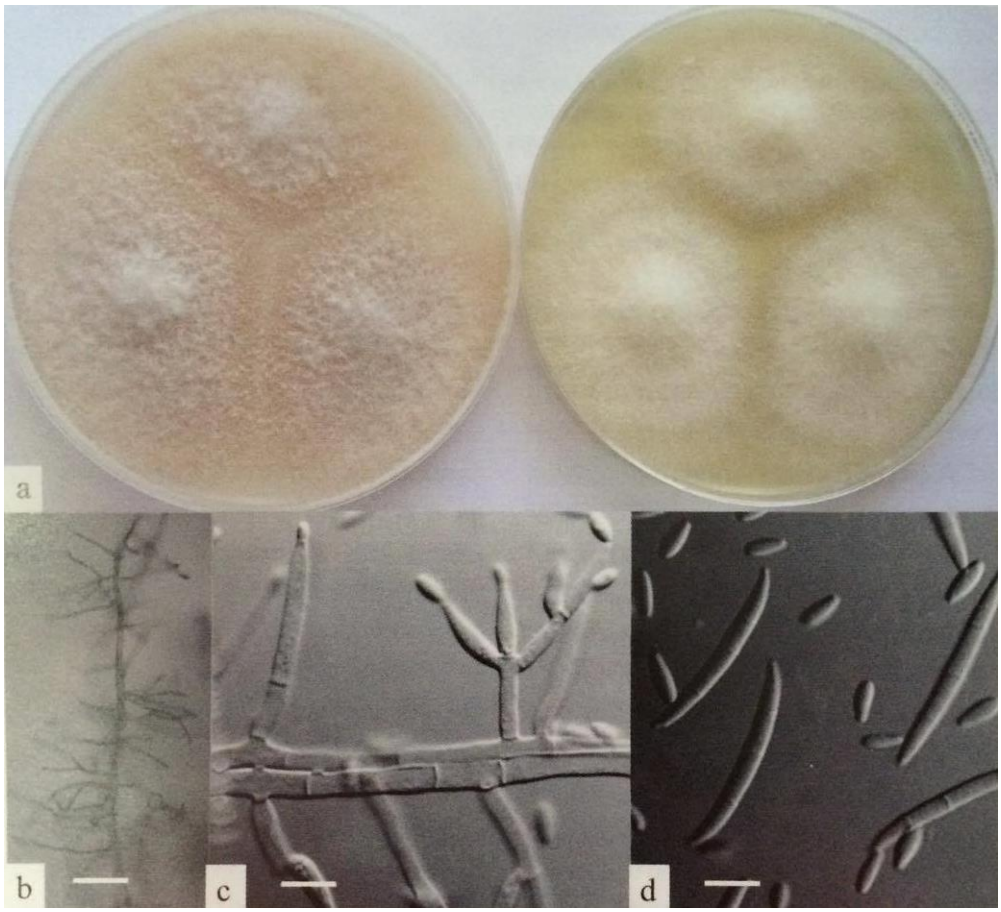

FIGURA 5. Macro e micromorfologia de *F. verticillioides*. Crescimento em meio BDA (a-esquerda) e DCPA (a-direita) por 7 dias a 25 ºC; (b) fiálides com cadeias de microconídios (barra = 50 µm); (c) fiálides em destaque (barra = 10 µm); (d) macro e microconidios (barra = 10 µm). Fonte: Pitt & Hocking, 2009.

A espécie *F. verticillioides* é inserida na seção *Liseola* dentro do gênero *Fusarium*, seção que engloba também *F. proliferatum* e *F. graminearum* (Magan & Ladred, 2007). O gênero *Fusarium* pertence ao filo Ascomycota e é inserido dentro do Sub-filo Pezizomycotina, classe Sordariomycetes (ÍNDEX FUNGORUM, 2016).

O *F. verticillioides* é amplamente distribuido no mundo e é particularmente associado ao milho, sendo considerado um importante patógeno desta cultura. A temperatura ótima para o crescimento do patógeno gira em torno de 30ºC em altitudes inferiores a 700 metros e, embora esta espécie seja difundida ao redor do mundo, é encontrado com maior frequência em regiões tropicais e subtropicais (Munkvold, 2003;

Ono *et al*., 2006). Nas plantas de milho pode causar podridões do colmo, raiz, sementes e da espiga, resultando em perdas significativas de rendimento e de qualidade do grão (Leslie & Summerell, 2006).

No campo, propágulos fúngicos originários principalmente do solo, mas também de resíduos vegetais de safras anteriores, sobrevivem na forma de micélio, alargando a parede das hifas para maior resistência, esporos ou peritécios durante grandes períodos entressafras (Figura 6) (OREN *et al*., 2003; YATES *et al*., 2003; Leslie & Summerell, 2006). Pelo solo, *F. verticillioides* pode atacar as plantas de milho desde o plantio das sementes até em fase de planta adulta, através da penetração no sistema radicular, causando podridão radicular ou crescendo endofiticamente de maneira assintomática e chegando até a espiga e os grãos, onde existe colonização completa (Leslie & Summerell, 2006; Nayaka *et al*., 2009; Munkvold *et al*., 2003). Pelo ar, *F. verticillioides* se dispersa e invade as plantas de milho através dos microconídios e pode penetrar as plantas de milho através de aberturas causadas por danos de insetos ou pelo elongamento do tubo germinativo no interior dos estigmas florais, chegando até a espiga e finalmente colonizando o interior dos grãos (Munkvold *et al*., 2003).

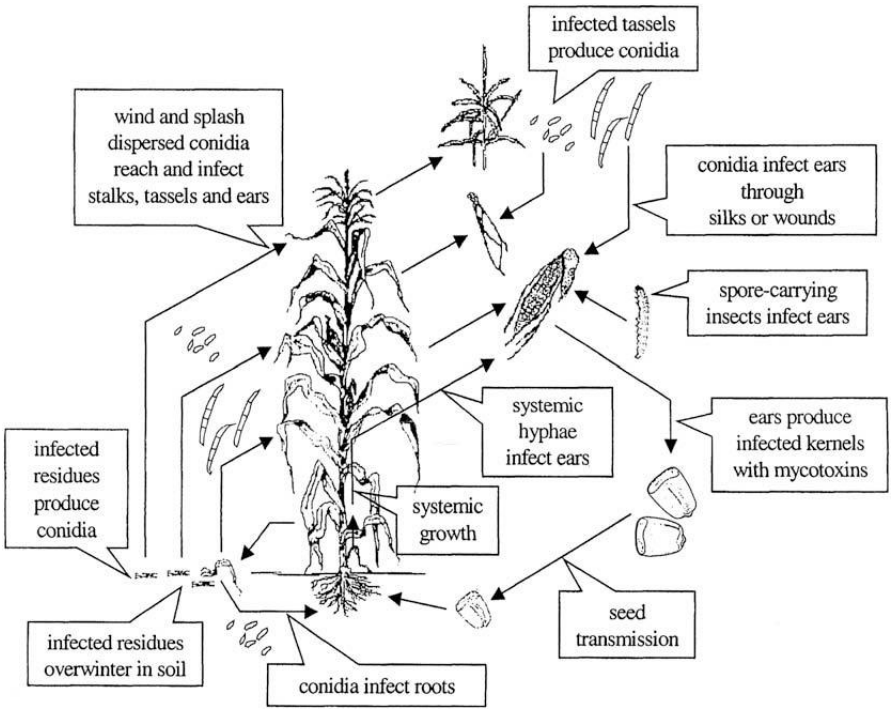

FIGURA 6. Ciclo de vida e vias de infecção de *F. verticillioides* no agroecossistema de cultivo de milho. Fonte: Battilani *et al*., 2003.

Este fungo foi seguidamente considerado um patógeno de campo apenas, mas atualmente sabe-se que o *F. verticillioides* pode prevalecer após a colheita, durante o transporte, o armazenamento (principalmente quando este é realizado de maneira incorreta) e, por fim, durante o processamento do alimento (Marín *et al*., 2004; Chulze, 2010).

2.2.2.2 Ocorrência de *F. verticillioides* em milho

F. verticillioides é considerado um importante patógeno do milho em função da sua capacidade destrutiva e de sua alta ocorrência. Etcheverry *et al*. (1999) analisaram a incidência de espécies do gênero *Fusarium* em produtos derivados de milho e relataram que 100% das amostras estavam contaminadas com este gênero, sendo que cerca de 50% dos isolados pertenciam a espécie *F. verticillioides*. O fungo comumente surge nos grãos assim que eles chegam na maturidade fisiológica e sua ocorrência cresce constantemente

até o final da época de plantio e inicio da colheita, período também crítico para o desenvolvimento deste fungo (Leslie & Summerell, 2006).

No Brasil, Rocha *et al*. (2012) isolaram diversas linhagens do gênero *Fusarium* em grãos de milho, onde 100% dos isolados foram identificados como pertencentes a espécie *F. verticillioides*. Ono *et al*. (2000) fizeram um levantamento de espécies do complexo *G. fujikuroi* em grãos de milho produzidos nos estados do sul do Brasil e relataram a prevalência de *F. verticillioides* entre 74 e 100% das amostras analisadas.

Mais recentemente, Stumpf *et al*. (2013) analisaram a incidência de espécies do complexo *G. fujikuroi* em grãos de milho produzidos no estado do Rio Grande do Sul e relataram alta incidência do gênero com evidente predominância da espécie *F. verticillioides* em relação as demais espécies do gênero, destacando a importância desta espécie para a cultura do milho. Em concordância, Lanza *et al*. (2014) relataram que 99% dos isolados pertencentes ao gênero *Fusarium* associados a grãos de milho no Brasil são pertencentes a espécie *F. verticillioides*. Os mesmos autores ainda relatam todos os *F. verticillioides* isolados de grãos de milho foram capazes de produzir Fumonisina B1, ressaltando o potencial toxigênico desta espécie.

2.2.2.3 Fumonisina B1: Toxicidade, ocorrência e legislação

Além de sua capacidade destrutiva, a ocorrência do patógeno é agravada por produzir metabólitos secundários potencialmente tóxicos para animais e seres humanos. Grãos aparentemente assintomáticos podem estar amplamente colonizados por *F. verticillioides* e em alguns casos, microconídios são produzidos dentro do grão. Estes grãos assintomáticos podem ser considerados grãos sadios e comercializados com a presença de micotoxinas produzidas pelo fungo, o que colocaria população consumidora em alto risco (Bacon *et al*., 1992; Leslie & Summerell, 2006). O *F.verticillioides* é

potencialmente produtor de diferentes micotoxinas, entre elas as fumonisinas, ácido fusárico e derivados, beauvericinas, fusarinas, nitrosometillisoamilamina e há relatos da produção eventual de giberelinas (Ji *et al*., 1986; Gelderblom *et al*., 1988; Bacon *et al*., 1996; Cantalejo *et al*., 1997; Leslie *et al*., 2004; Leslie & Summerell, 2006).

As fumonisinas são consideradas as mais importantes, podendo ser produzidas muitas vezes em altíssimas concentrações (Gelderblom *et al*., 1988). A fumonisina B1 (FB1) é a mais estudada e a de maior relevância em função de sua alta capacidade toxicológica e impacto econômico. A FB1, segundo Bezuidenhout *et al*. (1988), é um diéster de propano-1,2,3-ácido tricarboxílico e 2S-amino-12S,16R-dimetilo-3S,5R,10R,14S,15R-pentahidroxieicoisano, em que os grupos hidroxilas em C14 e C15 estão esterificados com um grupo carboxílico terminal do ácido tricarboxílico (Figura 7) (Sydenham *et al*., 1995; Kim *et al*., 2003; Lino *et al*., 2004). A fumonisina B1 é suficientemente termoestável, tanto que o aquecimento convencional durante o cozimento ou processamento do milho não destrói o composto (Dupuy *et al*., 1993).

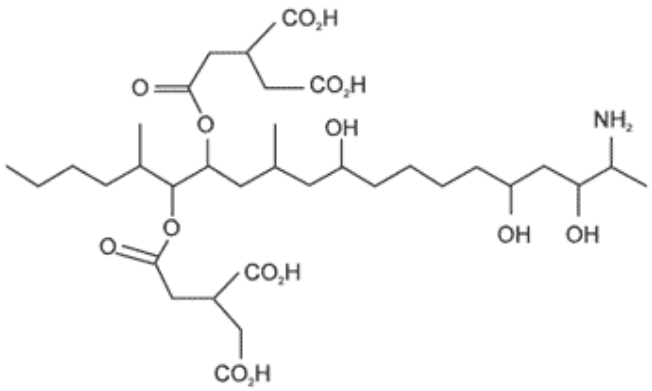

FIGURA 7. Estrutura química da Fumonisina B1. Fonte: Alltech Inc., 2015

A FB1 está associada a quebra do metabolismo de esfingolipídios, síndrome de edema pulmonar, câncer de fígado, renal e hepatotoxicidade, neurodegeneração, citoxicidade, inibição da síntese proteica e DNA, promoção do estresse oxidativo, indução da fragmentação de DNA e ainda é apontado como causa de

leucoencefalomalácia em equinos (Hopmans & Murphy, 1993; Shephard *et al*., 1996; Voss *et al*., 2001; Creppy *et al*., 2004; Lino *et al*., 2004; Westhuizen *et al*., 2004). Em humanos, a FB1 é possivelmente relacionada ao câncer esofágico e a implicação de defeitos congênitos (Meyer *et al*., 2003; Lino *et al*., 2004). Devido a dados epidemiológicos, a Agência Internacional de Pesquisa sobre o Câncer classifica a FB1 como grupo 2B, ou seja, possívelmente carcinogênica para humanos (IARC, 2002).

No Brasil, Queiroz *et al*. (2012) analisaram a ocorrência de fumonisina B1 em 40 amostras de milho e relataram a presença desta micotoxina em todas as amostras analisadas, em concentrações que variaram entre 230 e 6450 μg kg^{-1}. Mais recentemente, em estatísticas geradas pelo Laboratório de Análises Micotoxicológicas da Universidade Federal de Santa Maria, pode ser observado que o número de amostras de milho positivas para Fumonisina B1 variou entre 45 e 80% durante o período de Janeiro de 2010 até Março de 2015 (n = 4567) (LAMIC, 2015). Conforme a Figura 8 observa-se que o número de amostras positivas para esta micotoxina em milho ultrapassou os 35% anualmente desde o ano de 2000, com concentrações variando entre 250 até 3000 μg kg^{-1} no mesmo período.

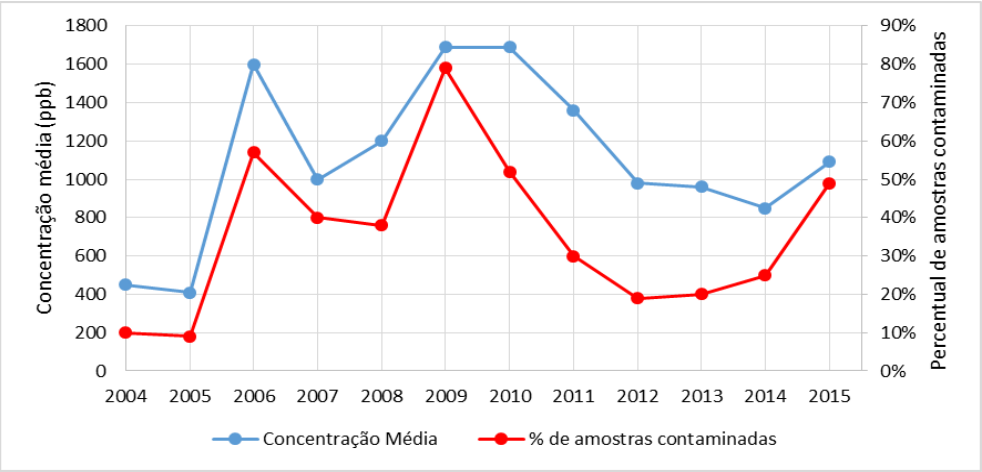

FIGURA 8. Contaminação anual de milho por fumonisinas entre os anos de 2004 e 2015. Fonte: Laboratório de Análises Micotoxicológicas (LAMIC), 2015.

Em função da sua alta ocorrência, toxicidade e dificuldade de remoção, as autoridades da União Européia e dos Estados Unidos fixaram limites máximos permitidos de FB1 para alimentos produzidos e importados para seus países. Nos Estados Unidos, o máximo permitido para a soma das Fumonisinas B1, B2 e B3 é de 2,0 mg kg^{1} para produtos de milho moído seco e sem germen; 4,0 mg kg^{-1} para produtos de milho moído, seco parcialmente, degerminados e farelo de milho seco e moído e; 3,0 mg kg^{-1} para milho limpo destinado a produção de pipoca (FDA, 2000).

A União Européia fixou como limite máximo permitido de Fumonisina B1 em milho não-processado de 2000 µg kg^{-1} e em farinha e óleo de milho de 1000 µg kg^{-1} (EUROPEAN UNION, 2006). Para alimentos para consumo direto, a restrição de FB1 é ainda maior: 400 µg kg^{-1} em alimentos a base de milho e 200 µg kg^{-1} em alimentos para bêbes e crianças (EUROPEAN UNION, 2006). No Brasil, segundo a resolução RDC N° 7 de 18 de Fevereiro de 2011, foram determinados limites máximos permitidos para a soma das Fumonisinas B1 e B2 com a última aplicação prorrogada para 1ºde janeiro de 2017 (ANVISA), conforme pode ser visualizado na Tabela 2.

TABELA 2. Limites máximos permitidos pela legislação brasileira para a soma das Fumonisinas B1 e B2 com aplicação prorrogada para 1 de janeiro de 2017.

Alimento	Limite Máximo Permitido (µg kg^{-1})*
Milho de pipoca	2000
Alimentos à base de milho para alimentação infantil (lactentes e crianças de primeira infância)	200
Milho em grão para posterior processamento	5000
Farinha de milho, creme de milho, fubá, flocos, canjica, canjiquinha	1500
Amido de milho e outros produtos à base de milho	1000

* ANVISA, RDC N° 7 de 18 de fevereiro de 2011.

Assim como para as aflatoxinas, embora existam limites máximos permitidos para a soma das fumonisinas B1 e B2, o ideal é a ausência completa destes compostos, levando em consideração o efeito altamente tóxico dos mesmos. Assim sendo, o controle dos patógenos é essencial para evitar a ocorrência de micotoxinas em alimentos. Como o *A. flavus* e o *F. verticillioides* são considerados os principais responsáveis pela ocorrência das micotoxinas ressaltadas em milho, seu controle é imprescindível para assegurar a qualidade do grão e a garantia da saúde dos animais e humanos consumidores (Magan & Ladred, 2007).

2.3 Métodos de controle fúngico

A descontaminação e detoxificação das micotoxinas é um processo caro e que não apresenta garantias de remoção completa destes compostos. Para evitar que a população consumidora esteja exposta a estas substâncias presentes no milho, a solução é a prevenção da ocorrência destes nos alimentos, realizada pelo controle da incidência de fungos. Diversas técnicas vêm sendo utilizadas para o controle fúngico no milho, não apenas para evitar uma possível contaminação por micotoxinas, mas também para evitar grandes perdas econômicas em função do potencial destrutivo dos fungos *F. verticillioides* e *A. flavus*. Atualmente, a utilização de compostos antifúngicos específicos é considerada o método primário para o controle de doenças fúngicas, em nível de pré- e pós-colheita (Korsten, 2006; Dal Bello *et al*., 2008).

O tratamento preventivo de sementes contra a contaminação de *A. flavus* e *F. verticillioides* já se demonstrou efetivo, onde compostos antifúngicos são utilizados extensivamente durante o período de cultivo do milho com o objetivo de controlar a incidência fúngica (Nayaka *et al*., 2009). Os fungicidas Thiram e Carbendazil se

mostraram efetivos como medida de controle a estes dois importantes fitopatógenos e por isso, são os produtos comerciais mais recomendados e utilizados (Nayaka *et al*., 2009).

Embora existam bons resultados obtidos com a utilização destes compostos, atualmente há uma demanda global que exige cada vez mais produtos ecologicamente seguros, ou seja, medidas de controle alternativas que não liberem resíduos tóxicos no ambiente (Janisiewicz & Korsten, 2002; Mari *et al*, 2007; Sharma *et al*., 2009). Estes resíduos, além da contaminação ambiental, podem estar presentes também nos grãos, podendo ter diversos efeitos agudamente tóxicos para a população consumidora dos produtos (Nayaka *et al*., 2009; Medeiros *et al*., 2012).

Além das desvantagens ecológicas, os antifúngicos químicos possuem alto custo e eficácia limitada, principalmente porque com o uso frequente, há a ocorrência de populações resistentes aos princípios ativos (Janisiewicz & Korsten, 2002; Lima *et al*., 2006; Sipiczki, 2006; Broders *et al*., 2007; Medeiros *et al*., 2012). Em adição, existem relatos do estímulo à produção de micotoxinas por diversos fungicidas químicos sintéticos, mesmo quando estes reduzem o desenvolvimento e proliferação fúngica (Magan & Olsen, 2004). Schmidt-Heydt & Geisen (2013) ao avaliarem o efeito de fungicidas comerciais na síntese de micotoxinas de diversas espécies fúngicas, relataram o estímulo da produção de ocratoxina A quando *P. verrrucosum* foi exposto ao fungicida Rovram. Van Rensburg *et al*. (2016) relataram estímulo à produção de fumonisinas por *Fusarium verticillioides* em milho na África do Sul quando expostos aos fungicidas Azoxystrobin, Difenoconazol e Carbendazim, mesmo quando a incidência fúngica em milho foi reduzida.

Métodos alternativos ao uso de fungicidas químicos sintéticos devem ser desenvolvidos, visando diminuir o impacto causado por estes compostos quando liberados no ambiente ao mesmo tempo em que controle fungos contaminantes e

fitopatógenos de maneira eficaz, permitindo o aumento da produtividade e da qualidade dos grãos e garantindo a ausência de micotoxinas nestes alimentos.

2.3.1 Controle biológico

A utilização de medidas alternativas de controle, como a utilização de organismos vivos como agentes de biocontrole vem ganhando atenção como um potencial método de controle destes fitopatógenos que seja ecológico, seguro e efetivo (Nayaka *et al*., 2009, Sultan & Magan, 2011; Medeiros *et al*., 2012).

A utilização de micro-organismos antagonistas aos fitopatógenos vem sendo bastante pesquisado nas últimas décadas, e os resultados obtidos, por diferentes pesquisadores frente a diferentes fungos toxigênicos, são bastante promissores, indicando reduzir e ou eliminar a contaminação por micotoxinas nos alimentos num curto-prazo (Dorner, 2004; Yin *et al*., 2008; Sultan & Magan, 2011). A utilização de diferentes organismos, como bactérias, actinomicetes, leveduras e fungos filamentosos demonstraram substancial progresso no manejo dos fitopatógenos e suas micotoxinas, e evidenciaram o amplo espectro de utilização de bioformulados no controle fúngico (Pereira *et al.* 2007; Nayaka *et al*., 2009; Etcheverry *et al*., 2009). Esta metodologia de controle tem alto potencial de utilização no campo, através do tratamento de sementes, grãos e no solo, e no armazenamento através da pulverização de bioformulados na massa de grãos (Weller, 1988; Janisiewicz & Korsten, 2002; Lima *et al*., 2006; Sipiczki, 2006; Broders *et al*., 2007; Nayaka *et al*., 2009; Etcheverry *et al*., 2009; Medeiros *et al*., 2012).

Há relatos de redução significativa da incidência fúngica e da produção de aflatoxinas e fumonisinas pela utilização de leveduras com atividade antagonista, bem como a utilização de cepas não-toxigênicas de *A. flavus*, provocando uma competição intraespecífica e reduzindo os níveis de aflatoxinas nos grãos (Hua *et al*., 1999; Masoud

& Kaltoft 2006; Dorner 2004, 2009; Pitt & Hocking, 2006; Horn & Dorner, 2009). A utilização de bactérias antagonistas destaca-se dos demais micro-organismos, em função da sua alta capacidade de inibição do crescimento fúngico e de suas micotoxinas, já havendo alguns bioprodutos registrados com diferentes espécies bacterianas (FormentI *et al*., 2012; Medeiros *et al*., 2012). Embora a utilização de bactérias antagonistas seja promissora, estes bioprodutos representam apenas 1% das vendas em relação a outros produtos (Figura 9) (Fravel, 2005; Medeiros *et al*., 2012).

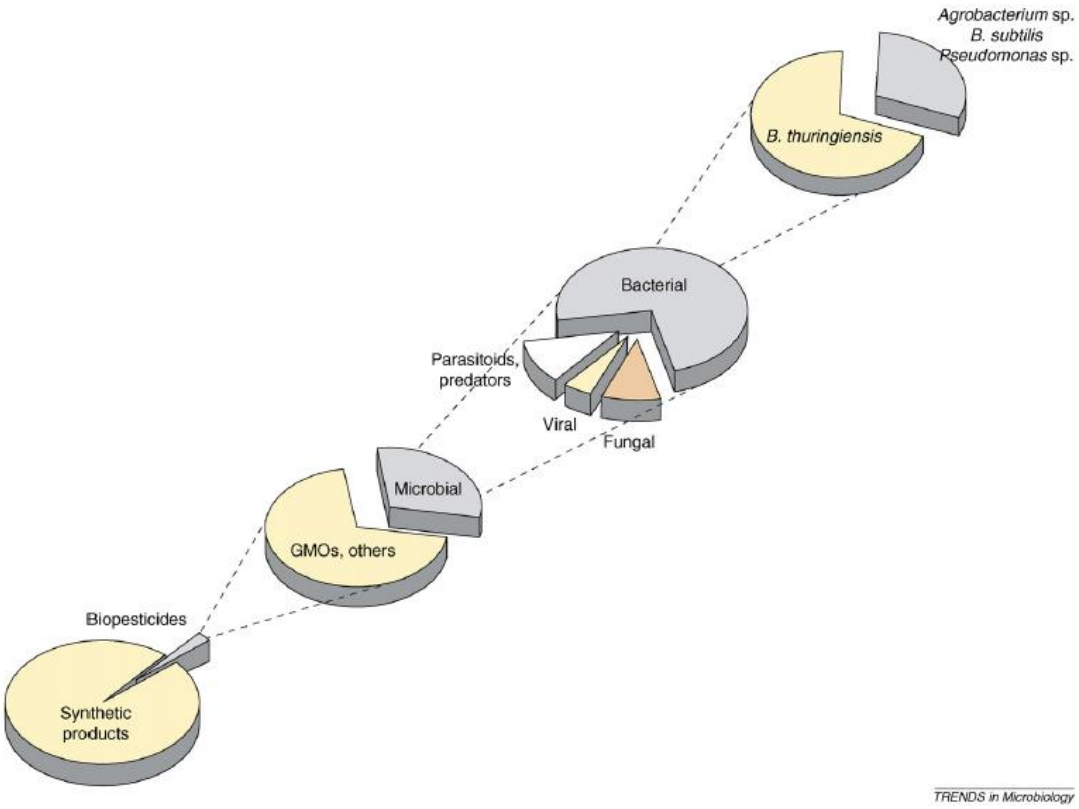

FIGURA 9. Fatia de mercado para agentes de biocontrole. Fonte: Ongena & Jacques, 2008.

2.3.1.1 Rizobacterias como agentes de biocontrole

Bactérias antagonistas podem produzir compostos naturais com capacidade antifúngica que atuam na síntese de micotoxinas, além de serem competidoras extremamente eficientes contra fungos filamentosos em função da facilidade e rapidez de

crescimento durante o processo competitivo (Bluma & Etcheverry, 2006; Palumbo *et al*., 2007). Dados de diversos trabalhos relatam superioridade da eficácia de bactérias antagonistas isoladas do solo, em função da imensa diversidade microbiana presente neste substrato (Baker *et al*., 1983; Kobayashi *et al*., 1988; Omura *et al*., 1988; Motomura *et al*., 1996; Bluma & Etcheverry, 2006). A superioridade da eficiência de micro-organismos isolados do solo sustenta a teoria da competitividade em ambientes adversos. Micro-organismos isolados do solo são facilmente cultiváveis (crescem com o mínimo de fontes de nutrientes) e possuem alto potencial antagonista aos outros organismos, pois estes dois fatores são indispensáveis para a sua própria sobrevivência, caracterizando assim, alta capacidade competitiva (Vining, 1990; Bluma & Etcheverry, 2006).

Rizobactérias encontradas no ecossistema do milho ainda apresentam uma vantagem adicional, pois estão adaptadas àquele ecossistema, e a sua utilização não causa o impacto ocasionado quando há a introdução de um micro-organismo proveniente de outro ambiente (Bluma & Etcheverry *et al*., 2006). As raízes criam um nicho protegido que pode ser um excelente ambiente para a obtenção de diferentes gêneros de rizobactérias que já demonstraram excelente potencial antimicrobiano (Palumbo *et al*., 2007; Bluma & Etcheverry *et al*., 2006; Cavaglieri *et al*., 2005).

De acordo com diversos autores, alguns dos principais atributos de um bom agente de biocontrole são: 1) estabilidade genética, 2) eficácia em baixas concentrações, 3) amplo espectro de ação, 4) requerimentos nutricionais simples, 5) sobrevivência em condições ambientais adversas, 6) ausência de patogenicidade para a planta hospedeira e, 7) ausência de produção de metabólitos potencialmente tóxicos para humanos (Spadaro & Gullino, 2004; Medeiros *et al*., 2012). A maioria das rizobactérias selecionadas como agentes de biocontrole possuem algumas ou várias destas características, sendo que a existência de todas é considerada como ótima para o desenvolvimento de um agente de

controle seguro e eficaz. Em adição, quando o micro-organismo alvo do biocontrole é um fungo toxigênico, o agente de biocontrole não deve estimular ou aumentar as concentrações finais de micotoxina, o que caracterizaria um grande perigo para a população consumidora (Magan & Olsen, 2004; Nesci *et al*., 2005; Bluma & Etcheverry, 2006).

Diversos autores já relataram o isolamento de rizobactérias capazes de inibir o crescimento e reduzir a produção de aflatoxinas e fumonisinas por *A. flavus* e *F. verticillioides*, respectivamente (Baker *et al*., 1983; Kobayashi *et al*., 1988; Omura *et al*., 1988; Motomura *et al*., 1996; Janisiewicz & Korsten, 2002; Bluma & Etcheverry, 2006; Lima *et al*., 2006; Sipiczki, 2006; Broders *et al*., 2007; Nayaka *et al*., 2009). Palumbo *et al*. (2007) relataram que rizobactérias dos gêneros *Achromobacter*, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Chryseobacterium*, *Pseudomonas*, *Wautersia*, *Stenotrophomonas* e *Variovorax* demonstraram-se promissoras para o biocontrole. Outros autores relatam que isolados do gênero *Bacillus* e *Pseudomonas* apresentaram atividade antagonista ao crescimento fúngico e à produção de micotoxinas em diversas condições ambientais, evidenciando a produção de um ou mais compostos com atividade antifúngica, que podem ser isolados e utilizados separadamente (MUNIMBAZI & BULLERMAN, 1997; CAVAGLIERI *et al*., 2005; Bluma & Etcheverry, 2006; Palumbo *et al*., 2007; Etcheverry *et al*., 2009; Formenti *et al*., 2012).

Das rizobactérias com maior potencial, destaca-se áquelas pertencentes ao gênero *Bacillus*, em função de sua grande capacidade de produção de compostos antimicrobianos, requerimentos nutricionais simples e alta capacidade competitiva e de sobrevivência.

2.3.1.2 *Bacillus* sp. como agentes de biocontrole

Os *Bacillus* são bactérias em forma de bastonetes, gram-positivas, aeróbias obrigatórias ou facultativas e catalase positivas (Figura 10) (Turnbull, 1996). São extremamente ubíquas na natureza em função de apresentarem crescimento muito rápido em relação a outras bactérias (tempo de geração médio de 25 minutos) e possuírem necessidades nutricionais muito baixas, propiciando inclusive o fácil cultivo em meios de cultura com baixo fator nutritivo (Bluma & Etcheverry, 2006; Todar, 2012).

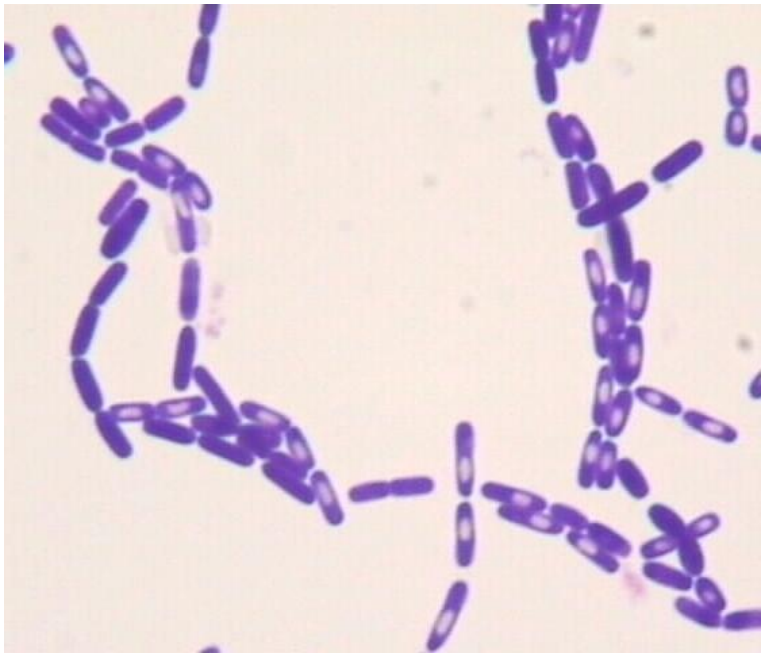

FIGURA 10. Microscopia de *Bacillus* sp. após coloração de Gram. Fonte: Todar, 2012.

Este gênero, que possui grande número de espécies nativas do solo, se caracteriza por apresentar excelentes potenciais agentes de biocontrole em função de características diferenciais que propiciam alta capacidade competitiva e de sobrevivência, mesmo em ambientes adversos. Bactérias pertencentes a este gênero tem a capacidade de formação de endósporos (Figura 11), uma estrutura dormente e extremamente resistente aos fatores físico-químicos, que confere a estas bactérias uma intensa adaptabilidade a condições ambientais adversas, principalmente às variações de temperatura, pH e atividade de água (Silo-Suh *et al*., 1994).

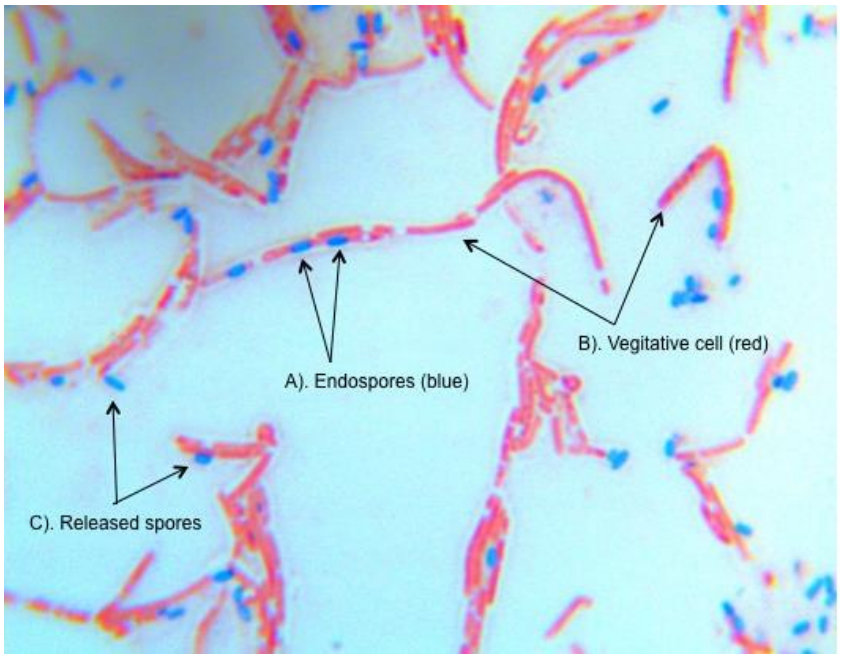

FIGURA 11. Microscopia de *Bacillus* sp. com endósporos (A, em azul), células vegetativas (B) e esporos desprendidos. Fonte: Sturm, 2012.

A alta capacidade de sobrevivência é uma excelente habilidade competitiva, porém, o que caracteriza este gênero como excelente agente de biocontrole é sua capacidade de produzir diversos metabólitos com atividade antimicrobiana (Pérez-García *et al.* 2011). Uma das espécies mais estudadas do gênero, o *Bacillus subtilis* têm de 4 a 5% de seu genoma dedicado a síntese de antibióticos e tem o potencial de produzir mais de duas dúzias de compostos antimicrobianos estruturalmente diversos (Stein, 2005).

Entre estes compostos produzidos por este gênero, merecem destaque, em função do seu potencial biotecnológico, os lipopeptídeos cíclicos, sufractinas, fengicinas e as iturinas. As fengicinas apresentam capacidade anti-fúngica e funcionam como sinalizadoras para induzir o *priming* da defesa vegetal sistêmica contra fitopatógenos; as iturinas também possuem atividade antifúngica e auxiliam no crescimento bacteriano, em função de suas propriedades surfactantes, e; as sufractinas apresentam capacidade antibacteriana, sinalizam a defesa vegetal e induzem o *priming* imunológico, auxiliam o

crescimento bacteriano e estão ligadas a formação de biofilmes, outra característica distintiva de bactérias deste gênero (Ongena & Jacques, 2008).

A atividade antifúngica destes compostos está muito relacionada a sua estrutura química (Figura 12) que é capaz de se ligar na membrana celular fúngica e formar poros, levando a um extravasamento citoplasmático e consequentemente, a morte celular, caracterizando a atividade antifúngica (Bonmatin *et al.* 2003).

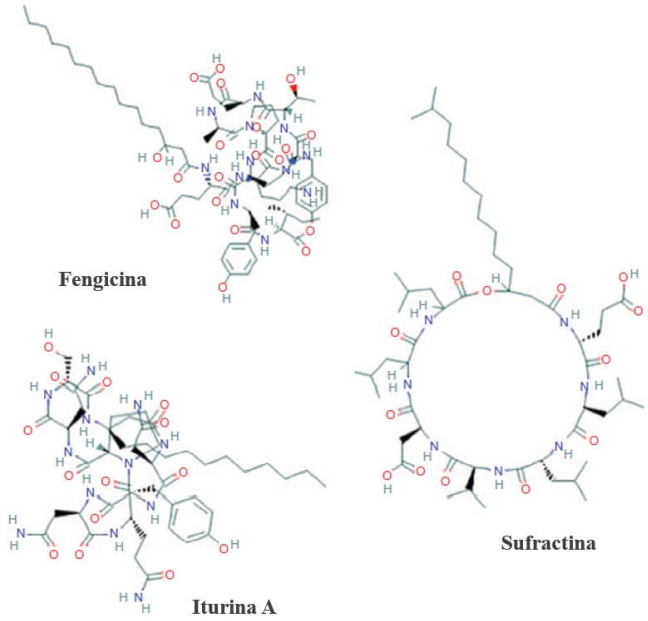

FIGURA 12. Estrutura química em 2D de representantes das três famílias de lipopeptídeos produzidos por *Bacillus* sp.: iturinas (iturina A), fengicinas (fengicina) e sufractinas (sufractina). Adaptado de Pérez-García *et al*., 2011.

Estes metabólitos podem ser utilizados isoladamente como compostos antifúngicos, porém, auxiliam diretamente o crescimento, sobrevivência e habilidade competitiva das bactérias, sendo mais eficazes contra fitopatógenos quando utilizados juntamente a um agente antimicrobiano (Ongena & Jacques, 2008). Diversos biofungicidas comerciais a base de *Bacillus* spp. já foram desenvolvidos, tendo como alvo

os principais fitopatógenos para culturas específicas, como pode ser observado na Tabela 3, a seguir:

TABELA 3. Formulação de alguns biofungicidas comerciais

Nome comercial	Espécie de *Bacillus*	Patógeno/Doença alvo	Cultura	Fabricante
Avogreen	*B. subtilis*	*Cercospora* sp.	Abacate	Stimuplant, South Africa
Ballad Plus	*B. pumilus*	Ferrugem, Oídio, Cercosporiose, Mancha-parda	Soja	AgraQuest, USA
Biobest	*B. subtilis*	Brusone, mancha-parda, *Rhizoctonia solani*	Arroz	Appliedchem, Thailand
Companion	*B. subtilis*	*Rhizoctonia, Pythium, Fusarium, Phytophthora, Sclerotinia*	Plantas ornamentais	Growth Products,USA
EcoGuard	*B. licheniformis*	Antracnose, *Sclerotinia homoeocarpa*	Turfa	Novozymes, Denmark
HiStick	*B. subtilis*	*Fusarium, Rhizoctonia, Aspergillus*	Soja e Amendoim	Becker Underwood, USA
Kodiak	*B. subtilis*	*Rhizoctonia, Fusarium, Pythium, Aspergillus*	Algodão, legumes e soja	Bayer CropScience, USA
Larminar	*B. subtilis*	*Alternaria, Botryodiplodia Colletotrichum, Corticium, Fusarium, Phytophthora*	Arvores frutíferas, arroz e culturas de campo	Appliedchem, Thailand
Serenade	*B. subtilis*	Ferrugens, Oídio, *Botrytis, Sclerotinia*	Vinhedos, nozes, legumes e frutíferas	AgraQuest, USA
Sonata	*B. pumilus*	Ferrugens, Oídios e Míldios	Legumes, ornamentais, culturas de campo	AgraQuest, USA
Subtilex	*B. subtilis*	*Rhizoctonia, Fusarium, Aspergillus*	Ornamentais, culturas de campo e legumes	Becker Underwood, USA
Taegro	*B. amyloliquefaciens*	*Rhizoctonia, Fusarium*	Sementes de árvoes, plantas ornamentais e arbustos	Novozymes, Denmark

Adaptado de Pérez-García *et al*., 2011.

A maioria dos bioprodutos envolve apenas uma espécie: *Bacillus subtilis*. Esta espécie é possivelmente a mais bem descrita do gênero e possui grande potencial biotecnológico. As espécies *B. amyloliquefaciens, B. pumilus* e *B. licheniformis* também

são utilizadas nos bioformulados comerciais e são espécies conhecidas por possuir grande potencial antifúngico (Pérez-Garcia *et al*., 2011). O que difere entre os diversos bioformulados são os isolados utilizados, que mesmo pertencentes as mesmas espécies, apresentam características competitivas únicas, diferenciando os isolados em capacidade de sobrevivência, adaptabilidade e eficácia antifúngica frente a um variado número de patógenos.

Embora a maioria dos bioformulados comerciais a base de *Bacillus* sp. não objetivem o controle micotoxicológico direto, alguns trabalhos foram realizados evidenciando o grande potencial de bactérias deste gênero como biocontroladores de fungos toxigênicos e suas micotoxinas nos alimentos. Lei *et al*., (2014) relataram redução de 88% de zearalenona em ração sob ação de *B. subtilis*. Há relatos de redução do crescimento de *Aspergillus*, *Penicillium* e *Fusarium* por *B. pumilus*, que também foi capaz de reduzir as concentrações finais de aflatoxinas, ácido ciclopiazônico, patulina e ocratoxina A (Munimbazi & Bullerman, 1998). Mais recentemente, Navya *et al*. (2015) demonstraram redução do crescimento de *A. flavus* e da produção de Aflatoxina B1 por *B. subtilis* BSP3 em diversos substratos. Semelhantemente, Kong *et al*. (2014) relataram redução *in vitro* da produção de Aflatoxina B1 e ácido ciclopiazônico por *A. flavus*, além da redução do seu desenvolvimento, por *B. megaterium* de origem marinha na China. Na Argentina, Chulze *et al*. (2015) relataram redução do acúmulo de deoxinivalenol em trigo contaminado com *Fusarium graminearum* sob ação de *Bacillus subtilis* RC 218 e Pereira *et al*. (2010) relataram redução do crescimento de *F. verticillioides* e acúmulo de fumonisinas em milho por *B. amyloliquefaciens*. Em adição, também na Argentina, Nesci *et al*. (2005) relataram redução de Aflatoxina B1 e da incidência de *A. flavus* quando interagindo com *B. subtilis* RCB 90. Estes trabalhos demonstram o grande potencial de

diferentes espécies do gênero *Bacillus* em influenciar tanto o crescimento e a propagação, quando o acúmulo de micotoxinas em diferentes alimentos e substratos.

2.4 Efeito das condições ambientais na interação entre organismos

No processo de seleção de potenciais agentes de biocontrole é de extrema importância considerar a relação existente entre o fungo toxigênico, o agente de biocontrole e as condições ambientais (Bluma & Etcheverry, 2006). Fatores ambientais são muito importantes na interação interespecífica, podendo ser decisivos na determinação da co-existência ou dominância de uma espécie em um nicho ecológico particular (Penna *et al*., 2004). Mudanças no ambiente afetam diretamente o metabolismo dos fungos toxigênicos e dos micro-organismos antagonistas, podendo favorecer um ou outro, ocasionalmente comprometendo o antagonismo gerado por agentes de biocontrole. O ideal é que a atividade inibitória permaneça estável mesmo em diferentes condições ambientais, garantindo que o agente não perca sua utilidade com as mudanças extrínsecas (Cotty & Mellon, 2006)

Os fatores ambientais de maior relevância neste contexto são a atividade de água (a_w) e temperatura ambiente (Pitt & Hocking, 2009; Cavaglieri *et al*., 2004; Penna *et al*., 2004; Nesci *et al*., 2005; Bluma & Etcheverry, 2006; Sultan & Magan, 2011).

2.4.1 Atividade de água

A atividade de água basicamente consiste na quantidade de água disponível em um substrato, não utilizada nas reações e livre para o uso pelos micro-organismos (Pitt & Hocking, 2009; Magan, 2007; Sultan & Magan, 2011). A atividade de água é considerada o fator determinante na sobrevivência ou não de um micro-organismo, afetando diretamente o seu metabolismo (Pitt & Hocking, 2009). Segundo Bluma & Etcheverry

(2006), mudanças na atividade de água de substratos de crescimento claramente afetaram a capacidade de desenvolvimento individual de rizobactérias antagonistas e sua capacidade de se relacionar e competir com outros micro-organismos. Os mesmos autores, ainda ressaltam que a análise do potencial antagonista em diferentes atividades de água deve ser o primeiro passo para a seleção ótima de agentes de biocontrole, pois simula diferentes situações de estresse que ocorrem naturalmente e que podem ser decisivos na interação entre estas rizobactérias e os fungos toxigênicos. Complementando, Penna *et al*. (2004) ainda ressaltaram que a determinação da atividade antagonista em diferentes atividades de água (e outros efeitos ambientais) é de extrema importância para a compreensão das estratégias ecológicas utilizadas por um agente e a sua capacidade de sobrevivência quando confrontado a outros micro-organismos.

Bluma & Etcheverry (2006), analisando a atividade antagonista de espécies de *Bacillus* sp. contra *A. flavus*, relataram que embora em altas atividades de água não houvesse inibição do crescimento micelial, a interação da rizobactéria com o fungo toxigênico proporcionou redução da produção de aflatoxina B1, sugerindo que a mudança ambiental causou alguma alteração metabólica no agente de biocontrole e no fungo toxigênico, levando a uma redução da produção de aflatoxinas ou a degradação do composto pelas espécies de *Bacillus* sp.

Segundo alguns autores, mudanças na atividade de água do substrato afetam ainda a relação dos micro-organismos com outros fatores ambientais (Marin *et al*., 1998; Lee & Magan, 1999; Penna *et al*., 2004). Lee & Magan (1999) observaram que com o aumento da atividade de água do substrato, maior a diversidade de fontes de carbono que foram utilizadas por diferentes micro-organismos. Ainda neste contexto, quando a atividade de água de um substrato de crescimento é considerada ótima para certo micro-organismo, maior é sua capacidade de tolerar outras mudanças ambientais, destacando

assim, a importância deste fator na sobrevivência (Pitt & Hocking, 2009; Taniwaki & Silva, 2001).

2.4.2 Temperatura

Embora a atividade de água seja considerada o principal fator interferente na proliferação, sobrevivência e interação microbiana, a temperatura pode causar mudanças importantes na relação interespecífica, podendo ter efeitos sinérgicos com a atividade de água (Pitt & Hocking, 2009; Taniwaki & Silva, 2001). Segundo diversos autores, assim como a atividade de água, diferentes temperaturas podem modular a atividade antagonista de diferentes grupos bacterianos (Whipps & Magan, 1987; Dickie & Bell, 1995; Slininger & Shea-Wilburg, 1995; Pitt & Hocking, 2009; Borowicz & Omer, 2000). Dickie & Bell (1995) relataram em seu estudo de seleção de agentes de biocontrole bacterianos, que a temperatura de teste tem um efeito profundo no crescimento e produção de antibióticos, podendo haver reduções significativas da atividade antagonista em determinadas temperaturas.

Demonstrou-se que a produção de compostos antifúngicos e o crescimento bacteriano foram modulados com a variação da temperatura (Reddy & Ranganathan, 1985; Batish *et al*., 1990; Sathe *et al*., 2007; Dalie *et al*., 2010). Reddy & Ranganathan (1985) relataram que o pico máximo de produção destes compostos por *Lactobacillus lactis* foi observado em 25ºC, enquanto Sathe *et al*. (2007) e Batish *et al*. (1990) relataram que o pico máximo de produção de antifúngicos por *L. plantarum* e *L. acidophilus* foi observado em 30ºC. De Curtis *et al*. (2012) demonstraram em seu trabalho de seleção de agentes de biocontrole para *Aspergillus carbonarius*, que a capacidade antagonista dos agentes selecionados aumentou conforme aumentou gradativamente de 20 para 30 ºC a temperatura de tratamento.

A temperatura ambiente não afeta apenas a produção de compostos microbianos, mas também a sua capacidade de ação. Segundo Narasimhan *et al*. (2013), a temperatura ótima de produção de enzimas quitinazes com ação antifúngica por *B. subtilis* foi diferente daquela observada para sua ação queratinolítica.

No processo de seleção de agentes de biocontrole é de extrema importância submeter o agente a diferentes fatores ambientais, com o objetivo de simular condições que este micro-organismo enfrentaria no campo quando sua atividade antagonista é posta à prova determinando, assim, o espectro de ação deste agente frente a diferentes condições ambientais.

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3 ARTIGOS

3.1 Biocontrol of fumonisin producer *Fusarium verticillioides* on non-rhizospherical soil and maize seedlings by *Bacillus* spp. isolated from maize root system

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Biocontrol of fumonisin producer *Fusarium verticillioides* on non-rhizospherical soil and maize seedlings by *Bacillus* spp. isolated from maize root system

T.C. Einloft[1], P.B. Oliveira[1], S. Hartke[1], R.G. Dionello[1].

1 Departamento de Fitossanidade, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

*Corresponding author: Tiago Centeno Einloft, Departamento de Fitossanidade, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 7712, Porto Alegre, Rio Grande do Sul, Brazil. Email: tiago.einloft@gmail.com

Abstract

AIMS: To isolate rhizobacteria with promising antifungal activity towards *Fusarium verticillioides*, investigate their ability to control fungal survival on non-rhizospherical

soil and to evaluate the effect of seed rhizobacterial treatments on agronomical parameters of maize seedling and *F. verticillioides* counts on maize root tissues.

METHODS AND RESULTS: Rhizobacteria were isolated from maize root system and tested *in vitro* for its antifungal ability towards *F. verticillioides*. Three isolates with intense antifungal activity showed ability to reduce fungal counts on non-rhizospherical maize field soil and positively affect agronomic parameters of maize seedlings while reducing the root colonization by *F. verticillioides* after seed bacterization treatments.

CONCLUSIONS: The isolate *Bacillus amyloliquefaciens* RP103 as well as the bacterial consortium formulated with the three *Bacillus* isolates showed promising ability to be used as biocontrol agents on maize field soil to control *F. verticillioides* before field seeding, reducing the primary fungal inoculum from the field to the plants. The RP103 isolate and the bacterial consortium showed the ability to be used also in seed treatments aiming the protection of maize seedlings against *F. verticillioides* root colonization, as well as the improvement of plant growth parameters like weight and length of root and aerial parts.

SIGNIFICANCE AND IMPACT OF STUDY: The description of three *Bacillus* isolates with major characteristics to be used commercially as biocontrol agents aiming maize plants protection towards *F. verticillioides* colonization.

Key-words Biological control, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, rhizobacteria, agronomic parameters.

Introduction

Maize (*Zea mays* L.) is the most important American originated cereal, with Brazil being the third main producer worldwide (85 million tons produced on 14/15 season) and its cultivation is mainly destined to animal and human consumption (NCGA 2015; CONAB 2016). A diverse number of soil-borne microorganisms, including phytopathogens and opportunistic contaminants, can colonize the maize plant. Among these, the filamentous fungi *Fusarium verticillioides* is an aggressive phytopathogen and its growth on maize tissues (root, stalk, leaves and ear rot) may lead to losses on plant health and consequentially grain yield (Robertson-Hoyt *et al*. 2007; Munkvold *et al*. 2003).

Fusarium verticillioides can survive on the soil through resistance structures or as mycelium and conidia on maize residue from remnant harvest. This ability characterize the soil as a primary source of fungal inoculum to maize kernels and to the root system (Nyvall and Kommedahl 1968). This fungus can invade the kernel and the root system and grow endophytically sometimes symptomless, causing a systemic infection and reaching maize ears and the grains (Desjardins *et al*. 1998). As *F. verticillioides* grow in the kernels, it may produce Fumonisin B1 (FB1), a neurotoxic secondary metabolite that when ingested may lead to hepatotoxicity, nefrotoxicity and immunosuppression in humans and carcinogenicity in animals (IARC 2002; Escrivá *et al*. 2015). In addition, FB1 ingestion causes leukoencephalomalacia on equines and pulmonary edema on swine's (WHO 2012; Caloni and Cortinovis 2010; Voss *et al*. 2007).

The most efficient way to avoid consumption of this toxic metabolite is prevention of fungal infection and growth. Traditionally, crop fungal diseases are controlled by resistant maize cultivars, good cultural practices and synthetic chemical fungicide applications. Usage of synthetic chemical fungicides often leads to pathogen resistance and in most cases represent toxicological hazards for the environment and the consumers when residues remain. Additionally, even when reducing fungal population, some chemical fungicides may stimulate mycotoxin production, leading to an increase of mycotoxin concentration on the kernels (Falcão *et al*. 2011; Miguel *et al*. 2015).

An alternative and promising strategy to reduce fungal infection and consequentially yield losses and FB1 contamination involves the usage of maize native microorganisms that can naturally compete and inhibit fungal growth, known as biocontrol agents (Bluma and Etcheverry 2006; Palumbo *et al*. 2007). The rhizosphere of crop plants has a rich biodiversity of microorganisms well adapted to that environment and highly capable to compete with plant pathogens and so show promising characteristics to be used ad biocontrol agents (Cavaglieri *et al*. 2005a; Etcheverry *et al*. 2009).

The biocontrol agent's ability to grow and compete with soil-borne plant pathogens on the rhizosphere and on non-rhizospherical soil may lead to reduction of direct infection on maize kernels and the fungal primary inoculum in the soil, respectively (Etcheverry *et al*. 2009). Thus, seed and field-soil treatments with native biocontrol agents is a promising method for biocontrol of soil-borne plant pathogens in their natural environment (Mao *et al*. 1998; Batson *et al*. 2000; Kerry 2000; Pereira *et al*. 2007). In this study, we report the

isolation, selection and identification of rhizobacterial isolates with antagonistic abilities against *F. verticillioides*. We also report the effect of interaction between the selected rhizobacterial isolates and *F. verticillioides* on fungal counts on non-rhizospherical soil and the effect of seed rhizobacterial treatments on maize seedling agronomical parameters and *F. verticillioides* counts on maize root tissues.

Materials and Methods

Fungal strains

Fumonisin B1 producer *F. verticillioides* previously isolated from maize grain was used. Stock cultures were stored in 150 mL L^{-1} aqueous glycerol.

Bacterial isolation from maize plants rhizosphere and rhizoplane

All bacterial strains were isolated from maize plants rhizosphere and rhizoplane from two different maize fields in Eldorado do Sul, Rio Grande do Sul, Brazil, during two stages: early and later harvest period.

On each field, two diagonals were traced comprehending a quadrant where the plants were cultivated. Five healthy Maize plants (cultivar 30A77HX) were sampled on each diagonals, totalizing 10 plants per field on each sampling period. Plants were lifted and shaken to remove rhizospheric soil, which was collected on one sterile plastic bag. The entire root system with adherent soil were collected on a separated sterile plastic bag (Cavaglieri *et al*. 2005a). Rhizospheric soil and root samples were transported to the laboratory and analyzed within 6 hours.

Bacterial isolations from the rhizosphere were carried out on solid 523 medium (Kado and Heskett 1970) by surface spreading method by diluting 10 g of rhizospheric soil with 90 ml phosphate-buffered saline (PBS) solution followed by agitation on orbital shaker (30 mins) and inoculation of 0.1 ml on the medium surface (Bluma and Etcheverry 2006). To conduct bacterial isolations from the root tissues, the roots + adherent soil were weighed, serially diluted on PBS solution and submitted to agitation on orbital shaker for 30 minutes. Aliquots of 0.1 ml were inoculated on the surface of 523 media to promote

bacterial isolation. All bacterial colonies recovered from rhizoplane or rhizosphere were isolated and tested for its antifungal ability.

Bacterial screening and selection for antagonism

All bacterial isolates were screened for antagonism against two *Fusarium verticillioides* isolates on maize meal extract agar (MMEA) (Marin *et al*. 1995). Briefly, spore suspensions (10^6 spores ml^{-1}) were pour plated on melted MMEA (~45 °C) and after the solidification, 10 µl of bacterial suspensions grown overnight on 523 broth were inoculated on the surface of the solidified media. Plates were incubated at 25 °C for 5 days and the bacterial isolates that presented inhibition of mycelial growth were selected for inhibition zone measurements. The isolates that presented inhibition zones were classified accordingly to the scale proposed by Bacon and Hinton (2002), which determines the strength of the antagonist by the inhibition zone size: -, no inhibition; +, weak (<3 mm) *in vitro* inhibition; ++, moderate (>3–9 mm) *in vitro* inhibition; +++, strong (>9–18 mm) *in vitro* inhibition; ++++, very strong (>18 mm) *in vitro* inhibition. Bacterial isolates that presented strong to very strong (+++/++++) *in vitro* inhibition were selected for the upcoming trials. Three isolates, nominated RF69, RP103 and RP242, presented strong and very strong inhibition zones and were selected.

Identification of selected isolates

Bacterial genomic DNA was extracted from colonies grown *overnight* on 523 broth at 28 °C according to Woo and Cheng (1992). The 16S rDNA gene fragments were amplified using two *primer* pairs for Eubacteria and one for *Bacillus* genus: 9F and 1510r (Hayat *et al*. 2013); 27f and 1525r (Palumbo *et al*. 2007) and PEU7 and DG74 (Vithanage *et al*. 2014), respectively. Sequence fragments were send to ACTGENE Molecular Analyzes (Porto Alegre, Brazil) for sequencing using the same *primers*.

Antagonism on non-rhizospherical soil

Inoculum preparation

Bacterial isolates were grown *overnight* on 523 broth and cell concentrations were adjusted for 10^9 CFU ml^{-1} by serial dilutions. Bacterial mix suspension was constructed by mixing 330 µl of each bacterial suspension. *Fusarium verticillioides* strains were grown on PDA media for 7 days at 25 °C and the colonies were washed with 10 ml peptone water (0.1%) to create spore suspensions. Spore suspensions were measured on a Neubauer chamber and adjusted for 10^3 and 10^6 spores ml^{-1} by serial dilutions (Bluma and Etcheverry 2006).

Inoculation

This experiment was conducted on sterile soil samples from maize experimental fields from Rio Grande do Sul, Brazil. Petri plates were filled with soil samples (50g), sterilized by autoclaving twice and inoculated on triplicates by central spraying 1 ml of fungal and bacterial isolates suspensions alone or in consortium (bacterial mix) (Bluma and Etcheverry 2006). Fungal inoculations were conducted on two spore concentrations (10^3 and 10^6 spores ml^{-1}) while bacterial inoculations were conducted with 10^9 cells ml^{-1}. The treatments tested are T1: RF69 control; T2: RP103 control; T3: RP242 control; T4: Bacterial mix control; T5: fungal control; T6: interaction *Fusarium* - RF69; T7: interaction *Fusarium* - RP103; T8 interaction *Fusarium* - RP242; T9 interaction *Fusarium* - Bacterial mix. Plates were incubated in triplicates at 25 °C for 7 days.

Fungal counts after 7 days of interaction

Ten gram of each soil sample were diluted on 90 ml of peptone water (0.1%) and agitated on rotary shaker for 1 minute. Each sample was serially diluted and 0.1 ml aliquots was inoculated in triplicates on the surface of Dichoran Rose Bengal Chloramphenicol Agar (DRBC) and Nash-Synder Agar (NSA) to perform fungal counts and 523 agar to perform bacterial counts (Nelson *et al*. 1983; Bluma and Etcheverry 2006; Pitt and Hocking 2009).

Rhizobacterial treatment of maize seedlings

Inoculum preparation

Bacterial isolates were grown *overnight* on 523 broth and cell concentration were adjusted for 10^9 CFU ml^{-1} by serial dilutions. Bacterial mix suspension was constructed by mixing 83.3 ml of each adjusted bacterial suspension. *Fusarium verticillioides* strains were grown on PDA media for 7 days at 25 °C and the colonies were washed with 10 ml

peptone water (0.1%) to create spore suspensions. Spore suspensions were measured on Neubauer chamber and adjusted for 10^6 spores ml^{-1} by serial dilutions.

Fungal inoculation and seed bacterization

Soil from maize experimental fields were sterilized by autoclaving twice and disposing on plastic cups for greenhouse studies. *Fusarium verticillioides* spore suspensions were centrally sprayed (2.5 ml) on soil to simulate the soil contamination by this species.

Seed bacterization was conducted according to Cavaglieri *et al*. (2005a) with a few modifications. Briefly, for each treatment, one hundred maize seeds (Cultivar Power Core 30A77PW) were submerged in 250 ml of bacterial inoculum in 500 ml Erlenmeyer flasks. Control seeds were submerged on 250 ml of 523 media. Flasks were incubated at 28 °C on a rotary shaker at 110 rpm for 2 hours for seed bacterization. After the incubation period, excess of media was removed from the seeds and immediately planted. The treatments used were: T1: *Fusarium verticillioides* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant (non-bacterized and planted on sterile soil); T7: *F. verticillioides* + RP69; T8: *F. verticillioides* + RP103; T9: *F. verticillioides* + RP242; T10: *F. verticillioides* + RF69+RP103+RP242.

Greenhouse studies

Bacterized seeds were planted on 2 cm holes made in the soil and rapidly covered. Each 700 ml plastic cup was filled with 5 seeds totalizing 50 seeds per treatment. All cups were placed in a greenhouse with 12h photoperiod for 20 days at 25 °C. Analysis of agronomical parameters and fungal counts on root tissues were conducted on days 10 and 20 after treatment. Agronomical parameters analyzed were stem, primary and adventitious root length; fresh and dry seedling and root weight; dry shoot:root ratio as an indicator of plant stress; seedling germination percentage and seedling emergence rate (Andrews *et al*. 1999; Kozdrój *et al*. 2004; Cavaglieri *et al*. 2005a; Pereira *et al*. 2009; 2011). To access the fungal counts on root tissues, root samples + adherent soil were weighed and serially diluted on peptone water (0.1%). To conduct fungal counts, aliquots of 0.1 ml were inoculated on the surface of DRBC and SNA to certify counts without external contamination (Nelson *et al*. 1983; Pitt and Hocking, 2009).

Statistical analyzes

Data were analyzed applying one-way ANOVA using Statistica 10.0 (StatSoft, Inc., 2011) software for Windows. Tukey test was used for average comparisons between treatments using $p \leq 0.05$ significance level throughout. Linear regression analyzes were conducted to calculate maize seedling emergence rate (seedling emergence per day).

Results

Bacterial isolation, screening and selection for antagonism

A total of 1283 bacterial colonies were isolated and 24 of those presented antagonism against two *F. verticillioides* strains tested (Table 1). Three isolates, named RF69, RP103 and RP242 presented strong or very strong antagonism on MMEA medium and were selected for the rest of the study.

Identification of selected isolates

All three isolates could be identified into species level with 100% of identity. Isolates RF69, RP103 and RP242 were identified as *Bacillus safensis*, *Bacillus amyloliquefaciens* and *Bacillus subtilis*.

Antagonism on non-rhizospherical soil

The results of interaction with rhizobacterial isolates on fungal counts on non-rhizospherical soil are shown on Table 2. All treatments using the rhizobacterial isolates resulted in bacterial growth after the incubating period (*data not shown*). All treatments reduced fungal counts in comparison to the counts observed on control treatment (T5). In both conidial concentrations, the bacterial mix treatment (T9) presented higher reduction on fungal counts when comparing with individual rhizobacterial treatments (T6, T7 and T8). Non-significant differences were found between fungal counts on SNA and DRBC media. None fungal counts were recovered on bacterial control treatments without fungal inoculation (T1, T2, T3 and T4).

Influence of maize treatments on agronomic parameters

The results of the maize seedling treatments with the rhizobacterial isolates on stem and root length, fresh seedling and root weight, dry seedling and root weight and the shoot:root ratio after 10 and 20 days of cultivation are shown on Tables 3 and 4, respectively. In comparison with the control treatment (T1), all rhizobacterial treatments presented increased values of stem and primary root length and fresh root and dry seedling weight after 10 days of cultivation. In general, treatments with *B. amyloliquefaciens* RP103 (T8) and the Bacterial Mix (T10) presented higher values of growth parameters of maize seedlings after 10 days of cultivation (Table 3).

After 20 days of cultivation, all rhizobacterial treatments presented increases on root length and seedling and root weight, both dry and fresh in comparison to control treatment (T1) (Table 3). As the results observed after 10 days of cultivation, in general, treatments with *B. amyloliquefaciens* RP103 (T8) and the Bacterial Mix (T10) presented higher values of growth parameters of maize seedlings. The increase of the shoot:root ratio was observed on all the treatments after 10 or 20 days of cultivation when compared to control treatment plants (T1) (Table 3 and 4). In addition, all the bacterial controls showed values similar to the healthy plant, indicating no phytotoxicity to the maize seedlings after 10 or 20 days of cultivation.

Seedling emergence rate and seed germination percentage are shown on Table 5 and Figure 1. All treatments were able to significantly increase the seedling emergence per day and the seeds germination when compared to control treatment (T1) (Table 5).

Fungal counts on root tissues of maize seedlings

Total fungal counts recovered from root tissues of maize seedlings growth for 20 days after seed bacterization are shown on Table 6. All rhizobacterial treatments significantly decreased the *Fusarium verticillioides* counts on root tissues after 10 and 20 days of cultivation. The bacterial mix treatment (T10) presented higher effectiveness on reducing fungal counts than the other treatments after 10 days, while after 20 days, *B. amyloliquefaciens* RP103 treatment (T8) presented the lowest fungal counts and therefore, the higher efficacy on controlling fungal growth.

Discussion

In this study, 1283 rhizobacterial isolates were obtained from healthy maize plants. From these isolates, about 2% presented antagonistic activity to *F. verticillioides* and three isolates showed great potential and intense antifungal activity. A diverse number of factors, such as soil type, plant cultivars and environmental conditions could interfere on the rhizobacterial population and therefore, result in a low number of isolates with antagonistic activity as seen in this study in comparison to other studies (Nesci *et al*. 2005; Palumbo *et al*. 2007; Lugtenberg and Kamilova 2009). The three selected isolates were identified as from the *Bacillus* genus, which is in agreement with other studies that reported that species within this genus are one of the main potential antagonistic rhizobacteria (Emmert and Handelsman 1999; Ongena and Jacques 2008). Species of this genus present great competitive advantages that enable their usage on bioformulated fungicides worldwide, like the formation of spores that allows them to resist adverse environmental conditions, the formation of biofilms that allow fast spreading and the production of diverse metabolites that interfere on the survival of other microorganisms (Branda *et al*. 2001; Ongena and Jacques 2008). The isolates RP69, RP103 and RP242 were identified as *Bacillus safensis*, *B. amyloliquefaciens* and *B. subtilis*, and these species are well known to have great potential for fungal biocontrol (Pérez-Garcia *et al*. 2011). Although the extensive use of these species in agriculture, the description of other strains with new competitive characteristics is extremely important.

The isolates obtained in this study showed the ability to grow, compete and reduce the *Fusarium verticillioides* counts on non-rhizospherical soil, regardless of the fungal density in soil, which characterizes a great potential usage of these isolates both individually or in a formulated mix. Since the main inoculum origin of this fungus to the maize seeds is the contaminated soil, the prophylactic treatment of the soil before seeding the field may be an effective method for reducing *Fusarium verticillioides* field counts and therefore, the first inoculum to the seeds (Munkvold 2003; Etcheverry *et al*. 2009). The higher effectiveness of the Bacterial Mix to control *F. verticillioides* on non-rhizospheric soil may be in due to the formation of a protocooperative interspecific population or a stimulant competition of the three isolates.

In both hypothesis, the presence of an interspecific interaction stimulate different metabolite productions that may have led to higher fungal control (Derewacz *et al*. 2015).

Etcheverry *et al*. (2009), in similar experiments using *Bacillus amyloliquefaciens* and other biological control agents, reported reduction of the fungal counts on non-rhizospheric soil. In a subsequent study, the same authors used the *B. amyloliquefaciens* previously tested on field experiments and the treatment was effective on the reduction of the final fumonisin content on maize grains (Pereira *et al*. 2007).

In the greenhouse studies, the inoculation of untreated maize seeds on *Fusarium* contaminated soil shows clear signs of disease and growth losses, which is in agreement with other studies (Yates *et al*. 2003; Cavaglieri *et al*. 2005b). The previous treatment of maize seed with the biocontrol agents significantly increased the stem and root length and seedling and root weight, as well as increased germination rates and the emergency rates. Similar growth was observed on seeds treated with the biocontrol agents and inoculated on sterile soil with the healthy plant control, which indicates absence of phytotoxicity of the biocontrol agents towards the maize seeds. Similar to what was observed on the non-rhizospheric soil experiments, the seed treatment with isolate *B. amyloliquefaciens* RP103 and the Bacterial Mix resulted in better growth parameters of the seedling after 10 or 20 days of cultivation. Although plant growth promotion was not observed (treatments T2, T3, T4 and T5 did not show higher values than the healthy plants, T6), the bacterial isolates were able to protect maize seedling, improving the agronomical parameters when exposed to *F. verticillioides* in relation to control without bacterial treatment (T1). These results are not in agreement with Pereira *et al*. (2009), which did not found improvement of agronomic parameters on seedlings treated with *B. amyloliquefaciens* and exposed to *F. verticillioides*.

Shoot/root ratio has been used as an indicator of plant stress, the lower the ratio, the higher the plant stress (Andrews *et al*. 1999; Kozdrój *et al*. 2004; Pereira *et al*. 2009). While the control with untreated seeds showed the lowest ratio and therefore higher stress, all bacterial treatments were effective in reducing the plant stress that resulted in less energy losses of the seedling during its growth.

The addition of the biocontrol agents significantly reduced fungal survival and therefore, fungal counts on maize seedling root tissues after 10 and 20 days of cultivation. Lowest fungal counts were observed after treatments with the Bacterial Mix and *B. amyloliquefaciens* RP103. Since colonizing the root system of maize seedlings is one of the main attack pathways of *F. verticillioides*, the reduction of fungal counts on this tissue

might indicate an effective method to reduce maize root rot and and the future endophytic growth of *Fusarium* that leads afterwards to ear infection and grain colonization (Sobek and Munkvold, 1999; Munkvold 2003).

In this study, we have shown that soil treatment and seed bacterization with the selected biocontrol agents are an effective method to reduce survival and colonization of the toxigenic *F. verticillioides* in greenhouse level. In addition to the reduction of fungal growth, the bacterial isolates tested as well as the formulated bacterial mix significantly increased maize seedling growth parameters and reduced plant stress when invaded by this phytophatogen, indicating a beneficial interaction of the maize seedling with the biocontrol agents. Although field studies need to be conducted, the selected biocontrol agents show promising characteristics to be used as a bioformulated fungicide aiming reduction of *Fusarium verticillioides* diseases and therefore the accumulation of fumonisin on maize grain.

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Conflict of Interest

None

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Table 1. Inhibition zone scores of rhizobacterial isolates antagonistic to *F. verticillioides* on MMEA medium, classified by the scale of Bacon and Hinton (2002).

Rhizobacterial isolate	Inhibition score
RF 69	+++
RF 96	+
RF 101	+
RF 138	+
RF 157	+
RF 160	+
RF 167	+
RF 187	+
RF 199	+
RP 13	+
RP 16	+
RP 17	+
RP 47	+
RP 66	+
RP 102	++
RP 103	++++
RP 106	+
RP 107	+
RP 112	+
RP 130	+
RP 131	+
RP 150	+
RP 242	+++
RP 692	+

Scale proposed by Bacon and Hinton (2002): -, no inhibition; +, weak (<3 mm) inhibition; ++, moderate (>3–9 mm) inhibition; +++, strong (>9–18 mm) inhibition; ++++, very strong (>18 mm) inhibition

Table 2. Effect of rhizobacterial treatments of non-rhizospherical soil on counts of *F. verticillioides* inoculated in different conidial concentrations.

	log CFU g^{-1} soil	
	10^3 conidia ml^{-1}	10^6 conidia ml^{-1}
T5	4.97 a	8.95 a
T6	4.20 b	7.89 c
T7	3.92 c	7.52 d
T8	3.97 c	7.97 b
T9	2.80 d	6.10 e
CV	0.1	0.1

T1: RF69 control; T2: RP103 control; T3: RP242 control; T4: Bacterial mix control; T5: fungal control; T6: interaction *Fusarium* - RF69; T7: interaction *Fusarium* - RP103; T8 interaction *Fusarium* - RP242; T9 interaction *Fusarium* - Bacterial mix. CV: Coefficient of variation (%).

Different letter within the same column indicate statistical difference ($p \leq 0.05$, Tuckey test).

Table 3. Growth parameters of maize seedlings sampled 10 days after rhizobacterial treatments.

Treatments	Lenght			Weight				S/R Index*
	Stem (cm)	Primary root (cm)	Adventitious roots (cm)	Fresh seedling (g plant^{-1})	Fresh root (g plant^{-1})	Dry seedling (g plant^{-1})	Dry root (g plant^{-1})	
T1	5.79 d	10.07 d	1.27 c	1.40 f	0.23 e	0.50 d	0.19 c	1.71 c
T2	8.80 bc	20.03 ab	5.77 ab	4.01 bc	0.87 ab	1.22 ab	0.31 a	3.00 ab
T3	11.91 a	21.23 a	7.26 a	5.04 a	0.99 a	1.30 a	0.32 a	3.03 ab
T4	11.37 a	21.17 a	6.70 a	4.22 ab	0.93 ab	1.23 ab	0.31 a	3.02 ab
T5	11.99 a	21.93 a	7.63 a	5.20 a	0.99 a	1.24 ab	0.31 a	3.02 ab
T6	11.77 a	20.60 ab	7.87 a	4.40 ab	0.93 ab	1.21 ab	0.3 a	3.00 ab
T7	7.71 c	12.78 c	3.53 bc	2.17 ef	0.57 cd	0.83 c	0.23 bc	2.65 b
T8	9.63 b	18.33 b	6.20 a	3.10 d	0.65 c	1.09 b	0.27 ab	3.15 ab
T9	9.23 b	14.87 c	3.70 bc	2.87 de	0.44 d	0.90 c	0.22 bc	3.02 ab
T10	9.80 b	18.37 b	6.84 a	3.47 cd	0.82 b	1.13 b	0.26 ab	3.37 a
CV	8.20	4.49	3.71	3.30	3.03	8.18	2.73	3.62

Data are means of samples analyzed in triplicates.*Dry Shoot/Root ratio. CV: Coefficient of variation (%).
Treatments: T1: *Fusarium verticillioides* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *F. verticillioides* + RP69; T8: *F. verticillioides* + RP103; T9: *F. verticillioides* + RP242; T10: *F. verticillioides* + RF69+RP103+RP242.
Different letters on the same column indicate statistical difference ($p \leq 0.05$, Tuckey test)

Table 4. Growth parameters of maize seedlings sampled 20 days after rhizobacterial treatments.

Treatments	Lenght			Weight				S/R Index*
	Stem (cm)	Primary root (cm)	Adventitious roots (cm)	Fresh seedling (g plant^{-1})	Fresh root (g plant^{-1})	Dry seedling (g plant^{-1})	Dry root (g plant^{-1})	
T1	12.59 d	17.97 d	9.27 f	2.68 g	1.44 f	1.44 e	0.27 e	4.29 b
T2	15.37 b	27.93 c	20.30 cde	9.05 c	3.27 b	5.72 ab	0.79 a	6.20 a
T3	18.67 a	35.03 a	25.60 a	11.14 a	3.92 a	6.35 a	0.83 a	6.63 a
T4	18.27 a	33.03 a	22.37 bc	9.91 b	3.31 b	6.12 a	0.81 a	6.58 a
T5	18.60 a	35.87 a	25.60 a	11.02 a	3.70 a	6.15 a	0.82 a	6.49 a
T6	18.72 a	32.53 ab	25.87 a	9.70 bc	3.24 b	5.96 a	0.81 a	6.38 a
T7	13.03 d	26.00 c	19.01 e	4.69 f	2.41 de	3.25 d	0.40 d	7.23 a
T8	15.43 b	29.44 bc	21.10 bcd	7.88 d	3.08 bc	5.11 b	0.73 b	6.01 a
T9	14.37 c	26.20 c	19.77 de	6.16 e	2.31 e	3.90 cd	0.56 c	5.97 a
T10	15.50 b	28.98 bc	22.40 b	7.50 d	2.75 cd	4.33 c	0.57 c	6.70 a
CV	1.77	2.31	3.08	2.57	3.04	4.56	4.94	1.88

Data are means of samples analyzed in triplicates.*Dry Shoot/Root ratio. CV: Coefficient of variation (%).
Treatments: T1: *Fusarium verticillioides* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *F. verticillioides* + RP69; T8: *F. verticillioides* + RP103; T9: *F. verticillioides* + RP242; T10: *F. verticillioides* + RF69+RP103+RP242.
Different letters on the same column indicate statistical difference ($p \leq 0.05$, Tuckey test)

Table 5. Germination percentage and seedling emergence rate of maize seeds treated with rhizobacterial isolates and cultivated on sterile or *Fusarium verticillioides* contaminated soil for 20 days.

Treatments	Seedling emergence rate (Seedling day^{-1})	Seed germination (%)
T1	0.60 d	30 e
T2	1.02 c	76 abc
T3	1.72 a	88 ab
T4	1.59 ab	76 abc
T5	1.14 c	84 abc
T6	1.34 bc	90 a
T7	1.21 bc	56 d
T8	1.71 a	70 cd
T9	1.73 a	72 bcd
T10	1.59 ab	72 bcd
CV	5.33	6.18

Treatments: T1: *Fusarium verticillioides* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *F. verticillioides* + RP69; T8: *F. verticillioides* + RP103; T9: *F. verticillioides* + RP242; T10: *F. verticillioides* + RF69+RP103+RP242. CV: Coefficient of variation (%).

Different letters on the same column indicate statistical difference ($p \leq 0.05$, Tuckey test)

Table 6. Total fungal counts recovered from rhizoplane tissue of maize seedlings sampled 10 and 20 days after rhizobacterial treatments.

Treatments	log CFU g^{-1} root			
	10 days		20 days	
	DRBC	SNA	DRBC	SNA
T1	7,13 a	7,09 a	7,05 a	7,01 a
T2	4,59f	4,58 f	3,65 f	3,61 f
T3	3,99 h	3,96 i	3,33 h	3,30 i
T4	4,11 g	4,10 j	3,63 f	3,61 f
T5	3,87 i	3,85 j	3,46 g	3,35 h
T6	3,99 h	3,99 h	3,51 g	3,48 g
T7	6,28 b	6,10 b	6,04 c	6,00 c
T8	5,38 d	5,36 d	5,33 e	5,11 e
T9	6,02 c	6,01 c	6,08 b	6,03 b
T10	5,08 e	5,07 e	5,78 d	5,68 d
CV	0.01	0.01	0.01	0.01

Treatments: T1: *Fusarium verticillioides* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *F. verticillioides* + RP69; T8: *F. verticillioides* + RP103; T9: *F. verticillioides* + RP242; T10: *F. verticillioides* + RF69+RP103+RP242. CV: Coefficient of variation (%).
Different letters on the same column indicate statistical difference ($p \leq 0,05$, Tuckey test)

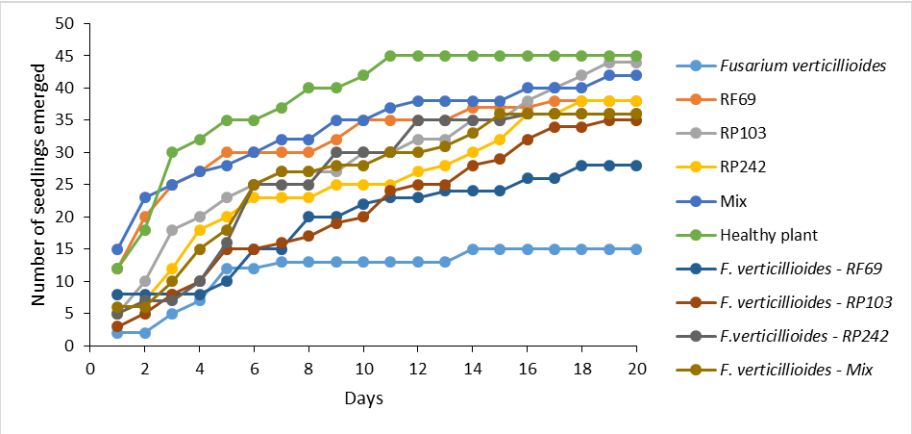

Figure 1. Seedling emergence of maize seeds treated with rhizobacterial isolates and cultivated on sterile or *Fusarium verticillioides* contaminated soil for 20 days.

3.2 Effect of maize seed and non-rhizospherical soil treatment with *Bacillus* spp. as a biocontrol agent for *Aspergillus flavus*

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Effect of maize seed and non-rhizospherical soil treatment with *Bacillus* spp. as a biocontrol agent for *Aspergillus flavus*.

Tiago C. Einloft [a,*], Patrícia B. Oliveira [a], Rafael G. Dionello [a]

[a] Departamento de Fitossanidade, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

*Corresponding author: Tiago Centeno Einloft, Departamento de Fitossanidade, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 7712, Porto Alegre, Rio Grande do Sul, Brazil. Email: tiago.einloft@gmail.com

Highlights:

Bacillus spp. as biocontrol agents against *A. flavus*

Reduction of *A. flavus* counts on treated maize soil

Reduction of *A. flavus* counts on maize seedling root tissues

Increase of maize agronomic parameters

B. amyloliquefaciens RP103 and Bacterial Formulated Mix shows promising characteristics as biocontrol agents

Abstract: *Aspergillus flavus* might infect maize plants and contaminate grains, producing Aflatoxin B1. The main inoculum source of this fungus is mycelium growing in the soils of maize field, from where the fungus can invade maize root system and ears. Biological control of *A. flavus* is an alternative method to chemical fungicides, lowering chemical residues and controlling fungal growth at their natural habitat. The aims of this study were to evaluate the effect of seed and soil *Bacillus* spp. treatments on *A. flavus* growth and maize agronomic parameters. All *Bacillus* spp. treatments reduced *A. flavus* counts on non-rhizospherical soil. After 20 days of maize seedling cultivation, *B. amyloliquefaciens* RP103 and a formulated Bacterial Mix reduced *A. flavus* counts on maize root tissues as well as increase seedling agronomic parameters, emergence rates and germination percentages. *B. amyloliquefaciens* RP103 and the bacterial mix presented promising characteristics to be used as biocontrol agents on maize field soil treatments and seed bacterization aiming *A. flavus* control on maize plants.

Key-words: Biological control, Aflatoxin B1, Corn, Rhizobacteria, Soil, Agronomic parameters

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereals produced worldwide and is mainly used for both human and animal consumption (USDA, 2014). Brazil is currently the third main maize producer in the world, with 85 million tons produced on 14/15 season, after USA and China, and one of the largest exporter (NCGA, 2014; CONAB, 2016). In the field, the maize plants are subject to contamination with phytophatogens and contaminants, such as *Aspergillus flavus* Link, an opportunistic fungus that can infect maize plants, often growing as a commensal on the plant roots, ears and on grains, leading to yield losses and decrease of plant health (IARC, 2012).

Aspergillus flavus can survive on the field soil or maize residues from crop remains through sclerotia, mycelium or latent spores (Nesci and Etcheverry, 2002). This ability characterizes the soil as a primary source of fungal inoculum to maize seeds and root systems (Abbas et al., 2009). *A. flavus* can invade maize seeds, causing direct spoilage, therefore decreasing seedling health and negatively affecting root and stem growth and elongation, seed germination ability and seedling emergence (Zablotowicz et al., 2007). As *A. flavus* grows on maize ears and kernels, it may produce aflatoxin B1 (AFB1), a

mycotoxin that has shown hepatotoxic, nefrotoxic and genotoxic characteristics, being considered by the International Agency for Research on Cancer as Group 1: carcinogenic to humans and animals (IARC, 2002; 2012).

The most efficient way to avoid exposure to AFB1 is prevention of fungal infection and growth. In a traditional way, resistant maize cultivars and synthetic chemical fungicides are the most used methods for fungal control. Biological control is an alternative control method that has shown great potential for both controlling fungal growth and lowering the fungicide residues on food and environment (Bluma and Etcheverry, 2006). When selecting biocontrol agents, the employment of native microorganisms often provides better results, since these microorganisms are already well adapted to with the target agroecosystem (Penna et al., 2004; Nesci et al., 2005).

Rhizobacteria from the *Bacillus* genus are the main antifungal-producing bacteria isolated from soil. Rhizobacteria are easily cultivated and their spore-forming ability favors survival under harsh environmental conditions, characterizing these rhizobacterial as attractive and promising biocontrol agents for *A. flavus* in their natural habitat (Bluma and Etcheverry, 2006; Ongena and Jacques, 2008). The ability to grow and compete with soil-borne pathogens on rhizosphere and maize field soils may lead to decreases in direct seed infection and of primary inoculum in soils, respectively (Etcheverry et al., 2009). Thus, seed and field soil treatments with native biocontrol agents is a promising method to control *A. flavus* in its natural environment (Mao et al., 1998; Batson et al., 2000; Kerry, 2000; Pereira et al., 2007).

In the present study the effects of non-rhizospherical soil treatment with three *Bacillus* spp. (*B. safensis* RF69, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242), applied single or in combination, on *A. flavus* counts are reported. We also report the effect of maize seed bacterization with *Bacillus* spp. on maize seedling agronomic parameters and on *A. flavus* counts on seedling root tissues.

2. Material and Methods

2.1 Fungal and rhizobacterial strains

An aflatoxin B1 producer *Aspergillus flavus* previously isolated from maize kernels was used on this study. Stock cultures were stored in 150 ml l^{-1} aqueous glycerol.

Bacillus safensis RF69, *Bacillus amyloliquefaciens* RP103 and *Bacillus subtilis* RP242 strains used in this study were previously isolated from maize root system as fungal antagonists. Bacterial strains were stocked on *deep freezer* (-80 °C), in tubes containing aqueous glycerol 300 ml l^{-1}.

2.2 Antagonism on non-rhizospherical soil

2.2.1 Inoculum preparation

For inoculum preparation, the fungi were grown on Malt Extract Agar (MEA) at 25 °C for 7 days and the colonies were washed with 10 ml peptone water (0.1%) to obtain spore suspensions (Pitt and Hocking, 2009). Spore suspensions were determined on Neubauer chamber and adjusted for 10^6 or 10^3 spores ml^{-1} by serial dilutions. Bacterial isolates were grown for 24h on 523 broth (Kado and Heskett, 1970) and cell concentrations were adjusted for 10^9 CFU ml^{-1} by serial dilutions. Bacterial mix suspension were prepared by mixing 330 µl of each bacterial suspension.

2.2.2 Inoculation

The assay was conducted on sterile soil samples collected from maize experimental fields, located at Eldorado do Sul, Rio Grande do Sul, Brazil. The experiment was conducted according to Bluma and Etcheverry (2006). Briefly, Petri dishes were filled with fifty grams of soil samples and sterilized twice by autoclaving. Microorganisms were inoculated by central spraying 1 ml of each spore suspension. Fungal inoculations were conducted on two spore concentrations (10^3 and 10^6 spores ml^{-1}) while bacterial inoculations were conducted with 10^9 cells ml^{-1}. Treatments are summarized on Table 1. Plates were incubated in triplicate at 25 °C for 7 days.

2.2.3 Fungal recovery and count

For fungal recovery and counts, 10g of each soil sample was diluted on 90 ml of peptone water (0.1%) and agitated on rotary shaker for 1 minute. Samples were serially diluted and 100 µl aliquots were inoculated in triplicates on Dichloran Rose Bengal Chloramphenicol Agar (DRBC), *Aspergillus flavus* and *parasiticus* Agar supplemented with chloramphenicol (AFPA-C) to perform fungal counts and on 523 agar to perform

bacterial counts (Kado and Heskett, 1970; Pitt et al., 1983; Bluma and Etcheverry, 2006; Pitt and Hocking, 2009).

2.3 Rhizobacterial treatment of maize seedlings and greenhouse studies

2.3.1 Inoculum preparation

Fungi were grown for 7 days on MEA at 25 °C and colonies were washed with 10 ml peptone water (0.1%) to formulate spore suspensions (Pitt and Hocking, 2009). Spore suspensions were measured on Neubauer chamber and adjusted for 10^6 spores ml^{-1} by serial dilutions. *Bacillus* spp. isolates were grown overnight on 523 broth and cell concentration was adjusted for 10^9 CFU ml^{-1} by serial dilutions. Bacterial mix suspension was prepared by mixing 83.3 ml of each adjusted bacterial suspension.

2.3.2 Fungal inoculation and seed bacterization

Soil from maize experimental fields was collected, sterilized twice by autoclaving and disposed on surface disinfected plastic cups for seed planting in greenhouse studies. *Aspergillus flavus* spore suspensions were centrally sprayed (2.5 ml) on soil to simulate soil colonization by this fungus. Seed bacterization was conducted as proposed by Cavaglieri et al. (2005) with a few modifications. For each treatment, 100 maize seeds (cultivar PowerCore 30A77PW) were submerged in 250 ml of bacterial inoculum within 500 ml Erlenmeyer flasks. For untreated control treatments, seeds were submerged in 250 ml of 523 broth. Treatments were incubated for 2 hours on rotary shaker (120 rpm) at 28 °C for seed bacterization. After treatment, the excess of media was removed and seeds were immediately planted. Treatments are summarized on Table 2.

2.3.3 Greenhouse studies

Treated seeds were planted on 2 cm holes made in the soil contaminated with *A. flavus* and covered. A total of 50 seeds were planted per treatment, disposed on ten plastic 700 ml cups (5 seeds per cup), which were placed in a greenhouse with 12h photoperiod for 20 days at 25 °C. Seedling growth agronomical parameters and fungal counts on root tissues were determined after 10 and 20 days of cultivation. The agronomical parameters

evaluated were stem, primary and adventitious root length, fresh and dry seedling and root weight, dry shoot:root ratio as an indicator of plant stress, seed germination percentages and seedling emergence rates (Andrews et al., 1999; Kozdrój et al., 2004; Cavaglieri et al., 2005; Pereira et al., 2009; 2011).

To obtain fungal contamination on seedling root tissues, root samples and adherent soil were aseptically weighed and serially diluted on peptone water (0.1%). Aliquots of 0.1 ml were inoculated on the surface of DRBC and AFP-C for fungal counts (Pitt and Hocking, 2009).

2.4 Statistical analyzes

One-way ANOVA was applied to data analyzes, using Statistica (StatSoft, Inc. (2011) software for Windows (Version 10.0). Tuckey test was performed for treatments mean comparisons using $p<0.05$ significance level and linear regression analyzes were conducted to calculate maize seedling emergence rates (number of seedling emerged per day).

3. Results

The effects of soil treatments with three *Bacillus* spp. in individual or combined application was evaluated and fungal counts recovered from non-rhizospherical soil after treatment period are presented on Table 3. On both fungal inoculum concentrations, all bacterial isolates were able to significantly ($p<0.05$) decrease fungal counts on non-rhizospherical maize soils. Treatment T9 (Bacterial mix with all *Bacillus* species combined) was the most effective treatment in both fungal concentrations in soil, being able to significantly reduce fungal counts when compared with both fungal control (T5) or the individual rhizobacterial treatments (T6, T7 and T8). No significant differences were found between fungal counts on DRBC and AFPA-C media.

The effect of maize seeds bacterization on agronomic parameters and fungal counts on seedling root tissues was also evaluated. Measurements of agronomical parameters of maize seedlings after 10 or 20 days of cultivation are shown on Tables 4 and 5, respectively. All bacterial seed treatments significantly increased maize seedling stem length and fresh and dry seedling weight after 10 days of cultivation. Treatment T7 (*B.*

safensis RF69) did not differ from fungal control without seed treatment (T1) on root length, and fresh and dry root weight. Treatments T8 and T10 (*B. amyloliquefaciens* RP103 and Bacterial mix, respectively) were the most effective in increasing stem and root length and weight (Table 4).

Similar results were obtained after 20 days of cultivation (Table 5). While treatment T7 presented no significant differences with fungal control without seed treatment on root length and fresh seedling weight, this treatment presented increased values for the other agronomic parameters. Treatments T8, T9 and T10 presented increased values for all agronomic parameters evaluated in comparison to control (T1), with T8 and T10 being significantly more effective enhancing stem and primary root length, fresh and dry seedling and dry root weight (Table 5). All treatments increased shoot:root ratios after 10 and 20 days of cultivation when compared to control treatments (T1).

The emergence of seedlings during 20 days of cultivation is represented on Fig. 1, and the emergence rate (number of seedlings emerged per day) and germination percentage are shown on Table 6. All treatments significantly increased ($p<0.05$) the seedling emergence rate and seed germination percentage after 20 days of cultivation (Table 6).

Total fungal counts recovered from maize seedling root tissues cultivated for 20 days are shown on Table 7. All rhizobacterial treatments significantly decreased ($p<0.05$) fungal counts on the seedling root tissues and adherent soil after 10 and 20 days of cultivation when compared to control treatment (T1). Treatment T10 (bacterial mix) presented significant lower values of fungal counts over the other treatments (T7, T8 and T9) after 10 days of cultivation, while after 20 days, the most effective treatment on controlling fungal survival on root tissues was T8 (*B. amyloliquefaciens* RP103).

4. Discussion

The present study evaluated the ability of *Bacillus* spp. from maize rhizosphere to control fungal development on non-rhizospherical soil and on maize root tissues. On non-rhizospherical soil, all three isolates were able to grow, compete and reduce the *Aspergillus flavus* counts on both fungal densities tested, highlighting the potential use of these biocontrol agents on direct application on field soil. The prophylactic treatment of field soil before seeding may be an effective strategy to reduce *A. flavus* counts on field

soil, since it represents the primary and main inoculum of this fungus to contaminate maize plants (Nesci and Etcheverry, 2002; Abbas et al., 2009; Etcheverry et al., 2009). Similar results were found by Etcheverry et al. (2009), which significantly reduced *A. flavus* counts using *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* strains. This is the first study to report the ability of *B. subtilis* and *B. safensis*, in addition to *B. amyloliquefaciens*, to compete and reduce *A. flavus* counts on non-rhizospherical soil.

Although individually, all strains presented reduction of fungal counts, the Bacterial Mix treatment was the most effective treatment for non-rhizospherical soil with the objective of *A. flavus* biocontrol. This results might be attributed to the formation of a protocooperative population that benefits all strains or a stimulant competition by the three species, inducing high metabolite production that may have led to higher fungal control (Derewacz et al., 2015).

The biocontrol agents ability to influence maize agronomical parameters as well as fungal counts on maize seedling root tissues at greenhouse level was also evaluated. After 10 and 20 days of cultivation, isolates *B. amyloliquefaciens* RP103 (T8) and *B. subtilis* RP242 (T9), as well as the Bacterial Mix (T10) resulted in increased root and shoot length and weight in comparison to the control treatment (T1). Similar results were observed by Aiyaz et al. (2015), which reported increased shoot and root weight and length of maize seedlings after *Bacillus* sp. treatment against *A. flavus*. The authors also found plant growth promotion ability by the used *Bacillus* strain, which was not observed in our study (treatments T2, T3, T4 and T5 did not show higher values than the healthy plant, T6). Nevertheless, bacterial treatments inoculated on sterile soil (without *A. flavus*), had similar results in comparison to treatment T6 (healthy plant), which characterizes the biocontrol agents as non-phytotoxic.

Also, maize seedling emergence rate and germination percentages were also significantly increased upon treatment with all rhizobacterial, which is an indication of vigor increase on the treated seeds as compared to the untreated seeds. This is in agreement with Aiyaz et al. (2015), that reported increasing germination percentages using a *Bacillus* sp. biocontrol agent towards *A. flavus*, highlighting that germination percentage and emergency rate increases lead to higher vigor due to the metabolically strengthened seedlings (Munizzi et al., 2010).

In addition, all bacterial treatments presented shoot:root ratio significantly different from the fungal control, which according to Pereira et al. (2009) indicates that the biocontrol

agents did not represent a stress condition on the absence of *A. flavus* and reduced the plant stress on the presence of the pathogen.

Under greenhouse conditions, all bacterial treatments decreased *A. flavus* counts on root tissues of maize seedlings after 10 and 20 days of cultivation. Since *A. flavus* can grow as a commensal on maize plants, the reduction of root contamination may lead to lower systemic invasion and therefore, low percentages of *A. flavus* and AFB1 on maize grains (IARC, 2012). Treatments with the Bacterial Mix (T10) and *B. amyloliquefaciens* RP103 (T8) presented the higher effectiveness on reducing *A. flavus* counts after 10 and 20 days of cultivation, respectively, and may indicate an effective method of seed infection control.

The present study provides evidence that soil or seed bacterization with mainly *B. amyloliquefaciens* RP103 or the formulated Bacterial Mix are an effective method to reduce *A. flavus* survival on non-rhizospherical maize field soil and seedling root colonization, improving agronomic parameters of maize seedlings. Although further studies will be needed to evaluate the effect of the rhizobacterial isolates on grain contamination and AFB1 production, the biocontrol agents showed promising characteristics to be used on a bioformulated fungicide aiming *A. flavus* reduction on grain and field and AFB1 accumulation.

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Table 1 Treatments conducted on non-rhizospherical soil experiment.

	Treatment
T1	Control *B. safensis* RF69
T2	Control *B. amyloliquefaciens* RP103
T3	Control *B. subtilis* RP242
T4	Control bacterial Mix
T5	Fungal control
T6	Interaction *A. flavus* - RF69
T7	Interaction *A. flavus* - RP103
T8	Interaction *A. flavus* - RP242
T9	Interaction *A. flavus* - Bacterial Mix

Table 2 Treatments conducted on greenhouse studies of bacterized maize seeds

	Treatment
T1	*A. flavus* Control
T2	*B. safensis* RF69
T3	*B. amyloliquefaciens* RP103
T4	*B. subtilis* RP242
T5	Bacterial Mix
T6	Healthy plant
T7	*A. flavus* + isolate RF69
T8	*A. flavus* + isolate RP103
T9	*A. flavus* + isolate RP242
T10	*A. flavus* + bacterial Mix

Table 3 Effect of rhizobacterial treatments of non-rhizospherical soil on counts of *A. flavus* inoculated in different conidial concentrations.

	log CFU g^{-1} soil	
	10^3 conidia ml^{-1}	10^6 conidia ml^{-1}
T1	0	0
T2	0	0
T3	0	0
T4	0	0
T5	5.93 a	8.35 a
T6	4.90 b	7.93 b
T7	4.94 b	7.78 c
T8	5.00 b	7.96 b
T9	4.23 c	7.05 d
CV	0.8	0.9

T1: RF69 control; T2: RP103 control; T3: RP242 control; T4: Bacterial mix control; T5: fungal control; T6: interaction *A. flavus* - RF69; T7: interaction *A. flavus* - RP103; T8 interaction *A. flavus* - RP242; T9 interaction *A. flavus* - Bacterial mix. CV: Coefficient of variation (%). Different letter within the same column indicate statistical difference ($p \leq 0.05$, Tuckey test)

Table 4 Growth parameters of maize seedlings sampled 10 days after rhizobacterial seed treatment.

Treatments	Lenght			Weight				S/R Index*
	Stem (cm)	Primary root (cm)	Adventitious root (cm)	Fresh seedling (g plant^{-1})	Fresh root (g plant^{-1})	Dry seedling (g plant^{-1})	Dry root (g plant^{-1})	
T1	6.57 d	10.50 e	2.07 d	1.75 e	0.30 e	0.44 e	0.18 e	1.42 b
T2	9.65 c	13.33 de	5.33 b	5.28 b	0.93 d	1.10 b	0.24 abc	3.68 a
T3	12.42 a	24.81 ab	9.28 a	6.74 a	1.28 a	1.24 a	0.28 a	3.43 a
T4	12.14 a	23.97 ab	9.17 a	5.35 b	0.90 ab	1.11 b	0.24 abc	3.68 a
T5	12.79 a	26.14 a	9.45 a	6.70 a	1.17 ab	1.21 ab	0.28 a	3.34 a
T6	12.35 a	23.54 ab	9.24 a	5.96 ab	0.99 abc	1.13 ab	0.26 ab	3.32 a
T7	9.23 c	12.98 de	4.90 cd	2.75 d	0.55 de	0.83 d	0.19 de	3.40 a
T8	9.40 c	17.87 c	5.13 bc	3.56 cd	0.74 cd	0.94 cd	0.23 bcd	3.23 a
T9	9.05 c	14.87 cd	3.13 d	3.37 cd	0.68 cd	0.85 d	0.21 cde	3.06 a
T10	10.68 b	22.13 b	8.36 a	4.19 c	0.86 bcd	0.98 c	0.24 abc	3.11 a
CV	2.44	3.12	1.21	1.04	2.78	2.76	1.09	3.20

Data are means of samples analyzed in triplicates.*Dry Shoot/Root ratio. CV: Coefficient of variation.

Treatments: T1: *Aspergillus flavus* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *Aspergillus flavus* + RP69; T8: *Aspergillus flavus* + RP103; T9: *Aspergillus flavus* + RP242; T10: *Aspergillus flavus* + RF69+RP103+RP242. Different letters on the same column indicate statistical difference ($p \leq 0.05$, Tuckey test).

Table 5 Growth parameters of maize seedlings sampled 20 days after rhizobacterial seed treatment.

Treatments	Lenght			Weight				S/R Index*
	Stem (cm)	Primary root (cm)	Adventitious root (cm)	Fresh seedling (g plant^{-1})	Fresh root (g plant^{-1})	Dry seedling (g plant^{-1})	Dry root (g plant^{-1})	
T1	12.01 d	21.08 f	10.10 d	3.98 f	1.86 e	1.47 g	0.30 d	4.07 c
T2	15.50 b	25.53 ef	19.77 b	8.61 c	4.07 b	6.04 c	0.93 a	5.48 ab
T3	18.60 a	39.06 a	22.10 ab	10.03 ab	5.63 a	6.41 a	0.97 a	5.58 ab
T4	18.07 a	36.13 ab	20.40 ab	9.33 abc	5.06 a	6.24 ab	0.96 a	5.52 ab
T5	18.53 a	39.73 a	22.40 a	10.27 a	5.51 a	6.29 ab	0.97 a	5.47 ab
T6	18.60 a	36.34 ab	22.63 a	9.20 bc	5.14 a	6.15 bc	0.95 a	5.45 ab
T7	13.90 c	22.30 f	12.20 d	4.58 f	2.72 d	3.82 f	0.63 c	5.04 b
T8	16.20 b	30.97 cd	16.70 c	7.40 d	3.52 bc	5.48 d	0.80 b	5.94 a
T9	14.47 c	28.52 de	15.40 c	6.35 e	3.04 cd	3.78 f	0.62 c	5.07 b
T10	16.13 b	33.47 bc	19.74 b	7.57 d	4.06 b	4.99 e	0.81 b	5.17 b
CV	3.71	2.91	1.08	1.44	0.97	3.12	1.07	5.22

Data are means of samples analyzed in triplicates.*Dry Shoot/Root ratio. CV: Coefficient of variation.
Treatments: T1: *Aspergillus flavus* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *Aspergillus flavus* + RP69; T8: *Aspergillus flavus* + RP103; T9: *Aspergillus flavus* + RP242; T10: *Aspergillus flavus* + RF69+RP103+RP242. Different letters on the same column indicate statistical difference ($p \leq 0.05$, Tuckey test).

Table 6. Germination percentage and seedling emergence rate of maize seeds treated with rhizobacterial isolates.

Treatments	Seedling emergence rate (Seedlings day^{-1})	Seed germination (%)
T1	0.38 e	30 c
T2	1.02 d	76 ab
T3	1.73 a	88 a
T4	1.59 ab	76 ab
T5	1.17 cd	84 ab
T6	1.15 cd	90 a
T7	1.05 cd	70 b
T8	1.18 cd	76 ab
T9	1.46 b	86 a
T10	1.25 c	80 ab
CV	9.77	6.82

Treatments: T1: *Aspergillus flavus* control; T2: isolate RF69 control; T3: isolate RP103 control; T4: isolate RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *Aspergillus flavus* + RP69; T8: *Aspergillus flavus* + RP103; T9: *Aspergillus flavus* + RP242; T10: *Aspergillus flavus* + RF69+RP103+RP242. CV: Coefficient of variation (%).

Different letters on the same column indicate statistical difference ($p \leq 0,05$, Tuckey test)

Table 7. Total fungal counts recovered from root tissues of maize seedlings sampled 10 and 20 days after rhizobacterial treatments.

Treatments	log CFU g^{-1} root			
	10 days		20 days	
	DRBC	AFPA-C	DRBC	AFPA-C
T1	7,20 a	6,11 a	6,98 a	6,89 a
T2	3,34 f	2,86 f	3,59 g	3,49 g
T3	3,24 f	2,64 f	3,05 i	3,01 i
T4	3,39 f	2,81 f	3,19 h	3,11 h
T5	3,17 f	2,53 f	3,05 i	3,02 i
T6	3,28 f	2,73 f	3,67 f	3,55 f
T7	5,90 b	5,72 b	5,54 b	5,32 b
T8	5,49 d	5,29 d	4,60 e	4,15 e
T9	5,72 c	5,65 c	5,36 c	5,02 c
T10	5,02 e	5,01 e	4,95 d	4,23 d
CV	0.06	0.01	0.07	0.01

Treatments: T1: *Aspergillus flavus* control; T2: *B. safensis* RF69 control; T3: *B. amyloliquefaciens* RP103 control; T4: *B. subtilis* RP242 control; T5: RF69+RP103+RP242 control; T6: Healthy plant; T7: *A. flavus* + RP69; T8: *A. flavus* + RP103; T9: *A. flavus* + RP242; T10: *A. flavus* + RF69+RP103+RP242. CV: Coefficient of variation (%).

Different letters on the same column indicate statistical difference ($p \leq 0.05$, Tuckey test)

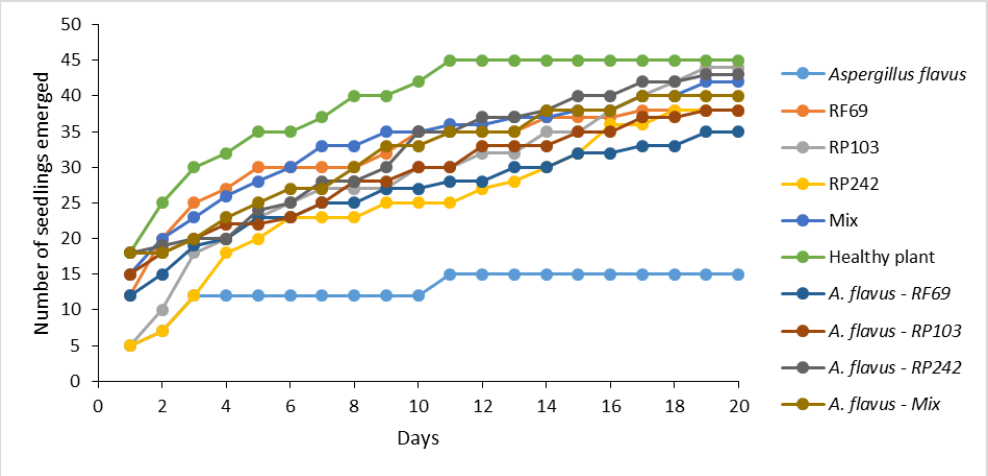

Fig. 1. Seedling emergence of maize seeds treated with *Bacillus safensis* RF69, *B. amyloliquefaciens* RP103, *B. subtilis* RP242 and Bacterial Mix and cultivated on sterile or *Aspergillus flavus* contaminated soil for 20 days

Table captions:

Table 1 Treatments conducted on non-rhizospherical soil experiment.

Table 2 Treatments conducted on greenhouse studies of bacterized maize seeds.

Table 3 Effect of rhizobacterial treatments of non-rhizospherical soil on counts of *A. flavus* inoculated in different conidial concentrations.

Table 4 Growth parameters of maize seedlings sampled 10 days after rhizobacterial seed treatment.

Table 5 Growth parameters of maize seedlings sampled 20 days after rhizobacterial seed treatment.

Table 6. Germination percentage and seedling emergence rate of maize seeds treated with rhizobacterial isolates.

Table 7. Total fungal counts recovered from root tissues of maize seedlings sampled 10 and 20 days after rhizobacterial treatments.

3.3 Biocontrol of *Aspergillus flavus* by *Bacillus* spp. *in vitro* and on maize grain.

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Biocontrol of *Aspergillus flavus* by *Bacillus* spp. *in vitro* and on maize grain

T. C. Einloft [a,*], P. B. Oliveira [a], F. F. Veras [b], A. Brandelli [b], J. E. Welke [b], L. L. Radunz [c], C. A. Mallmann [d], P. Dilkin [d], R. G. Dionello [a]

[a] Departamento de Fitossanidade, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[b] Instituto de Ciencia e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[c] Departamento de Engenharia Agrícola, Universidade Federal da Fronteira Sul campus Erechim, Brazil.

[d] Laboratório de Análises Micotoxicológicas (LAMIC), Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul, Brazil.

*Corresponding author: Tiago Centeno Einloft, Departamento de Fitossanidade, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 7712, Porto Alegre, Rio Grande do Sul, Brazil. Email: tiago.einloft@gmail.com

Highlights: Reduction of *A. flavus in vitro* mycelial growth rate, conidial germination, production and survival.

Production of iturin A by *B. amyloliquefaciens* RP103 and *B. subtilis* RP242.

Isolates RP103 and RP242 presented intense *in vitro* dominance towards *A. flavus* at different temperatures and water activities

Reduction of *A. flavus* growth and Aflatoxin B1 production on maize grains treated with biological control agents.

Abstract:

The toxigenic fungus *Aspergillus flavus* may colonize maize grains in the field and during storage, leading to yield and quality losses, mainly by the production of Aflatoxin B1, a hepatotoxic and carcinogenic mycotoxin. Biological control is a promising alternative control method and the use of soil bacteria as biocontrol agents has great potential due to excellent survival and competitive abilities of this microorganisms. In this study, we evaluated *Bacillus* spp. effect on *in vitro* growth of *A. flavus* and dispersal parameters, dominance interspecific interactions and bacterial ability to produce iturin A. The effect of maize grain bacterial treatments on fungal growth and aflatoxin B1 production were also evaluated. All *Bacillus* spp. were able to reduce *A. flavus in vitro* mycelial growth rate, conidial germination and survival and *A. flavus* growth and aflatoxin B1 production on maize grain. Isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 maintained a dominant pattern towards *A. flavus* in all water activities and temperatures evaluated and were able to produce iturin A at the tested conditions. The results indicate the promising characteristics of these isolates to be used as biocontrol agents aiming the control of *A. flavus* growth and aflatoxin B1 production on maize grains.

Key-words: Index of Dominance, Rhizobacteria, mycotoxin, Corn, Aflatoxin B1

1. Introduction

Maize grain (*Zea mays* L.) can be contaminated by *Aspergillus flavus* during pre-harvest and in storage. Apart from grain degradation and yield losses, *A. flavus* may produce

aflatoxin B1 (AFB1), a mycotoxin with hepatotoxic, genotoxic and teratogenic characteristics (Binder et al., 2007; IARC, 2012). In addition, the International Agency for Research on Cancer classified AFB1 as group 1: carcinogenic to humans and animals (IARC, 2002).

Pre-harvest colonization of maize grain by *A. flavus* can occur mainly through insect damage or direct penetration during flowering or during storage, where variations of water activity (a_w), temperature and biotic interactions may led to fungal proliferation on grain mass and AFB1 production (Lacey and Magan, 1991). Control strategies are necessary to minimize fungal invasion and the contamination of maize grain with AFB1.

Among different methods to prevent fungal colonization, biological control using native microorganisms emerged with great potential due to its application during both at pre-harvest, through direct application on maize ears, and in storage, by application on the grain mass (Abadías et al., 2001; Penna et al., 2004; Khafanari et al., 2007).

Soil bacteria generally present promising characteristics to be used as biocontrol agents, mainly because they are strong competitors and produce several antifungal compounds (Vining, 1990; Bluma and Etcheverry, 2006). The *Bacillus* spp. are the main antifungal-compounds producer among these bacteria and due to its spore-forming ability, they are capable to resist harsh environmental changes and still be able to grow and inhibit fungal growth (Silo-Suh et al., 1994; Bluma and Etcheverry, 2006; Ongena and Jacques, 2008; Pérez-García et al., 2011).

Possible changes in the environmental conditions affect directly microbial physiology and metabolite production. Both are decisive in determining the competitive ability, survival and dominance of one species to another in a particular agroecological niche (Marín et al., 1998; Nesci et al., 2005). Thus, it is important that during selection of potential biocontrol agents, their interaction and the target toxigenic fungi should be clarified under a variety of environmental conditions (Petterson et al., 1998; Nesci et al., 2005; Bluma and Etcheverry, 2006). In addition, both the biocontrol agent and its metabolites, should be able to reduce fungal proliferation while not enhancing toxin production, in a way to assure food safety when using this control method.

The present study report the *in vitro* effect of three *Bacillus* sp. isolates on *A. flavus* mycelial growth rate and dispersion parameters as well as interspecific dominance interactions under a different set of environmental condition variations. The effect of

maize grain treatments with *Bacillus* sp. isolates on *A. flavus* growth and aflatoxin B1 production is also reported.

2. Materials and Methods

2.1 Fungal and bacterial strains

Two isolates of aflatoxin B1 producer *Aspergillus flavus* previously isolated from maize grains were used in this study. Stock cultures were stored in 150 ml l^{-1} aqueous glycerol. *Bacillus safensis* RF69, *Bacillus amyloliquefaciens* RP103 and *Bacillus subtilis* RP242 strains used in this study were previously isolated from maize root system as fungal antagonists. Bacterial strains were stocked on *deep freezer* (-80 °C), in tubes containing aqueous glycerol 300 ml l^{-1}.

2.2 Inoculum preparation

For inoculum preparation, bacterial isolates were grown for 24h on 523 broth (Kado and Heskett, 1970) and cell concentrations were adjusted for 10^9 CFU ml^{-1} by serial dilutions. Fungi were grown on Malt Extract Agar (MEA) at 25 °C for 7 days and the colonies were washed with 10 ml peptone water (0.1%) to create spore suspensions or used for removal of agar plugs (5 mm) from the border of growing colonies with a cork borer (Marín et al., 1995, Pitt and Hocking, 2009). Spore suspensions were measured on Neubauer chamber and adjusted for 10^6 or 10^3 spores ml^{-1} by serial dilutions.

2.3 In vitro interaction studies

2.3.1 Fungal mycelial growth rate

For the evaluation of the effect of *Bacillus* sp. isolates on *A. flavus* mycelium growth rate, the method proposed by Bluma and Etcheverry (2006) was conducted with a few modifications. Briefly, aliquots (1 ml) of bacterial suspensions were inoculated on Petri plates (final concentration of 10^9 cells ml^{-1}) and after that, 19 ml of melted Maize Meal Extract Agar (MMEA) was inoculated and homogenized. After solidification, agar plugs of *A. flavus* mycelium were inoculated in the center of the plate. Treatment and control plates (*A. flavus* growing alone) were incubated on polyethilene bags for 7 days at 25 ºC in triplicates and repeated three times.

Fungal growth in diameter, was evaluated daily by measuring two radii at right angles one to another with a ruler (Cavaglieri et al., 2005, Bluma and Etcheverry, 2006). The radial growth rate (mm day^{-1}) was calculated by linear regression and the relative reduction of mycelial growth was calculated by the following formula proposed by Gong et al. (2014):

$$\text{Inhibition (\%)} = [(CD-TD)/CD] \times 100$$

Where CD is control diameter and TD is treatment diameter.

2.3.2 Conidial production

Seven day cultures from the experiment described in item 2.3.1 were used to evaluate the effect of *Bacillus* spp. on *A. flavus* conidial production. Cultures were washed twice with 5 ml of peptone water (0.01%) and harvested 3 times using a glass handle. Conidial counts of the 10 ml suspensions were performed on a Neubauer chamber and the number of conidia ml^{-1} was calculated. Subsequently, total conidial number was calculated and determined the concentration of conidia per cm^2 of mycelium. The assays were repeated three times.

2.3.3 Conidial germination and survival

The experiments were carried out on 2 ml tubes containing 500 µl of sterile peptone water (0.01%). Aliquots of 250 µl of bacterial suspensions (10^9 cells ml^{-1}) and 250 µl of filtered *A. flaus* conidial suspension (10^6 conidia ml^{-1}) were inoculated simultaneously on the tubes and cultures were incubated at 25 ºC for 24h (Chang et al., 2009). Treatment and control tubes (without the bacterial aliquot, which was substituted for sterile 523 broth) were conducted in triplicates and repeated three times. After 24h of treatment, 200 conidia were counted per replicate and the proportion of germinated conidia was determined. A conidium was considered geminated when the germinative tube presented the double of the conidial length. The formula proposed by Gong et al. (2014) was used to calculate the germination inhibition percentage, substituting mycelial growth to number of germinated conidia.

After conidial counts, 100 µl of each replicate was inoculated in duplicates on the surface of plates containing Dichloran Rose Bengal Chloramphenicol Agar (DRBC) with the objective to determine the conidial survival percentage. Plates were incubated at 25 ºC

for 5 days and after that, colony forming units (CFUs) were counted. The assays were repeated three times.

2.3.4 Index of Dominance (I_D)

Basic MMEA medium (0.999 a_w) was adjusted to 0.982, 0.955 and 0.937 by addition to known amounts of glycerol in accordance with Dallyn and Fox (1980) and Nesci et al. (2005). Ten microliters of bacterial suspensions were inoculated on one border of the petri plate and an agar plug (5 mm) containing growing *A. flavus* mycelium was inoculated on the opposite border of the plate. Plates were incubated in triplicates at 25 ºC for 7 days and after that the interaction of each dual cultures were examined macroscopically. The type of interaction was determined using the following I_D scores: 1/1: mutual intermingling, 2/2: mutual inhibition on contact, 3/3: mutual inhibition at a distance, 4/0: dominance of one species on contact, 5/0: dominance at a distance (Magan and Lacey, 1984; Penna et al., 2004; Nesci et al., 2005). The assays were repeated three times for validation of dominance interactions.

2.4 Iturin A production

The ability of *Bacillus* spp. to produce iturin A was evaluated. Extraction and quantification of iturin A was conducted according to Yuan et al. (2011). Briefly, tubes of the experiment described on 2.3.3 with *Bacillus* spp. grown for 36 h were centrifuged and supernatant was extracted with n-butanol. The extracts were rested for 24h, the organic phase was deposited on Petri plates, which were transferred to a stove for drying at 30 ºC for 24h. Dried extracts were resuspended on 2 ml methanol, filtered and applied on HPLC to quantification. A C_{18} HPLC column was used (150 x 4.6 mm, 5 mm pore, 20 ºC), with acenotrile:acetic acid (0.1%) (40:60) as mobile phase on an isocratic system, with injection volume of 20 µl and flow rate of 0.6 ml min^{-1}.

2.5 Interactions on maize grain

2.5.1 Inoculation and culture media

Petri plates were filled with 70 g of autoclaved maize grains, centrally sprayed with 1 ml of *A. flavus* spore suspensions (10^6 conidia ml^{-1}) and bacterial suspensions (10^9 cells ml^{-1}) and incubated in triplicates at 25 ºC for 10 days (Bluma and Etcheverry, 2006). Control plates were incubated without the bacterial suspension.

2.5.2 A. flavus and Bacillus spp. viable cell counts

Dilution plating and surface spreading techniques were used for fungal and bacterial enumeration. 10 grams of inoculated maize was diluted on 90 ml of peptone water (0.01%), shaken on an orbital shaker for 30 minutes, serially diluted and 0.1 ml aliquots were inoculated on plates containing DRBC and 523 medium for fungal and bacterial enumeration, respectively (Bluma and Etcheverry, 2006). Colonization of maize was assessed as CFU g^{-1} maize.

2.5.3 Aflatoxin extraction, clean-up and quantification

AFB1 extraction was conducted by the method proposed by Garcia et al. (2012) with a few modifications. Briefly, 10 g of milled inoculated maize samples were extracted with 15 ml of acetonitrile:water (60:40, v.v.) and shaken on orbital shaker for 20 minutes. Extract was filtered, mixed with 15 ml of phosphate buffered saline (PBS) solution and cleaned through an immunoaffinity column AflaTest (VICAM ,Watertown, MA, USA). Column was washed with 20 ml of PBS, left to dry for 15 minutes, eluted with 3 ml of methanol and evaporated to dryness.

Samples were resuspended on 0.6 ml ammonium acetate (10mM) and injected on HPLC/MS. The liquid chromatography column used was a Zorbax Eclipse C18, 4.6 x 50 mm, 1.8 μm pore (Agilent Technologies Inc., USA). HPLC run parameters were the following: flow rate of 0.6 mL/min; column temperature of 40 ºC and injection volume of 5 μm. Gradient elution was performed using water:ammonium acetate (10mM) as eluent A and methanol as eluent B. The MS source dependent parameters were: source temperature 350 ºC, dry gas 10 L/min and the spray voltage was set to 1000 V. Detection (LOD) and quantification (LOQ) limits were 0.06 and 0.2 ng g^{-1} and recovery rate was 98.2%.

2.6 Statistical analyzes

One-way ANOVA was applied to data analyzes, using Statistica (StatSoft, Inc. (2011) software for Windows (Version 10.0). Tuckey test was conducted for treatment mean comparison using $p<0.05$ significance level and linear regression analyzes were conducted to calculate fungal mycelial growth rate.

3. Results

Results of the *in vitro* interaction of *Bacillus* spp. biocontrol agents and *A. flavus* on fungal mycelial growth rate, conidial production, germination and survival are summarized on Table 1. All bacterial biocontrol agents significantly ($p \leq 0.05$) reduced fungal mycelial growth on MMEA medium, reducing growth rate in a range between 73-78% (Table 1). All bacteria also significantly affected conidial production (conidia per cm^2); isolates *B. safensis* RP69 and *B. amyloliquefaciens* RP103 reduced conidial production while isolate *B. subtilis* RP242 significantly increased production, stimulating sporulation when compared to control treatment. Reduction of conidial germination was also observed on all bacterial treatments, ranging in between 79 and 100% of germination inhibition (Table 1). Conidial survival was significantly ($p<0.05$) affected by all biocontrol agents; isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 resulted in lower fungal counts, presenting more effectiveness on reducing conidial survival after 24h of treatment (Table 1). Isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 were able to produce iturin A on the conditions tested, while non detectable levels of iturin A was observed on *B. safensis* RF69 extracts. Quantified concentrations of Iturin A are also showed on Table 1.

The I_D between *A. flavus* and bacterial biocontrol agents on MMEA medium are presented on Table 2. Two dominance patterns were observed on the interaction between bacteria and *A. flavus* under a different set of environmental changes. The first pattern was fungal inhibition at distance (5/0) as a predominant interaction between *B. amyloliquefaciens* RP103 x *A. flavus* and *B. subtilis* RP242 x *A. flavus*, where the bacteria maintained its dominance status after the environmental changes. The second pattern was a decrease of dominance of *B. safensis* RF69 towards *A. flavus* in order that the temperature and water activity was reduced. The final I_D scores between bacterial strains and *A. flavus* are also shown on Table 2.

Results obtained on the evaluation of maize grain treatment with bacterial strains on fungal counts and AFB1 accumulation are shown on Table 3. All bacterial isolates were able to grow (data of bacterial counts not shown) and significantly reduce ($p \leq 0.05$) fungal counts on maize grain on a range between 19% (*B. safensis* RF69) and 47% (*B. amyloliquefaciens* RP103). Detectable levels of AFB1 were significantly reduced ($p<0.05$) by grain treatment with biocontrol agents, which decreased AFB1 accumulation between 19 and 47% when compared to control plates with untreated maize grains. In addition, AFB1 per CFU was also reduced by all bacterial strains in a range between 44

and 89%. Isolate *B. amyloliquefaciens* RP103 was the most effective bacterial strain in the control of both growth and AFB1 production by *A. flavus* when compared with *B. safensis* RF69 and *B. subtilis* RP242, presenting a reduction of 94% of final AFB1 content and 89% of AFB1 per CFU.

4. Discussion

The present study provides information about the interaction of previously isolated *Bacillus* spp. from maize rhizosphere on *in vitro* growth parameters of *A. flavus* and on maize grain. When selecting potential biocontrol agents, it is necessary to elucidate the interaction characteristics of both microorganisms in a controlled environmental, so that the effect of predictable environmental changes can be tested.

The isolates evaluated on this study presented extremely promising characteristics, being able to reduce both the fungal growth and dispersion ability by decreasing mycelial growth rate, conidial production, germination and survival. Inhibition of fungal growth rate often delays microbial *lag* phase, and therefore, decrease the competitiveness and survivability, characterizing a possible control method for this fungus (Cavaglieri et al., 2005).

The ability to reduce spore production, germination and survival was also observed and is an interesting characteristic to potential biocontrol agents, since in the field, *A. flavus* spores carried by the air are the main infection pathway to infect the maize plants (Abbas et al., 2009). Air-carried spores invade the plant by insect damaged areas or penetrating the ear silk during flowering through the germ tube emerged from the spores, reaching the ear and maize grains (Horn, 2003; Abbas et al., 2008, 2009). This bacterial ability has been previously described by Gong et al. (2014), which reported reduction of *A. flavus* sporulation and spore germination by an isolate of *B. subtilis*. The authors identified the production of Bacillomycing D, a *Bacillus* sp. metabolite with high antifungal activity, being capable to injure fungal cell walls and membranes.

Although Bacillomycin production was not investigated, we observed the production of Iturin A by *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 when in contact with *A. flavus*. Iturin A is a well known bacterial metabolite with high antifungal activity, also with the capacity to injure fungal cell walls and membranes causing citoplasmatic overflow and therefore, cell death (Bonmatin et al., 2003; Aranda et al., 2005; Ongena

and Jacques, 2008). Certainly, this metabolite is one of the main responsible for the high antifungal activity observed, since the decrease of conidial survival indicates fungicide and not fungistatic ability, which is in agreement with the Iturin A mode of action. The Iturin A production pattern is in agreement with what previous authors concluded, with *B. subtilis* producing the higher concentration of this metabolite in relation with *B. amyloliquefaciens* RP103 (Koumoutsi et al., 2004; Ongena and Jacques, 2008).

Despite the fact that isolate *B. subtilis* RP242 increased conidial production, the enhancement of aflatoxin B1 was not observed on maize grain when treated with this isolate. Taking into consideration that the conidial production and mycotoxin synthesis are metabolically closely related and the increase of one is often accompanied with the increase of the other, the enhancement of sporulation did not characterize a higher risk for aflatoxin B1 when *A. flavus* is interacting with *B. subtilis* RP242 (Hyuk-Yu and Keller, 2005).

The I_D experiments carried clearly shown high dominance by *Bacillus amyloliquefaciens* RP103 and *B. subtilis* RP242 towards *A. flavus*. Water and temperature stress did not affect bacterial physiology in a way that reduced its competitiveness. Probably due to the spore-formation ability of RP103 and RP242, these strains were able to survive the harsh environmental conditions while secreting antifungal compounds. This is in agreement with the theory that microorganisms derived from adverse environments are more suitable to be used as biocontrol agents because of the ability to survive and compete even under extreme environmental conditions (Vining, 1990; Bluma and Etcheverry, 2006). The I_Ds observed from *B. safensis* RF69 shows dominance only at 30 ºC and high humidity levels, indicating that this isolate may not be an ideal biocontrol agent since it tend to lose its antifungal activity as the environmental conditions change. The results of isolates RP103 and RP242 are not in agreement with Nesci et al. (2005), which reported predominance of mutual intermingling (I_D 1/1) and no clear dominance by the *Bacillus* spp. under the tested conditions. This clearly shows that our *Bacillus* spp. isolates are better competitors then those reported by Nesci et al. (2005), probably being more suitable to be used as biocontrol agents.

All isolates were able to reduce *A. flavus* growth on maize grains, suggesting a possible application on maize ears on the field and even on stored systems. *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 are capable to resist temperature and water stress and reduce

fungal growth on maize grain, which indicates that they could be used allied to silo temperature decrease and grain drying aiming storage fungal control. Similar results were found by Bluma and Etcheverry (2006). The authors reported significant reduction of *Aspergillus* section *Flavi* on maize grains treated with *Bacillus* spp. highlighting two strains of *B. amyloliquefaciens*, which presented high fungal inhibition. In addition, Etcheverry et al. (2009) reported reduction of *A. flavus* growth after application of one strain of *B. amyloliquefaciens* on maize ears apex and base, showing that ear treatment is viable when used with high competitive isolates like the isolates reported in our study.

Aflatoxin B1 accumulation and AFB1 per CFU was also significantly reduced by all bacterial strains evaluated, with *B. amyloliquefaciens* RP103 being the most effective on controlling AFB1 production. Similar results were obtained by Bluma and Etcheverry (2006), which found reduction of AFB1 levels on maize grains treated with strains of *B. amyloliquefaciens*. This suggests that even different strains of *B. amyloliquefaciens* have the ability to highly influence on AFB1 production and accumulation leading to lower levels of this mycotoxin when this species is present. Apart of the study conducted by Bluma and Etcheverry (2006), the ability of *Bacillus* spp. to reduce AFB1 production has been reported previously only on *in vitro* evaluations. Since discrepancies between *in vitro* and on grain evaluations may occur, the inhibition of AFB1 production on maize grain have important implications on biological control both in the field and on storage (Nesci et al., 2005; Palumbo et al., 2007).

The reduction of AFB1 per CFU is an important observation, mainly because it assures that production of AFB1 was effected by the biocontrol agents instead of reduction of final concentrations only in response to a decrease on growth levels. Since stimulation of toxin production may occur even when growth is reduced, the biocontrol agents evaluated on this study were capable to reduce both growth and AFB1 production, leading to a decrease on final concentrations (Magan & Olsen, 2004; Al-Saad et al., 2016). Accordingly to Choudhary et al (1992), reduction of final AFB1 levels can be the result of one or a combination of several factors: physical competition for space and nutrition; competition for a specific substrate required for toxin production but not growth; induction to a change in the biochemical environment affecting the toxin production metabolic pathway and direct degradation of aflatoxin after its formation. Further experiments need to be conducted to clarify if AFB1 production is being directly affected

by competition, changes in environment or toxin detoxification is responsible for a decrease in final concentrations of AFB1.

In summary, the results obtained in this study present the promising characteristics of *Bacillus* spp. to be used as biocontrol agents to *Aspergillus flavus* on maize agroecological systems. The isolates were able to reduce fungal growth and dispersion ability *in vitro* and on maize grain, as well as reduce AFB1 production on the conditions tested. In addition, isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 were able to maintain dominance towards *A. flavus* even on temperature and water stress conditions, characterizing these isolates as promising biocontrol agents to *A. flavus* for usage on both maize ear in the field and during storage. Future studies with direct application on maize ear under greenhouse conditions and during storage need to be conducted in order to confirm the potential use of these biocontrol agents.

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Table 1
Effect of *in vitro* interaction of *Bacillus* spp. and *A. flavus* on fungal mycelial growth rate, conidial production, germination and survival and *in vitro* production of iturin A.

	Colony diameter (cm)	Mycelial growth rate (cm day^{-1})	Mycelial growth reduction (%)	Conidial production (log conidia per cm^2 of mycelium)	Conidial germination inhibition (%)	Conidial survival (log UFC ml^{-1})	Iturin A (µg ml^{-1})
Control	8.2 ± 0.18 a	1.10 ± 0.02 a	0 a	5.80 ± 2.59 b	0 a	5.98 ± 2.50 a	-
B. safensis RF69	2.2 ± 0.44 b	0.20 ± 0.06 b	73.2 ± 5.33 b	5.07 ± 1.53 c	79 ± 0.9 b	5.00 ± 2.45 b	ND
B. amyloliquefaciens RP103	1.9 ± 0.23 b	0.12 ± 0.01 b	76.8 ± 0.31b	5.16 ± 1.28 c	97 ± 0.8 b	4.87 ± 1.54 c	74.72 ± 7.75 b
B. subtilis RP242	1.8 ± 0.15 b	0.10 ± 0.01 b	78.2 ± 0.07 b	5.91 ± 1.98 a	100 ± 0.4 b	4.83 ± 2.32 c	143.52 ± 37.74 a

ND: Not detectable levels found. Different letters within the same column indicate significant difference ($p \leq 0.05$)

Table 2

Indices of Dominance (I_D) between bacterial strains and aflatoxigenic *Aspergillus flavus* under different water activities and temperatures on Maize Meal Extract agar.

Rhizobacterial isolates	a_w	Temperature			
		20 °C	25 °C	30 °C	I_D
B. safensis RF69	0.999	2/2	4/0	5/0	11/2
	0.982	2/2	2/2	4/0	8/4
	0.955	2/2	1/1	4/0	7/3
	0.937	1/1	1/1	4/0	6/2
	I_D	7/7	8/4	17/0	64/22
B. amyloliquefaciens RP103	0.999	5/0	5/0	5/0	15/0
	0.982	5/0	5/0	5/0	15/0
	0.955	5/0	5/0	5/0	15/0
	0.937	5/0	4/0	4/0	13/0
	I_D	20/0	19/0	19/0	116/0
B. subtilis RP242	0.999	5/0	5/0	5/0	15/0
	0.982	5/0	5/0	5/0	15/0
	0.955	5/0	5/0	5/0	15/0
	0.937	4/0	5/0	5/0	14/0
	I_D	19/0	20/0	20/0	118/0

Index of Dominance (I_D):

1/1: Mutual intermingling

2/2: Mutual inhibition on contact

3/3: Mutual inhibition at a distance

4/0: Dominance of one species on contact

5/0: Dominance at a distance.

I_D: Bacterial isolates/*Aspergillus flavus*

Table 3
Effect of bacterial treatment of maize grain on *Aspergillus flavus* counts and Aflatoxin B1 accumulation.

	Fungal counts (log CFU g^{-1} maize)	Aflatoxin B1 (µg kg^{-1})	Aflatoxin B1 per CFU (µg CFU^{-1})
Control	8.67 ± 2.08 a	761 ± 123 a	87.73 a
B. safensis RF69	6.96 ± 2.64 b	339 ± 80 b	48.67 b
B. amyloliquefaciens RP103	4.57 ± 1.91 d	41,5 ± 21 d	9.05 d
B. subtilis RP242	5.65 ± 1.96 c	216 ± 30 c	38.30 c

Different letters within the same column indicate significant difference ($p \leq 0.05$, Tuckey test).

3.4 *In vitro* and *in situ* biological control of *Fusarium verticillioides* by *Bacillus* spp.

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In vitro and *in situ* biological control of *Fusarium verticillioides* by *Bacillus* spp.

T. C. Einloft [a,*], P. B. Oliveira [a], F. F. Veras [b], L. L. Radunz [c], C. A. Mallmann [d], P. Dilkin [d], R. G. Dionello [a]

[a] Departamento de Fitossanidade, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[b] Instituto de Ciencia e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

[c] Departamento de Engenharia Agrícola, Universidade Federal da Fronteira Sul campus Erechim, Brazil.

[d] Laboratório de Análises Micotoxicológicas (LAMIC), Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul, Brazil.

*Corresponding author: Tiago Centeno Einloft, Departamento de Fitossanidade, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 7712, Porto Alegre, Rio Grande do Sul, Brazil. Email: tiago.einloft@gmail.com

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Abstract: *Fusarium verticillioides* is a phytopathogen capable to cause maize root and ear rot, leading to yield and grain quality losses, and produce Fumonisin B1, a hepatotoxic and potential carcinogenic mycotoxin. Biological control of phytopathogens using native microorganisms is a promising control method, with high efficacy and low environmental impact. Three *Bacillus* spp., candidate as biocontrol agents isolated from maize root system were evaluated in this study. The biocontrol agents significantly reduce *in vitro F. verticillioides* mycelial growth rate and conidial production, germination and survival. All *Bacillus* spp. also significantly reduced fungal growth and fumonisin B1 production on maize grain. Isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 were able to maintain dominance towards *F. verticillioides* at all evaluated water activities and temperatures, highlighting the competitiveness of these rhizobacteria. The results presented in this study shows the promising characteristics of these *Bacillus* spp. isolates and confirms their potential to be used as biocontrol agents aiming reduction of *F. verticillioides* growth and Fumonisin B1 accumulation on maize grain.

Key-words: Rhizobacteria, Biocontrol, Fumonisin B1, Mycotoxin, Corn, Index of Dominance.

Introduction

Maize is an important cereal worldwide and grain production is mainly destined for animals and humans consumption (NCGA, 2015). In the field, highly specialized phytopatogens may invade and colonize maize plants, leading to diseases and plant death. Ear and root rot of maize plants (*Zea mays* L.) caused by *Fusarium verticillioides* is one of the most destructive diseases, causing yield and quality losses worldwide (Fandohan et al., 2003; Presello et al., 2008). In addition to direct damage to the plant, this phytopathogen may produce fumonisin B1 (FB1), an hepatotoxic, genotoxic and cytotoxic mycotoxin, that has been classified by the International Agency of Research on Cancer as group 2: possibly carcinogenic to humans (IARC, 2002; Escrivá et al., 2015).

Pre-harvest colonization of maize plants by *F. verticillioides* occurs mainly by root infection and systemic invasion, through insect damaged areas or direct penetration on the ear silks during flowering (Munkvold et al., 2003). Although less common, during storage, variations on grain water activity (a_w), silo temperature and native microbiota can led to fungal proliferation on the grain mass (Lacey & Magan, 1991; Mylona et al.,

2012). Methods of fungal control are extremely necessary to minimize fungal damage and mycotoxin accumulation, avoiding human and animal exposure to FB1 (Chulze, 2010).

Alternative control methods emerged to minimize the impacts caused by synthetic fungicides, aiming a more environmental friendly agricultural production while restraining the phytopathogens damage. Biological control using native microorganisms is one of the most promising control methods due to its low environmental impact and high efficacy on fungal control (Chulze et al., 2014). Although this method is mostly used at pre-harvest by direct application of the biocontrol agents on soil or plant leaves and ear, post-harvest usage is also an option via application into grain mass in storage (Nesci et al., 2005).

Soil bacteria present several of the important characteristics a biocontrol agent needs to succeed: high competitive profile and antifungal production ability (Vining, 1990; Bluma & Etcheverry, 2006). Rhizobacteria belonging to *Bacillus* genus are excellent biocontrol agent candidates, since they are one of the most antifungal-compound producing bacteria and have high competitive characteristics, being able to survive adverse environmental changes due to its spore-forming ability (Silo-Suh et al., 1994; Bluma & Etcheverry, 2006; Ongena & Jacques, 2008; Pérez-García et al., 2011).

Changes in environmental conditions are decisive to the survival and dominance of one microbial species over another, since they profoundly affect physiology and metabolite production (Nesci et al., 2005). On selecting potential biocontrol agents, the interaction between them and the target phytopathogen must be elucidated under a different set of environmental conditions, to predict possible dominance and survival changes that directly interfere on biocontrol efficacy (Petterson et al., 1998; Nesci et al., 2005; Bluma and Etcheverry, 2006). In addition, when aiming the control of toxigenic fungi, the biocontrol agent and its metabolites should not enhance mycotoxin production to avoid jeopardizing toxicological safety (Nesci et al., 2005).

The objective of the present study was evaluate the *in vitro* biological interactions between three *Bacillus* spp. biological control candidates and fumonisin B1 producer *Fusarium verticillioides*, evaluating fungal growth rate, conidial production, germination and survival as well as dominance status on a different set of environmental conditions.

Also were evaluated the effect of the biocontrol agent candidates on *F. verticillioides* growth and fumonisin B1 production on maize grain *in situ*.

Material and Methods

Fungal and bacterial isolates

Bacillus safensis RF69, *Bacillus amyloliquefaciens* RP103 and *Bacillus subtilis* RP242 strains used in this study were previously isolated from maize root system as fungal antagonists. Bacterial strains were maintained in *deep freezer* (-80 °C), in tubes containing aqueous glycerol 300 ml l^{-1}. Two isolates of fumonisin B1 producer *Fusarium verticillioides* previously isolated from maize grains were used on this study. Stock cultures were stored in 150 ml l^{-1} aqueous glycerol.

Inoculum preparation

For preparation of inoculum, bacterial isolates were grown overnight on 523 broth (Kado & Heskett, 1970) and cell concentration were adjusted for 10^9 CFU ml^{-1} by serial dilutions. Fungi were grown on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) at 25 °C for 7 days and the colonies were washed with 10 ml peptone water (0.1%) to create spore suspensions or used for removal of agar plugs (5 mm) from the border of growing colonies with a cork borer (Pitt & Hocking, 2009). Spore suspensions were measured on Neubauer chamber and adjusted for 10^6 or 10^3 spores ml^{-1} by serial dilutions.

In vitro interaction studies

Effect on mycelial growth rate

The effect of interaction with bacterial isolates on *F. verticillioides* mycelial growth rate was evaluated by the method proposed by Bluma and Etcheverry (2006). Briefly, 1 ml aliquots of *Bacillus* spp. suspensions were inoculated on Petri plates on final concentration of 10^9 cells ml^{-1} and 19 ml of melted Maize Meal Extract Agar (MMEA) was mixed on the plates (Marín et al., 1995). After solidification, *F. verticillioides* mycelium agar plugs were inoculated on the center of the plates. Treatments and control

plates (*F. verticillioides* growing alone) were incubated for 7 days at 25 ºC in triplicates and the assay was repeated three times.

Diameter of growing mycelium was evaluated daily by measurement of two radii at right angles to another with a common ruler (Cavaglieri et al., 2005a; Bluma & Etcheverry, 2006). Mycelium radial growth rate (cm day^{-1}) was subsequently calculated by linear regression and the relative reduction of mycelial growth was calculated by the formula proposed by Gong et al. (2014).

Conidial production

Cultures from mycelial growth rate experiment grown for seven days were subsequently used to evaluate the effect of *Bacillus* spp. on *F. verticillioides* conidial production. Cultures were washed two times with 5 ml peptone water (0.01%) and harvested three times using a glass handle. Conidial suspensions (10 ml) were submitted to counts (conidia ml^{-1}) on a Neubauer Chamber. After counting, total conidial number was calculated and the concentration of conidia per cm^2 of mycelium was determined.

Conidial germination and survival

Effect of *Bacillus* spp. on conidial germination assay was conducted on 2 ml Eppendorf-type tubes containing 500 µl of sterile peptone water (0.01%). Bacterial suspensions aliquots (250 µl, 10^9 cells ml^{-1}) and filtered *F. verticillioides* conidial suspensions (250 µl, 10^6 cells ml^{-1}) were simultaneously inoculated on tubes and the cultures were incubated at 25 ºC for 24h (Chang et al., 2009). Control tubes with addition of sterile 523 broth instead of bacterial inoculum. Treatments were conducted in triplicates and the assay was repeated three times. After treatment 200 conidia were counted per replicate and proportion of germinated conidia was determined. A conidium was considered germinated when the germinative tube presented the double of conidial length. The formula proposed by Gong et al. (2014) was used to calculate germination inhibition percentage

After conidial counting, 100 µl of each replicate was inoculated in duplicates on the surface of plates containing Dichloran Rose Bengal Chloramphenicol Agar (DRBC) to determine conidial survival percentage (Pitt & Hocking, 2009). Plates were incubated at 25 ºC for 5 days and after that, colony forming units (CFUs) were counted.

Index of Dominance (I_D)

Water activity of basic MMEA medium (0.999 a_w) was adjusted to 0.982, 0.955 and 0.937 by addition of known amounts of non-ionic glycerol (Dallyn & Fox, 1980; Nesci et al., 2005). *Bacillus* spp. suspensions (10 µl, 10^9 cells ml^{-1}) were inoculated on one border of the Petri plate and a 5 mm agar plug containing growing *F. verticillioides* mycelium was inoculated on the opposite border of the plate, on a dual culture model. Plates (in triplicates) were incubated at 25 ºC for 7 days and the interaction of each dual culture was examined macroscopically. The type of interaction was determinaded using the following I_D scores: 1/1: mutual intermingling, 2/2: mutual inhibition on contact, 3/3: mutual inhibition at a distance, 4/0: dominance of one species on contact, 5/0: dominance at a distance (Magan & Lacey, 1984; Penna et al., 2004; Nesci et al., 2005). The assay was repeated three times.

Maize grain interaction studies

Inoculation and culture media

Petri plates were filled with 70 g of autoclaved maize grains and centrally sprayed with 1 ml of each *F. verticillioides* suspensions (10^6 conidia ml^{-1}) and *Bacillus* spp. suspensions (10^9 cells ml^{-1}). Treatment and control plates (sprayed with sterile 523 broth instead of bacterial suspensions) were incubated in triplicates at 25 ºC for 10 days.

F. verticillioides viable cell counts

Dilution plating and surface spreading techniques were used for fungal counts. Samples of inoculated maize (10 g) was diluted on 90 ml of peptone water (0.01%), shaken of orbital shaker for 30 minutes, diluted serially and aliquots were inoculated on Petri plates containing DRBC for fungal counts (Bluma & Etcheverry, 2006). Colonization of maize was assessed as CFU g^{-1} maize.

Fumonisin B1 extraction, clean up and quantification

FB1 extraction was conducted by the method proposed by Garcia et al. (2012) with a few modifications. Briefly, 10g of milled inoculated maize kernels were extracted with 15 ml of methanol:acetonitrile:water (25:25:50, v/v/v) + 1 g of NaCl and shaken on orbital shaker for 20 minutes. Extract was filtered, mixed with 40 ml of phosphate buffered saline (PBS) solution and cleaned on an imunoaffinity column FumoniTest (VICAM, Watertown, MA, USA). Column was washed with 20 ml of PBS solution, left to dry for

15 minutes, eluted with 3 ml of methanol:water (50:50, v/v) and evaporated to dryness. Samples were resuspended on 1% formic acid acetonitrile/water solution (1:1, v/v) before liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

The method of Oliveira et al. (2015) was used for fumonisin B1 analysis by liquid chromatography-mass spectrometry (LC-MS/MS). Briefly, a Zorbax C18, 150 x 4.6 mm, 5 mm (Agilent Technologies Inc., USA) column with a C18 pre-column cartridge was used, with a flow rate of 0.8 mL/min, column temperature of 40 ºC and injection volume of 5 mL. Gradient elution was performed using water as eluent A and acetonitrile as eluent B, both acidified with 1% formic acid. The MS source dependent parameters were: curtain gas (CUR) 20 L/min; collision-activated dissociation gas (CAD) was set to medium; source temperature 650 ºC, dry gas 1 (GS1) 50 L/min, dry gas 2 (GS2) 45 L/min, and the spray voltage was set to 5500 V. The recovery obtained by this method was 98.8% for fumonisin B1 (Oliveira et al., 2015).

Statistical analyzes

One-way ANOVA was applied to data analyzes, using Statistica 10.0 (StatSoft, Inc. (2011) software for Windows. Tuckey test was conducted for treatments mean comparison using $p<0.05$ significance level and linear regression analyzes were conducted to calculate fungal mycelial growth rate.

Results

The results of *in vitro* interaction between *Bacillus* spp. and *Fusarium verticillioides* are shown on Table 1. All bacteria significantly ($p<0.05$) reduced final mycelium diameter, as well as mycelial growth rate between 53 and 92%. All biocontrol agents also significantly reduced conidial production, with *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 being more effective in comparison with control treatment and isolate *B. safensis* RF69.

Microscopic observation showed that all bacterial treatments significantly impaired conidial germination, resulting in short fungal tube germs or no tube emergence. The inhibition of conidial germination ranged between 98 and 99% after 24h of treatment

(Table 1). Conidial survival was also significantly reduced by all *Bacillus* spp. treatments, which reduced in 100 times conidia survival when exposed to bacterial isolates (Table 1).

The I_D scores between *F. verticillioides* and *Bacillus* spp. isolates on MMEA medium are shown on Table 2. Two different profiles of dominance were observed: major dominance at distance of *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 towards *F. verticillioides* on all water activities and temperatures tested and the reduction of dominance by *B. safensis* RF69 when situations of water stress and temperature decrease were imposed. Biocontrol agents *B. amyloliquefaciens* RP103 and *B. safensis* RP242 shown to be more stable to environmental changes, maintaining antifungal production on all temperatures with the exception of 20 ºC, where only dominance at contact (4/0) was observed. On contrary, *B. safensis* RF69 shown dominance on contact or at distance (5/0) only on high water activities and temperatures, presenting mutual intermingling (1/1) or mutual inhibition on contact (2/2) as a predominant score (Table 2).

Results from interaction evaluation on maize grain are shown on Table 3. All biocontrol agents were able to significantly reduce ($p<0.05$) fungal colonization on maize grain on ranges between 20 and 42%. Isolate *B. amyloliquefaciens* presented the lowest fungal counts and therefore, higher efficiency on controlling fungal growth when comparing to the other treatments. Although some mycelial growth was observed, maize grains treated with biocontrol agents presented a healthier aspect when compared to the control treatment, which showed extensive fungal growth after 10 days of incubation. Biocontrol agents also significantly decrease Fumonisin B1 content on maize grains inoculated with *F. verticillioides* (Table 3). Reduction of 45, 81 and 82% of F B1 content was observed when maize grain was treated with *B. safensis* RF69, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242, respectively, when compared with control treatments, which produced high amounts of FB1 on untreated maize samples. In addition to reduction of final FB1 numbers, FB1 per fungal CFU was also reduced (Table 3), indicating reduction of fumonisin production by evaluated the biocontrol agents.

Discussion

The present work provides information regarding the interactions of three *Bacillus* spp. potential biocontrol agents and fumonisin B1 producer *F. verticillioides in vitro* and *in situ* on maize grain. The isolates used as biocontrol agents on this study showed promising

characteristics to be used aiming reduction of *F. verticillioides* growth, dissemination and mycotoxin production.

All biocontrol agents were able to reduce *F. verticillioides* growth rate and therefore, final mycelium diameter. Isolates *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 showed to be extremely effective on controlling *F. verticillioides* growth, reducing mycelial growth by 90 and 92%, respectively. On the remaining mycelium growing, morphological alterations on hyphae and colony characteristics were observed (*data not shown*), indicating relevant effects of the biocontrol agents towards the fungal physiology. Inhibition of fungal growth rate often delays *lag phase* (adaptation to the environment phase), reducing change to survival and fungal competitiveness (Cavaglieri et al., 2005a).

The ability to reduce conidial production, germination and survival was also observed to all evaluated biocontrol agents. *Fusarium verticillioides* can infect maize grain by root colonization or direct penetration on maize silks and ears by air-carried conidia, and therefore, reduction of both production and availability of conidia is an effective control method since the fungal initial inoculum is highly affected by the biocontrol agents (Munkvold et al., 2003). In addition, decrease of spore survival indicates fungicide rather than fungistatic activity, which resulted in better control due to cell death instead of interruption of growth. Similar results were observed by Alvindia et al. (2013), which reported intense conidial germination reduction of *F. verticillioides* and other fungi by a strain of *B. amyloliquefaciens*. The authors highlighted that inhibition of conidial germination is one of the main control methods for fungal diseases, since it prevents further development and infection of inner tissues.

The index of dominance show the interspecific interactions between microbial species under a set of environmental conditions, these interactions were greatly influenced by water activity and temperature (Cavaglieri et al., 2004). The I_D experiments conducted clearly showed two dominance patterns by the biocontrol agents. While *B. safensis* RF69 had its dominance towards *F. verticillioides* reduced on decreasing temperature and water activity, *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 maintained a dominant behavior even on low temperatures and water stress conditions. *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 shown fungal inhibition at distance (5/0) on higher temperatures and water activities and inhibition on contact (4/0) mainly on 0.937 a_w. Probably due to the spore-forming ability, *B. amyloliquefaciens* RP103 and *B. subtilis*

RP242 were able to survive and compete well under harsh environmental conditions, suggesting that both isolates probably will not lose efficacy if used as biocontrol agents on the field if subjected to drastic environmental changes. Our results are in agreement with Vining's theory (1990), which affirms that microorganisms derived from adverse environments are more suitable to be used as biocontrol agents due to their ability to survive and compete even on extreme environmental conditions.

All biocontrol agents were also able to significantly reduce ($p<0.05$) *F. verticillioides* growth and fumonisin production on maize grains, suggesting that application of a bioformulated fungicide directly on maize grains may be an effective control method. Growth reduction between 20 and 42% was observed on maize grains treated with biocontrol agents in comparison to control plates without bacterial treatment. That is in agreement with other studies that reported fungal growth reduction on maize grains treated with *B. amyloliquefaciens* and other strains of *Bacillus* sp. (Cavaglieri et al., 2005b; Pereira et al., 2007, 2010).

When reducing fungal growth, often toxin production is increased or not affected, leading to decreased fungal counts but no alteration on final toxin levels on grains (Magan & Olsen, 2004; Al-Saad et al., 2016). The results obtained in this study shown that both final concentrations of FB1 and FB1 concentration per CFU were significantly reduced ($p<0.05$), indicating that the biocontrol agents are effective on controlling *Fusarium* growth and FB1 production on maize grains. Biocontrol agents *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 showed to be the most effective affecting FB1 production, being able to reduce FB1 final concentrations by 81 and 82% and FB1 per CFU by 70 and 77%, respectively. Our results are in agreement with those obtained by Pereira et al. (2010). The authors reported reduction of FB1 on maize ears treated by a strain of *B. amyloliquefaciens*, although their experiments were conducted with direct application of the biocontrol agents on maize ears during flowering instead of *in situ* maize grains.

Accordingly to Choudhary (1992), reduction of toxins levels can be attributed to one or a combination of the following reasons: Physical competition for space and nutrition, competition with the fungus for a substrate required for toxin production but not growth, induction for a change in the biochemical environment influencing the metabolic pathway available to the toxigenic fungi and degradation of FB1 following its formation.

In addition, although FB1 content on control plates (maize grains inoculated only with *F. verticillioides*) were lower than those limited by actual Brazilian legislation, the values observed were higher than those limited by the European Union for unprocessed maize grains (2000 μg kg^{-1}). The biocontrol agents were able to reduce FB1 to values permitted by both legislations implicating in the acceptance of the grains treated by these bacteria on both commercial regions (European Union, 2006; ANVISA, 2011).

The results presented in this study clearly indicate that the biocontrol agents evaluated have promising characteristics for the control of *F. verticillioides* growth and dissemination *in vitro* and on grains, being effective on reducing final concentrations of FB1 on maize. In addition, biocontrol agents *B. amyloliquefaciens* RP103 and *B. subtilis* RP242 were able to maintain a high competitive profile on harsh environmental conditions, being suitable for usage both on the field and on storage aiming fungal and mycotoxin control. Further studies need to be conducted to evaluate the effect of the biocontrol agents on *F. verticillioides* colonization and FB1 accumulation on maize plants on a field scale to confirm the potential usage of these biocontrol agents.

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Table 1. Effect of *in vitro* interaction of *Bacillus* spp. and *F. verticillioides* on fungal mycelial growth rate, conidial production, germination and survival.

	Colony diameter (cm)	Mycelial growth rate (cm day^{-1})	Mycelial growth reduction (%)	Conidial production (log conidia per cm^2 of mycelium)	Conidial germination inhibition (%)	Spore survival (log UFC ml^{-1})
Control	9.07 ± 0.06 a	1.26 ± 0.01 a	0 a	6.24 ± 2.75 a	0 a	5.74 ± 2.34 a
B. safensis RF69	4.23 ± 0.21 b	0.74 ± 0.03 b	53.36 ± 2.29 b	5.49 ± 2.57 b	98 ± 0.45 b	3.81 ± 1.04 b
B. amyloliquefaciens RP103	0.82 ± 0.02 c	0.10 ± 0.01 c	90.96 ± 0.17 c	5.04 ± 2.35 c	98 ± 0.43 b	3.49 ± 1.72 b
B. subtilis RP242	0.70 ± 0.01 c	0.11 ± 0.01 c	92.28 ± 0.06 c	5.14 ± 2.48 c	99 ± 0.42 b	3.39 ± 1.28 b

Different letters within the same column indicate significant difference ($p \leq 0.05$)

Table 2. Indices of Dominance (I_D) between bacterial strains and fumonisin B1 producer *Fusarium verticillioides* under different water activities and temperatures on Maize Meal Extract agar.

Rhizobacterial isolates	a_w	Temperature			
		20 °C	25 °C	30 °C	I_D
B. safensis RF69	0.999	2/2	5/0	4/0	11/2
	0.982	2/2	4/0	5/0	11/2
	0.955	1/1	2/2	4/0	7/3
	0.937	1/1	2/2	2/2	5/5
	I_D	6/6	13/4	15/2	68/24
B. amyloliquefaciens RP103	0.999	5/0	5/0	4/0	14/0
	0.982	5/0	5/0	5/0	15/0
	0.955	4/0	5/0	5/0	14/0
	0.937	4/0	4/0	5/0	13/0
	I_D	18/0	19/0	19/0	112/0
B. subtilis RP242	0.999	5/0	5/0	5/0	15/0
	0.982	4/0	5/0	5/0	14/0
	0.955	4/0	5/0	5/0	14/0
	0.937	4/0	5/0	5/0	14/0
	I_D	17/0	20/0	20/0	114/0

Index of Dominance (I_D):

1/1: Mutual intermingling

2/2: Mutual inhibition on contact

3/3: Mutual inhibition at a distance

4/0: Dominance of one species on contact

5/0: Dominance at a distance.

I_D: Bacterial isolates/*Fusarium verticillioides*

Table 3. Effect of bacterial treatments of maize grains on *Fusarium verticillioides* counts and Fumonisin B1 accumulation.

	Fungal counts (log CFU g^{-1} maize)	Growth reduction (%)	Fumonisin B1 (µg kg^{-1})	Fumonisin B1 per CFU (µg CFU^{-1})
Control	7.84 ± 2.07 a	0	2840 ± 280 a	361.92 a
B. safensis RF69	6.91 ± 2.64 b	20.2	1550 ± 71 b	224.15 b
B. amyloliquefaciens RP103	4.96 ± 1.91 d	42.7	535 ± 121.6 c	107.77 c
B. subtilis RP242	6.36 ± 1.93 c	26.6	511.5 ± 75.7 c	80.40 d

Different letters within the same column indicate significant difference ($p \leq 0.05$)

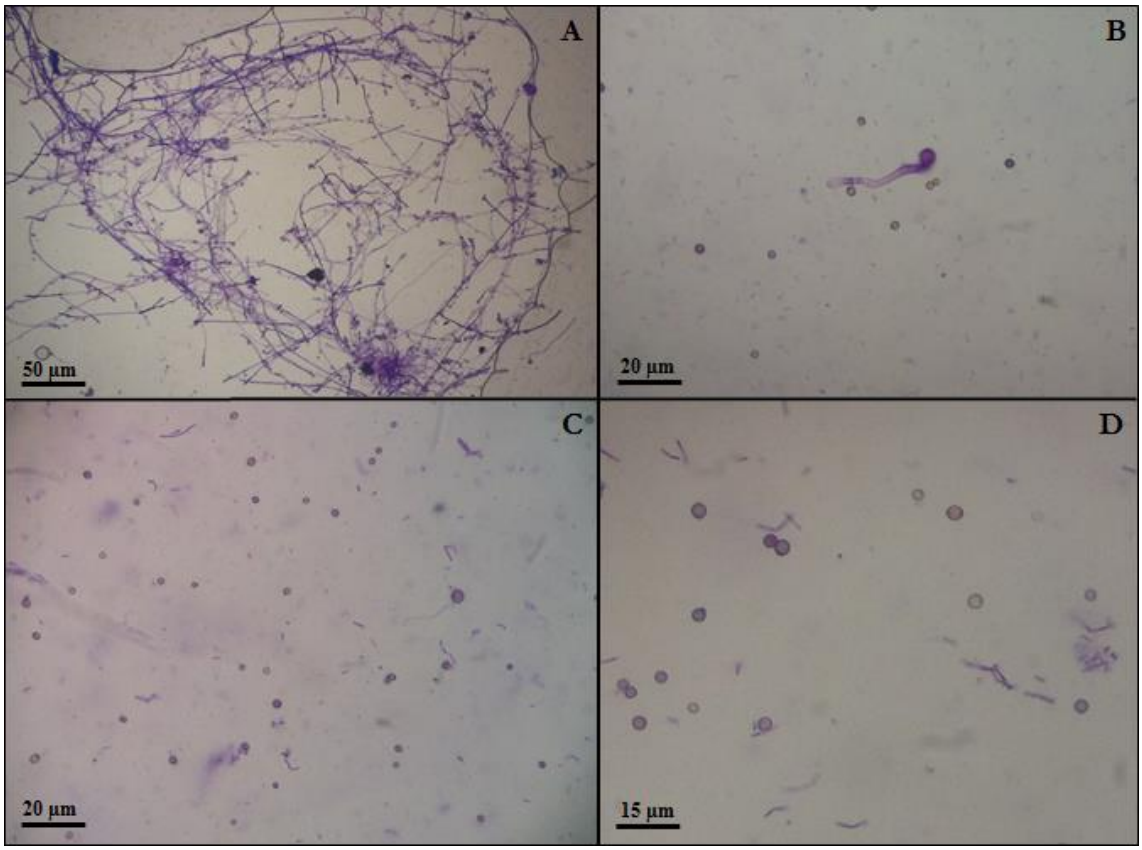
4 CONCLUSÕES

- A hipótese inicial do projeto, ou seja, a existência de bactérias no sistema radicular de plantas de milho com grande potencial para o biocontrole de *A. flavus* e *F. verticillioides* foi confirmada;
- Isolados apresentam as características ideais para o desenvolvimento de um bioformulado objetivando o controle micotoxigênico: Requerimento nutricional baixo, tempo de geração curto, rápida multiplicação e proliferação, alta capacidade competitiva e de sobrevivência, intensa atividade antifúngica em diferentes substratos, estabilidade fisiológica e manutenção da eficácia antifúngica.
- Os isolados demonstraram-se extremamente promissores para o biocontrole dos fungos toxigênicos por restringirem o crescimento, reprodução e disseminação fúngica, sendo eficazes em todas as etapas do ciclo de vida de *A. flavus* e *F. verticillioides*;
- A iturina A é um dos mecanismos antifúngicos utilizados pelos isolados *B. amyloliquefaciens* RP103 e *B. subtilis* RP242;
- A estabilidade fisiológica de *B. amyloliquefaciens* RP103 e *B. subtilis* RP242 garantem a manutenção da eficácia antifúngica em condições de stress hídrico e térmico;

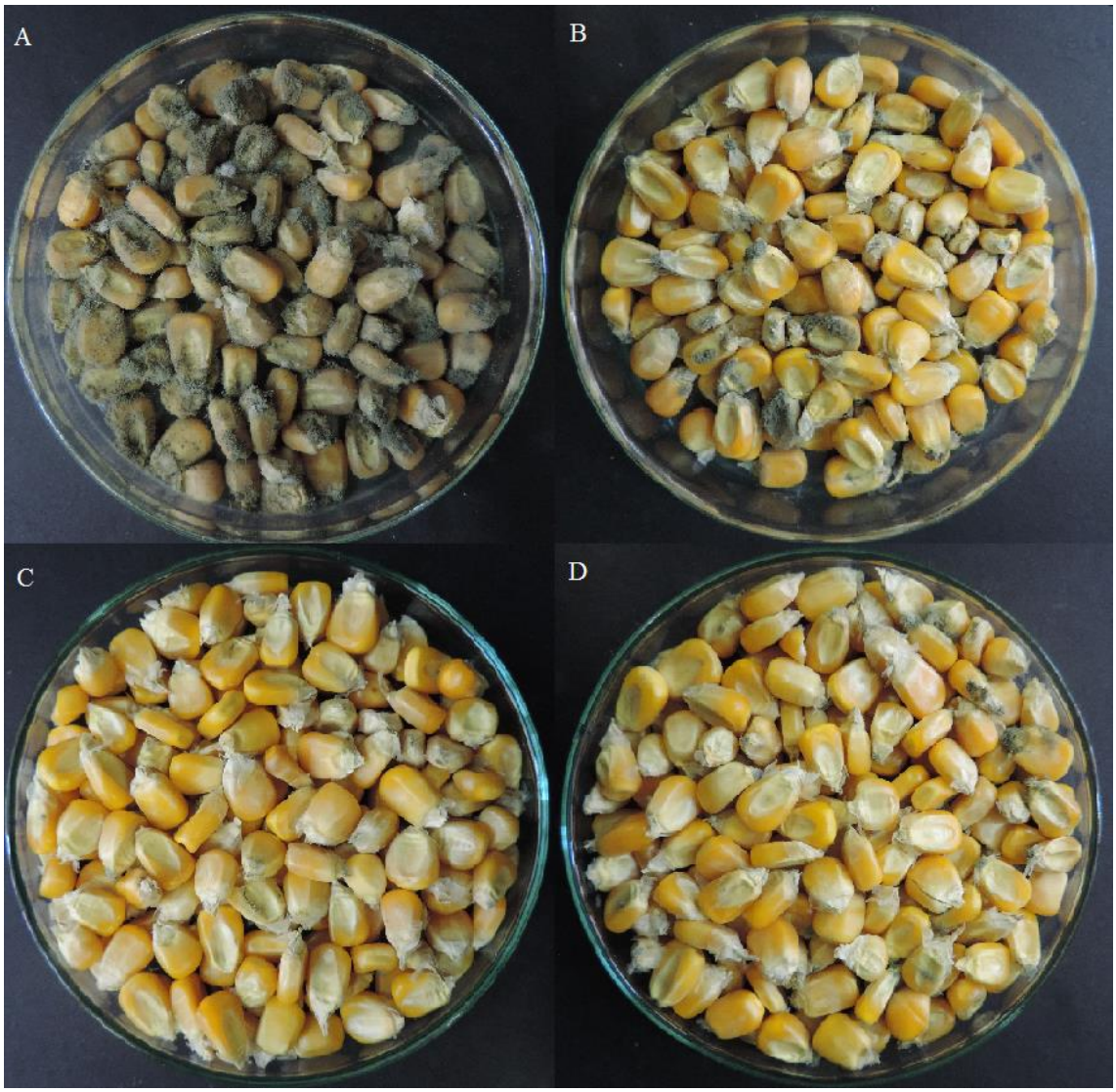
- Os três isolados selecionados são eficazes em reduzir a contaminação fúngica e o acúmulo de aflatoxina B1 e fumonisina B1 nos grãos de milho até níveis permitidos pelas legislações Brasileira e Europeia;
- Os resultados observados *in vitro, in situ* e em solo não-rizosférico, indicam a aplicação dos agentes de biocontrole no campo, através da pulverização no solo objetivando a redução do inoculo fúngico inicial e a contaminação ambiental; no tratamento de sementes, objetivando proteção direta das plântulas e reduzindo a colonização radicular e consequentemente sistêmica e; no armazenamento por pulverização na massa de grãos buscando reduzir o desenvolvimento fúngico e o acúmulo de micotoxinas;

5 APÊNDICES

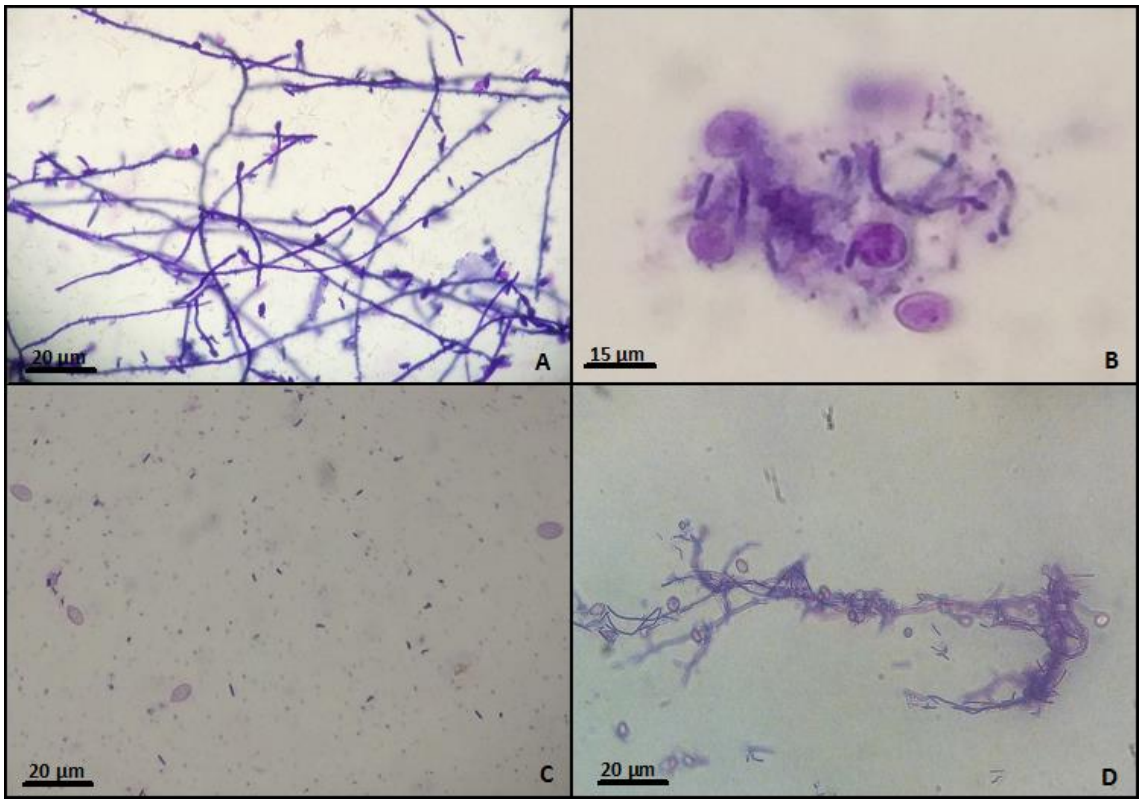
APÊNDICE 1. Efeito *in vitro* dos agentes de biocontrole na germinação de conídios de *Aspergillus flavus*. A: Controle sem o tratamento bacteriano; *B: Bacillus safensis* RF69; C: *B. amyloliquefaciens* RP103 e; D: *B. subtilis* RP242.



APÊNDICE 2. Placas contendo grãos de milho pulverizadas com os agentes de biocontrole e *Aspergillus flavus*. A: Placas controle sem a inoculação bacteriana; B: *Bacillus safensis* RF69; C: *Bacillus amyloliquefaciens* RP103; D: *Bacillus subtilis* RP242.



APÊNDICE 3. Efeito *in vitro* dos agentes de biocontrole na germinação de conídios de *Fusarium verticillioides*. A: Controle sem o tratamento bacteriano; *B: Bacillus safensis* RF69; C: *B. amyloliquefaciens* RP103 e; D: *B. subtilis* RP242.



APÊNDICE 4. Placas contendo grãos de milho pulverizadas com os agentes de biocontrole e *Fusarium verticillioides*. A: Placas controle sem a inoculação bacteriana; B: *Bacillus safensis* RF69; C: *Bacillus amyloliquefaciens* RP103; D: *Bacillus subtilis* RP242.