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DESENVOLVIMENTO DE FORMULAÇÕES DE *Trichoderma* PARA USO NA PROMOÇÃO DE CRESCIMENTO DE PLANTAS E CONTROLE DE *Sclerotinia sclerotiorum*

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Tese apresentada à Faculdade de Ciências Agronômicas da Unesp Câmpus de Botucatu, para obtenção do título de Doutor em Agronomia/Proteção de Plantas.

Orientador: Dr. Wagner Bettiol

Coorientadora: Cristiane Sanchez Farinas

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TÍTULO DA TESE: DESENVOLVIMENTO DE FORMULAÇÕES DE *Trichoderma* PARA USO NA PROMOÇÃO DE CRESCIMENTO DE PLANTAS E CONTROLE DE *Sclerotinia sclerotiorum*

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RESUMO

Fungos do gênero *Trichoderma* apresentam um complexo arsenal de mecanismos envolvidos na proteção de plantas, os quais incluem supressão de fitopatógenos, promoção de crescimento e mitigação de estresses abióticos em plantas. Para tanto, a seleção dos isolados é de fundamental importância, pois *Trichoderma* spp. são altamente diversificadas em eficácia na supressão de patógenos de plantas, apresentando respostas variadas de acordo com as cepas que estão sendo confrontadas. Outros desafios são relacionados à multiplicação, ao armazenamento, e ao desenvolvimento de formulações estáveis e com vida de prateleira adequada. Vencer esses desafios colaborará para a disponibilização de produtos com qualidade adequada no mercado. O presente trabalho teve como objetivos selecionar isolados de *Trichoderma* spp. promotores de crescimento em algodoeiro e inibidores da germinação de escleródios de *Sclerotinia sclerotiorum;* otimizar a produção de *Trichoderma asperelloides* em farinha de arroz e; desenvolver formulações granulares à base de farinha de arroz. *Trichoderma asperelloides* CMAA 1584 apresentou maior eficiência no controle de *Sclerotinia sclerotiorum*, enquanto o efeito bioestimulante no crescimento do algodoeiro foi mais pronunciado com *Trichoderma lentiforme* CMAA 1585. Na otimização da produção de *Trichoderma asperelloides* na farinha de arroz, o teor de nitrogênio (0,1% p/p) e o tipo de fermentador (Erlenmeyer) tiveram efeitos significativos na obtenção de maiores rendimentos, enquanto a melhor fonte de nitrogênio foi a levedura hidrolisada (Hilyses®). As formulações $G_{Controle}$, $G_{Break\text{-}Thru}$, $G_{Bentonita}$ e $G_{Composto\ orgânico\ +\ Break\text{-}Thru}$ foram as que formaram o maior número de unidades formadoras de colônia g^{-1} (UFC g^{-1}) após reidratação em ágar-água. A viabilidade à temperatura ambiente foi mantida estável por até 3 meses nas formulações $G_{Controle}$ e $G_{Bentonita}$, enquanto em condições refrigeradas a viabilidade foi mantida por 12 meses nas formulações $G_{Bentonita}$ e $G_{Composto\ orgânico\ +\ Break\ Thru}$. Não foram observadas diferenças significativas na inibição da germinação miceliogênica de escleródios de *Sclerotinia sclerotiorum* no solo pela aplicação da formulação $G_{Controle}$ nas doses de 5×10^4, 5×10^5 ou 5×10^6 UFC g^{-1} de solo, mantendo um índice de controle de escleródios em 79,2; 87,5; e 93,7%, respectivamente. Desta forma, pode ser considerado que *Trichoderma asperelloides* CMAA 1584 apresenta a maior eficiência no controle de *Sclerotinia sclerotiorum*, enquanto *Trichoderma lentiforme* CMAA 1585 apresenta a maior promoção de crescimento das plantas, podendo a

mistura de ambos ser usada para o controle do patógeno e como bioestimulante em plantas de algodão.

Palavras-chave: biofungicida, biofertilizante, formulação, mofo branco e fermentação sólida

ABSTRACT

Fungi of the genus *Trichoderma* present a complex arsenal of mechanisms involved in plant protection, which include suppression of plant pathogens, growth promotion and mitigation of abiotic stresses in plants. Therefore, the selection of potential isolates must be performed carefully, as *Trichoderma* spp. are highly diversified in effectiveness in suppressing plant pathogens, showing varied responses according to the strains being confronted. Other challenges are related to multiplication, storage, and development of stable formulations with adequate shelf life. Overcoming these challenges will help make products of adequate quality available on the market. The objectives of this study were to select *Trichoderma* spp. strains growth promoters in cotton and with biocontrol activity against sclerotia of *Sclerotinia sclerotiorum*, to optimize the production of *Trichoderma asperelloides* in rice flour, and to develop granular formulations based on rice flour. *Trichoderma asperelloides* CMAA 1584 is more efficient in controlling *Sclerotinia sclerotiorum*, while the biostimulating effect on cotton growth was more pronounced with *Trichoderma lentiforme* CMAA 1585. In optimizing the production of *Trichoderma asperelloides* in rice flour, the nitrogen content (0.1% w/w) and the type of fermenter (Erlenmeyer flasks) had significant effects in obtaining higher yields, while hydrolyzed yeast (Hilyses®) was the best source of nitrogen. The formulations $G_{Control}$, $G_{Break\text{-}Thru}$, $G_{Bentonite}$ and $G_{Organic\ compost\ +\ Break\text{-}Thru}$ were those that formed the highest number of colonies forming unit g^{-1} (CFU g^{-1}) after rehydration in water-agar. Viability at room temperature was maintained stable for up to 3 months in $G_{Control}$ and $G_{Bentonite}$ formulations while under refrigerated conditions, viability was maintained for 12 months in $G_{Bentonite}$ and $G_{Organic\ compost\ +\ Break\text{-}Thru}$ formulations. No significant differences were observed in the inhibition of the mycelogenic germination of *Sclerotinia sclerotiorum* in soil by the application $G_{Control}$ formulation at doses of 5×10^4, 5×10^5 or 5×10^6 CFU g^{-1} of soil, maintaining a sclerotia control index of 79.2; 87.5; and 93.7%, respectively. Thus, *Trichoderma asperelloides* is more efficient in controlling *Sclerotinia sclerotiorum*, while *Trichoderma lentiforme* is more suitable as a biostimulant in cotton plants.

Keywords: biofungicide, biofertilizer, formulation, white mold and solid-state fermentation

SUMÁRIO

INTRODUÇÃO GERAL

Aliar compromissos ambientais e a necessidade de crescente oferta de alimentos, fibras, bioenergia e uma variedade de matérias primas e produtos é apontado como um dos principais desafios do agronegócio global (GODFRAY et al., 2010). Segundo estimativas, o agronegócio contemporâneo deverá sustentar uma população mundial de cerca de oito a nove bilhões de pessoas entre 2022 e 2050 (SMITH; GREGORY, 2013; GU et al., 2021), como resultado, a demanda mundial por calorias e proteínas para este mesmo período deverá mais que dobrar (TILMAN et al., 2011).

Nesse contexto, as doenças de plantas têm papel fundamental, pois são consideradas como uma das mais sérias ameaças à produção de alimentos e à segurança alimentar em todo mundo (FAO, 2017; ZAKI et al., 2020). Embora seja difícil quantificar precisamente as perdas em produtividade, segundo a Organização das Nações Unidas para a Alimentação e a Agricultura (FAO), é estimado que 20 a 40% da produção global de alimentos seja perdida anualmente devido a ação de pragas e doenças (FAO, 2017). Desse modo, visando proteger as lavouras de possíveis perdas de safra e quedas na qualidade de seus produtos, os pesticidas químicos são extensivamente utilizados nos sistemas agrícolas globais (DAMALAS, 2009). Contudo, em virtude de seus recorrentes efeitos adversos à saúde humana (VAN MAELE-FABRY et al., 2010; KIM et al., 2017), à segurança alimentar (VERGER; BOOBIS, 2013) e à manutenção da biodiversidade (BEKETOV et al., 2013) a redução de sua dependência no manejo de pragas e doenças é apontada como um dos pilares para o desenvolvimento de uma agricultura mais sustentável.

Diante disso, a busca por alternativas de manejo fitossanitário menos agressivas ao meio ambiente tem crescido de interesse entre cientistas, sociedade e indústria. Dentre as medidas propostas para auxiliar nesse processo, a utilização de microrganismos tem se mostrado como uma abordagem promissora, pois é um método seguro, economicamente vantajoso, de baixo impacto no meio ambiente e na saúde humana, e com risco mínimo para organismos benéficos não alvo, como abelhas, minhocas e predadores naturais, que são alguns dos principais agentes fornecedores de serviços ecossistêmicos (GLARE et al., 2010; VAN LENTEREN et al., 2018). Além disso, muitas espécies de microrganismos possuem importantes funções ecológicas e contribuem de forma significativa no crescimento de diversas

culturas agrícolas (SHARMA et al., 2013; ALORI et al., 2017), participando da decomposição e mineralização dos resíduos vegetais (RICHARDSON et al., 2009; BONONI et al., 2020), aumentando a biomassa vegetal e os teores de nutrientes no solo (BONONI et al., 2020), assim como controlando diversos fitopatógenos e pragas (MANIANIA et al., 2003; ZHANG et al, 2016), os quais impactam diretamente a obtenção de maiores produtividades.

De acordo com o relatório publicado pela Research and Market (2022), o mercado global de biopesticidas está projetado para crescer a uma taxa de 13,7% ao ano, saltando de um valor estimado de US$ 12,9 bilhões em 2022 para US$ 24,6 bilhões em 2027. Segundo o Business Intelligence Panel, análise realizada anualmente pela consultoria Spark Inteligência Estratégica, a comercialização de bioinsumos no Brasil cresceu 37% na safra 2020/2021 em relação à 2019/2020, e já é responsável por movimentações financeiras da ordem de R$ 1,7 bilhão. Como resultado 21% das áreas cultivadas com soja no Brasil já fazem uso de biodefensivos, totalizando aproximadamente 7,9 milhões de hectares (SPARK INTELIGÊNCIA ESTRATÉGICA, 2020).

Dentre os fitopatógenos de maior importância agrícola, o fungo *Sclerotinia sclerotiorum* (Lib.) De Bary, agente etiológico do mofo-branco, é considerado como um dos mais devastadores e cosmopolitas, sendo capaz de infectar mais de 400 espécies de plantas (BOLAND; HALL, 1994; BOLTON et al., 2006). Apontado como a segunda doença mais importante da sojicultura no mundo (PELTIER et al., 2012), somente nos EUA, 2,8 milhões de toneladas de perdas foram estimadas entre os anos de 2010 e 2014, o que custou aos agricultores cerca de US$ 1,2 bilhão (ALLEN et al. 2017; USDA-NASS, 2017). No Brasil, maior produtor mundial do grão, a doença causa perdas significativas com epidemias de alta prevalência e severidade, especialmente em regiões com altitudes acima de 600 m (MEYER et al., 2014). Outro agravante, é que *S. sclerotiorum* é endêmico em aproximadamente 27% das áreas de produção de soja no Brasil (MEYER et al., 2020), o que pode resultar em perdas econômicas de até US$ 1,47 bilhão anualmente (LEHNER et al., 2017). As perdas de rendimento são causadas principalmente pela redução da quantidade e do peso dos grãos, resultante do apodrecimento dos tecidos da planta. Para cada ponto percentual de aumento da incidência de mofo-branco ocorre uma redução média na produtividade da soja de 17,2 kg ha^{-1}, e um incremento na produção de escleródios de 100 g ha^{-1} (LEHNER et al., 2017). Não obstante, em razão do grande número de plantas susceptíveis ao

patógeno e a maioria dos cotonicultores brasileiros cultivarem o algodão na segunda safra, isto é, após a colheita da soja, a doença também tem infligido perdas na cotonicultura nacional (SILVA et al., 2019; IMEA, 2021). Dessa forma, devido a sucessão de cultivos suscetíveis ao mofo branco, a incidência da doença em áreas de ocorrência do patógeno tem aumentado consideravelmente.

De acordo com a Portaria nº 5, de 21 de agosto de 2015, do Departamento de Sanidade Vegetal/Ministério da Agricultura, Pecuária e Abastecimento (DSV/MAPA), *S. sclerotiorum* é considerada como uma das oito pragas/doenças de maior risco fitossanitário para o Brasil, para as quais o desenvolvimento e o registro de tecnologias de controle devem ser priorizados (BRASIL, 2015). Dentre os maiores desafios no manejo da doença, a redução do número de escleródios no solo está entre os principais, haja vista sua grande persistência e alta produção de ascósporos (inóculo inicial) (ADAMS; AYERS, 1979; WILLETTS; WONG, 1980; MEYER et al., 2022). Escleródios são estruturas de resistência formados por agregados de hifas e consistem principalmente de uma camada externa com células melanizadas, o que confere alta resistência às condições ambientais adversas e a degradação química, e um componente interno formado de carboidratos (principalmente β-1,3-glucanos) e proteínas (LE TOURNEAU, 1979).

Diante disso, o uso do método químico de forma não integrada a outras estratégias de manejo tem apresentado efeito limitado e inconsistente, principalmente devido às dificuldades em alcançar uma boa cobertura com fungicidas e o tempo de aplicação em relação à liberação de ascósporos (MEYER et al., 2014; MEYER et al., 2022). Além disso, a ausência de resistência genética em cultivares comerciais e a adoção de estratégias intensivas de manejo baseadas unicamente em fungicidas, resultaram no desenvolvimento de cepas resistentes para muitos ingredientes ativos (LIANG et al., 2015; MAO et al., 2018). Portanto, é imperativo explorar medidas alternativas como o controle biológico para o manejo da doença.

Como mais um importante aliado no manejo do mofo branco em cultivos agrícolas, espécies do gênero *Trichoderma* vêm sendo utilizadas há vários anos e com sucesso. O impacto do uso do *Trichoderma* no manejo de *S. sclerotiorum* está relacionado à sua capacidade de parasitar e degradar escleródios no solo, havendo uma correlação inversamente proporcional da frequência de aplicação de *Trichoderma* com a viabilidade de escleródios no solo (FERRAZ; NASSER; CAFÉ-FILHO, 2011; MEYER et al., 2022).

Fungos do gênero *Trichoderma* apresentam um complexo arsenal de mecanismos envolvidos na proteção de plantas, os quais incluem micoparasitismo, competição por nutrientes, antibiose e produção de enzimas hidrolíticas (LORITO et al., 2010; DRUZHININA et al., 2011; HERMOSA et al., 2012; MONTE et al., 2019). Além disso, devido à plasticidade de seus genomas em expressar múltiplas funções ecológicas, várias espécies de *Trichoderma* promovem o crescimento de plantas (RUBIO et al. 2017; MONTE et al. 2019), contribuem para a melhor utilização de nutrientes (HARMAN 2011; DOMÍNGUEZ et al. 2016) e induzem respostas de defesa contra estresses bióticos e abióticos (HERMOSA et al. 2012; BROTMAN et al. 2012; RUBIO et al. 2017; MONTE et al. 2019).

As espécies/isolados de *Trichoderma* são altamente diversificados em eficácia na supressão de patógenos de plantas (HARMAN et al., 2004, VERMA et al., 2007), apresentando respostas variadas de acordo com as cepas que estão sendo confrontadas (ATANASOVA et al., 2013). Desse modo, a seleção dos isolados consiste no primeiro passo no desenvolvimento de produtos à base deste antagonista. Além disso, atributos como virulência, persistência e tolerância à estresses abióticos (temperatura, umidade e radiação UV), assim como baixas exigências nutricionais, alta produção de propágulos infectivos e capacidade de se desenvolver em substratos simples e baratos, são de extrema importância, pois o conceito de produção em massa se baseia nas necessidades de uso inundativo, logo requerem um elevado número de propágulos a fim de atingir o alvo ou colonizar o habitat (FARIA; WRAIGHT, 2001).

A produção massal de *Trichoderma* spp. pode ser realizada de três formas: via fermentação sólida, líquida ou bifásica (MASCARIN et al., 2019). A primeira delas, também conhecida como fermentação semi-sólida ou fermentação sólida estática, o crescimento microbiano ocorre na ausência de água livre, ou seja, a umidade necessária ao seu crescimento se encontra absorvida ou complexada no interior da matriz sólida (LONSANE et al. 1985; SOCCOL 1996). Enquanto na fermentação líquida ou submersa, como o próprio nome sugere, o crescimento microbiano é realizado em soluções nutritivas líquidas (JACKSON, 1997).

No Brasil, a maioria das biofábricas utiliza o sistema de fermentação bifásica, na qual o inóculo é inicialmente produzido em cultura líquida e, posteriormente, transferido para substratos sólidos para a produção de conídios aéreos (KUMAR et al., 2007; LI et al., 2010; MASCARIN et al., 2010; WOO et al., 2014; MASCARIN et al., 2019). Nesse processo, grãos de arroz são majoritariamente utilizados como substrato

e se realiza a incubação em sacos de polipropileno ou em bandejas por um período de 10 a 14 dias, com posterior remoção dos conídios (FARIA; WRAIGHT, 2007; LI et al., 2010; MASCARIN et al., 2019). No entanto, devido às características hidrofílicas dos conídios aéreos de *Trichoderma* (JIN; CUSTIS, 2011) e a necessidade de extrair os conídios dos grãos, muitos fabricantes lavam os substratos colonizados com soluções surfactantes antes da formulação para concentrar a biomassa (FARIA; WRAIGHT, 2007; LI et al., 2010; MASCARIN et al., 2019). Entretanto, ao longo deste processo, os metabólitos que possuem propriedades antimicrobianas e/ou atuam como estimulantes vegetais são inevitavelmente perdidos. Adicionalmente, o resíduo sólido após a extração dos esporos necessita de destinação adequada, sendo geralmente explorada na produção de energia (Elias et al., 2022) ou compostagem.

Para contornar parte desse problema e reduzir os custos de produção, tendo em vista que os substratos podem representar mais de 50% dos custos de produção (ELTEM et al., 2014; STANBURY et al., 2017), diversos subprodutos e resíduos agroindustriais são frequentemente avaliados em processos fermentativos, pois são abundantes e muitas vezes subutilizados (FARINAS, 2015; SOCCOL et al., 2017). Além disso, a reutilização de subprodutos agroindustriais para a geração de novos produtos de alto valor agregado é extremamente benéfica e fomentada internacionalmente, como pode ser observado no plano de ação da União Europeia para uma economia circular (COMISSÃO EUROPEIA, 2020).

O arroz (*Oryza sativa* L.) é uma das principais culturas de cereais, bem como alimento básico para quase metade da população mundial, especialmente nos países asiáticos (BIRD et al., 2000). Contudo, até chegar à mesa do consumidor, uma série de processos são empregados para o beneficiamento do grão, os quais combinados produzem diversos subprodutos (ESA et al., 2013). O arroz quebrado, um dos subprodutos do beneficiamento do grão, representa cerca de 10-15% do arroz beneficiado (NUNES et al., 2017) e é comercializado por 30-50% do valor do grão inteiro (NUNES et al., 2017; LI et al., 2019), sendo pouco aproveitado para a alimentação humana e majoritariamente utilizado para a alimentação animal (NUNES et al., 2017).

Assim, devido ao seu baixo custo, alta disponibilidade e valor nutricional (74% amido e 7% proteína) (LIU et al., 2016), várias tecnologias têm sido propostas para aumentar seu uso na indústria (AHMED et al., 2015; BICH et al., 2018; MYBURGH et al., 2019; NAKANO et al., 2012). Dentre elas, o uso como substrato para produção

massal e como inerte em formulações de *Trichoderma* tem potencial em garantir bons sistemas de entrega e proporcionar vantagens competitivas em relação à comunidade nativa do solo, uma vez que o *Trichoderma* spp. pode hidrolisar o amido em açúcares simples e de metabolização rápida (GUIMARÃES et al., 2018; KLAIC et al., 2018) para sua nutrição. Além disso, melhorias na viabilidade e vida de prateleira de microrganismos formulados com compostos amiláceos são relatadas, em razão de seu suporte estrutural e proteção contra estresses térmicos, oxidativos e osmóticos (CHAN et al., 2011; SCHOEBITZ et al., 2012; TAL et al., 1999).

Assim, a moagem do arroz quebrado em farinha pode se tornar uma alternativa simples, barata e livre de resíduos para o desenvolvimento de novos produtos à base de *Trichoderma*, além de remover a etapa de extração de conídios e manter os metabólitos no produto final. Contudo, para maximizar os rendimentos, a otimização das condições de cultivo é imprescindível, pois a descoberta das condições que levam a uma esporulação mais rápida, pode ser o fator mais importante na redução dos custos de produção (JACKSON, 1997).

Além dos métodos de produção, as formulações desempenham um papel fundamental na determinação do sucesso de um produto. Os componentes de uma formulação geralmente são categorizados em três partes: ingrediente ativo, veículo e adjuvantes (ASH, 2010; BURGES, 1998). O ingrediente ativo é a forma infecciosa do microrganismo (conídios, microescleródios, blastósporos, endósporos, micélios, etc.); os veículos são inertes utilizados para diluir o agente ativo; e os adjuvantes compreendem uma ampla variedade de agentes que aprimoram uma ou mais características da formulação (BURGES, 1998).

Segundo Lewis e Papavizas (1985), a adição de amido como veículo em formulações de *Trichoderma viride, Trichoderma harzianum* e *Trichoderma hamatum* aumentou em até 100 vezes o número de UFC g^{-1} de solo. Portanto, há evidências que sustentam a hipótese de que a utilização de farinha de arroz para a produção de *Trichoderma,* seguida pela formulação via extrusão/granulação, tem potencial em fornecer condições adequadas ao estabelecimento do *Trichoderma* no solo, uma vez que, o grânulo atuará como um mini reator ao seu crescimento inicial.

A apresentação dos estudos desenvolvidos nesta tese de doutorado está estruturada em dois capítulos, organizados da seguinte forma: no capítulo 1 são apresentados os resultados obtidos na seleção dos isolados, na qual foram avaliados a eficiência no parasitismo a *Sclerotinia sclerotiorum*, na germinação e o vigor de

sementes de algodão, na promoção de crescimento do algodoeiro e na solubilização de fosfato. Esse primeiro capítulo está publicado na Frontiers in Plant Science: Silva L.G., Camargo R.C., Mascarin G.M., Nunes P.S.O., Dunlap C., Bettiol W. Dual functionality of *Trichoderma*: Biocontrol of *Sclerotinia sclerotiorum* and biostimulant of cotton plants. Frontiers in Plant Science, 13:983127, 2022. doi: 10.3389/fpls.2022.983127

No capítulo 2 são apresentados os resultados da otimização da produção de *Trichoderma asperelloides* em farinha de arroz, no desenvolvimento de formulações granulares e na avaliação da vida de prateleira em condições refrigeradas e à temperatura ambiente, eficiência de biocontrole a escleródios de *Sclerotinia sclerotiorum*, e conidiação. Este capítulo é intitulado: Bioreactor-in-a-granule designed for *Trichoderma asperelloides* using rice flour and its efficacy against *Sclerotinia sclerotiorum*

CHAPTER 1

DUAL FUNCTIONALITY OF *Trichoderma*: BIOCONTROL OF *Sclerotinia sclerotiorum* AND BIOSTIMULANT OF COTTON PLANTS

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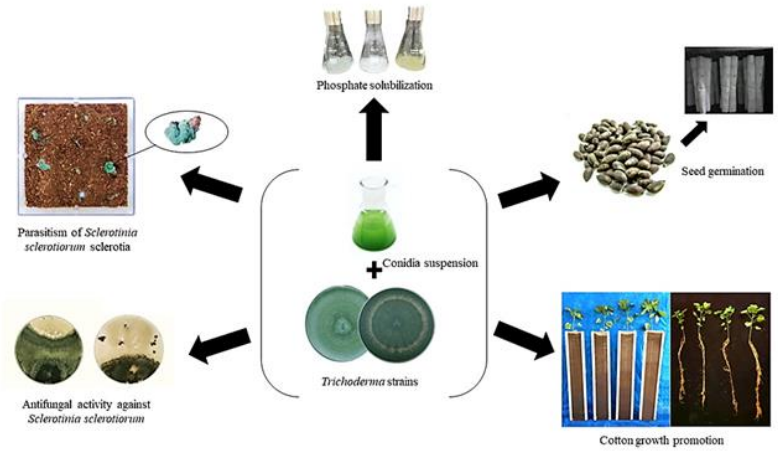
Graphical Abstract



Abstract

Microbial crop protection products based on *Trichoderma* have the ability to display multifunctional roles in plant protection, such as; pathogen parasitism, enhance nutrient availability and stimulate plant growth, and these traits can be used to enhance the overall agronomic performance of a variety of crops. In the current study, we explored the multifunctional potential of two indigenous Brazilian strains of *Trichoderma* (*T. asperelloides* CMAA 1584 and *T. lentiforme* CMAA 1585) for their capability of controlling *Sclerotinia sclerotiorum*, a key plant pathogen of cotton, and for their ability of growth promotion in cotton plants (*Gossypium hirsutum*). Both strains were able to solubilize mineral phosphorus ($CaHPO_4$), to release volatile organic compounds that impaired the mycelial growth of *S. sclerotiorum*, and to promote the growth of cotton plants under greenhouse conditions. In dual culture, *Trichoderma* strains reduced the growth rate and the number of sclerotia formed by *S. sclerotiorum*. By treating sclerotia with conidial suspensions of these *Trichoderma* strains, a strong inhibition of the myceliogenic germination was observed, as a result of the marked mycoparasitic activity exerted on the sclerotia. The parasitism over *S. sclerotiorum* was more effective with *T. asperelloides* CMAA 1584, whereas the effect of biostimulants on cotton growth was more pronounced with *T. lentiforme* CMAA 1585 that also showed a higher capacity of phosphate solubilization. Thus, *T. asperelloides* CMAA 1584 displays higher efficiency in controlling *S. sclerotiorum*, while *T. lentiforme* CMAA 1585 is more suitable as a biostimulant due to its ability to promote cotton plants growth. Overall, these *Trichoderma* strains may be used in mixture to provide both pathogen control and promotion of plant growth, and this strategy will support growers in minimizing the use of synthetic fertilizers and fungicides against white mold in cotton crops.

Keywords: Bioprotectant; biofungicide; white mold; biofertilizer; phosphate solubilization ability.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is the most important source of natural fibers in the world (Sivakumar et al., 2021; Wu et al., 2022). According to Tarazi et al. (2019), approximately 150 countries are directly involved in the cotton industrial chain, being an income source for more than 100 million families worldwide. Brazil stands out as the fourth largest producer of cotton worldwide, attaining a cultivated area of 1.6 million hectares with an estimated crop production of 6.7 million tons in 2021/22 (CONAB, 2022).

Since 1990s, the cotton growing area has dramatically expanded throughout the savannah Central-West region of Brazil (known as biome 'Cerrado'), mainly due to breeding efforts for developing locally adapted high-yielding cultivars and improving agronomic practices (Morello et al., 2015; Silva Neto et al., 2016; Barroso et al., 2017; Silva et al., 2019). As a result, the cultivated area in the Mato Grosso State has increased by approximately 1,500% in the last 30 years (ABRAPA, 2021). However, the high incidence of pests and diseases remain inflicting high productivity losses, accounting for approximately 35% of production costs (IMEA, 2019).

Among several diseases that limit cotton growth and yield, white mold, also known as Sclerotinia stem rot, caused by the ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Ascomycota: Sclerotiniaceae), is one of the most devastating and yield-limiting diseases. This plant pathogen cause billions of dollars of crop losses and is of great economic importance to several agricultural and vegetable crops worldwide, notably including cotton and soybean (Boland and Hall, 1994; O'Sullivan et al., 2021). According to the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA), *S. sclerotiorum* is considered one of the eight diseases/pests with the highest phytosanitary risk for Brazil (Brazil, 2015). This disease poses a serious threat to cotton plants at all phenological stages, and pathogen forms a dark-pigmented and hardened mycelial threads known as sclerotium capable of surviving for several years in

soil (Tourneau, 1979; Schwartz and Singh, 2013). Symptoms associated with the white mold in cotton include wilt, necrosis and rotting of stems, bolls, petioles and leaves (Charchar et al., 1999; Suassuna et al., 2019).

Currently, the Mato Grosso State is responsible for approximately 71% of the Brazilian cotton production, following an integrated cropping system with soybean and corn (Silva et al., 2019; IMEA, 2021). According to Meyer et al. (2020), *S. sclerotiorum* is endemic in approximately 27% of soybean production areas in Brazil, and 87% of cotton growing areas in Mato Grosso, in which cotton is cultivated as a second crop after soybean crop (IMEA, 2021). Thus, the succession of susceptible crops to *S. sclerotiorum* has been responsible for the continuous increase of the incidence of white mold, leading to an overuse of chemical fungicides as a means to alleviate crop yield losses. However, over-reliance of broad-spectrum chemical fungicides poses a serious risk to the environment (Komárek et al., 2010), health of growers (Kniss, 2017), and accelerate the selection of resistant strains of *S. sclerotiorum* (Zhou et al., 2014), all of which requires urgent alternative measures that include the development bio-rational solutions for the integrated management of white mold disease.

Despite the fact that chemical fungicides are effective in protecting plants from the white mold, their stand-alone use has inconsistent and unsatisfactory results. This is mainly due to difficulties in achieving adequate application coverage of the target pathogen, coupled with the best timing of application when ascospores are discharged (Meyer et al., 2014). Additionally, the lack of genetically resistant plants and the adoption of intensive management strategies based solely on synthetic fungicides have resulted in the development of resistant *S. sclerotiorum* strains to many chemical active ingredients (Liang et al., 2015; Mao et al., 2018). In this sense, it is imperative to explore alternative measures such as biological control strategies against this cosmopolitan plant pathogen (Bettiol et al., 2021).

Among biological control agents, *Trichoderma* spp. are considered effective in controlling *S. sclerotiorum* across several crops (Li et al., 2005; Sharma and Sain, 2010; Elias et al., 2016; Sumida et al., 2018), including cotton. The efficacy of using the necrotrophic mycoparasite *Trichoderma* in the management of *S. sclerotiorum* is related to its ability to parasitize and degrade sclerotia, resulting in an inversely proportional relationship between the frequency of *Trichoderma* application and the viability of sclerotia in the soil (Ferraz et al., 2011; Geraldine et al., 2013; Smolińska and Kowalska, 2018). Notably, *Trichoderma* spp. antagonize a myriad of plant pathogens by distinct mechanisms of action (Lorito et al., 2010; Druzhinina et al., 2011; Hermosa et al., 2012; Monte et al., 2019; Monte and Hermosa, 2021). Furthermore, due to the plasticity of their genomes in expressing multiple ecological functions and diverse biochemical machinery, several *Trichoderma* species promote plant growth (Rubio et al., 2017; Monte et al., 2019; Monte and Hermosa, 2021) and induce plant defenses against biotic and abiotic stresses (Brotman et al., 2012; Hermosa et al., 2012; Rubio et al., 2017; Monte et al., 2019).

The effectiveness of plant pathogen suppression as well as growth promotion mediated by *Trichoderma* are species and strain dependent (Harman et al., 2004; Verma et al., 2007; Atanasova et al., 2013; Haddad et al., 2017; Sumida et al., 2018). Owing to the lack of studies exploring the biocontrol and biostimulant abilities of *Trichoderma* strains in association with cotton plants, this study aimed to investigate the potential of two novel indigenous Brazilian strains, *Trichoderma asperelloides* CMAA 1584 and *Trichoderma lentiforme* CMAA 1585, against *S. sclerotiorum* along with their role as cotton growth promoters.

MATERIAL AND METHODS

Microorganisms

Trichoderma asperelloides CMAA 1584 (BRM 065723, GenBank accession ON542481) and *Trichoderma lentiforme* CMAA 1585 (BRM 065775, GenBank accession ON542480), both isolated from soil in Jaguariúna, SP, Brazil (22º43'43" S and 47º01'04" W), and deposited in the Collection of Microorganisms of Agricultural and Environmental Importance (CMAA) from Embrapa Environment (Jaguariúna, SP, Brazil), were used in these studies. These strains were reactivated and grown on potato-dextrose-agar medium (PDA; Acumedia Manufacturers®, Michigan, USA) in Petri dishes (9 × 1.5 cm) for 14 days at 25 ± 2 ºC and 12:12 hours photoperiod. For preservation, 7-day-old sporulated colonies grown on PDA were cut into 5 mm pieces, placed in cryovials containing 1.5 mL of sterile solution of 20% (v/v) glycerol (Dinâmica®, São Paulo, SP, Brazil) prepared with double deionized water, and stored at –80 ºC as stock cultures. Five-day-old PDA-grown cultures of these two *Trichoderma* strains were morphologically characterized based on colony growth aspects, conidiophores, and conidia size. Conidia size measurements were recorded with a light phase-contrast microscope (Olympus CS43 microscope and Olympus EP50 camera). The strains were identified phylogenetically using the translation elongation factor 1-α gene through direct comparison with data from reference type strains.

The plant pathogen *Sclerotinia sclerotiorum* CMAA 1105 (GenBank accession OM348513) was cultured on PDA in Petri dishes through myceliogenic germination from surface-sterilized sclerotia, and the newly-formed sclerotia were stored at 4 ºC. This *S. sclerotiorum* strain was isolated in Jaguariúna, SP, Brazil (22º43'43" S and 47º01'04" W) in 1992, and was then deposited in the Collection of Microorganisms of Agricultural and Environmental Importance (CMAA) from Embrapa Environment (Jaguariúna, SP, Brazil). All fungal strains used in this study are registered under the Brazilian genetic heritage – SisGen – protocol A135E26.

Ability of *Trichoderma* strains to solubilize phosphate

The ability of *Trichoderma* strains to solubilize inorganic phosphate (P) was evaluated by quantifying the solubilized P in liquid NBRIP (National Botanical Research Institute's Phosphate) medium, which contained per liter: 10.0 g glucose, 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl and 0.1 g $(NH_4)_2SO_4$ (Nautiyal, 1999). In the medium, 50 mL of K_2HPO_4 (10%) and 100 mL of $CaCl_2$ (10%) were added to form an insoluble calcium phosphate ($CaHPO_4$) precipitate. For inoculum production, 7-day-old sporulated cultures of each *Trichoderma* strain were rinsed with 10 mL of a sterile solution containing 0.04% polyoxyethylene sorbitan mono-oleate (Tween® 80, Synth, SP, Brazil) and calibrated using a hemocytometer (improved Neubauer chamber, 400× magnification) under a microscope (DM 500, Leica Microsystems GmbH®, Germany) to provide a final inoculum size of 5×10^6 conidia mL^{-1} in the medium. These liquid cultures were then incubated at 28 ± 1 °C in an orbital rotary shaker (TE-1401, Tecnal®, Piracicaba, SP, Brazil) at 180 rpm for 5 days with a 12:12 hours photoperiod. The amount of calcium phosphate in the medium before inoculation of *Trichoderma* strains were approximately 150 µg mL^{-1}. Aliquots of 1 mL were taken at the 5th day and centrifuged at 7,000 rpm and 22 ºC for 5 minutes to determine the concentration of soluble phosphorus, according to the colorimetric method described by Murphy and Riley (1962). The concentration of solubilized P in the supernatant was calibrated based on a standard curve of $CaHPO_4$ (Sigma-Aldrich®, St. Louis, MO, USA) at concentrations of 0.5, 1.0, 2.0, 2.5, and 5.0 mg mL^{-1}. The experiments were carried out with four biological repetitions to each fungal strain. Untreated control group (blank) was performed without the presence of microorganisms, whose values obtained were subtracted from those obtained in the presence of the fungal inoculum as a means to normalize the absorbance reads.

Antifungal activity of *Trichoderma* strains against *S. sclerotiorum*

The ability of *Trichoderma* strains to antagonize *S. sclerotiorum* was evaluated by dual culture tests. Mycelial plugs (5 mm diameter) from the colony margin of an actively growing *Trichoderma* culture in PDA were placed on the edge of the Petri dish, and another plug of 7-day-old colony of *S. sclerotiorum* cultured on PDA was placed on the opposite side, maintaining 7 cm apart from colony discs. The plates were incubated at 25 ± 2 ºC and the mycelial growth of both fungi was measured daily until *Trichoderma* strains have overgrown or surrounded the *S. sclerotiorum* colony. After 14 days of incubation under dual culturing, the antagonistic potential of *Trichoderma* strains inhibiting the pathogen's growth was measured and the development of sclerotia was also evaluated. Furthermore, a diagrammatic scale proposed by Bell et al. (1982) was used to score the antagonistic capacity, where: 1 - *Trichoderma* overcomes the pathogen and grows in 100% of the plate; 2 - *Trichoderma* grows on at least 75% of the plate; 3 - *Trichoderma* and the pathogen colonize approximately 50% of the plate; 4 - The pathogen colonizes at least 75% of the plate and resists to *Trichoderma*; 5 - The pathogen completely overlaps *Trichoderma* and occupies the entire surface of the plate. As a control, Petri dishes inoculated only with the pathogen served as the reference to calculate the percent inhibition of pathogen's colony growth exerted by *Trichoderma* strains. The experiment was performed with five biological replicates for each strain.

To assess the effect of volatile organic compounds (VOCs) released by *Trichoderma* strains on *S. sclerotiorum* mycelial growth, two Petri dish bottoms, one containing the pathogen and the other with a *Trichoderma* strain, all plated in the center and grown on PDA, were superimposed (Muthukumar et al., 2011). As a control, Petri dishes containing the pathogen were overlaid with another containing only PDA. These paired cultures were maintained in a growth chamber under the same environmental conditions described above. After 2 days of incubation, due to the rapid mycelial growth of *S. sclerotiorum*, the percentage of inhibition of

the pathogen was assessed and further calculated by the equation: Inhibition (%) = $(D_1 – D_2)/D_1 \times 100$, where D_1 represents the radial diameter of the pathogen in the control treatment, and D_2 the radial diameter of the pathogen confronted with *Trichoderma*. The experiment was performed with five biological replicates for each strain.

Parasitism of *S. sclerotiorum* sclerotia by *Trichoderma* strains

The ability of both *Trichoderma* strains in parasitizing *S. sclerotiorum* sclerotia was evaluated in polypropylene boxes (11 cm × 11 cm × 3.5 cm) (Gerbox®) containing 200 g of a dystroferric dark red latosol, collected at Embrapa Environment and autoclaved at 121 ºC for 60 minutes on three consecutive days. Dark-pigmented mature *S. sclerotiorum* sclerotia were produced in 500 mL Erlenmeyer flasks containing carrot and cornmeal, according to Garcia et al. (2012). Each autoclaved flask received three 5-mm-PDA discs of *S. sclerotiorum* mycelium, taken from the edge of a 7-day-old colony and incubated at 25 ± 2 ºC. After 30 days of growth on carrot-cornmeal substrate, mature sclerotia were removed, placed on absorbent paper inside a laminar flow chamber, left drying for 24 hours, and then kept in a refrigerator at 4 °C prior to using in bioassays. In each polypropylene box, 12 sclerotia were randomly distributed on the soil surface, and 10 mL suspensions containing 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1} of each *Trichoderma* strain were evenly applied with a pipette over the soil surface. All groups were incubated for 15 days at 25 ± 2 ºC with a photoperiod of 12:12 hours (Geraldine et al., 2013). A control group was set up with sterile distilled water. After 15 days of incubation, all sclerotia were removed from the soil, surface-sterilized with ethanol (70%) and sodium hypochlorite (2%) for 2 minutes, and subsequently rinsed three times in sterile distilled water prior to plating them on a selective media. Soft and disintegrated sclerotia due to colonization by *Trichoderma* strains were counted after slight pressure with a tweezer (Henis et al., 1983). Sclerotia viability was evaluated by incubating them on Neon medium (Napoleão et al., 2006)

for 7 days at 25 ± 2 ºC, then observing for the formation of a yellow halo around the sclerotia, which were deemed to be viable. The experiment was performed in a completely randomized design for each strain, with three treatments (inoculum size) and four biological replicates, in addition to a mock control treated only with water.

Germination and vigor of cotton seeds treated with *Trichoderma*

Seeds of cotton cv. FM 975 WS® provided by Instituto Mato-Grossense do Algodão (IMA, Mato Grosso, Brazil) were used in the interaction studies involving *Trichoderma* strains and cotton plants. The seeds were surface disinfected in 70% ethanol followed by 2% sodium hypochlorite solution for 2 minutes and washed in sterile distilled water three times. Surface-sterilized cotton seeds were soaked in an aqueous *Trichoderma* suspension containing 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1} for 60 minutes, and then layered on a Petri dish to air-dry for 1 hour inside a laminar flow hood. *Trichoderma*-treated cotton seeds were sown in germitest® paper (Cienlab Equipamentos Científicos Ltda, Campinas, SP, Brazil) using a roller system moistened with distilled water and incubated at 25 ± 2 ºC. The experiment was set up in a completely randomized design with three treatments (inoculum concentrations) and four independent biological replicates, with 20 seeds each (i.e., total of 80 seeds per treatment). The number of germinated seeds was determined on the 4^{th} day after sowing, being expressed as a percentage of germinated seeds. Afterwards, the seedlings were dried in an oven at 105 ºC until constant weight, and the vigor index was determined according to Abdul-Baki and Anderson (1973) by the equation: Vigor Index = Germination (%) × Seedling dry weight (g).

Effect of *Trichoderma* on cotton growth

Cotton seeds cv. FM 975 WS® were treated with crescent concentrations of *Trichoderma* conidia as described above and sown in rhizotron made of polyvinyl chloride

(PVC) half-longitudinal tubes (100 cm height × 17.5 cm diameter), containing a mixture of a dystroferric dark red latosol and sand in a ratio of 2:1 (v/v). The soil exhibited the following chemical and physical attributes analyzed at 0 - 20 cm depth: pH in H_2O = 4.3; OM = 32.3 g kg^{-1}; P = 9.36 mg dm^{-3}; Ca = 3.09 cmolc dm^{-3}; Mg = 1.48 cmolc dm^{-3}; K = 128.55 mg dm^{-3}; SB = 4.95 cmolc dm^{-3}; H + Al = 6.10 cmolc dm^{-3}; t = 4.99 cmolc dm^{-3}; V% = 44.54. In addition to seed treatment, 10 mL of the same conidial suspensions were applied in the planting furrow via drench at 15, 30 and 45 days after sowing (DAS). The experiment was set up in a randomized block design with three treatments (inoculum concentrations) and five independent biological replicates, in addition to mock cotton seeds as a control. The assay was carried out in a greenhouse for 60 days and the following growth parameters of the cotton plants were evaluated: height (14, 24, 31 and 55 DAS), root length (at 7, 14, 24 and 60 DAS) and leaf area of the first non-cotyledonary leaf (at 24 DAS), as described by Grimes and Carter (1969). At 60 DAS the following parameters were determined: stem diameter (2 cm above the soil surface), fresh and dry weights for both the aboveground portion and roots of the plants.

Statistical analysis

Homogeneity of variances and normality tests were performed by Bartlett's and Shapiro-Wilk tests. Data were fitted to linear models and analyzed by analysis of variance (ANOVA) using original data sets to identify significant differences between means of the treatments (Tukey's test, $P < 0.05$). Statistical analyses were performed using Minitab® software version 19.1.

RESULTS

Morphological characterization of indigenous *Trichoderma* spp. strains

According to the phylogenetic analysis based on tef-alpha 1 gene, the strain CMAA 1584 was confirmed to be *Trichoderma asperelloides*, while the strain CMAA 1585 was identified as *Trichoderma lentiforme*. Purified monosporic cultures of these two *Trichoderma* spp. strains were very divergent from each other in terms of growth, color, conidial size, and conidiophores. As depicted in Figure 1, cultures of *T. asperelloides* CMAA 1584 exhibited profuse growth on PDA with dark green color when fully sporulated and forming ovoid conidia averaging 3.60 × 3.59 µm (length and width) with a resultant area estimated in 10.10 μm^2 (standard error: ± 0.17 μm^2, n = 20). When looking at *T. lentiforme* CMAA 1585 cultures, its sporulated colony assumed pale greenish color and produced conidia averaging 2.54 × 2.44 µm (length and width) with an estimated average area of 4.88 μm^2 (standard error: ± 0.18 μm^2, n = 20). The area size of *T. asperelloides* was noted to be twice larger than conidia of *T. lentiforme*. The morphological phenotypes and conidia sizes are consistent with values previously reported for these species (Samuels et al., 2010; Chaverri et al., 2015).

Phosphate solubilization

Trichoderma lentiforme CMAA 1585 and *T. asperelloides* CMAA 1584 were both capable of solubilizing inorganic phosphate, resulting in about 31.7% and 5.2% of $CaHPO_4$ remaining in the medium, respectively, in comparison to control (Table 1). Phosphate solubilization was significantly ($P < 0.05$) higher in NBRIP medium, in which *T. lentiforme* solubilized significantly more phosphate than *T. asperelloides* (Table 1).

Antifungal activity of *Trichoderma* strains against *S. sclerotiorum*

In general, volatile organic compounds (VOCs) released by *Trichoderma* strains significantly reduced ($P < 0.05$) the mycelial growth of *S. sclerotiorum* (Table 2). Compared to

the control, VOCs emitted by *T. lentiforme* CMAA 1585 and *T. asperelloides* CMAA 1584 significantly reduced the growth rate of *S. sclerotiorum* by 55% and 53%, respectively (Table 2). However, there was no difference between these *Trichoderma* strains in their ability to inhibit this pathogen by means of released VOCs.

In dual culture assay for direct confrontation, *T. asperelloides* CMAA 1584 and *T. lentiforme* CMAA 1585 reduced the growth rate (mm day^{-1}) of *S. sclerotiorum* in 10% and 13%, respectively, when compared to control (Table 2) ($P < 0.05$). The inhibition of mycelial growth was 9.5% and 12.2% for *T. asperelloides* CMAA 1584 and *T. lentiforme* CMAA 1585, respectively (Table 2). Notably, *T. asperelloides* CMAA 1584 and *T. lentiforme* CMAA 1585 remarkably decreased by 96% and 47% the number of sclerotia formed by *S. sclerotiorum* colony in comparison to control, respectively (Table 2, $P < 0.05$). *Trichoderma lentiforme* CMAA 1585 received higher scores (3.6) when compared to *T. asperelloides* CMAA 1584 (2.2) according to Bell's diagrammatic scale, indicating that the former was less aggressive in parasitizing sclerotia than the latter (Figure 2).

Parasitism of *S. sclerotiorum* sclerotia by *Trichoderma* strains

The myceliogenic germination of sclerotia was significantly ($P < 0.05$) reduced by both *Trichoderma* strains (Figure 3). All concentrations of *T. asperelloides* CMAA 1584 colonized 100% of sclerotia and thus strongly inhibited the myceliogenic germination of all sclerotia (Figure 3A). *Trichoderma lentiforme* CMAA 1585, despite colonizing 100% of sclerotia, only 69% sclerotia were found ungerminated or non-viable based on the Neon selective medium test. The degradation of sclerotia did not reveal significant differences ($P > 0.05$) between *Trichoderma* strains for all concentrations tested (Figure 3B).

Germination and vigor of cotton seeds treated with *Trichoderma*

Trichoderma asperelloides CMAA 1584 and *T. lentiforme* CMAA 1585 applied in cotton seeds, at 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1}, did not show any significant differences ($P > 0.05$) for seed germination and initial vigor of cotton seedlings, when compared to control (Table 3).

Effect of *Trichoderma* strains on cotton growth promotion

Under greenhouse conditions, growth of cotton plants derived from seeds coated with spores of *T. asperelloides* CMAA 1584 and *T. lentiforme* CMAA 1585 strains were compared with mock control plants. Notably, *T. lentiforme* CMAA 1585 outperformed *T. asperelloides* CMAA 1584 in promoting growth of cotton plants (Tables 4 and 5, Figures 4 and 5). Looking at *T. asperelloides* CMAA 1584, this strain incited cotton growth promotion only for leaf area ($P < 0.05$), reaching indexes of 98.9% and 42.0% higher than the mock control plants, when applied to seeds at 1×10^7 and 1×10^8 conidia mL^{-1}, respectively (Table 4, Figure 5AB). Plant height, stem diameter, aboveground and root fresh and dry weights increased ($P < 0.05$) with the application of *T. lentiforme* CMAA 1585 at 1×10^8 conidia mL^{-1} (Table 5, Figure 4). Notably, cotton plants derived from seeds treated with *T. lentiforme* CMAA 1585 at 1×10^8 conidia mL^{-1} increased stem diameter, height, aboveground and root fresh and dry weights of 23.7%, 35.2%, 69.3%, 86.7%, 46.0%, and 30.4% (Table 5, Figures 4 and 5), when compared to control plants ($P < 0.05$), respectively.

DISCUSSION

The present study reveals the ability of two indigenous Brazilian strains, *T. lentiforme* CMAA 1585 and *T. asperelloides* CMAA 1584 to solubilize inorganic phosphorus, a macronutrient of low availability in tropical soils. Furthermore, these strains are capable of

emanating VOCs which inhibit the mycelial growth of *S. sclerotiorum*. In addition, these strains reduce the growth rate and total number of sclerotia of *S. sclerotiorum* in dual culture assay, and they inhibit the myceliogenic germination due to degradation of sclerotia through direct parasitism.

The difference in biocontrol performance between the *T. lentiforme* CMAA 1585 and *T. asperelloides* CMAA 1584 lies in their ability to suppress the myceliogenic germination of sclerotia, as reported in the present study (Figure 2), which may be related to secondary metabolites, including antifungal compounds, and the direct capacity of parasitism using an arsenal of well-known cuticle-degrading enzymes, where both mechanisms have been correlated with the virulence strategies employed by *Trichoderma* species (Harman et al., 2004; Geraldine et al., 2013; Monte et al., 2019). Previous studies have shown that *T. harzianum*, *T. koningii*, *T. pseudokoningii*, *T. koningiopsis*, *T. asperellum*, *T. atroviride*, and *T. virens* displayed excellent inhibitory effect on the myceliogenic germination of *S. sclerotiorum* in the range of 62% to 100%, when applied directly to the sclerotia (Haddad et al., 2017; Sumida et al., 2018). As noted in our study, evidence of interspecific variation in biocontrol efficacy among *Trichoderma* spp. is common and should be a key criterion to be incorporated into screening studies for biocontrol of plant pathogens.

There is a tremendous diversity among *Trichoderma* species and strains in their ability to produce and release biogenic volatile organic compounds (BVOCs) with remarkable roles in mediating plant growth and antagonism towards plant pathogens (Siddiquee et al., 2012; Contreras-Cornejo et al., 2014; Li et al., 2018). In this study, we noted that both of our *Trichoderma* strains imposed similar detrimental effects on *S. sclerotiorum* growth under *in vitro* conditions through emission of VOCs, whose compounds remain elusive. Given the importance of some VOCs emitted by *Trichoderma* playing pivotal roles in plant growth and biocontrol activity against plant pathogens, further research is needed to elucidate the emission

profiles of VOCs by these *T. asperelloides* and *T. lentiforme* strains in view of providing new insights and applications of their metabolites in cotton growth enhancement and protection against white mold disease.

Trichoderma asperelloides CMAA 1584 showed a great potential for use in biological control of *S. sclerotiorum* as it inhibited 100% myceliogenic germination of all sclerotia exposed to their conidia, as well as decreasing the sclerotia formation by 96.1%, when compared to the mock control. According to Atanasova et al. (2013), *Trichoderma* spp. craft distinct strategies to combat and outcompete other host fungi. These authors observed host sensing in *T. atroviride* and *T. virens* through expression of genes involved in the attack, whereas *T. reesei* was keener to outcompete the pathogen for nutrients. Thus, screening studies for potential biocontrol candidates of *Trichoderma* can reveal interesting phenotypical traits between species and strains and differential pattern of gene expression linked to biocontrol during the parasitism process of targeted hosts (Atanasova et al., 2013; Troian et al., 2014). Our results strengthen the need to select the antagonist strain according to the desired targeted pathogen taking into account its biology and epidemiology in the crop system (Köhl et al., 2011; Bettiol et al., 2021).

Sclerotia that failed myceliogenic germination and were colonized by *Trichoderma* were classified as unviable, as this was the same criterion employed by Abdullah et al. (2008) and Görgen et al. (2009). Such mycotrophic lifestyle is one of the most remarkable antagonistic mechanisms expressed by *Trichoderma* spp. and is implicated in the direct attack of one fungal species to another (Sood et al., 2020). In this sequential process, the first step involves recognition by chemical cues of the targeted pathogenic fungus by *Trichoderma*, which then its hyphae attach and coil around the prey fungal hyphae (Harman et al., 2004), followed by the onset production of lytic enzymes that cause the dissolution of fungal cell walls (Druzhinina et al., 2011). Hence, it may be expected that antagonists with increased secretion of extracellular

enzymes should be responsible for a more pronounced decline in the *S. sclerotiorum* inoculum levels in soil (Woo et al., 2006).

Among 20 strains of *Trichoderma* spp. evaluated for management of *S. sclerotiorum* in common beans, *T. asperellum* (cryptic sister species of *T. asperelloides*) exhibited the highest secretion of cell wall-degrading enzymes (CWDE) activity (Lopes et al., 2012). These data are consistent with those observed by Qualhato et al. (2013), where *T. asperellum* was effective against *Fusarium solani*, *Rhizoctonia solani* and *S. sclerotiorum*, and its antagonistic activity was associated with high activity of chitinase, β-1,3-glucanase and acid phosphatases. In our study, *T. asperelloides* CMAA 1584 displayed a great ability to inhibit myceliogenic germination and further degrade sclerotia of *S. sclerotiorum* by direct parasitism, outperforming *T. lentiforme* CMAA 1585 in this particular attribute. According to Geraldine et al. (2013), NAGase (N-β-acetylglucosaminidase) and β-1,3-glucanase enzymes play a key role in reducing the number of apothecia and the chain of events in the field that account for white mold severity, underlining the importance of these CWDEs in the control of white mold.

On the other hand, *T. lentiforme* CMAA 1585 demonstrates to be more suitable as a biostimulant due to its ability to boost growth of cotton plants (Table 5, Figures 4 and 5). The high phosphate solubilization in the soil (Table 1) displayed by this strain and better development of cotton roots are possible mechanisms associated with plant growth enhancement. Many authors have detailed the ability of *Trichoderma* spp. to modulate physiological, biochemical, and molecular mechanisms in a wide assortment of plants under various growth conditions (Hermosa et al., 2013; Rubio et al., 2014, 2017; Elkelish et al., 2020), by the production phytohormones and a plethora of secondary metabolites (Jaroszuk-Ściseł et al., 2019).

Using *in vitro* bioassay, Contreras-Cornejo et al. (2009) showed that *T. virens* Gv29.8 and *T. atroviride* IMI206040 can synthesize indole-acetic acid [IAA] (and some of its

derivatives), and suggests that the higher lateral root development observed in *Arabidopsis* wildtype plants is mediated by auxins. IAA synthesized by plant root-associated microorganisms can interfere with plant development by disturbing the auxin balance in plants, which can modify root architecture, increase root mass, and consequently, increase nutrient uptake by well-developed root system (Contreras-Cornejo et al., 2009). Sofo et al. (2011) reported that cherry rootstocks treated with *T. harzianum* commercial strain T-22 resulted in increased root and shoot growth by 76% and 61%, respectively. Furthermore, in mass spectrometry analyses these authors found that IAA and gibberellic acid (GA) levels were significantly increased by 40% and 143% in the roots, and by 49% and 71% in leaves, respectively.

Gravel et al. (2007) suggested that growth promotion, in tomato seedling, is associated with the reduced ethylene (ET) production resulting from a decrease in its precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and/or through the ACC deaminase (ACCD) activity present in the microorganism. Another possible mechanism arises from increased plant tolerance to abiotic stresses and/or by mitigation of damages caused by the accumulation of reactive oxygen species (ROS) in stressed plants (Mastouri et al., 2010). Thus, it can be hypothesized that the absence of significant results ($P > 0.05$) in seed germination and seedling vigor index in our assay under laboratory conditions, using the germitest paper method, the growth promotion may be related to the absence of environmental stresses. When evaluating the germination and vigor of wheat seedlings (*Triticum aestivum* L.) after seed treatment with different strains of *Trichoderma* spp., Anjum et al. (2020) obtained results that corroborate this scenario, because in a greenhouse trial, the positive outcomes were significantly more expressive than those observed in *in vitro* test conducted in the laboratory. In the present study, although the germination index was similar for these *Trichoderma* strains in both growth conditions, cotton plants from the mock control group exhibited less growth in the greenhouse

trial when compared with plants derived from the *Trichoderma* treatments, most likely due to the exposition of mock plants to suboptimal environmental conditions in contrast to higher resilience and improved growth promotion afforded by *Trichoderma* as a biological inoculant.

Both *Trichoderma* strains may be further tested under field conditions, but with different purposes. As such, we propose that *T. asperelloides* CMAA 1584 should be designated to control sclerotia of *S. sclerotiorum*, while *T. lentiforme* CMAA 1585 would assume a role as a biostimuant due to its ability to promote better growth of cotton plants. Overall, these selected *Trichoderma* strains are suitable for application in consortium targeting both pathogen control and growth promotion in cotton crops with a consequent contribution to diminishing the reliance on chemical fertilizers and fungicides.

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Author contributions

WB, GMM, and LGS conceived and designed the laboratory and greenhouse experiments. LGS, RCC and PSON performed the laboratory and greenhouse experiments, and analyzed the data. WB, and GMM contributed with reagents/materials/analysis tools. CD provided critical analysis and editorial enhancements. All authors wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

All authors declare that there is no conflict of interest in this original article.

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Table 1. Phosphate solubilization by *Trichoderma asperelloides* CMAA 1584 and *Trichoderma lentiforme* CMAA 1585.

Strain	Phosphate solubilization (%)
CMAA 1584	5.2 ± 0.79 b
CMAA 1585	31.7 ± 4.08 a

*Values represent means (± standard error) and when followed by the same letter do not differ significantly (Tukey's test at $p < 0.05$).

Table 2. Antagonistic activity of *Trichoderma asperelloides* CMAA 1584 and *Trichoderma lentiforme* CMAA 1585 against *Sclerotinia sclerotiorum* by dual culture test and production of volatile organic compounds (VOCs).

Treatments	Volatile organic compounds		Dual culture		
	Growth rate (mm day^{-1})	Inhibition (%)	Growth rate (mm day^{-1})	Inhibition (%)	Number of sclerotia
CMAA 1584	23.6 ± 1.2 b (53%)	41.9 ± 2.7 a	27.4 ± 0.1 a (10%)	9.5 ± 0.4 a	0.6 ± 0.6 a
CMAA 1585	22.8 ± 1.5 b (55%)	43.8 ± 3.3 a	26.5 ± 0.6 a (13%)	12.2 ± 1.8 a	7.4 ± 1.5 b
Control	42.5 ± 0.0 a	-	30.5 ± 0.5 b	-	15.6 ± 1.6 c

*Values in each column represent means (± standard error) and when followed by the same letter do not differ significantly from each other (Tukey $p < 0.05$).
Values between parentheses indicate the inhibition growth rate when compared to control.

Table 3. Effect of *Trichoderma asperelloides* CMAA 1584 and *Trichoderma lentiforme* CMAA 1585 in cotton seeds germination and vigor index.

Treatments (conidia mL^{-1})	*T. asperelloides* CMAA 1584		*T. lentiforme* CMAA 1585	
	Germination (%)	Vigor index[1]	Germination (%)	Vigor index[1]
Control	92.5 ± 1.4 a	163.4 ± 1.5 a	92.5 ± 1.4 a	163.4 ± 1.5 a
1×10^6	91.2 ± 2.4 a	165.6 ± 4.6 a	92.5 ± 1.4 a	164.2 ± 3.6 a
1×10^7	88.7 ± 2.4 a	155.5 ± 8.3 a	92.5 ± 2.5 a	162.0 ± 3.8 a
1×10^8	93.7 ± 3.1 a	163.6 ± 6.1 a	91.2 ± 1.2 a	164.7 ± 2.3 a

*Values in each column represent means (± standard error) and when followed by the same letter do not differ significantly (Tukey's test at $p < 0.05$).
[1]Calculated according to Abdul-Baki and Anderson (1973) by the equation: Vigor Index = Germination (%) × Seedling Dry Weight (g).

Table 4. Leaf area, stem diameter, root length, plant height and fresh and dry weight of the root and aboveground of cotton plants treated with different concentrations of *Trichoderma asperelloides* CMAA 1584.

Treatment	Leaf area (cm^2)	Stem diameter (mm)	Root fresh weight (g)	Root dry weight (g)	Aboveground fresh weight (g)	Aboveground dry weight (g)	Root length (cm)	Plant height (cm)
							60 DAS	55 DAS
Control	22.6 ± 3.7 a	4.2 ± 0.4 a	16.2 ± 0.9 a	8.9 ± 0.7 a	16.0 ± 1.8 a	11.5 ± 1.0 a	107.4 ± 2.2 a	27.8 ± 3.1 a
1×10^6	25.7 ± 2.6 a	4.5 ± 0.2 a	16.9 ± 1.0 a	9.2 ± 0.4 a	15.4 ± 1.8 a	11.6 ± 0.6 a	108.4 ± 4.7 a	32.8 ± 1.5 a
1×10^7	44.9 ± 2.0 b	5.0 ± 0.3 a	19.8 ± 1.0 a	10.4 ± 0.7 a	19.4 ± 1.9 a	13.1 ± 1.2 a	105.8 ± 0.8 a	33.3 ± 2.9 a
1×10^8	32.0 ± 5.0 ab	5.0 ± 0.2 a	19.2 ± 1.4 a	9.5 ± 0.2 a	19.8 ± 2.0 a	12.9 ± 0.6 a	105.2 ± 2.5 a	34.2 ± 1.3 a
CV (%)	24.6	14.0	14.0	13.1	26.2	17.5	6.4	16.7

*Values in each column represent means (± standard error) and when followed by the same letter do not differ significantly (Tukey's test at $p < 0.05$). DAS = day after sowing.

Table 5. Leaf area, stem diameter, root length, height and fresh and dry weight of the root and aboveground of cotton plants treated with different concentrations of *Trichoderma lentiforme* CMAA 1585.

Treatment	Leaf area (cm^2)	Stem diameter (mm)	Root fresh weight (g)	Root dry weight (g)	Aboveground fresh weight (g)	Aboveground dry weight (g)	Root length (cm)	Plant height (cm)
							60 DAS	55 DAS
Control	22.6 ± 3.7 a	4.2 ± 0.4 a	16.2 ± 0.9 a	8.9 ± 0.7 a	16.0 ± 1.8 a	11.5 ± 1.0 a	107.4 ± 2.2 a	27.8 ± 3.1 a
1×10^6	26.1 ± 1.8 a	4.5 ± 0.1 ab	19.4 ± 2.3 a	9.9 ± 0.6 a	16.5 ± 0.7 a	12.7 ± 0.6 a	108.2 ± 3.8 a	33.8 ± 2.1 ab
1×10^7	26.4 ± 2.8 a	4.4 ± 0.1 ab	17.5 ± 0.2 a	8.6 ± 0.3 a	15.4 ± 0.4 a	10.8 ± 0.4 a	112.0 ± 2.4 a	30.0 ± 1.2 ab
1×10^8	33.7 ± 2.9 a	5.2 ± 0.3 b	30.3 ± 2.1 b	11.6 ± 0.3 b	27.1 ± 3.3 b	16.8 ± 1.1 b	110.2 ± 3.9 a	37.7 ± 2.4 b
CV (%)	24.9	11.2	18.7	8.5	22.2	14.0	7.3	14.6

*Values in each column represent means (± standard error) and when followed by the same letter do not differ significantly (Tukey's test at $p < 0.05$). DAS = day after sowing.

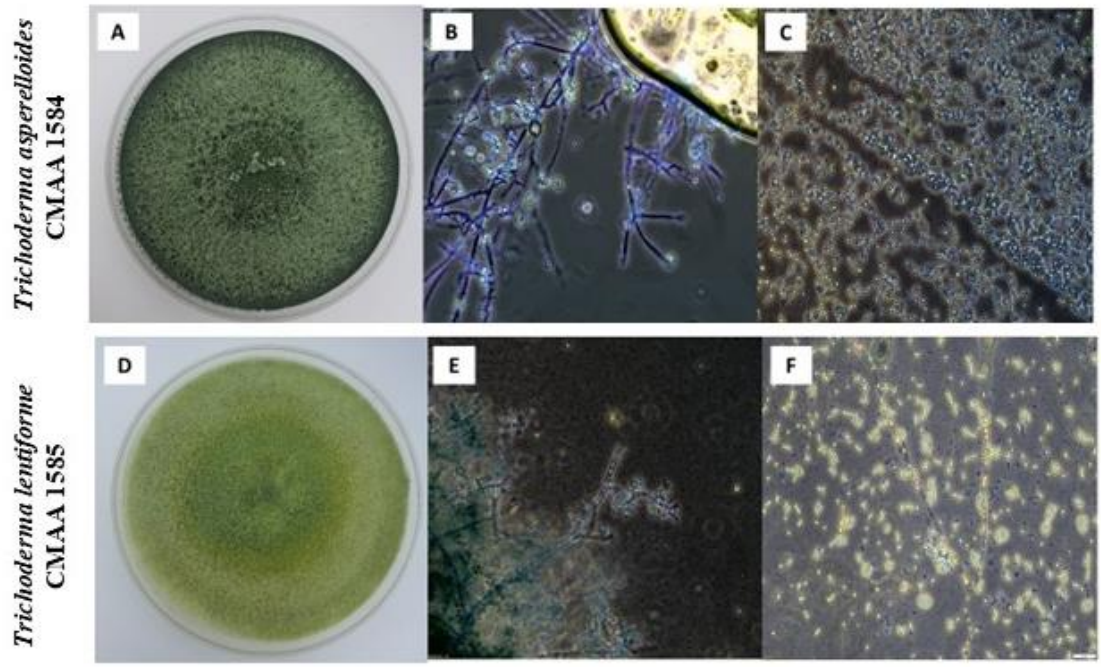

Figure 1. Morphological characterization of indigenous *Trichoderma* spp. (A) Five-day-old PDA-grown cultures of *Trichoderma asperelloides* (CMAA 1584); (B) Microscopic images showing conidiophores of CMAA 1584 (magnification at 200 ×); (C) Typical conidia of CMAA 1584 (magnification at 200 ×); (D) Five-day-old PDA-grown cultures of *Trichoderma lentiforme* (CMAA 1585); (E) Microscopic images showing conidiophores of CMAA 1585 (magnification at 200 ×); (F) Typical conidia of CMAA 1585 (magnification at 200 ×).

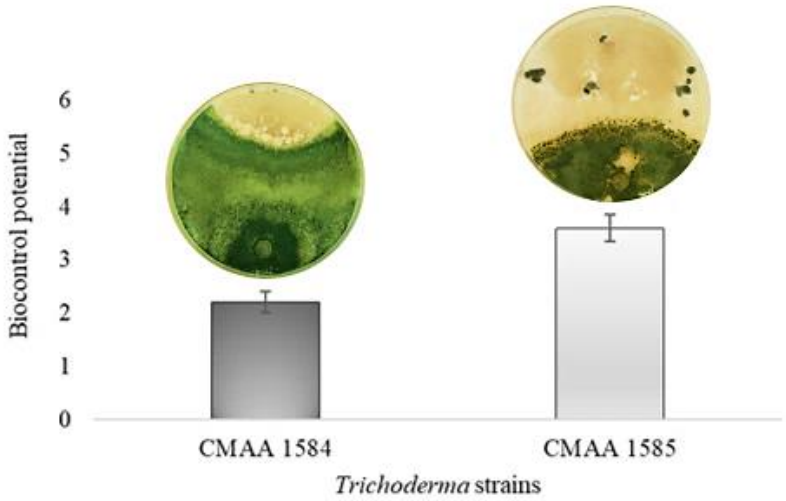

Figure 2. Biocontrol potential of *Trichoderma* strains against *Sclerotinia sclerotiorum* according to Bell scale. Bars indicate mean ± standard error.

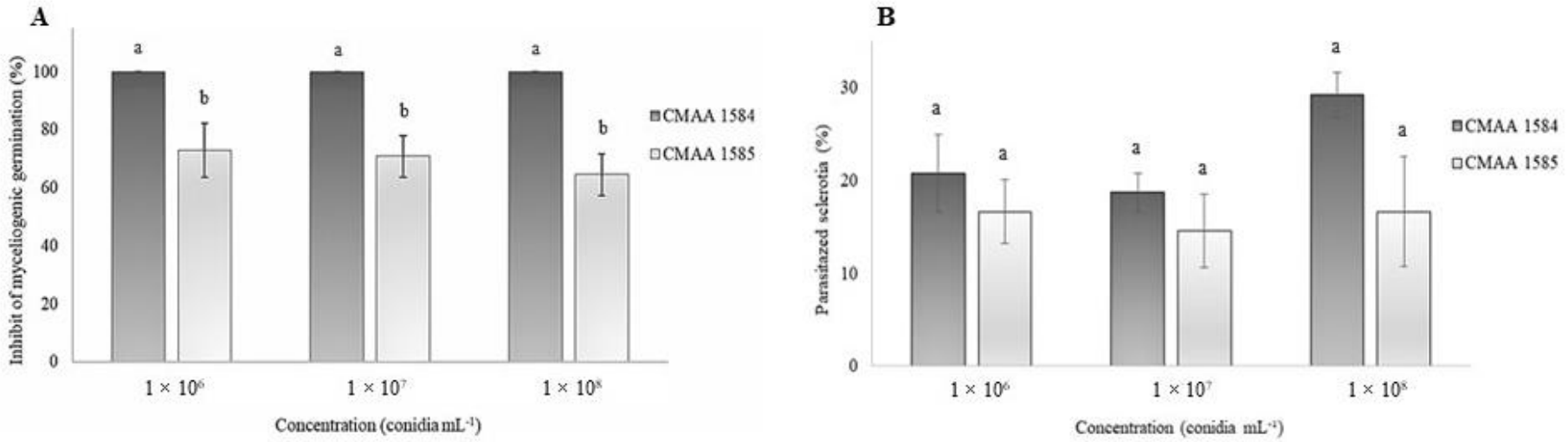

Figure 3. Ability of *Trichoderma asperelloides* CMAA 1584 and *Trichoderma lentiforme* CMAA 1585 strains to inhibit myceliogenic germination (A) and to parasitize (B) *Sclerotinia sclerotiorum* sclerotia by direct antagonism at different inoculum concentrations. *Black diamond symbol represents means (± standard error) and the dashed line represents the fitted quadratic curve.

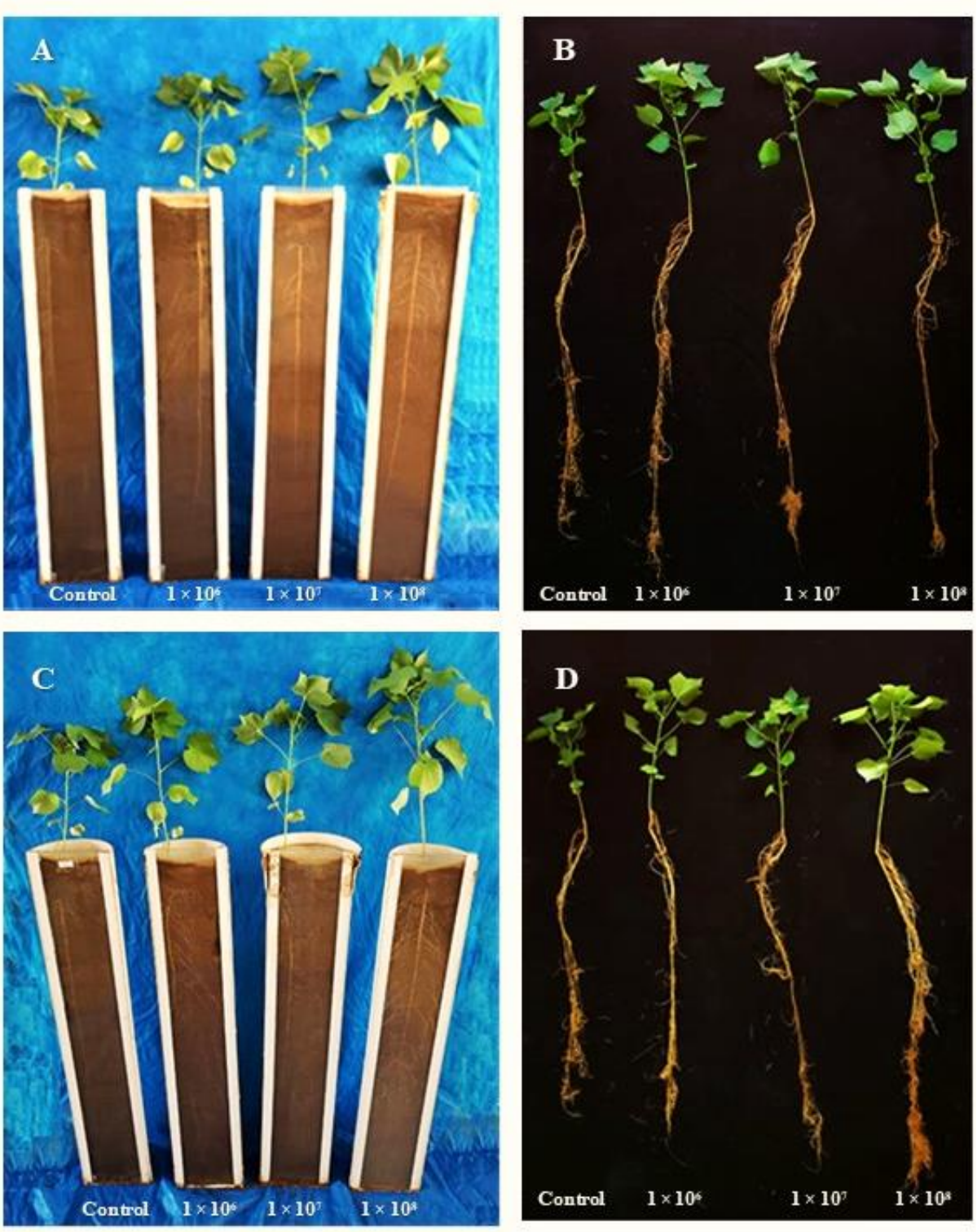

Figure 4. Cotton plants treated with *Trichoderma asperelloides* CMAA 1584 (A, B) and *Trichoderma lentiforme* CMAA 1585 (C, D) after 60 days of sowing in rhizotron.

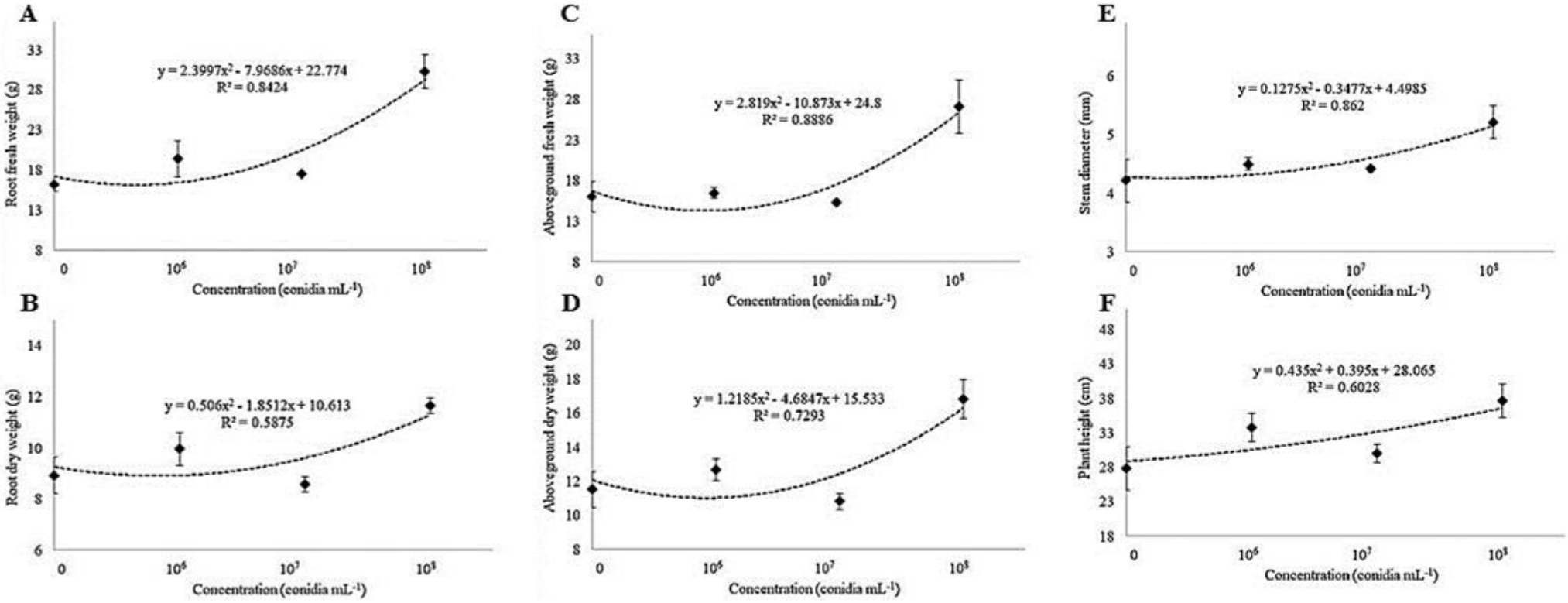

Figure 5. Fresh and dry weight of the root (A and B) and aboveground (C and D), stem diameter (E), and plant height (F) of cotton cultivar FM 975 WS® with *Trichoderma lentiforme* CMAA 1585. Bars indicate standard error.

CHAPTER 2

BIOREACTOR-IN-A-GRANULE DESIGNED FOR *Trichoderma asperelloides* USING RICE FLOUR AND ITS EFFICACY AGAINST *Sclerotinia sclerotiorum*

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Abstract

The replacement of cereal grains by agro-industrial by-products (wastes) for mass production of biological control agents (BCA) allied to the development of stable formulations with extended shelf-life represents a critical step for advancing the use of BCA. This study aimed to optimize the *Trichoderma asperelloides* stationary fermentation in rice flour, an inexpensive and abundant residue in Brazil, to sustain high yields of conidial production and to subsequently develop a rice flour-based formulation designed to simulate a microbioreactor that affords *in-situ* conidiation, extended shelf-life, and effective control of *Sclerotinia sclerotiorum*, the most devastating fungal pathogen of annual legume crops. The conidial yield of *T. asperelloides* in rice flour was mainly influenced by nitrogen content (0.1% w/w) and by the fermentor type (Erlenmeyer flask). Hydrolyzed yeast (Hilyses®) was the best source of nitrogen combined with rice flour with a resultant yield of 2.6×10^9 CFU g^{-1} within 14 days. Subsequently, five formulations ($G_{Control}$, $G_{Lecithin}$, $G_{Break\text{-}Thru}$, $G_{Bentonite}$ and $G_{Organic\ compost\ +\ Break\text{-}Thru}$) were obtained by extrusion and assessed for their potential to induce secondary sporulation *in situ*, storage stability, and efficacy against *S. sclerotiorum*. Formulations $G_{Control}$, $G_{Break\text{-}Thru}$, $G_{Bentonite}$ and $G_{Organic\ compost\ +\ Break\text{-}Thru}$ stood out with the highest number of CFU after sporulation upon re-hydration on water-agar medium. The shelf-life at room temperature (~25 °C) was maintained invariably for up to 3 months with formulations $G_{Control}$ and $G_{Bentonite}$, while the fungus remained viable for 12 months with $G_{Bentonite}$ and $G_{Organic\ +\ Break\text{-}Thru}$ formulations during refrigerated storage (4 °C). Formulations exhibited similar efficacy in suppressing the myceliogenic germination of *S. sclerotiorum* irrespective of application rates (i.e., 5×10^4, 5×10^5 or 5×10^6 CFU g^{-1} of soil), resulting in 79.2, 87.5 and 93.7% of sclerotial inhibition, respectively. Significant number of degraded and dead sclerotia was observed with increased dose of $G_{Control}$ formulation, ranging from 2.1 to 23.0%.

Keywords: Bioprotectant, extrusion, solid-state fermentation, white mold, shelf-life.

INTRODUCTION

Trichoderma is a well-known genus for its multi-beneficial roles in agriculture, including its wide biocontrol activity against several plant pathogens, plant growth promotion, and mitigation of abiotic stresses in plants (Hermosa et al., 2012; Lorito et al., 2010; Morán-Diez et al., 2020; Rubio et al., 2017). *Trichoderma*-based products, such as bioprotectors and biofertilizers, play pivotal role in food production worldwide as they can potentially minimize the chemical fertilizer inputs in agricultural crops (Bettiol et al., 2019; Harman et al., 2010; Woo et al., 2014). According to Bueno et al. (2020), *Trichoderma* was applied in more than 5.5 million hectares (ha) of soybean in Brazil in 2017 for the management of soilborne diseases. This area has increased in recent years, possibly reaching 20 million ha, and it is by far the most sold fungal biofungicide in Brazil due to its easiness in mass production allied to its multiple properties in plant protection and health.

Among the soilborne plant pathogens that affect soybean growth and yield, white mold, also known as Sclerotinia stem rot, caused by the ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most globally destructive diseases (Boland; Hall, 1994). According to Meyer et al. (2019), *S. sclerotiorum* is endemic in approximately 27% of soybean production areas in Brazil, and its damages can result in economic losses of up to US$ 1.47 billion annually (Lehner et al., 2017). This fungus infects leaves, flowers, stems, and pods of the host plants, forming resistant structures, known as sclerotia, which are able to survive in soil or on crop debris for several years (Bolton et al., 2006). Thus, the management of *S. sclerotiorum* occurs at several stages of crop development and usually requires integration of multiple methods, including biological control specifically directed to targeting the pathogen's sclerotia remained in soil (Smolińska; Kowalska, 2018).

The bioproduct manufacturers can use three different fermentation strategies in order to mass produce *Trichoderma* spp.: solid, liquid, and biphasic fermentation (Jin; Custis, 2011;

Mascarin et al., 2019). Currently, manufacturers employ mostly biphasic fermentation systems, in which the inoculum is initially produced by liquid culture media and subsequently transferred to solid substrates (rice, barley, wheat, oat, or millet) for induction of aerial conidiation or simply sporulation that leads to a sheer number of conidia, the main active ingredient for different types of formulation (Li et al., 2010; Jin; Custis, 2011; Mascarin et al., 2010, 2019; Woo et al., 2014). In this system, harvesting the spores is mandatory to concentrate the fungal biomass for the formulation process (Faria; Wraight, 2007; Li et al., 2010; Mascarin et al., 2019). However, throughout this process, metabolites which harbor antimicrobial properties and/or act as plant stimulants are inevitably lost. Additionally, the solid residue after spore extraction needs proper destination, which may be explored in energy production (Elias et al., 2022) or composting.

Growth substrate is the bottleneck for the production of cost-effective and quality BCAs, representing up to 50% of production costs (Eltem et al., 2014; Stanbury et al., 2017). Thus, the recent increases in paddy rice cost in Brazil, which reached more than 90% between January and December 2020 (CEPEA, 2021), directly impacts the production of fungal biocontrol agents that rely on this cereal grain for their growth. The use of agricultural by-products (wastes) for the production of fungal bioagents by the biopesticide industry offers a valuable alternative to the use of rice grains, as it allows the reduction of production costs and a more noble destination of this residue, especially in Brazil where these agricultural by-products are plenty and inexpensive (Farinas, 2015; Soccol et al., 2017).

Another concern that arises from using rice as fungal growth substrate resides in its social importance as staple food and main source of energy for more than half of the world's population, especially in Asian countries (Bird et al., 2000). Before reaching the consumer, several processing steps are carried out with the grain, which eventually generate several by-products (Esa et al., 2016). Broken rice, one of these by-products, represents between 10 to 15

% of all rice processed. Due to its low cost (about 1/3 to 1/2 of the brown rice), high availability, and good nutritional value (74% starch and 7% protein) (Liu et al., 2016), several technologies have been proposed to increase its use in the industry (Ahmed et al., 2015; Myburgh et al., 2019; Nakano et al., 2012), including in biological control manufacturers (Jaronski 2014; Bich et al., 2018).

On another important aspect that must be taken into account in the production pipeline of microbial biopesticides concerns the development of waste-free and low-cost formulations that enable prolonged shelf-life, improved efficiency, and easy application of microbial biocontrol agents. Of particular interest, starchy compounds have successfully been used as a carrier or additive in formulations of plant beneficial microorganisms (Vassilev et al., 2020). In the bioencapsulation matrix, starch reduces the physical stress to microbial cells and significantly improves their survival (Bashan et al., 2002), as it provides structural support and protection against thermal, oxidative, and osmotic stresses (Chan et al., 2011; Schoebitz et al., 2012; Tal et al., 1999). Moreover, considering that *Trichoderma* has the ability to hydrolyze starch into simple sugars (Schellart et al., 1976; Asis et al., 2021), the use of starchy products for mass production and as a carrier in *Trichoderma*-based formulations may provide a competitive advantage in relation to the competing native soil microbial community, corroborating the concept of a "bioreactor-in-a-granule" system proposed by Guimarães et al. (2018) and Klaic et al. (2018).

Grinding broken rice into flour to use as substrate and carrier in formulations can become a simple, inexpensive, and waste-free alternative for the development of new *Trichoderma*-based products, besides avoiding the conidia extraction step while keeping useful metabolites in the final product. Thus, the main objectives of this study focused on i) the optimization of *T. asperelloides* stationary fermentation in rice flour to sustain high conidial yields; ii) the development of a rice flour-based granule formulation designed to simulate a

bioreactor-in-a-granule prototype capable of supporting high conidiation, extended shelf-life, and effective biocontrol activity against sclerotia of *S. sclerotiorum*; and iii) the influence of storage temperature on the shelf stability of such developed formulations.

MATERIAL AND METHODS

Microorganisms

Trichoderma asperelloides strain CMAA 1584 (BRM 065723, GenBank accession ON542481), used throughout this study, was selected based in its ability to control *S. sclerotiorum* (Silva et al., 2022; Rezende et al., 2020). *Trichoderma asperelloides* CMAA 1584 was obtained from the Collection of Microorganisms of Agricultural and Environmental Importance (CMAA) of Embrapa Environment, Jaguariúna, SP, Brazil. For preservation, sporulated colonies were cut into 5-mm-diameter disks, transferred into cryovials containing 1.5 mL sterile solution of 20% (v/v) glycerol (Dinâmica®, Indaiatuba, SP, Brazil) prepared with double deionized water. These stock cultures were then stored at -80 ºC. Frozen stock cultures of this fungus served as the primary source of inoculum to grow it on Potato-Dextrose-Agar (PDA, Acumedia Manufacturers®, Michigan, USA) in Petri dishes (Pleion®, polystyrene, 90 × 10 mm) at 25 ± 2 °C with 12:12 h photoperiod for 10 days until fully sporulated. This fungal strain has been registered under the Brazilian genetic heritage – Sisgen – protocol A135E26.

Sclerotinia sclerotiorum strain CMAA 1105 (GenBank accession OM348513), used throughout this study as the target plant pathogen in our bioassays, was obtained from the Collection of Microorganisms of Agricultural and Environmental Importance (CMAA). The pathogen was grown on PDA medium through induction of myceliogenic germination from surface-sterilized sclerotia, and the newly formed sclerotia were stored at 4 ºC until use in bioassays.

Optimization of solid-state fermentation in rice flour

The optimization of *T. asperelloides* stationary fermentation in rice flour was performed using the statistical design of experiments based on Plackett-Burman Design (PBD 12) with two levels (+1 and -1), which allowed to investigate the effect of the following five factors: substrate moisture (%), inoculum density (conidia g^{-1} of substrate), substrate weight (g), fermentor type (polypropylene bags and Erlenmeyers flasks), and nitrogen content (% w/w) (Tables 1 and 2). The experiments were independently repeated three times using different fungal batches.

The stationary fermentation process was carried out and compared based on two systems: 1000-mL Erlenmeyer flasks (Pyrex®, Corning, SP, Brazil) versus in polypropylene bags (35 × 25 cm). *Trichoderma asperelloides* CMAA 1584 was grown on PDA medium at 25 ± 2 °C and 12:12 h photoperiod in a growth chamber. After 10 days, conidia were suspended in 10 mL of a sterile solution containing 0.04% polyoxyethylene sorbitan mono-oleate (Tween® 80, Synth, Diadema, SP, Brazil). The concentration was adjusted with a hemocytometer under a phase-contrast microscope at 400× magnification (DM 500, Leica Microsystems GmbH®, Germany) to provide a final inoculum size of 1×10^5 and 1×10^6 conidia g^{-1} of substrate, according to the inoculum density level (Table 1).

In each trial, an autoclaved urea solution (20% C and 46.6% N, Hexapur®, Holland), previously adjusted to delivery 1 and 0.1% of the substrate weight in nitrogen, was added to the rice flour. The substrate moisture was adjusted to 40 and 60% with sterile deionized water (Table 1), and incubated for 14 days at ambient temperature (~ 25 ºC). Afterwards, 1 g of fungus-colonizing substrate was collected to determine the colony-forming units (CFU) by serial dilutions and plating 100 µL aliquots on Petri dishes containing PDA + 0.1% Triton X-100 (Synth®, Diadema, SP, Brazil). Data were expressed as CFU g^{-1}.

Screening nitrogen sources

After optimizing the mass production of *T. asperelloides* in rice flour, the impact of different nitrogen sources on the concentration of propagules was evaluated. Therefore, the non-significant factors were kept constant in levels which provided greater economy [moisture (40%), inoculum density (1×10^5 conidia g^{-1} of substrate) and substrate weight (150 g)], while the significant factors were kept constant at the levels that provided the highest yield of CFU g^{-1} of substrate [nitrogen content (0.1%) and fermentor type (Erlenmeyer flasks)].

Five nitrogen sources were evaluated: autolysed yeast (8.0% N, LysCell®, ICC Brazil, São Paulo, SP, Brazil), ammonium sulfate (21.2% N, Vetec®, Duque de Caxias, RJ, Brazil), corn steep liquor (3.4% N, Ingredion®, Mogi Guaçu, SP, Brazil), cottonseed flour (9.3% N, Pharmamedia®, Archer Daniels Midland Company, USA), and hydrolyzed yeast (6.6% N, Hilysis®, ICC Brazil, São Paulo, SP, Brazil). The propagule production was evaluated after 14 days of incubation, as previously described, and expressed in CFU g^{-1}.

Mass production and formulations

Mass production of *T. asperelloides* CMAA 1584 was performed under optimized conditions. Five formulations were prepared by adding: bentonite (Sigma-Aldrich®, St. Louis, MO, USA), soy lecithin (Quimisul® SC, Joinville, SC, Brazil), Break-Thru S301 (Evonik®, Essen, Germany), and organic compost (Ribumin® C, Technes Agrícola, Guatapara, SP, Brazil), as shown in Table 5. A formulation with only colonized substrates was used as control treatment. Formulations were extruded in an extruder cylinder (CL22, Do Cheff®, Erechim, RS, Brazil) adjusted to a thickness of 2.5 mm (Figure 2). The resulting material was dried at room temperature, 25 cm from an exhaust hood (Vidy®, Taboão da Serra, SP, Brazil), equipped with three micro exhaust motors (Elco do Brasil®, Taboão da Serra, SP, Brazil). The granules were

dried until final moisture reached 5 ± 1% (w/w) after measurement with a moisture analyzer (ID-200, Marte Científica®, São Paulo, SP, Brazil).

Storage stability

In order to evaluate shelf-life, each formulation was stored in the dark, without vacuum packaging and in two conditions: under refrigeration at 4 ºC and under ambient temperature at 25 ± 2 ºC. Samples were taken every month and contained 1 g of each formulated product, and then mixed in 9-mL sterile solution of 0.04% Tween® 80 (Synth®, Diadema, SP, Brazil) followed by serial dilutions. An aliquot of 100 µL of the final dilution was then plated on Petri dishes containing PDA + 0.1% Triton X-100 (Synth®, Diadema, SP, Brazil) and incubated for 48 to 72 h at 25 ± 2 °C and 12:12 h photoperiod. For each formulation and evaluation date, six replicates were performed. Results were expressed in CFU g^{-1}.

Conidiation of *T. asperelloides* formulations

Conidiation of *T. asperelloides* was evaluated in accordance with the concept of a "bioreactor-in-a-granule" system proposed by Guimarães et al., 2018 and Klaic et al., 2018. Samples of 0.1 g of each formulation was collected, gently macerated in a pestle mortar, and then spread over the surface of water-agar medium at 2% (w/v) (Kasvi®, São José dos Pinhais, SP, Brazil) in Petri dishes. After incubation at 25 ± 2°C in 12:12 h photoperiod for 10 days, the entire surface of the plate was washed with 10 mL of a sterile solution containing 0.04% of polyoxyethylene sorbitan mono-oleate (Tween® 80) and the number of colony-forming units was determined, as previously described. Results were expressed in CFU g^{-1}. The initial CFU g^{-1} of all formulations were determined before incubation and subtracted from those obtained on the 10^{th} day of incubation.

Effectiveness of *T. asperelloides* formulation against *S. sclerotiorum*

Dark-pigmented mature *S. sclerotiorum* sclerotia were produced in 500-mL Erlenmeyers flasks containing carrot and cornmeal, according to Garcia et al. (2012). Each autoclaved flask received three 5-mm PDA discs of *S. sclerotiorum* mycelium, taken from the edge of a 7-day-old colony and were incubated at 25 ± 2 ºC. After 30 days of growth on the carrot-cornmeal substrate, mature sclerotia were removed, placed on absorbent paper inside a laminar flow chamber, left drying for 24 hours, and then kept in a refrigerator at 4 °C before being used in the bioassays.

In order to evaluate the effectiveness of *T. asperelloides* formulation against *S. sclerotiorum* sclerotia, 12 sclerotia were randomly placed in polypropylene boxes (11cm × 11cm × 3.5cm) (Gerbox®) containing 200 g of dystroferric dark red latosol previously autoclaved at 121 ºC for 60 minutes on three consecutive days. The soil was then inoculated with $G_{Cont.}$ formulation of *T. asperelloides* in the concentrations of 5×10^4, 5×10^5 and 5×10^6 CFU g^{-1} of soil. The boxes were incubated at 25 ± 2 ºC with a photoperiod of 12:12 h for 15 days (Geraldine et al., 2013). One of the controls was set up with sterile distilled water, while the other with conidia suspension of *T. asperelloides* in the concentration of 5×10^6 conidia g^{-1} of soil. Soft and disintegrated sclerotia, due to colonization by *Trichoderma* strains, were counted as degraded after slight pressure with a tweezer (Henis et al., 1983). After 15 days of incubation in those polypropylene boxes, all sclerotia were removed, surface-disinfested with ethanol (70%) and sodium hypochlorite (2%) for 2 min, and subsequently rinsed three times in sterile distilled water. Sclerotia was incubated on Neon medium for 7 days at 25 ± 2 ºC to evaluate viability. Viable sclerotia presented the formation of a yellow halo (Napoleão et al., 2006). The test was carried out in a completely randomized design, with three treatments (inoculum size) and four replicates, in addition to the control treatments.

Statistical analysis

Data generated with Plackett-Burman experiments were analyzed with Pareto diagram to select the meaningful factors at 5% probability level of significance. In order to meet homoscedasticity assumptions, variables were $\log_{10}$-transformed prior to analysis when necessary. Data were submitted to analysis of variance (ANOVA) and comparison of means by the Tukey test ($P < 0.05$) in order to evaluate the effects of nitrogen sources on *T. asperelloides* yield, conidiation, and efficacy against *S. sclerotiorum*. Storage stability data were analyzed by Dunnett test

RESULTS

Selection of key variables for the production of *T. asperelloides* in rice flour

The highest concentrations of CFU were observed in trial 2 (moisture = 60%, inoculum density = 1×10^6 conidia g^{-1} of substrate, substrate weight = 150 g, fermentor type = Erlenmeyer flask, nitrogen content = 0.1%) and 4 (moisture = 60%, inoculum density = 1×10^5 conidia g^{-1} of substrate, substrate weight = 100 g, fermentor type = Erlenmeyer flask, nitrogen content = 0.1%). Under these conditions, *T. asperelloides* produced, on average, 1.2×10^9 and 1.1×10^9 CFU g^{-1} of rice flour after 14 days of cultivation, respectively (Table 2). The lowest CFU production was observed in trials 5 (6.9×10^5 CFU g^{-1}) and 6 (1.8×10^7 CFU g^{-1}) (Table 2).

Among the five parameters analyzed in the PBD 12, the nitrogen content and the fermentor type were statistically significant ($P < 0.05$). The nitrogen content ($P = 0.006$) added to rice flour had a negative effect on increasing the concentration in the substrate (Table 3 and Figure 1). Increasing nitrogen content from level -1 (0.1%) to +1 (1.0%), with all other conditions held constant, resulted in a decrease from 1.2×10^9 in trial 2 to 6.9×10^5 CFU g^{-1} in trial 5 (Table 2). Erlenmeyer flasks, used as fermentor type, proved to be more efficient than polypropylene bags ($P = 0.018$; Table 3 and Figure 1), as the former was associated with higher

number of CFU g^{-1} from 6.7×10^7 in trial 1 to 1.1×10^9 in trial 4 (Table 2). These results are also illustrated in the Pareto diagram (Figure 1), where bars extending beyond the reference line are considered significant ($P < 0.05$).

Screening of nitrogen sources for *T. asperelloides* productions in rice flour

Hydrolyzed yeast, corn steep liquor, and autolyzed yeast produced the highest concentrations of *T. asperelloides* (2.62×10^9, 2.19×10^9, and 1.68×10^9 CFU g^{-1}, respectively), without significant differences among them ($P < 0.05$) (Table 4). Ammonium sulfate produced the lowest CFU yields of *T. asperelloides* g^{-1} from rice flour (2.6×10^8 CFU g^{-1}) differing significantly from the hydrolyzed yeast, corn steep liquor, and autolyzed yeast ($P < 0.05$) (Table 4), but similarly to the production using cottonseed flour (8.7×10^8 CFU g^{-1}).

Storage stability

Storage temperature significantly ($P < 0.05$) affected the viability of *T. asperelloides* formulations over time (Figure 3, Tables 6 and 7). Formulations $G_{Cont.}$ and $G_{Bent.}$ showed the best shelf-life results at ambient temperature (25 ± 2 ºC), with no significant drops ($P < 0.05$) of viability for 3 months, while under refrigerated conditions (4 ºC), the viability was maintained for 7 and 12 months, respectively (Tables 6 and 7). Formulations $G_{Lec.}$ and $G_{Org. + BT.}$ could be stored for up to 2 months at ambient temperature (25 ± 2 ºC), without significant ($P < 0.05$) drops in the viability, and for 10 and 12 months in refrigerated conditions (4 ºC), respectively (Tables 6 and 7). The $G_{BT.}$ formulation did not show significant differences between storage temperatures, and could be stored in both conditions for only 1 month (Tables 6 and 7).

Conidiation of *T. asperelloides* formulations

The use of starchy compounds as major components in granular formulations of *T. asperelloides* resulted in increases in concentrations of CFU in all formulations, as seen for $G_{Lecithin.}$ (from 2.9×10^8 to 5.9×10^9 CFU g^{-1}) and for $G_{Break\text{-}Thru.}$ (from 1.6×10^8 to 1.2×10^{10} CFU g^{-1}) (Table 8). Among the five formulations, $G_{BT.}$ was the one with the highest increments ($P < 0.05$) (1.2×10^{10} CFU g^{-1}), followed by formulations $G_{Organic\ compost\ +\ Break\text{-}Thru}$, $G_{Control}$ and $G_{Bentonite}$, with 7.3×10^9, 6.8×10^9 and 6.7×10^9 CFU g^{-1}, respectively (Table 8). The $G_{Lecithin}$ formulation showed the lowest increments in CFU, increasing from 2.9×10^8 to 5.9×10^9 CFU g^{-1} (Table 8).

Effectiveness of *T. asperelloides* formulations against *S. sclerotiorum*

Trichoderma asperelloides formulated in granules ($G_{Control}$) significantly ($P < 0.05$) inhibited the myceliogenic germination of sclerotia of *S. sclerotiorum* (Figure 5). *Trichoderma asperelloides* at 5×10^4, 5×10^5, and 5×10^6 CFU g^{-1} of soil inhibited the myceliogenic germination of *S. sclerotiorum* sclerotia at 79.2, 87.5, and 93.7%, respectively (Figure 4 and 5B). However, no significative differences ($P < 0.05$) were observed between the different *Trichoderma* dosages applied to the soil surface (Figure 5B). The number of soft and degraded sclerotia showed significant differences ($P < 0.05$) among the different concentrations of *T. asperelloides* applied. Increasing dosages provided linear increases for this variable, with mean values of 2.1, 8.3 and 23.0%, at doses of 5×10^4, 5×10^5 and 5×10^6 CFU g^{-1} of soil, respectively (Figure 5A). The high control rates observed in the application of the conidia suspension are probably due to the greater infiltration of *T. asperelloides* conidia in the soil, initiating the infection process prior to formulation.

DISCUSSION

In this study, a new approach was designed for the mass production and formulation of *Trichoderma* in a simple, affordable and waste-free way, resulting in high conidiation and improved shelf-life under refrigerated conditions. Among the five factors analyzed for the stationary fermentation of *T. asperelloides* in rice flour (Table 1), the fermentor type ($P = 0.018$) and the N content ($P = 0.006$) added to the substrate significantly influenced the achievement of higher yields (Table 3 and Figure 1). The nitrogen supplementation at a rate of 0.1% (w/w) provided the highest concentration of CFU, emphasizing the importance of maintaining the nutritional balance of the culture medium.

In order of magnitude, carbon, hydrogen, oxygen and nitrogen are the main nutrients required for microbial growth (Zabriskie et al., 2008), and variations in the nutrient sources and C:N ratio has been reported as directly influencing the propagule yield, desiccation tolerance and propagules type (Jackson et al., 1991; Verma et al., 2007; Mascarin et al., 2018; Rezende et al., 2020). Among the nutrients required, nitrogen sources are the constituent with the highest added value in culture media. However, cost may be lowered with the use of a plethora of complex organic nitrogen sources, derived from agricultural commodities (Jackson 1997; Mascarin et al., 2018). In this study, we observed that complex organic nitrogen sources, such as hydrolyzed yeast, corn steep liquor, autolyzed yeast and cottonseed flour provided the highest production of conidia upon growth in rice flour rather than the inorganic nitrogen source (ammonium sulfate) (Table 4).

An important feature in the microbial growth is related to the content of soluble amino N and the total amount of free aminoacids (Mascarin et al., 2018). In our study, we used four organic nitrogen sources containing, on average, 3.4 to 9.3% total nitrogen. It is noteworthy highlighting that several other nutrients, such as carbohydrates, organic acids, trace metals and vitamins (Zabriskie et al., 2008) are also found in these organic complex compounds derived

from agro-industrial by-products, and they may play a role as driving factors for the highest yields observed in this study.

According to our optimized conditions for stationary fermentation by *T. asperolloides*, we found that hydrolyzed yeast, corn steep liquor, autolyzed yeast, and cottonseed flour were able to deliver yields of 2.6×10^9, 2.1×10^9, 1.6×10^9 and 8.7×10^8 CFU g^{-1}, respectively (Table 4). These results were higher than those observed by Muniz et al. (2018), Cavalcante et al. (2008) and Hewavitharana et al. (2018), who used different species of *Trichoderma* and several agricultural by-products as low-cost substrates in solid-state fermentation. Regarding the fermentor type, it is hypothesized that the highest yields obtained in Erlenmeyers flasks is related to better surface-area ratio that led to increased gas exchange and thus more efficient heat dissipation (Figure 1 and Table 3) in comparison to the polypropylene bag system.

Increase shelf-life of BCAs formulations is one of the main bottlenecks in the development of fungal-based products (Jackson et al., 2009; Faria et al., 2022). However, factors such as microorganism species (Hong et al., 1997; Hong et al., 2001), type of propagules (Cliquet; Zeeshan, 2008), storage temperature (Hong et al., 1999), humidity (Hong et al., 2001) and packaging system (Faria et al., 2012) play singly or combined a critical role in fungal shelf stability. As expected, the higher the temperature, the lower was the viability of *T. asperelloides* formulations over time (Figure 3, Tables 6 and 7). According to Swaminathan et al. (2016), the interaction among formulations, storage temperatures and relative humidity suggests that there is no suitable formulation for storage under all conditions and, particularly, changes in RH affect spore survival in some formulations much more than in others. Finally, these authors concluded that among the five formulations of *Trichoderma atroviridae*, the one containing starch was the least influenced by high temperatures and humidity.

Starch is a multi-purpose additive in formulations of BCAs (Vassilev at al., 2020), which, besides acting as an energy source for microbial cells (Lewis; Papavizas 1985; Klaic et

al., 2018), enhances the stability of the formulation (Przyklenk et al., 2017) by providing protection against thermal, oxidative, and osmotic stresses (Chan et al., 2011; Schoebitz et al., 2012; Tal et al., 1999). Recently, in a techno-economic assessment of the industrial feasibility of a biofertilizer production, based on *T. asperelloides* CMAA 1584, starch, and phosphatic rock, Elias et al. (2022) estimated that the capital investment and annual operating cost for spore extraction would be 18.6% and 0.9%, respectively. Thus, when comparing with the use of rice grains, the rice flour used as a substrate as well as a carrier in granular formulations is not only cost attractive, but most importantly, a waste-free alternative for the development of new *Trichoderma*-based bioformulations.

The $G_{Bentonite}$ and $G_{Organic\ compost\ +\ Break\text{-}Thru}$ formulations obtained the best viability results for storage stability under refrigerated conditions (Tables 6 and 7). According to Stotzky and Burns (1982), soil with concentrations greater than 2% of montmorillonite (main component of bentonite) significantly reduced fungal respiration, radial growth, and conidia germination. Scanning electron microscopy showed that the clay particles were tightly bound to the hyphae of *Histoplasma capsulatum*, suggesting that the clays reduced respiration rate by adhering to the mycelial surface, and thereby interfering with the movement of nutrients, metabolites, and gases across the mycelial wall (Lavie; Stotzky, 1986a). Further, the reduction of fungal respiration seemed to have been indirectly impacted by montmorillonite through its absorption of an iron-transporting siderophore produced by the fungus (Lavie; Stotzky, 1986b). Thus, results obtained in storage stability of $G_{Bentonite}$ formulation may be due to similar effects in *T. asperelloides* propagules.

As observed in this study for $G_{Organic\ compost\ +\ Break\text{-}Thru}$ formulation, which is rich in humic substances, Young et al. (2006) reported that survival and storage rates of *Bacillus subtilis* formulated in alginate beds were increased by the addition of humic acid. Humic acid can oxidize or reduce elements, photosensitize chemical reactions, and either enhance or retard the

uptake of toxic compounds or micronutrients in plants and microorganisms (Nardi et al., 2002; Bacilio et al., 2003). Hence, they suggest that the humic acid, once entrapped in the beads, can bring benefits to plant when the composite is applied to the soil, and to the microorganism by offering supplementary nutrients during encapsulation.

Given the growing interest in the use of BCAs and their inundative delivery requirements, which means that the active propagules must reach the target or colonize the habitat (van Lenteren et al., 2018), the use of nutrient sources to microbial cells in formulations for production of new infective propagules is very advantageous. Lewis and Papavizas (1985) obtained similar results in the conidiation of *Trichoderma* formulations with the addition of starch, in which the number of CFU g^{-1} of soil increased up to 100 times (Table 8). Furthermore, according to Przyklenk et al. (2017), starch is not fully degraded by *Metarhizium brunneum* after four weeks of incubation in Petri dishes, indicating that it can, not only provide long-term nutrients in the field, but also contribute to the continuous flow of the microorganism to the soil until its total consumption.

Trichoderma spp. stationary fermentation in rice flour followed by granulation/extrusion presents a simple, cost-effective, and most importantly waste-free alternative for the development of new formulated product based on aerial conidia of this fungus. Additionally, studies of persistence under field conditions and effectiveness against a broad spectrum of plant pathogens are warranted to fully explore the potential of this type of formulation.

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Author contributions

WB, GMM, CSF, CRO and LGS conceived and designed the experiments. LGS, RCC and CPF performed the experiments, and analyzed the data. WB, GMM, CSF and CRO contributed with reagents/materials/analysis tools and provided critical analysis. All authors wrote the manuscript. All authors read and approved the final manuscript

Conflict of interest

All authors declare that there is no conflict of interest in this original article.

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Table 1. Values of factors and levels (low and high) of the experimental planning according to Plackett-Burman Design 12 (PBD 12).

Factors	Code	Level	
		-1 (low)	+1 (high)
Moisture (%)	X_1	40	60
Inoculum density (conidia g^{-1})	X_2	1×10^5	1×10^6
Substrate weight (g)	X_3	100	150
Fermentor type	X_4	Polypropylene bag	Erlenmeyer flask
Nitrogen content (% w/w)	X_5	0.1	1

Table 2. Effect of moisture, inoculum density, substrate weight, fermentor type, and nitrogen content on colony-forming units of *Trichoderma asperelloides* CMAA 1584 per gram (on wet basis) of rice flour (CFU g^{-1}).

Trial	Factors					*T. asperelloides* yield (CFU g^{-1})
	X_1	X_2	X_3	X_4	X_5	
1	1	-1	1	-1	-1	6.7×10^7
2	1	1	-1	1	-1	1.2×10^9
3	-1	1	1	-1	1	2.6×10^7
4	1	-1	1	1	-1	1.1×10^9
5	1	1	-1	1	1	6.9×10^5
6	1	1	1	-1	1	1.8×10^7
7	-1	1	1	1	-1	3.5×10^8
8	-1	-1	1	1	1	8.3×10^7
9	-1	-1	-1	1	1	4.7×10^8
10	1	-1	-1	-1	1	7.5×10^7
11	-1	1	-1	-1	-1	5.0×10^8
12	-1	-1	-1	-1	-1	1.9×10^8

X_1 = moisture (%); X_2 = inoculum density (conidia g^{-1} of substrate); X_3 = substrate weight (g); X_4 = fermentor type (polypropylene bag and Erlenmeyer flask); X_5 = nitrogen content (% w/w).

Table 3. Estimated values of the effects and *p*-values for moisture, inoculum density, substrate weight, fermentor type, and nitrogen content on the colony-forming units (CFU) of *Trichoderma asperelloides* CMAA 1584 per gram (on wet basis) of rice flour).

Variable	Effect value	*p*-value
Moisture	155632778	0.347
Inoculum density	10132778	0.951
Substrate weight	-125488333	0.447
Fermentor type	406710556	0.018
Nitrogen content	-477733889	0.006

Table 4. Effect of various nitrogen sources on colony-forming units (CFU) of *Trichoderma asperelloides* CMAA 1584 per gram (on wet basis) of rice flour under optimized conditions.

Nitrogen sources	*T. asperelloides* yield ($\times 10^8$ CFU g^{-1})
Hydrolyzed yeast	26.2 (± 4.5) a
Corn steep liquor	21.9 (± 4.2) ab
Autolyzed yeast	16.8 (± 3.0) ab
Cottonseed flour	8.7 (± 0.2) bc
Ammonium sulfate	2.6 (± 0.5) c

*Values represent means (± standard error) and when followed by the same letter do not differ significantly from each other (Tukey $p < 0.05$).

Table 5. Composition of granular (G) formulations of *Trichoderma asperelloides* CMAA 1584.

Formulations	Break-Thru S301 (%)	Soy lecithin (%)	Bentonite (%)	Organic compost (%)
G_{Cont}	-	-	-	-
G_{Lec}	-	5.0	-	-
G_{BT}	2.5	-	-	-
$G_{Ben.}$	-	-	5.0	-
$G_{Org + BT}$	5.0	-	-	43.0

Bentonite ($_{Bent.}$, Sigma-Aldrich®, St. Louis, MO, USA), soy lecithin ($_{Lec.}$, Quimisul® SC, Joinville, SC, Brazil), Break-Thru S301 ($_{BT.}$, Evonik®, Essen, Germany), and organic compost ($_{Org.}$, Ribumin® C, Technes Agrícola, Cabreúva, SP, Brazil).

Table 6. Conidiation of *Trichoderma asperelloides* CMAA 1584 in granular formulations upon re-hydration after 10 days of incubation at 25 ± 2 °C and 12:12 h photoperiod.

Formulations	Conidiation ($\times 10^8$ CFU g^{-1})
$G_{Control}$	68.2 (± 5.3) ab
$G_{Lecithin}$	59.1 (± 11.1) b
$G_{Break\text{-}Thru}$	119.5 (±16.2) a
$G_{Bentonite}$	67.3 (± 14.0) ab
$G_{Organic\ compost\ +\ Break\text{-}Thru}$	73.6 (± 4.6) ab

*Values represent means (± standard error) and when followed by the same letter do not differ significantly from each other (Tukey $p < 0.05$).

Table 7. Shelf-life of *Trichoderma asperelloides* CMAA 1584 formulations storage at cool temperature (4 ºC).

Formulation	Formulation viability (× 10^7 CFU g^{-1})							
	0 month	1 month	2 months	3 months	4 months	6 months	9 months	12 months
G_{Cont}	25.13 (± 1.4) a	21.75 (± 0.9) a	19.72 (± 2.3) a	20.87 (± 0.9) a	20.18 (± 0.5) a	19.68 (± 1.7) a	17.57 (± 0.6)	9.33 (± 0.4)
G_{Lec}	21.33 (± 0.8) a	15.05 (± 1.3) a	14.85 (± 1.4) a	17.47 (± 1.6) a	17.02 (± 1.7) a	21.18 (± 3.1) a	25.87 (± 0.9) a	12.23 (± 1.1)
G_{BT}	17.07 (± 1.4) a	15.22 (± 0.9) a	11.42 (± 0.9)	11.47 (± 0.6)	11.62 (± 0.9)	13.03 (± 0.5)	10.88 (± 0.3)	9.75 (± 0.3)
G_{Bent}	21.05 (± 2.1) a	22.52 (± 1.7) a	17.12 (± 1.0) a	16.35 (± 1.1) a	25.62 (± 2.4) a	20.32 (± 1.2) a	17.87 (± 0.8) a	16.48 (± 1.1) a
$G_{Org + BT}$	21.83 (± 2.7) a	24.97 (± 1.2) a	25.67 (± 2.4) a	25.62 (± 2.4) a	18.83 (± 3.1) a	22.28 (± 1.3) a	22.70 (± 0.5) a	15.65 (± 2.4) a

*Values represent means (± standard error) and when followed by the same letter in each line do not differ significantly from the time 0 (month 0) (Dunnet $p < 0.05$).

Table 8. Shelf-life of *Trichoderma asperelloides* CMAA 1584 formulations storage at ambient temperature (25 ± 2 ºC).

Formulation	Formulation viability (× 10^7 CFU g^{-1})							
	0 month	1 month	2 months	3 months	4 months	6 months	9 months	12 months
G_{Cont}	17.88 (± 2.3) a	21.47 (± 0.9) a	14.10 (± 1.6) a	8.53 (± 0.4) a	6.55 (± 0.8)	4.70 (± 0.9)	0.27 (± 0.1)	0.03 (± 0.0)
G_{Lec}	19.07 (± 3.4) a	13.30 (± 0.6) a	9.68 (± 1.3) a	7.27 (± 0.5)	5.72 (± 0.4)	3.28 (± 0.8)	0.10 (± 0.0)	0.00 (± 0.0)
G_{BT}	9.10 (± 1.1) a	13.45 (± 1.8) a	2.52 (± 0.3)	1.63 (± 0.3)	0.87 (± 0.1)	0.78 (± 0.1)	0.02 (± 0.0)	0.0 0 (± 0.0)
G_{Bent}	20.10 (± 1.5) a	17.40 (± 0.9) a	18.02 (± 1.5) a	14.88 (± 1.9) a	10.87 (± 1.4)	4.58 (± 0.8)	2.03 (± 0.6)	0.11 (±0.0)
$G_{Org + BT}$	21.83 (± 2.7) a	16.13 (± 1.4) a	12.42 (± 1.8)	4.82 (± 1.2)	1.90 (± 0.3)	1.00 (± 0.1)	0.00 (± 0.0)	0.00 (± 0.0)

*Values represent means (± standard error) and when followed by the same letter in each line do not differ significantly from the time 0 (month 0) (Dunnet $p < 0.05$).

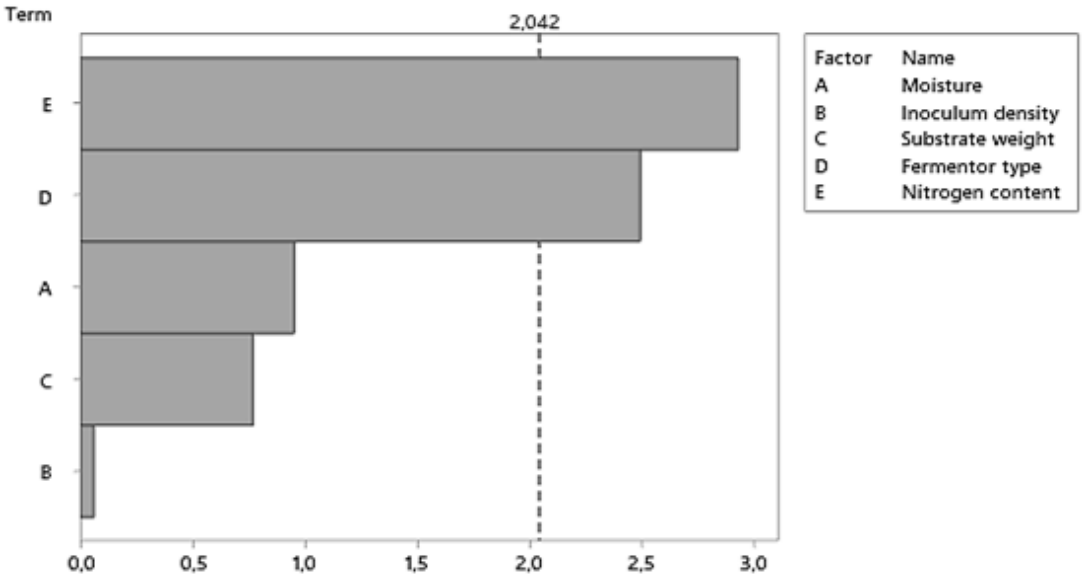

Figure 1. Pareto diagram (α = 0.05) demonstrating the significance of the moisture, inoculum density, substrate weight, fermentor type and nitrogen content variables in production of colony-forming units (CFUs) of *Trichoderma asperelloides* CMAA 1584 in optimized culture medium.

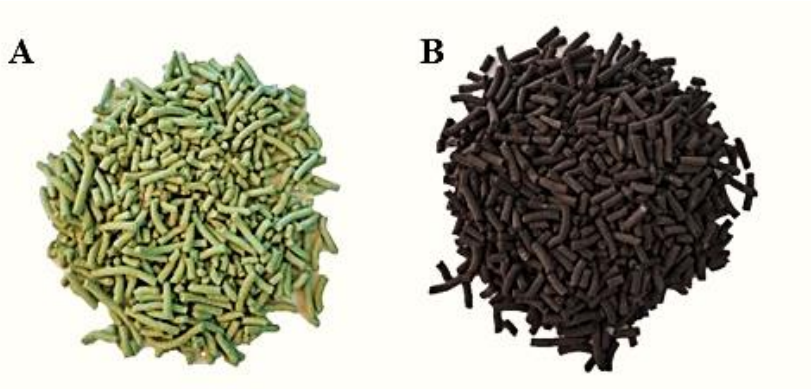

Figure 2. Visual aspect of $G_{Control}$ (A) and $G_{Organic + Break\text{-}Thru}$ (B) granular formulations of *Trichoderma asperelloides* CMAA 1584.

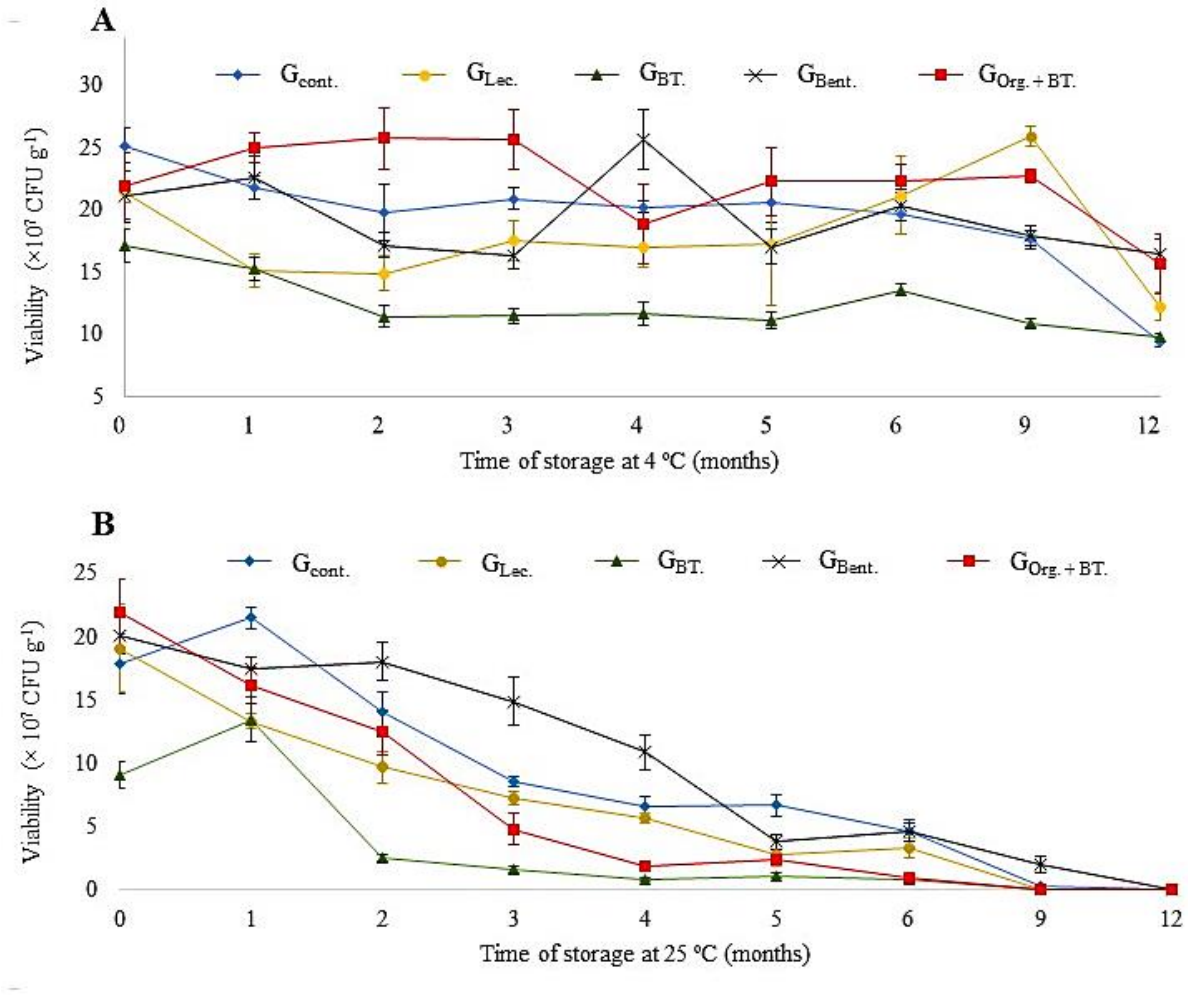

Figure 3. Shelf-life of *Trichoderma asperelloides* formulations storage in ambient temperature (25 ± 2 ºC) (A), and in cool temperature (4 ºC) (B). Mean (± standard error).

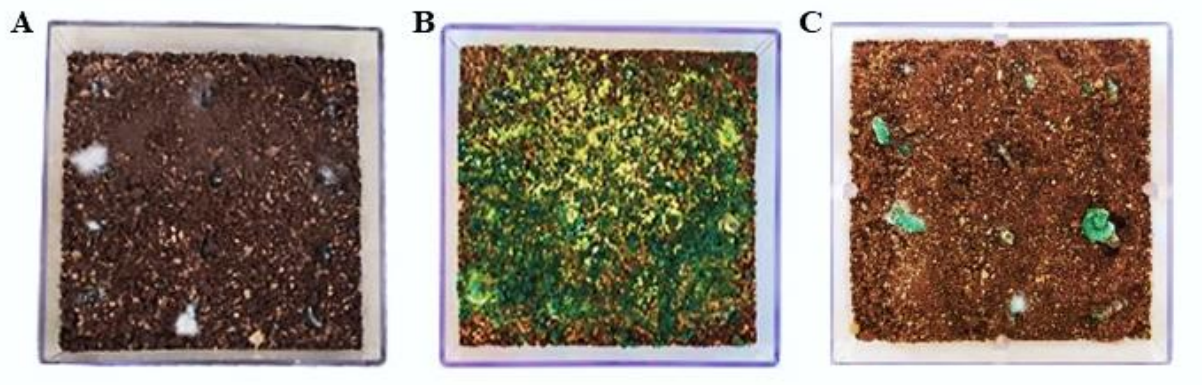

Figure 4. Myceliogenic germination of *Sclerotinia sclerotiorum* sclerotia in control (untreated) group (A) versus sclerotia exposed to 5 × 10^6 UFC g^{-1} soil of $G_{Control}$ formulation (B), versus sclerotia exposed to conidia suspension adjusted to 5 × 10^6 conidia g^{-1} soil of *Trichoderma asperelloides* CMAA 1584 (C).

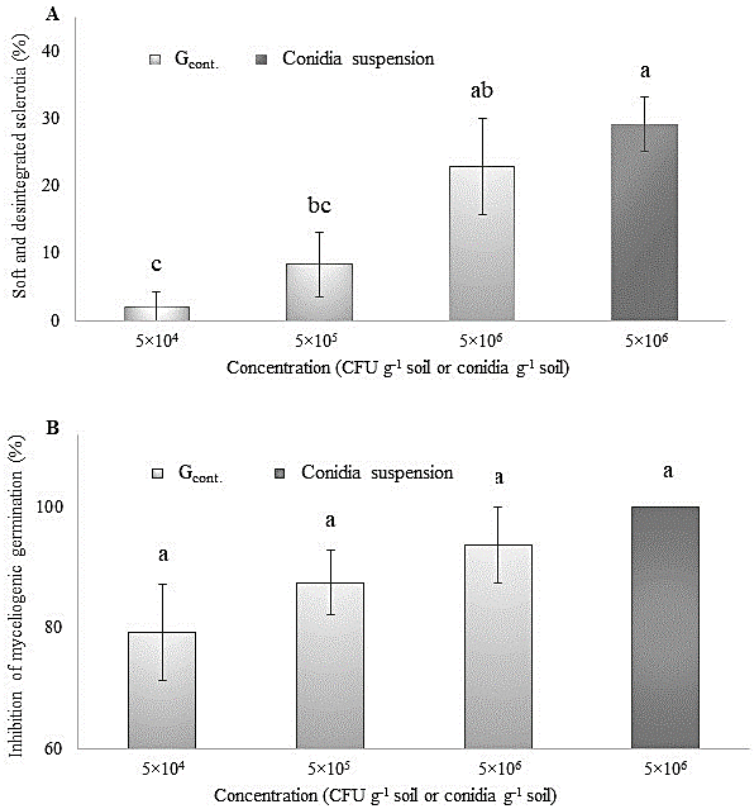

Figure 5. Soft and disintegrated *Sclerotinia sclerotiorum* sclerotia (A) and inhibition of myceliogenic germination of *Trichoderma asperelloides* CMAA 1584 at different concentrations applied to the soil. Untreated group (control) is not shown as all sclerotia were healthy and germinated. Bars indicate the mean (± standard error) and different letters indicate significant differences ($P < 0.05$).

CONSIDERAÇÕES FINAIS

Trichoderma asperelloides CMAA 1584 apresenta eficiência no controle de escleródios de *Sclerotinia sclerotiorum* e em parasitar hifas do patógeno *in vitro*.

Trichoderma lentiforme CMAA 1585 solubiliza fosfato e promove o crescimento de plantas de algodão.

A fermentação de *Trichoderma asperelloides* CMAA 1584 em farinha de arroz apresenta bons rendimentos de propágulos (UFC g^{-1}), sendo uma alternativa simples e barata para utilização como substratos para a fermentação sólida.

A fermentação sólida de *Trichoderma asperelloides* CMAA 1584 em Erlenmeyers utilizando farinha de arroz suplementada com 0,1% (p/p) de levedura hidrolisada tem efeito direto no rendimento de propágulos (UFC g^{-1}).

A formulação de *Trichoderma asperelloides* CMAA 1584 por extrusão da biomassa com o substrato foi capaz de aumentar o número médio de UFCs, após reidratação e incubação por 10 dias, em pelo menos 20 vezes.

As formulações $G_{Bentonite}$ e $G_{Organic\ compost\ +\ Break\text{-}Thru}$ podem ser armazenadas a temperatura de 4 ºC por 12 meses, sem haver quedas significativas de viabilidade.

A formulação de *Trichoderma asperelloides* em grânulos não altera ou dificulta a colonização e a colonização do solo e; não alterando a eficiência de controle a escleródios de *Sclerotinia sclerotiorum* quando aplicado formulado e não formulado.

Ambos isolados de *Trichoderma* têm potencial para serem promissores agentes de biocontrole, mas com diferentes propósitos. *Trichoderma asperelloides* CMAA 1584 para o controle de doenças, enquanto que *T. lentiforme* CMAA 1585 é indicado como bioestimulante.

Ambos os isolados de *Trichoderma* são adequados para uso em campo, podendo ser utilizados em consórcio visando tanto ao controle de patógenos quanto à promoção do crescimento em lavouras de algodão, com consequente contribuição para diminuir a dependência de fertilizantes químicos e fungicidas.

A fermentação sólida e a formulação de *Trichoderma asperelloides* CMAA 1584 por extrusão utilizando a farinha de arroz apresenta bons resultados, além de ser um método simples, barato e livre de resíduos.

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