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Caracterização do agente de controle biológico *Cladosporium* spp. na cultura do arroz de terras altas

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Tese apresentada à Universidade de Brasília como requisito parcial para a obtenção do título de Doutora em Fitopatologia pelo Programa de Pós-graduação em Fitopatologia

Orientadora
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CARACTERIZAÇÃO DO AGENTE DE CONTROLE BIOLÓGICO *CLADOSPORIUM* SPP. NA CULTURA DO ARROZ DE TERRAS ALTAS.

AMANDA ABDALLAH CHAIBUB

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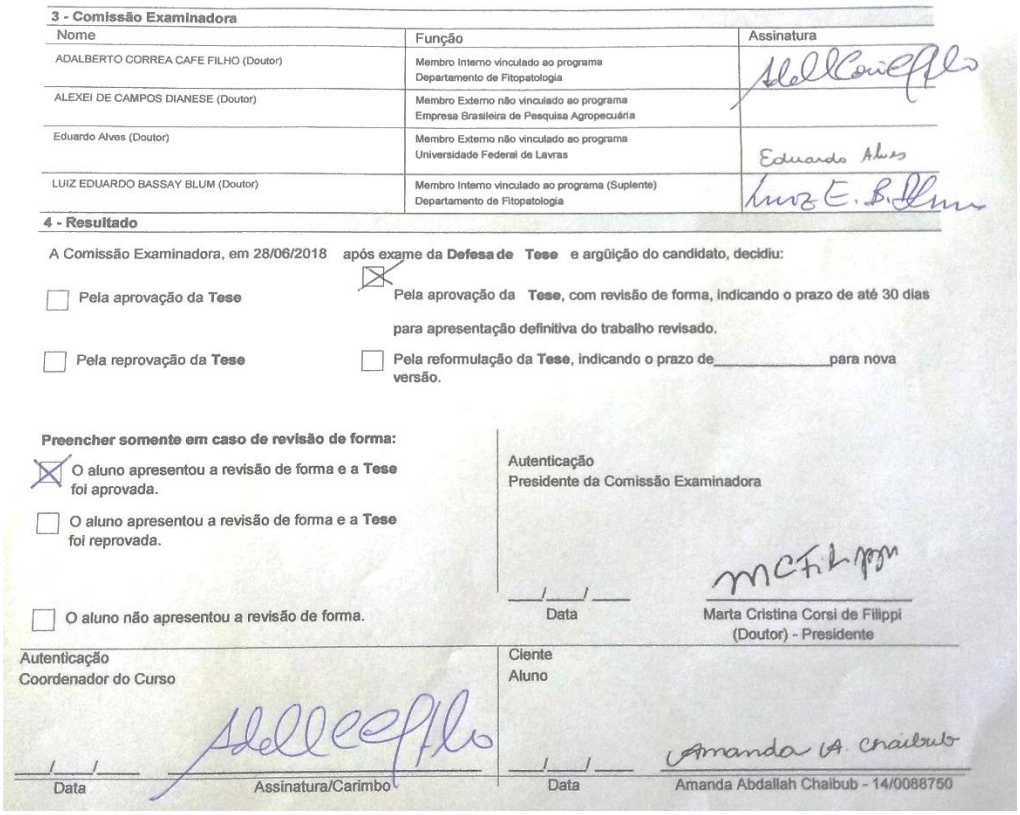

3 - Comissão Examinadora

Nome	Função	Assinatura
ADALBERTO CORREA CAFE FILHO (Doutor)	Membro Interno vinculado ao programa Departamento de Fitopatologia	
ALEXEI DE CAMPOS DIANESE (Doutor)	Membro Externo não vinculado ao programa Empresa Brasileira de Pesquisa Agropecuária	
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4 - Resultado

A Comissão Examinadora, em 28/06/2018 após exame da **Defesa de Tese** e argüição do candidato, decidiu:

- ☐ Pela aprovação da **Tese**
- ☒ Pela aprovação da **Tese**, com revisão de forma, indicando o prazo de até 30 dias para apresentação definitiva do trabalho revisado.
- ☐ Pela reprovação da **Tese**
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Preencher somente em caso de revisão de forma:

- ☒ O aluno apresentou a revisão de forma e a **Tese** foi aprovada.
- ☐ O aluno apresentou a revisão de forma e a **Tese** foi reprovada.
- ☐ O aluno não apresentou a revisão de forma.

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CHAIBUB, Amanda Abdallah. **Caracterização do agente de controle biológico *Cladosporium* spp. na cultura do arroz de terras altas**. 106p. Tese (Doutorado em Fitopatologia) - Universidade de Brasília, Brasília, DF.

O arroz é uma cultura de grande importância e a ocorrência de doenças, principalmente a brusone (*Magnaporthe oryzae*), é a principal causa de redução da produção de arroz. A inclusão do controle biológico no manejo integrado da brusone é uma alternativa para reduzir a aplicação de pesticidas. E o uso abusivo de pesticidas trouxe prejuízos que levaram ao aumento da demanda por produtos livres de resíduos. Além disso, o controle biológico pode ser utilizado na fase de transição entre os sistemas convencional e agroecológico, trazendo benefícios e facilitando o processo. *Cladosporium* spp. tem sido relatado como um agente biológico para vários patógenos de plantas, mas seu mecanismo de ação nunca foi esclarecido, tampouco foi identificado a nível de espécie, em arroz. O isolado C24G foi isolado do filoplano da planta de arroz, adaptado às condições naturais do seu habitat original. O presente estudo teve como objetivo identificar isolados de *Cladosporium* spp., verificar sua potencial atividade antagônica frente aos principais patógenos que afetam a cultura do arroz, identificar o melhor método e momento de aplicação, bem como a expressão gênica e atividade enzimática das plantas durante a interação com C24G e sua eficiência em suprimir a brusone em condições de campo, em sistema de produção agroecológico. Nove isolados de *Cladosporium* spp. foram identificados e caracterizados como antagônicos aos principais patógenos fúngicos do arroz de terras altas. A identificação foi realizada e seu efeito antagônico aos patógenos do arroz determinado. Investigou-se a aplicação na semente (microbiolização), no solo (incorporando e regando) e na pulverização foliar, antes (preventiva) e após (curativa) a inoculação com o patógeno (*M. oryzae*). O melhor tratamento identificado foi a pulverização foliar de C24G com aplicações preventivas. Este tratamento foi investigado para atividade enzimática e expressão gênica, por PCR em tempo real e em condições de campo em sistema agroecológico. Os nove isolados foram identificados como *C. cladosporioides*, *C. tenuissimum* e *C. subuliforme*. Quatro isolados, identificados como *C. cladosporioides*, inibiram o crescimento micelial, tais como C1H (68,59%) para *Sarocladium oryzae*, C5G (74,32%) para *Cochliobolus miyabeanus*, C11G (75,97%) para *M. oryzae* e C24G (77,39%) de *Monographella albescens*. O isolado C24G apresentou maior atividade de enzimas líticas e, posteriormente, testado para *C. miyabeanus* e *M. oryzae*, e mais de 59,36% de inibição da germinação de conídios e formação de apressórios. Suprimiu 83,78% da severidade da brusone nas folhas, aumentou a expressão relativa dos genes

JIOsPR10, *LOX-RLL* e *PR1b* e aumentou a atividade de quitinase, β-1,3-glucanase, lipoxigenase e fenilalanina amônia-liase. Verificamos a supressão de até 85,58% e 79,63% da brusone nas folhas e panículas, respectivamente, e foram detectadas alterações na biomassa e em parâmetros fisiológicos como taxa fotossintética (*A*), transpiração (*E*), condutância estomática (*gs*) e eficiência do uso da água (*WUE*). Além de um aumento significativo no rendimento de grãos, que não refletiu aumento com aplicações combinadas ou um maior número de aplicações. A caracterização de *C. cladosporioides* elege-o como um potencial bioagente para o manejo de diversas doenças do arroz, principalmente a brusone. Essa é a primeira vez que *Cladosporium* spp. isolado do filoplano de arroz é identificado a nível de espécie e que demonstrou-se seu mecanismo de ação, como a atividade de enzimas líticas e expressão gênica. Conclui-se que C24G elicita as respostas de defesa da planta e deve ser componente do manejo sustentável da brusone, reduzindo a aplicação de fungicidas, gerando maior rentabilidade ao produtor e ao ambiente e grãos de arroz livres de contaminações e sua inserção no sistema agroecológico é vantajosa, reduzindo a severidade da brusone e facilitando a transição agroecológica com considerável rendimento de grãos e levando a um produto final sem resíduos.

Palavras-chaves: *Oryza sativa*, controle biológico, antagonismo, indução de resistência, agroecologia, desenvolvimento sustentável.

Orientadora - Dra. Marta Cristina Corsi de Filippi - EMBRAPA Arroz e Feijão, Sto. Antônio de Goiás-GO.

GENERAL ABSTRACT

CHAIBUB, Amanda Abdallah. **Characterization of the biological control agent *Cladosporium* spp. in upland rice**. 106p. Thesis (PhD in Phytopathology) - University of Brasília, Brasília, DF.

Rice is a crop of great importance and the occurrence of diseases, especially the blast (*Magnaporthe oryzae*) is the main cause of rice production reduction. The inclusion of biological control in the integrated management rice blast is an alternative to reduce the application of pesticides. And, the abusive use of pesticides has brought losses that have led to increased demand for waste-free products. Moreover, biological control can be used in the transition phase between the conventional and agroecological systems, bringing benefits and facilitating the process. *Cladosporium* spp. has been reported as a biological agent for several plant pathogens, but its mechanism of action has never been clarified, nor was it identified at the species level in rice. The isolated C24G was isolated from rice plant phylloplane, thus adapted to the natural conditions of its original habitat. The present study aims to identify isolates of *Cladosporium* spp., verify their potential antagonistic activity against the main pathogens that affect rice culture, identify the best method and moment for application, as well as the gene expression and enzymatic activity of plants during interaction with C24G and, its efficiency to suppress rice blast under field conditions, in agroecological production system. Nine isolates of *Cladosporium* spp. were identified and characterized as antagonistic to the main fungal pathogens from upland rice. The identification was performed and determined its antagonistic effect with the rice pathogens. It was investigated the application in the seed (microbiolization), soil (incorporating and drenching) and foliar spraying, before (preventive) and after (curative) challenge inoculation with the pathogen (*M. oryzae*). The best treatment identified was the foliar sprayed of C24G as preventive applications. This treatment was further investigated for enzymatic activity and gene expression, by Real-time PCR and in fields conditions in agroecological system. The nine isolates were identified as *C. cladosporioides*, *C. tenuissimum* and *C. subuliforme*. Four isolates, identified as *C. cladosporioides*, were inhibit mycelial growth such as C1H (68.59%) of *Sarocladium oryzae*, C5G (74.32%) of *Cochliobolus miyabeanus*, C11G (75.97%) of *M. oryzae* and C24G (77.39%) of *Monographella albescens*. The C24G isolate showed higher activity of lytic enzymes and later tested for *C. miyabeanus* and *M. oryzae*, and higher than 59.36% conidial germination and appressorium formation inhibition. It suppressed 83.78% leaf blast severity, increased relative expression of the *JIOsPR10*, *LOX-RLL* and *PR1b* genes and increased Chitinase, β-1,3-Glucanase, Lipoxygenase

and Phenylalanine ammonia-lyase activity. We verified the suppression of up to 85.58% and 79.63% of leaf and panicle blast, respectively, and changes were detected in biomass and in physiological parameters such as photosynthetic rate (*A*), transpiration (*E*), stomatal conductance (*gs*) and water use efficiency (*WUE*). In addition to a significant increase in grain yield, which did not reflect increase with combined applications or a greater number of applications. The characterization of *C. cladosporioides* elect it as a potential bioagent for management of several rice diseases, especially rice blast. This is the first time a *Cladosporium* spp. isolated from rice phylloplane is identified at the species level and demonstrated its mechanism of action, such as the activity of lytic enzymes and gene expression. We conclude that C24G elicits the plant defense responses and should be component of sustainable blast management, reducing the application of fungicides, generating greater rentability to producer and environment and rice grains free of contaminations and its insertion in the agroecological system is advantageous, reducing the severity of the blast and facilitating the agroecological transition with considerable yield of grains and leading to a final product without residues.

Keywords: *Oryza sativa*, biological control, antagonism, induced resistance, agroecology, sustainable development.

Advisor: Dra. Marta Cristina Corsi de Filippi - EMBRAPA Rice and Beans, Sto. Antônio de Goiás-GO

INTRODUÇÃO GERAL

O arroz (*Oryza sativa* L.), é uma gramínea herbácea anual, autógama e essencial para a alimentação e nutrição de mais da metade da população mundial. Por ser um dos cereais mais consumidos do mundo, a demanda está prevista para expandir e alcançar 503,9 milhões de toneladas em 2017/18 (FAO, 2018).

O Brasil é o maior produtor de arroz fora do continente asiático, e seu cultivo ocorre em todo o território nacional, em dois sistemas. Na região Sul, concentra-se 77% da produção nacional (CONAB, 2016), em sistema de cultivo irrigado por inundação, e nas demais regiões, o sistema de cultivo predominante é em terras altas.

O arroz de terras altas, apesar de apresentar seu cultivo reduzido (Guimarães *et al*., 2006), apresenta vantagens em relação ao irrigado, devido ao seu menor custo de produção, consumo reduzido de água e menor efeito negativo na camada de ozônio. Mesmo com grande potencial para aumento de sua produção, o arroz de terras altas possui alguns entraves como a baixa resposta a adubação, vigor inicial, instabilidade climática e uma alta ocorrência de doenças, resultando numa baixa produtividade quando comparado ao arroz irrigado (Filippi *et al*., 2012).

A produtividade, em ambos os sistemas de plantio é afetada pela ocorrência de doenças, destacando-se as doenças causadas por fungos como a brusone (*Magnaporthe oryzae*), a mancha parda (*Cochliobolus miyabeanus*), a escaldadura (*Monographella albescens*) e a podridão da bainha (*Sarocladium oryzae*), que são as principais causas de perdas de rendimento e qualidade de grãos. As perdas causadas pela brusone podem chegar a 100% (Prabhu *et al*., 2009), pela mancha parda 90% (Mew & Gonzales, 2002), pela escaldadura mais de 30% (Prabhu & Filippi, 2006) e pela podridão da bainha até 80% (Muralidharan & Venkata Rao, 1980).

A brusone, principal doença do arroz ao redor do mundo, é causada por um fungo, denominado *Pyricularia oryzae* Cav. e seu teleomorfo um ascomiceto, *Magnaporthe oryzae* B. Couch. Os conídios são piriformes, obclavados, com a base circular e o ápice fino, levemente escuros ou hialinos, com pequeno hilo na base e a maioria possui um ou dois septos transversais. Porém, fatores fisiológicos e ambientais contribuem para a variação na forma dos conídios. Durante a fase de amadurecimento, ainda ligado ao conidióforo, o conídio aumenta de tamanho e libera uma gota de mucilagem, que permite a sua aderência a qualquer superfície, mesmo molhada (Prabhu & Filippi, 2006). É um fitopatógeno hemibiotrófico (Jia *et al*., 2000) que pode atacar as plantas de arroz em todas as fases de desenvolvimento, podendo infectar folhas, caules, colmos, bainhas e panículas (Wilson & Talbot, 2009).

O ciclo de vida inicia-se com a produção dos conídios, geralmente liberados e disseminados durante a noite. Depois de aderidos ao hospedeiro, os conídios germinam e formam o tubo germinativo. Na extremidade do tubo germinativo inicia-se a diferenciação celular que leva a formação da célula gancho, um inchaço considerado como início da formação do apressório, estrutura desenvolvida por vários fungos para romper a superfície foliar do hospedeiro. Posteriormente, a penetração ocorre na epiderme da folha do hospedeiro através do rompimento da cutícula da planta e formação da hifa de infecção. Após 72 horas da inoculação, as lesões começam a aparecer e crescem em tamanho e número até coalescerem (Prabhu & Filippi, 2006).

A doença ocorre em todo território brasileiro e também em todas as áreas produtoras de arroz do mundo, desde a plântula até a fase de maturação da cultura. Os sintomas nas folhas iniciam-se com a formação de pequenas lesões necróticas, de coloração marrom e centro cinza ou esbranquiçado. Em condições favoráveis, as lesões coalescem causando morte das folhas e, muitas vezes, da planta inteira (Prabhu *et al*., 1989).

A intensificação do cultivo de arroz altamente mecanizado em áreas contíguas extensas proporcionou aumento da brusone e sérios prejuízos na produtividade e qualidade dos grãos (Prabhu & Filippi, 2006).

A brusone ocorre em todas as fases de desenvolvimento da planta, mas torna-se muito severa quando ocorre na fase vegetativa, entre os 20 e 40 dias após o plantio (V2 e V5), e na fase reprodutiva, logo após a emissão do cacho (R2 a R4), até o enchimento dos grãos. As fontes de inóculo de brusone para as lavouras ocorrem através de sementes infectadas, de restos culturais e de conídios levados pelo vento (Filippi *et al*., 2015).

Em muitos patossistemas, a adoção de técnicas isoladas de controle de doenças tem-se mostrado ineficiente, havendo, portanto, a necessidade de combinação de métodos de controle, denominado manejo integrado. O manejo integrado é uma mistura de práticas agronômicas com práticas de controle da doença que regulam a população do patógeno a níveis toleráveis, sem causar danos econômicos (Prabhu & Filippi, 2006).

O manejo integrado da brusone requer o uso de práticas culturais, resistência genética e controle químico (Pooja & Katoch, 2014). A nutrição das plantas, a substituição de cultivares, menor densidade de semeadura e irrigação adequada, diminuem o inóculo e consequentemente, o estabelecimento da doença (Prabhu & Filippi, 2006). Além dessas práticas, o uso de fungicidas e cultivares geneticamente resistentes também são amplamente adotados (Pagliaccia *et al*., 2017; Selisana *et al*., 2017).

A resistência genética é uma das estratégias mais viáveis, por ser econômica, porém,

diversas cultivares de arroz melhoradas para resistência a brusone, não apresentam resistência estável, quando plantada em larga escala, causando reflexos negativos na produtividade (Ou *et al*., 1980). Muitos genes de resistência para *M. oryzae* já foram identificados, mas a duração da resistência depende da complexa biologia de *M. oryzae*, que rapidamente é capaz de produzir novos patótipos (Parleviet & Zadoks, 1977). A diversificação de cultivares dentro da área também é recomendada, se possível, pois com diferentes cultivares, maior a diversidade de raças de *M. oryzae*, sem a prevalência de uma raça, ocasionando menos doença ao longo do tempo (Filippi et al., 2015).

O uso de fungicidas torna-se essencial quando o grau de resistência das cultivares é ineficaz. Dentre os fatores limitantes para o uso de fungicidas, é o aumento considerável no custo da produção, que podem chegar a mais de $ 70 ha^{-1} (Nalley *et al*., 2016) e a capacidade dos patógenos adquirirem resistência aos princípios ativos utilizados (Castroagudín *et al*., 2015; Pooja & Katoch, 2014), levando assim a um uso indiscriminado.

O aumento da conscientização pública sobre a produção sustentável levou a práticas de manejo agrícola de baixo insumo, com uma demanda crescente por alimentos livres de pesticidas (Pieniak *et al*., 2010). No entanto, para realizar a transição dos sistemas agrícolas convencionais para os agroecológicos, algumas ferramentas, como o controle biológico, auxiliam no processo e ajudam a reduzir a incidência de pragas e doenças.

O arroz orgânico representa aproximadamente 3 milhões de hectares e está se tornando cada vez mais popular na China (Huang *et al*., 2016). No entanto, há uma falta de pesquisa e tecnologia para sua produção (Huang *et al*., 2016). No Brasil, a produção de arroz orgânico ainda é baixa e está concentrada nos assentamentos da reforma agrária no Rio Grande do Sul, embora ainda seja a maior da América Latina. A área cultivada com arroz em sistema agroecológico totalizou 5 mil hectares na safra 2014/15. Ao todo, 502 agricultores cultivam arroz agroecológico no Rio Grande do Sul, o equivalente a 4,5% dos 11 mil produtores de arroz (Gonçalves *et al*., 2017).

Segundo Prabhu *et al*. (2002), os danos ocasionados pelos métodos do manejo integrado devem ser mínimos ao ambiente e à saúde humana e deve evitar ações desfavoráveis, como o uso indiscriminado de agrotóxicos, com possíveis efeitos sobre microrganismos não alvos, diminuindo a atividade antagonista no filoplano (Gonçalves *et al*., 2012).

A combinação de agentes biológicos e fungicidas pode ser um dos meios de se otimizar o uso das demais táticas de manejo, aumentando a eficiência do controle químico e permitindo a inserção do controle biológico em um sistema de manejo integrado de doenças (Duffy, 2000; Sanjay *et al*., 2008).

O controle biológico, segundo Cook & Baker (1983), é a redução da densidade do inóculo ou das atividades determinantes da doença, mediada por um ou mais organismos que não o homem. É um método de controle que está ganhando espaço a cada dia nos sistemas agrícolas e pode ser inserido no manejo integrado de diversas doenças, reduzindo os riscos ambientais e à saúde humana.

O controle biológico tem sido usado há mais de um século, com primeiro relato em 1880, com a introdução da mosca, *Cryptochaetum iceryae* Williston (Diptera: Cryptochaetidae) e do besouro *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) para o controle de *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) em pomares de citrus (Barrat et al., 2018). No entanto, o crescimento e sucesso da indústria de pesticidas causou o desaparecimento do uso do controle biológico até a publicação de Rachael Carson "Silent Spring", que denunciou o uso de pesticidas enfatizando os danos ao meio ambiente (Barrat *et al*., 2018).

Esta abordagem consiste na aplicação de uma população benéfica para reduzir uma população patogênica em um determinado hospedeiro (Lenteren *et al*., 2018). Agentes biológicos podem atuar por mecanismos diretos e indiretos. Mecanismos diretos incluem: antibiose, parasitismo e competição. Já um exemplo de um mecanismo indireto é a indução de resistência (Francesco *et al*., 2016). Este processo ocorre no hospedeiro após o contato entre o agente biológico e a planta e consiste em estimular o sistema imune da planta (Burketová *et al*., 2015).

Na literatura, a indução de resistência é denominada como resistência sistêmica adquirida (SAR) e resistência sistêmica induzida (ISR). SAR pode ser desencadeada após uma infecção local por um patógeno e é dependente de ácido salicílico (SA) e seus análogos que ativam genes relacionados à patogênese, que se expressam através das proteínas relacionadas à patogênese (PRPs) (Vlot *et al*., 2009). Já ISR é tratada por ser ativada por microrganismos benéficos (bactérias e fungos) e ser regulada por vias de sinalização dependentes de jasmonatos (JA) e etileno (ET) (Pieterse *et al*., 2014). Mas diversos estudos mostram que microrganismos benéficos podem ativar ambas as vias (SAR and ISR) e regular uma complexa rede de sinais que envolvem SA, JA e ET e o crosstalk entre elas, demonstrando como as vias estão interconectadas na indução da rede de sinalização de respostas de defesa (Vitti *et al*., 2015; Hermosa *et al*., 2012; Salas-Marina *et al*., 2011).

Os microrganismos da rizosfera e da filosfera se destacam no controle biológico, tanto por mecanismos diretos e indiretos citados acima. Uma maneira de controlar patógenos de parte

aérea é através da introdução de antagonistas na folha, um ambiente onde antibiose, competição, e parasitismo são exercidos, resultando em um controle da doença.

O filoplano é a superfície da parte aérea das plantas e a filosfera é a área que circunda o filoplano, local que há a disponibilidade de nutrientes que as plantas exudam, como açúcares, íons minerais e compostos nitrogenados, portanto a capacidade de um microrganismo estabelecer-se, multiplicar-se e sobreviver no filoplano é função também da sua capacidade de utilizar esses exudatos (Romeiro, 2007).

Residentes do filoplano são alternativas ainda pouco exploradas, principalmente quando comparadas às rizobactérias, podendo essa diferença ser atribuída, em grande parte às características do habitat onde cada um se localiza. O filoplano, do ponto de vista eco e microbiológico é um ambiente instável, que sofre grandes alterações de temperatura, umidade, radiação, ventilação, composição e quantidade de nutrientes disponíveis e isso dificulta o estabelecimento de populações a serem introduzidas para fins de biocontrole. Esses fatores fazem com que muitas vezes antagonistas promissores sejam ineficientes por não serem capazes de sobreviver e se manter em alta densidade, impedindo o controle das doenças (Romeiro, 2007).

Entre esses microorganismos, podemos citar as bactérias (Filippi *et al*., 2011; Halfeld-Vieira *et al*., 2015) e os fungos (Kawamata *et al*., 2004; Sena *et al*., 2013; Chaibub *et al.*, 2016; Harish *et al*., 2007; Raja & Mahalakshmi, 2014).

O gênero *Cladosporium* spp. é cosmopolita e habita o filoplano de vários hospedeiros, sendo facilmente isolado de vários substratos, que incluem a superfície foliar de várias espécies de plantas (Bensch *et al*., 2010; Bensch *et al*., 2012). As espécies do gênero *Cladosporium* spp. exibem uma ampla variedade de estilos de vida, desde saprófitas, endofíticos, agentes de biocontrole e patógenos humanos e vegetais (Bensch *et al*., 2015).

Como agentes de biocontrole, habitantes do filoplano ou hiperparasitas, *Cladosporium* spp. foi relatado com efeito de controle para vários patógenos, como *Botrytis fabae*, *Phytophthora capsici*, *Venturia inaequalis*, *Puccinia striiformis* f. sp. *tritici*, *Puccinia horiana*, *Pyrenophora tritici-repentis*, *Sclerotinia sclerotiorum*, *Exobasidium camelliae* var. *gracilis*, *Cronartium flaccidum*, *Peridermium pini*, *Uromyces appendiculatus* e *Melampsora medusae* (Jackson *et al*., 1997; Sakagami *et al*., 1995; Köhl *et al*., 2015; Zhan *et al*., 2014; Torres *et al*., 2017; Larran *et al*., 2016; Boland & Hunter, 1988; Mims *et al*., 2007; Moricca *et al*., 2001; Assante *et al*., 2004; Nasini *et al*., 2004; Sharma & Heather, 1980). Além disso, *Cladosporium* spp. foi capaz de reduzir a micotoxinas prejudiciais para a qualidade do café (Chalfoun *et al*., 2010; Chalfoun *et al*., 2007; Pereira *et al*., 2005; Angelico, 2012; Angelico *et al*., 2017).

Além de *Cladosporium* spp. atuar como antagonista e indutor de resistência, Paul & Park (2013) relataram que o agente biológico *C. cladosporioides* promoveu significativamente o crescimento de mudas de fumo *in vitro* e identificaram compostos voláteis de *C. cladosporioides*, como α-pineno, (-)-*trans*-cariofileno, tetra-hidro-2,2,5,5-tetramethylfuran, dehydroaromadendrene e (+)-sativene. Porém, são necessários estudos sobre estes compostos para garantir seu papel efetivo na promoção de crescimento. Hamayun *et al*. (2009) e Hamayun *et al*. (2010) identificaram *Cladosporium sphaerospermum* and *Cladosporium* sp. como produtores de giberelinas, que promoveram o crescimento de plantas de soja e pepino.

A produção de arroz orgânico, permite um desenvolvimento sustentável e a expansão da cadeia produtiva, por apresentar menor custo de produção que o sistema convencional. No entanto, é necessária dedicação intensiva para manter todas as atividades neste sistema de produção (Zanon *et al*., 2015).

A utilização de *Cladosporium* spp. em sistemas agroecológicos deve ser investigada, pois pode reduzir a severidade de doenças, assim como aumentar a produção. Mas para isto, são necessários estudos sobre *Cladosporium* spp. para investigar seu modo de vida, como eles interagem com o hospedeiro, com os patógenos e com outras culturas associadas ao sistema, assim como o que produzem quando estão interagindo.

Existem extensivas informações genômicas, transcriptômicas, proteômicas e metabolômicas para fungos do gênero *Trichoderma* spp. (Lorito *et al*., 2010; Mukherjee *et al*., 2013). Em contrapartida, apesar de existirem muitos relatos do potencial de *Cladosporium* spp. como agente de controle biológico seus mecanismos ainda não foram totalmente compreendidos.

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OBJETIVO GERAL

O presente estudo teve como objetivo caracterizar isolados de *Cladosporium* spp. e determinar sua capacidade em suprimir a brusone e aumentar o rendimento de grãos provocando alterações na atividade enzimática, expressão gênica e trocas gasosas.

Objetivos específicos

1) Identificar isolados de *Cladosporium* spp. e verificar sua potencial atividade antagônica frente aos principais patógenos que afetam a cultura do arroz;

2) Identificar o melhor método e momento de aplicação, bem como a expressão gênica e atividade enzimática das plantas durante a interação com *Cladosporium* spp.

3) Determinar eficiência em suprimir a brusone e aumentar o rendimento de grãos em condições de campo, em sistema de produção agroecológico.

CAPÍTULO 1

Molecular and morphological characterization of rice phylloplane fungi and determination of the antagonistic activity against rice pathogens.

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Molecular and morphological characterization of rice phylloplane fungi and determination of the antagonistic activity against rice pathogens

Abstract

Cladosporium spp. is a cosmopolitan fungus genus. It has been reported as a biological agent for several plant pathogens, but their mechanism of action and identification at the species level have never been clarified. The present study aims for the identification based on DNA phylogeny of nine isolates of *Cladosporium* spp. obtained from the phylloplane of rice in different regions of Brazil and their potential antagonistic activity against the main fungal pathogens that affect rice crop. Nine isolates of *Cladosporium* spp. isolated from rice phylloplane from Brazil were identified, based on DNA phylogeny and characterized as antagonistic to the main fungal pathogens from upland rice. The molecular and morphological identification of the nine isolates of *Cladosporium* spp. and determined its antagonistic effect with the rice pathogens *C. miyabeanus*, *M. oryzae*, *M. albescens* and *S. oryzae*. The best four isolates were selected for studies of lytic enzymes such as β-1,3-glucanase, chitinase and protease and only one was selected for a conidial germination and appressoria formation assay. The nine isolates were identified as *C. cladosporioides*, *C. tenuissimum* and *C. subuliforme*. Four isolates, identified as *C. cladosporioides*, inhibited mycelial growth of rice pathogens such as C1H (68.59%) of *S. oryzae*, C5G (74.32%) of *C. miyabeanus*, C11G (75.97%) of *M. oryzae* and C24G (77.39%) of *M. albescens*. The C24G isolate showed higher activity of lytic enzymes and later tested for *C. miyabeanus* and *M. oryzae*, and higher than 59.36% conidial germination and appressorium formation inhibition. The characterization of *C. cladosporioides* elect it as a potential bioagent for management of several rice diseases, especially rice blast. This is the first time a isolate from the rice phylloplane from *Cladosporium* spp. complex is identified at the species level and demonstrated its mechanism of action, including as the activity of lytic enzymes.

Keywords *Oryza sativa*, biological agent, antagonism, mycoparasitism, enzyme activity, taxonomy.

INTRODUCTION

The rice phylloplane is inhabited by a large variety of microorganisms that are able to survive and colonize foliar tissues without causing damage to the host plant. Among these microorganisms, we can mention bacteria from the genus *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. (Filippi et al. 2011; Halfeld-Vieira et al. 2015; Kumaravel et al. 2018; Thapa et al. 2017; Harikrishnan et al. 2014); yeasts such as *Yarrowia lipolytica*, *Rhodotorula taiwanensis*, and *Candida tropicalis* (Limtong et al., 2014), Khunnamwong et al 2018); and various fungi such as *Epicoccum* spp., *Cladosporium* spp., *Penicillium* spp., and *Aspergillus* spp. (Kawamata et al., 2004, Sena et al., 2013, Chaibub et al, 2016, Harish et al., 2007, Raja and Mahalakshmi, 2014).

The genus *Cladosporium* spp. is considered the largest and most heterogeneous genus of Hyphomycetes and is composed of three large species complexes (*C. cladosporioides* complex, *C. herbarum* complex and *C. sphaerospermum* complex) that differ genetically and morphologically (Bensch et al., 2012). It has a cosmopolitan distribution and inhabits the phylloplanes of several hosts, including rice, being easily isolated from several substrates, including leaves of several hosts (Bensch et al. 2010; Bensch et al. 2012).

Several species of *Cladosporium* spp. were reexamined and reorganized based on the compatibilization of morphological and phylogenetic characteristics of DNA, published by Bensch et al. (2012). Among the 993 species attributed to *Cladosporium s. lat.*, only 170 species were recognized in *Cladosporium s. str.* Subsequently, Bensch et al. (2015, 2018) described another 19 new species belonging to this complex. With new collection and continuous isolation, including indoor environments, on several continents, the number of species of the genus has increased to 218 species and 16 new species.

The species of the genus *Cladosporium* spp. exhibit a wide variety of lifestyles, ranging from saprophytes, endophytes, mycophilic species, biocontrol agents and human and plant pathogens (Bensch et al. 2015).

As biocontrol agents, both as natural inhabitants of the phylloplane and hyperparasites, *Cladosporium* spp. have been reported in studies of various pathogens, such as *Botrytis fabae*, *Phytophthora capsici*, *Venturia inaequalis*, *Puccinia striiformis* f. sp. *tritici*, *Puccinia horiana*, *Pyrenophora tritici-repentis*, *Sclerotinia sclerotiorum*, *Exobasidium camelliae* var. *gracilis*, *Cronartium flaccidum*, *Peridermium pini*, *Uromyces appendiculatus*, and *Melampsora medusae* (Jackson et al. 1997; Sakagami et al. 1995; Köhl et al. 2015; Zhan et al. 2014; Torres et al. 2017; Larran et al. 2016; Boland and Hunter 1988; Mims et al. 2007; Moricca et al. 2001; Assante et al. 2004; Nasini et al. 2004; Sharma and Heather 1980). In addition, *Cladosporium*

spp. are able to reduce ochratoxin A (mycotoxin), which is detrimental to coffee quality by increasing the sensory quality of the drink (Chalfoun et al. 2010; Chalfoun et al. 2007; Pereira et al. 2005; Angelico 2012; Angelico et al. 2017).

Cladosporium spp. have been reported as biological agents *in vitro* for pathogens affecting rice, such as *Magnaporthe oryzae* (rice blast), *Cochliobolus miyabeanus* (brown spot) and *Sarocladium oryzae* (sheath rot). *In vivo*, these species suppress leaf blast but, to date, have not been identified at the species level (Chaibub et al. 2016; Raja and Mahalakshmi 2014).

Rice blast, brown spot, sheath rot and leaf scald (*Monographella albescens*) are the main causes of grain yield and quality losses. Losses caused by rice blast can reach 100% (Prabhu et al., 2009), by brown spot 90% (Mew and Gonzales 2002), by scald more than 30% (Filippi and Prabhu 2006) and by sheath rot up to 80% (Muralidharan and Venkata Rao 1980).

Rice fungal disease control is usually carried out through the application of fungicides, which may have deleterious effects on nontarget microorganisms inhabiting the phylloplane (Gonçalves et al., 2012), an environment in which a highly competitive and antagonistic activity occurs, thus reducing the possibility of natural disease control.

Studies of *Cladosporium* spp. need to investigate their way of life, how they interact with plant host and pathogens, how and what they produce when in antibiosis. There is information about genomic, transcriptomic, proteomic and metabolomic approaches, similar to the extensive information available for fungi of the genus *Trichoderma* spp. (Lorito et al. 2010; Mukherjee et al. 2013). As basic knowledge is needed to initiate an extensive investigation of this bioagent, the present study aims for the identification based on DNA phylogeny of nine isolates of *Cladosporium* spp. obtained from the phylloplane of rice in different regions of Brazil and their potential antagonistic activity against the main fungal pathogens that affect rice culture.

MATERIAL AND METHODS

Isolates

Nine isolates of *Cladosporium* spp. (C1H, C2Pe, C5G, C5H, C11G, C11Rb, C18Pa, C19G and C24G), isolated from rice phylloplane collected at commercial fields, cultivated in different cropping systems in the years 2009 and 2010, in Brazil (Table 1).

Molecular characterization

DNA extration

Colonies of each *Cladosporium* spp. isolates, maintained on filter paper stored at -20 °C, were transferred and grown on Petri dishes containing culture medium (PDA). Genomic DNA extraction was proceed according to Dellaporta (1983) with modifications. Mycelium from each isolate was transferred to microtubes and added 500 µl of Dellaporta extraction buffer (1.57g Tris-HCl 100 mM; 1.86g EDTA 50 mM; 2.92g NaCl 500 mM; 100 mL H_2O e pH 8.0). Subsequently, 30 µL of SDS (2.8 g dodecyl sodium sulfate, 14 mL H_2O ultrapure) was homogenized and incubated at 65 °C for 30 minutes. 160 µL of potassium acetate 5M was added, stirring for 2 minutes and centrifugation for 10 minutes at 18,626 x g.

The supernatant was transferred to a new microtube where 330 µl of isopropanol was added, homogenized and then centrifuged for 10 minutes at 18,626 x g. The supernatant was discarded and 500 µl of 70% ethanol was added twice, consecutively, followed by 30 seconds centrifugation and discard of the supernatant again. 250 µl of ethanol PA was added and centrifuged for 5 minutes at 18,626 x g. The supernatant was discarded and the pellet was placed for drying at room temperature. The DNA was resuspended in 100 µL ultrapure water and then quantified in a spectrophotometer (NanoDrop 2000) at wavelength (λ) equal to 260 nm, diluted to a concentration of 50 ng/µL and stored at -20 °C.

Amplification

ITS-1, 5.8S rDNA, ITS-2 amplification region used the primers ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (3'-TCCTCCGCTTATTGATATGC–5') (White et al. 1990). To obtain the species-level resolution for *Cladosporium* spp. the ITS region was supplemented with partial sequences of elongation factor genes (EF-1α) using the primers EF1-728F (5'–CATCGAGAAGTTCGAGAAGG–3') and EF1-986R (5'–TACTTGAAGGAACCCTTACC–3') and actina (ACT) gene with primers ACT-512F (5'–ATGTGCAAGGCCGGTTTCGC–3') and ACT-783R (5'–TACGAGTCCTTCTGGCCCAT–3') (Carbone and Kohn 1999). PCR reactions were performed as described by Bensch et al. (2012) with modifications using the Qiagen® PCR Multiplex Kit, following the manufacturer's instructions for a final reaction volume of 10 µL and using 50 ng of DNA from each isolate and 0.2 µmol of each primer.

The amplification program for ACT and EF-1α consisted of initial denaturation at 95 °C for 15 minutes followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 20 s, extension at 72 ° C for 20 °C final extension at 72 °C for 10 minutes. For amplification of the ITS region, the program consisted of initial denaturation at 95 °C for 15 minutes followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, extension at 72 °C

for 20 if final extension at 72 ° C for 10 minutes. Amplifications were performed in a thermocycler (SwiftTM Max Pro, Esco) and the PCR products separated by electrophoresed on 1% (w/v) agarose gel in TBE 1 X buffer at 90 V for 1.5 h. Gel was stained with ethidium bromide and the bands were visualized in UV light on the Universal Hood II Photodocumentator (BIO-RAD, CA, USA).

PCR products were purified with ExoSAP-IT^{TM} PCR Product Cleanup Reagent and sequenced with primers used in PCR with BigDye Terminator Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA) in ABI 3130 DNA Sequencer (Applied Biosystems, Foster City, CA) in Center for Functional Genomics of ESALQ-USP.

Sequence Analysis

Sequences were edited manually using SeqAssem® software 07/2008 and blasted with sequences from GenBank (NCBI, USA). The most similar sequences were added to the alignment, which was performed using the MUSCLE - Multiple Sequence Comparison by Log-Expectation (Edgar 2004) implemented in MEGA software version 7.0.26 (Kumar et al., 2016).

The maximum parsimony tree was generated with all three genes sequence alignment using the Tree-Bisection-Regrafting (TBR) algorithm involved 23 nucleotide sequences. The robustness of the branches was determined with 1000 replicas of bootstrap. The sequences were deposited in GenBank and the isolates used in the tree are described in Table 2.

Morphological characterization

The isolates of *Cladosporium* spp. were transferred to plates containing PDA medium at 25 °C for 10 days, under dark and light conditions, to make cultural characteristics observations such as pigmentation, texture, mycelial growth and sporulation. The assay was performed using a completely randomized design in a factorial arrangement (isolated x treatments - light regimes: light and dark conditions) nine treatments, and three replicates.

To evaluate the amount of conidia production by each isolate, the conidia was removed and from the plate using a brush and 20 µL of sterile distilled water and suspension was collected. Conidia suspension concentration was estimated using a Neubauer-type haemocytometer. For mycelial growth, 15 µL of conidial suspensions each isolate was adjusted for $1x10^5$ conidia.mL^{-1}, was distributed in a straight line from one end to the other of the plate. Growth measurements were collected in the horizontal diameter of the colonies (HDC).

The isolates were also cultured in synthetic nutrient-poor agar (SNA) medium for 10 days in under dark conditions to mount slides using transparent adhesive tape.

The observations were performed in immersion oil (1000x) under a Nikon Eclipse 80i microscope and images captured with the DXM 1200C camera and the NIS-Elements AR 2.20 software.

To obtain quantitative data, 30 measurements of fungal structures, such as width of hyphae, width and length conidiophores, ramoconidia and conidia each isolate were performed according to Bensch et al. (2012) and Walker et al. (2016).

Antagonism between *Cladosporium* spp. and rice pathogens

The nine isolates of *Cladosporium* spp. described above and the four pathogens *Cochliobolus miyabeanus*, *Magnaporthe oryzae*, *Monographella albescens* and *Sarocladium oryzae* were grown in Petri dishes containing PDA medium and kept at 27 °C in the presence of light for ten days.

Fifty µl of each conidial suspension (*Cladosporium* spp. and pathogens), adjusted at the concentration of $1x10^5$ con.mL^{-1} were transferred to 6 cm diameter Petri dishes containing PDA.

The experiment was carried out with 40 treatments (9 isolates of *Cladosporium* spp. *versus* 4 pathogens and the respective controls) in a completely randomized design and three replicates.

The Petri dishes were incubated for 10 days at 27 °C under continuous white light. Measurements were made of the horizontal diameter of the colonies of the pathogens (HDC) and also determined the area of the colonies based on the area of the circle.

The reduction of colony diameter (RCD) was determined using the horizontal diameter of the colonies (HDC) and for the colony area (RCD AREA) using the formula: [100 - (diameter of pathogens with *Cladosporium* spp. × 100 / diameter of pathogens without *Cladosporium* spp.)].

β-1,3-glucanase (GLU), chitinase (CHI) and e protease (PRO)

The detection of lytic enzyme activity was performed according to Lopes et al. (2012), One mL of a conidial suspension (10^6 con.mL^{-1}) of four *Cladosporium* spp. isolates (C1H, C5G, C11G and C24G) were cultivated during seven days in solid BDA medium and transferred to Erlenmeyrs containing 25 mL of minimal medium TLE ($CaCl_2.2H_2O$, 0.3 g l^{-1}; KH_2PO_4, 2.0 g l^{-1}; $(NH_4)_2SO_4$, 1.4 g l^{-1}; $MgSO_4.7H_2O$, 0.3 g l^{-1}; ureia, 0.3 g l^{-1}; peptona, 1.0 g l^{-1}; and 0.1 % of elements solution [Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}]) containing 0.5% macerated and lyophilized cell wall of the pathogens (*C. miyabeanus*, *M. oryzae*, *M. albescens* and *S. oryzae*)

as the only source of carbon. Every 24 hours the cultures were collected and used as a source of enzymes for up to 96 hours.

CHI activity was determined using 1% colloidal chitin as substrate and sodium acetate buffer (50 mM, pH 5.0); GLU activity was determined using laminarin as substrate in the same conditions (Ramada et al., 2010). After incubation at 50 °C for 10 minutes, 100 µl of 3,5-dinitrosalicylic acid (DNS) was added, and the samples were again incubated at 95 °C for 5 minutes. The amount of reducing sugars was determined in a spectrophotometer at 540 nm. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 mmol of reducing sugar per minute.

Protease activity was determined using azocasein as a substrate at 0.25% concentration in 50 mM phosphate buffer (pH 5.0). Samples were incubated at 37 °C for 30 min, then 100 µl of 10% w/v trichloroacetic acid (TCA) were added and the mixtures were re- incubated at 4 °C for 10 min. Samples were centrifuged at 2500 rpm for 30 min, then 100 µl of the supernatant was transferred to microplates containing 100 µl of 1 M NaOH. The amount of protease was determined in a spectrophotometer at 450 nm. One unit of enzyme activity was defined as the amount of enzyme required to raise 1 unit of absorbance per minute.

Conidium germination and appressorium formation

Conidium germination and appressorium formation of *M. oryzae* and *C. miyalbeanus*. isolates were induced on a hydrophobic artificial surface, previously sterilized with sodium hypochlorite and 70% alcohol and run in high humidity conditions (Filippi 2004; Sena et al. 2013).

The conidial suspension of C24G was prepared at $6x10^6$, $5x10^5$, $2x10^5$, $5x10^3$ and $5x10^1$ con.mL^{-1}, and conidia suspensions of *C. miyalbeanus* and *M. oryzae* at $1x10^5$ con.mL^{-1}.

An aliquot of 10 µL of each suspension was deposited over the surface totaling 20 µL. The evaluations were performed after 4, 6 and 24 h, observing the slides in a light microscope. The controls consisted of a 20 µL drop of pathogens conidial suspension deposited over the same surface, but without C24G suspension.

The experiment was carried out in a completely randomized design with four replicates. Conidia germination and appressoria formation were evaluated after 4, 6 and 24 hours after incubation at 27 °C. The percentage of conidia germination and appressoria formation was determined counting 100 conidia per replicate under a microscope.

Statistical Analyses

Analyzes of variance of the collected data were performed. The averages were compared by the Tukey test ($p < 0.05$) in the Statistical Package for the Social Sciences (SPSS), version 18.0.

The quantitative data of the morphological characterization (measurement of fungal structures) were used for subsequent clustering of *Cladosporium* spp. isolates by the UPGMA method, based on the Euclidian distance matrix (Unweighted Pair Group Method using Arithmetic Averages) in the PAST program version 2.17c.

RESULTS

Molecular characterization

The ACT, EF-1α and ITS regions were amplified, sequenced and compatibilized for a combined analysis of *Cladosporium* spp., which supported the identification of isolates C1H, C5G, C11G, C18Pa, C19G and C24G as *Cladosporium cladosporioides*; C2Pe and C5H as *Cladosporium tenuissimum*; and C11Rb as *Cladosporium subuliforme*. According to the clustering of the three species complexes of *Cladosporium* spp. that make up the tree, all isolates from this study belong to the complex of the species *C. cladosporioides* (Fig. 1).

Morphological characterization

Colonies of *Cladosporium* spp. behaved differently depending on the light regime. The increase in the horizontal diameter of the colonies (HDC) was higher under light conditions, and isolate C11Rb showed the highest growth (Fig. 2a). Conidia production was higher under dark conditions, except for in the isolate C11Rb, which presented lower production of conidia in the dark than the others. In general, the isolates of *Cladosporium* spp. in this study presented better growth and sporulation under light and under dark conditions, respectively (Fig. 2b).

There was no difference among the isolates in pigmentation and texture (Table 3). The quantitative data from morphological characterization shown in Table 3 were used for subsequent clustering of *Cladosporium* spp. isolates by the UPGMA and corroborated the molecular characterization data. These isolates formed three distinct groups, the first composed of the isolates C1H, C5G, C11G, C18Pa, C19G and C24G identified in the phylogenetic tree as *C. cladosporioides*, the second composed of the isolate C11Rb identified as *C. subuliforme* and the third composed of the isolates C2Pe and C5H identified as *C. tenuissimum* (Fig. 3).

Antagonism of *Cladosporium* spp. against rice pathogens

All rice pathogens from this study when co-cultivated with *Cladosporium* spp. presented smaller horizontal diameter of the colonies (HDC) and colony area compared to the respective controls (Table 4).

When co-cultivated with *C. miyabeanus*, isolates C5G and C5H promoted 74.32% of the colony diameter reduction (RCD AREA) (Table 4), which was the greatest reduction (Figs. 4a, 4b). All tested isolates decreased *M. oryzae* and *M. albescens* mycelial growth (Table 4), but the greatest colony diameter reduction (RCD AREA) of *M. oryzae* was 75.97% promoted by C11G (Figs. 4c, 4d) and of *M. albescens* was 77.39% promoted by C24G (Figs. 4e, 4f).

C1H promoted the greatest colony diameter reduction (RCD AREA) of *S. oryzae*, at 68.59% (Table 4; Figs. 4g, 4h).

β-1,3-glucanase, chitinase and acid protease activities

The four isolates of *Cladosporium* sp. presented differences in chitinase (CHI), β-1,3-glucanase (GLU) and acid protease (PROT) activities during the evaluated period (Fig. 5).

GLU: The C24G isolate showed higher activity (865.27 $U.mg^{-1}$) when cultivated with the *C. miyabeanus* cell wall (Fig. 5a) and when cultivated with the *M. oryzae* cell wall for 24 h (511.8 $U.mg^{-1}$) but did not differ from the C1H isolate, which retained the highest activity after 48 h (Fig. 5d). When cultivated with the *S. oryzae* cell wall, the isolate C1H was highlighted (177 $U.mg^{-1}$), as well as the isolates C24G and C5G, after 24 h, returning to high activity again after 96 h (Fig. 5j). When cultivated with the *M. albescens* cell wall, C24G (167.61 $U.mg^{-1}$) presented the highest activity after 48 h, and C1H and C11G did so after 72 h (112.88 $U.mg^{-1}$) (Fig. 5g).

CHI: When cultivated in the presence of *C. miyabeanus*, *M. oryzae*, *M. albescens* and *S. oryzae* cell walls, C24G presented higher activity (Figs. 5b, 5e, 5h, 5k).

PROT: In the first 24 and 48 h when cultivated in the presence of the *C. miyabeanus* cell wall, C24G presented the highest activity after 24 and 48 h (0.064 $U.mg^{-1}$) (Fig. 5c). When cultivated in the presence of *M.* oryzae and *M. albescens* cell walls, C1H (0.021 $U.mg^{-1}$) and C11G (0.011 $U.mg^{-1}$) presented the highest activities after 24, 48 and 72 h (Fig. 5f, 5i). When cultivated in the presence of the *S. oryzae* cell wall, C5G (0.012 $U.mg^{-1}$) and C1H (0.008 $U.mg^{-1}$) presented higher activity after 24, 48 and 96 h (Fig. 5l).

Germination of conidium and formation of appressorium

The C24G (*C. cladosporioides*) isolate at concentrations $6x10^6$, $5x10^5$ and $2x10^5$ con.mL^{-1} significantly decreased the percentage of conidia germination and appressorium formation of the pathogens *C. miyabeanus* and *M. oryzae* (Fig. 6).

The concentration of $6x10^6$ con.mL^{-1} was more efficient because it inhibited germination and appressorium formation of *M. oryzae* by 54.85% and 59.36%, respectively (Figs. 6a, 6b), while $5x10^5$ and $2x10^5$ con.mL^{-1} concentrations inhibited conidia germination by 34.34% and 25.23%, respectively, and appressorium formation by 37.23% and 32.85%. The others did not inhibit germination and appressorium formation (Figs. 6a, 6b).

The same tendency was observed for *C. miyabeanus*, as $6x10^6$ con.mL^{-1} inhibited germination and appressorium formation by 42.4% and 46.49%, respectively (Figs. 6c, 6d), while $5x10^5$ and $2x10^5$ con.mL^{-1} inhibited conidia germination in 26.61% and 21.85%, respectively, and appressorium formation in 28.0% and 28.34%, The others did not inhibit germination and appressorium formation (Figs. 6c, 6d).

We observed that after 24 h, in treatments with the highest concentration ($6x10^6$ con.ml^{-1}), C24G conidia agglomerated around the conidia of *M. oryzae* and *C. miyabeanus* (Figs. 7a, 7b; Figs. 7c, 7d).

DISCUSSION

This is the first report in which different isolates of *Cladosporium* spp. inhabitants of rice plant phylloplanes were identified as biological agents of the main pathogens of the rice, unveiling some of their mechanisms of action and classification at the species level. All nine *Cladosporium* spp. isolates significantly inhibited rice pathogen growth *in vitro* by direct antagonism. Among the nine isolates, four presented better mycelial growth inhibition. The isolates C1H (68.59% inhibition), C5G (74.32%), C11G (75.97%) and C24G (77.39%) presented antagonism with *S. oryzae*, *C. miyabeanus*, *M. oryzae* and *M. albescens*, respectively, all 4 isolates identified as *C. cladosporioides.*

We believe that the ability of isolates to adapt and maintain themselves as an effective biological agent is far greater than the ability of an organism isolated from another crop to do so because they are natural inhabitants of the rice phylloplane. *C. cladosporioides* was not pathogenic to rice in previous studies (Chaibub et al., 2016), although it is pathogenic to some other crops such as pecan trees (*Carya illinoinensis*) (Walker et al. 2016) and strawberry (Nam et al. 2015).

Regarding the phylogeny of the nine isolates studied, six (C1H, C5G, C11G, C18Pa, C19G and C24G) were identified as *C. cladosporioides*, two as *C. tenuissimum* (C2Pe and C5H) and one (C11Rb) as *C. subuliforme*, all belonging to the complex of *C. cladosporioides* species.

According to the polyphasic approach used to determine *Cladosporium* spp. species, morphological characterization supported the molecular identification of the isolates from the phylloplane of the rice, showing that analyses of the morphological characters grouped the isolates into the same species as did the molecular analyses.

Considering the genus *Cladosporium* spp., to identify species of *Cladosporium* spp., it is important to choose an approach that combines morphological characterization, from measurement of fungal structures to molecular characterization based ACT, EF-1α and ITS sequencing regions, as delineated by Bensch et al. (2012, 2015) and mentioned by other authors (Zhan et al. 2014; Torres et al. 2017).

Bensch et al. (2015) showed that the sequence of the ITS region can distinguish species from the *C. sphaerospermum* complex, as well as ACT and EF-1α. However, in regard to the distinction between *C. cladosporioides* and *C. herbarum* complexes, only the sequences of the ITS are no longer efficient and sufficient. ACT and EF-1α sequences are currently more informative and equally adept at distinguishing species.

Other parameters for morphological characterization, in addition to the measurements of fungal structures, such as texture and pigmentation, did not vary among the species in this study; however, colony growth and sporulation varied depending on the light regime. For *Cladosporium* spp., there are few studies about the best light regime for conidiogenesis. Sussman et al. (1963) reported that *Cladosporium mansoni* produces more aerial mycelium in the light, while in the dark, the colonies are practically devoid of mycelial aggregates.

The isolates of *Cladosporium* spp. grew better under light conditions but poorly produced conidia. Under dark conditions, we observed the opposite results: the isolates produced conidia very well and showed a lower rate of growth, except for the isolate C11Rb, which produced the same amount of conidia under light and dark conditions.

The light regime influences the production of conidia of different fungal species. For the biological agent *Dicyma pulvinata*, conidia production was favored when exposed to continuous light (Melo and Mello 2009), but for *Metarhizium robertsii*, conidia production was the same when grown under light or dark conditions (Rangel et al. 2011). For *Exserohilum monoceras*, the best conidiogenesis occurred under dark conditions (Zhang and Watson 1997). Kohl et al. (2008) reported that conidia of the biological agent *C. cladosporioides* (H39) have some sensitivity to light because the wettable powder formulation showed less susceptibility to

ultraviolet light exposure than the conidia produced and formulated under other conditions. The optimization of conditions favoring conidiogenesis is important for future mass production considering application under field conditions, as shown by Kohl et al. (2009, 2015).

We identified isolates from *Cladosporium* spp.-secreted enzymes related to the process of mycoparasitism (β-1,3-glucanase, chitinase, protease) when grown under poor nourishment conditions.

The four isolates (C1H, C5G, C11G and C24G) that significantly reduced mycelial growth of rice pathogens showed remarkable activity of the evaluated enzymes. C24G presented the highest activity for the three enzymes CHI, GLU and PRO at 24 hours. The composition of the pathogen cell wall induces an increase in the activity of these enzymes because the fungal cell wall is a complex structure composed of glucans, chitin and cellulose (Elad et al. 1983).

Similar results are widely found in the literature, mainly for *Trichoderma* spp., in which the activity of enzymes that degrade the cell wall of pathogens such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium solani* and *Aspergillus niger* increased (Almeida et al. 2007; Ramada et al. 2010; Lopes et al. 2012; Qualhato et al. 2013; Gajera and Vakharia 2012). Torres et al. (2017) demonstrated that *C. cladosporioides* and *C. pseudocladosporioides* were able to grow in culture media containing laminarin as the sole source of carbon, evidencing it ability to digest glucans.

The formation of an inhibition halo also indicates the role of some compounds produced by *Cladosporium* spp., which may be toxic to rice pathogens. These compounds likely confer specificity of action because the isolates behave differently in relation to the pathogens, as has been verified for enzyme activity.

Sakagami et al. (1995) reported that *C. cladosporioides* exhibited strong activity in the malformation of *Phytophthora capsici* hyphae and named cladosporol as the biological active substance extracted from *C. cladosporioides*.

Jackson et al. (1997) tested the potential of *C. cladosporioides* in controlling *in vitro Botrytis fabae* and observed the activity of extracellular metabolites in the reduction of radial growth of *B. fabae* with distinct areas of growth inhibition as well as the discoloration of the mycelium of the pathogen.

In addition to the production of antifungal compounds and lytic enzyme activity (demonstrated in our study), we also observed inhibition of conidial germination and appressorium formation in *M. oryzae* (54.85% and 59.36%) and *C. miyabeanus* (42.4% and 46.49%).

M. oryzae conidia secrete a mucilage after deposition and hydration to adhere to the inductive surface and thus initiate germination followed by appressorium formation (Howard and Valent 1996). This mucilage is composed of carbohydrates, lipids and proteins (Prabhu and Filippi 2006), which can be a fast and attractive source for *C. cladosporioides* conidia. We believe that this mucilage attracted *C. cladosporioides* conidia and, if consumed by them, interfered with adequate adhesion and the recognition of the inductive surface by *M. oryzae* conidia. Conidia stores in their interior reserves, such as mannitol, which when degraded provides energy for the germination process. The availability of this energy source may also have attracted the conidia of *C. cladosporioides*. We believe that the conidia of *C. cladosporioides* were attracted by mucilage and if consumed interfered in adequate adhesion and in the recognition of the inductive surface of *M. oryzae*. Conidia is stored in their interior reserves, such as mannitol, which when degraded provides energy for the germination process.

The evaluation of conidia germination and appressorium formation is important because it connects us to the disease cycle, where the interference of *C. cladosporioides* in the initial stage of germination and formation of appressorium will collaborate with the reduction in the initial inoculum of rice blast. The recognition of the surface, release of mucilage for conidia adhesion, germination and appressorium formation by pathogen conidia are such complex mechanisms that involve 2912 conidial proteins of *M. oryzae* (Gokce et al. 2012).

Regarding the concentration of conidia, we observed a gradient. The highest concentration ($6x10^6$ con.mL^{-1}), followed by the following two higher concentrations ($5x10^5$ and $2x10^5$ con.mL^{-1}), was more efficient. When we increase the concentration of *C. cladosporioides* conidia, there is a greater demand for space and nutrients and greater consumption of the energetic sources involved in adhesion, germination and appressorium formation. Therefore, we believe that competition is primarily involved in the inhibition of germination and formation of appressoria and thus followed by parasitism (secretion of lytic enzymes for degradation of the pathogen wall) and antibiosis (production of antifungal compounds causing malformation of pathogen structures).

Larran et al. (2016) verified that some endophytes, identified as *Cladosporium herbarum* and wheat isolates, inhibited the germination of conidia of *Drechslera tritici-repentis*. Jackson et al. (1997) showed that the filtrate obtained from *C. cladosporioides* reduced *B. fabae* conidial germination and germ tube elongation by 36%.

Cladosporium spp. has been extensively reported in the literature as a possible biological agent for several crops, including in field trials with *Venturia inaequalis* (Kohl et al. 2015). However, the mechanisms of action involved, such as competition and parasitism, were

demonstrated here, and thus, by having more than one mechanism of action, *Cladosporium* spp. chances of success as an antagonist are increased.

Microorganisms identified as inhabitants of the rice phylloplane include *Epicoccum* sp., *Cladosporium* sp., *Penicillium* sp., *Aspergillus niger*, *Pseudomonas fluorescens*, *A. flavus*, *Curvularia* sp., *Fusarium* sp. and *Bacillus subtilis* (Harish et al., 2007; Sena et al. 2013; Raja and Mahalakshmi, 2014). The competition for space and nutrients and the production of lytic enzymes and toxins may also be the key to the successful survival *C. cladosporioides* in rice phylloplanes and to several substrates being well adapted to them because the phylloplane is an environment in which organisms undergo sudden changes in temperature, humidity and radiation. *C. cladosporioides* probably withstands large variations in UV radiation from direct sunlight and damage from radiation because it has melanin present in its conidia and hyphae cell walls (Segers et al., 2016).

C. cladosporioides has been reported as a pathogen for some other crops, but none of these crops are grown together with rice. The importance of the identification and classification of biological agents is to avoid doubts regarding a potential bioagent. *C. cladosporioides* has already been formulated into a commercial product and widely tested under field conditions in Europe (Kohl et al. 2015). In Brazil, a commercial product named "Cladosporin" has been denominated as a bioprotector of coffee quality. Researchers confirmed that *C. cladosporioides* is a promising alternative for application when coffee fruits are still attached to the plant and that this microorganism has characteristics that protect the quality of the coffee, mainly by antagonistic action against fungi that are harmful to the quality of the final product (Angélico 2012; Chalfoun et al. 2013).

Therefore, due to the increase in the consumption of agrochemicals and the need for a more sustainable management approach, we hope that our study will bring light to the mechanisms involved in the control of pathogens exerted by *C. cladosporioides*. This is important information for the management of several diseases, especially rice diseases, because it can interfere with the sporulation and disease cycle of many pathogens.

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TABLES

Table 1 *Cladosporium* spp. isolates isolated from rice phylloplane collected from commercial rice fields in Brazil in the years 2009 and 2010.

Isolates	City - State	Farming systems	Rice Cultivars	Year
C1H	Hidrolândia - Goiás	upland rice/organic	Cateto	2009
C2Pe	Perolândia - Goiás	upland rice/conventional	Sertaneja	2009
C5G	Goianira - Goiás	upland rice/conventional	Primavera	2009
C5H	Hidrolândia - Goiás	upland rice/organic	Cateto	2009
C11G	Goianira - Goiás	upland rice/conventional	Primavera	2009
C11Rb	Rio Brilhante - Mato Grosso do Sul	irrigated/conventional	SCS Epagri 114	2010
C18Pa	Paranatinga - Mato Grosso	upland rice/conventional	Sertaneja	2009
C19G	Goianira - Goiás	upland rice/conventional	Primavera	2009
C24G	Goianira - Goiás	upland rice/conventional	Primavera	2009

Table 2 GenBank accession numbers of *Cladosporium* spp. used in the molecular characterization.

Species	Accession number	GenBank accession numbers			Substrate	Country	Reference
		ACT	EF-1α	ITS			
C. cladosporioides	C1H	--	--	--	Rice leaves	Brazil	This work
C. cladosporioides	C5G	--	--	--	Rice leaves	Brazil	This work
C. cladosporioides	C11G	--	--	--	Rice leaves	Brazil	This work
C. cladosporioides	C18Pa	--	--	--	Rice leaves	Brazil	This work
C. cladosporioides	C19G	--	--	--	Rice leaves	Brazil	This work
C. cladosporioides	C24G	--	--	--	Rice leaves	Brazil	This work
C. cladosporioides	CPC 14271	HM148532	HM148286	HM148045	Twigs of an unidentified tree	France	Bensch et al., 2010; 2018
C. cladosporioides	CPC 14024	HM148530	HM148284	HM148043	*Asimina* sp.	South Africa	Bensch et al., 2010; 2018
C. cladosporioides	CPC 14021	HM148529	HM148283	HM148042	Wheat	South Africa	Bensch et al., 2010; 2015
C. tenuissimum	C2Pe	--	--	--	Rice leaves	Brazil	This work
C. tenuissimum	C5H	--	--	--	Rice leaves	Brazil	This work
C. tenuissimum	CPC 14370	HM148711	HM148466	HM148221	Soil, Bat cave	Bali	Bensch et al., 2010; 2018
C. tenuissimum	CPC 14196	HM148708	HM148463,	HM148218	*Basella alba*	Laos	Bensch et al., 2010
C. subuliforme	C11Rb	--	--	--	Rice leaves	Brazil	This work
C. subuliforme	CPC 16318	KT600652	KT600554	KT600455	*Eucalyptus* sp.	South Africa	Bensch et al., 2015
C. subuliforme	CBS 126500	HM148196	HM148441	HM148686	*Chamaedorea metallica*	Thailand	Bensch et al., 2010; 2012
C. subuliforme	CPC 18243	KT600653	KT600555	KT600456	Cotton	Brazil	Bensch et al., 2015
C. sphaerospermum	CBS 193.54	EF101380	EU570261	DQ780343	Man, nails	Netherlands	Bensch et al., 2012; 2015
C. fusiforme	EXF-449	EF101372	JN906988	DQ780388	Hypersaline water	Slovenia	Bensch et al., 2012; 2015
C. herbarum	CBS 121621	EF679516	EF679440	EF679363	*Hordeum vulgare*	Netherlands	Bensch et al., 2012; 2015
C. herbaroides	CBS 121626	EF679509	EF679432	EF679357	Hypersaline water from salterns	Israel	Bensch et al., 2012; 2015
C. salinae	EXF-335	EF101390	JN906993	DQ780374	Hypersaline water	Slovenia	Bensch et al., 2012; 2015
Cercospora beticola	CBS 116456	AY840458	AY840494	AY840527	*Beta vulgaris*	Italy	Bensch et al., 2012; 2015

ACT: partial actin gene;

EF-1α: partial translation elongation factor 1-alpha gene;

ITS: internal transcribed spacer regions with 5.8S nrRNA gene.

-- : sequence submitted to Genbank awaiting codes.

Table 3 Morphological characteristics and measurement of *Cladosporium* spp. structures.

Isolate	Species	Pigmentation	Texture	Conidiophore		Ramoconidia		Conidia		Hyphae
				Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)
C1H	*C. cladosporioides*	Olivaceous-grey	Velvety	3.84+0.22	42.14+0.22	2.52+0.32	16.44+0.27	2.61+0.37	3.86+0.58	2.03+0.24
C2Pe	*C. tenuissimum*	Olivaceous-grey	Velvety / floccose	1.88+0.22	80.12+0.56	1.72+0.38	15.04+0.22	2.08+0.24	3.66+0.38	2.53+0.29
C5G	*C. cladosporioides*	Olivaceous-grey	Velvety	2.94+0.32	40.11+0.31	2.63+0.30	14.52+0.38	2.72+0.38	3.72+0.42	2.83+0.39
C5H	*C. tenuissimum*	Olivaceous-grey	Velvety / floccose	1.94+0.32	84.13+0.57	1.63+0.44	15.62+0.33	2.49+0.38	3.81+0.41	3.23+0.26
C11G	*C. cladosporioides*	Olivaceous-grey	Velvety	2.96+0.37	39.15+0.35	2.61+0.32	17.43+0.22	2.87+0.42	3.93+0.58	2.17+0.38
C11Rb	*C. subuliforme*	Olivaceous-grey	Velvety / floccose	2.84+0.17	19.14+0.32	2.12+0.33	18.4+0.28	2.50+0.41	3.99+0.34	1.87+0.38
C18Pa	*C. cladosporioides*	Olivaceous-grey	Velvety	2.87+0.42	42.17+0.42	2.76+0.34	16.88+0.21	2.66+0.37	3.78+0.43	2.05+0.25
C19G	*C. cladosporioides*	Olivaceous-grey	Velvety	2.82+0.23	39.18+0.33	2.67+0.41	17.21+0.34	2.72+0.38	3.75+0.33	2.43+0.39
C24G	*C. cladosporioides*	Olivaceous-grey	Velvety	2.88+0.21	41.09+0.41	2.54+0.36	16.5+0.33	2.63+0.37	3.86+0.55	2.33+0.27

Means followed by standard deviation (± SD).

Table 4 Horizontal diameter of the colonies (HDC), colony area (AREA), reduction of colony diameter based on HDC (RCD HDC) and reduction of colony diameter based on AREA (RCD AREA) of rice pathogens (*Cochliobolus miyabeanus*, *Magnaporthe oryzae*, *Monographella oryzae* and *Sarocladium oryzae*) against nine *Cladosporium* spp. isolates (C1H, C2Pe, C5G, C5H, C11G, C11Rb, C18Pa, C19G e C24G).

Isolate	***Cochliobolus miyabeanus***				***Magnaporthe oryzae***			
	HDC* (cm)	**AREA (cm2)**	**RCD HDC* (%)**	**RCD AREA* (%)**	**HDC* (cm)**	**AREA (cm2)**	**RCD HDC* (%)**	**RCD AREA* (%)**
C1H	3.13 bc	3.85 b	44.04 ab	69.47 ab	2.80 b	3.07 b	45.09 a	70.63 a
C2Pe	3.16 bc	3.93 b	43.45 ab	68.82 ab	2.83 b	3.16 b	44.44 a	69.85 a
C5G	2.86 c	3.24 b	48.80 a	74.32 a	2.83 b	3.15 b	44.44 a	69.92 a
C5H	2.93 bc	3.38 b	47.61 a	73.16 a	2.80 b	3.09 b	45.09 a	70.48 a
C11G	3.26 bc	4.19 b	41.66 ab	66.78 ab	2.53 b	2.51 b	50.32 a	75.97 a
C11Rb	3.0 bc	3.53 b	46.42 ab	72.0 ab	2.56 b	2.59 b	49.67 a	75.24 a
C18Pa	3.16 bc	3.94 b	43.45 ab	68.78 ab	2.66 b	2.79 b	47.71 a	73.38 a
C19G	3.23 bc	4.10 b	42.26 ab	67.45 ab	2.80 b	3.07 b	45.09 a	70.63 a
C24G	3.40 b	4.53 b	39.28 b	64.04 b	2.56 b	2.58 b	49.67 a	75.33 a
Control	5.66 a	12.62 a	-	-	5.16 a	10.48 a	-	-
Isolate	***Monographella albescens***				***Sarocladium oryzae***			
	HDC* (cm)	**AREA (cm2)**	**RCD HDC* (%)**	**RCD AREA* (%)**	**HDC* (cm)**	**AREA (cm2)**	**RCD HDC* (%)**	**RCD AREA* (%)**
C1H	2.83 b	3.17 b	39.71 a	64.63 a	1.56 c	1.01 b	44.04 a	68.59 a
C2Pe	3.03 b	3.61 b	35.46 a	59.73 a	2.23 abc	1.96 b	20.23 ab	39.16 ab
C5G	2.5 b	2.50 b	46.80 a	72.10 a	2.0 bc	1.57 b	28.57 ab	51.25 ab
C5H	2.6 b	2.67 b	44.68 a	70.22 a	2.30 ab	2.07 b	17.85 b	35.77 ab
C11G	3.0 b	3.53 b	36.17 a	60.59 a	2.26 abc	2.01 b	19.04 ab	37.67 ab
C11Rb	2.73 b	2.96 b	41.84 a	66.89 a	2.13 bc	1.82 b	19.04 ab	43.65 ab
C18Pa	2.93 b	3.37 b	37.58 a	62.34 a	2.30 ab	2.09 b	17.85 b	35.28 b
C19G	2.43 b	2.43 b	48.22 a	72.88 a	2.20 abc	1.90 b	21.42 ab	41.22 ab
C24G	2.20 b	2.02 b	53.19 a	77.39 a	1.96 bc	1.53 b	29.76 ab	52.66 ab
Control	4.76 a	8.97 a	-	-	2.86 a	3.23 a	-	-

Means followed by the same letters were not significantly different from each other according to Tukey's test ($P < 0.05$).

FIGURES / FIGURE CAPTIONS

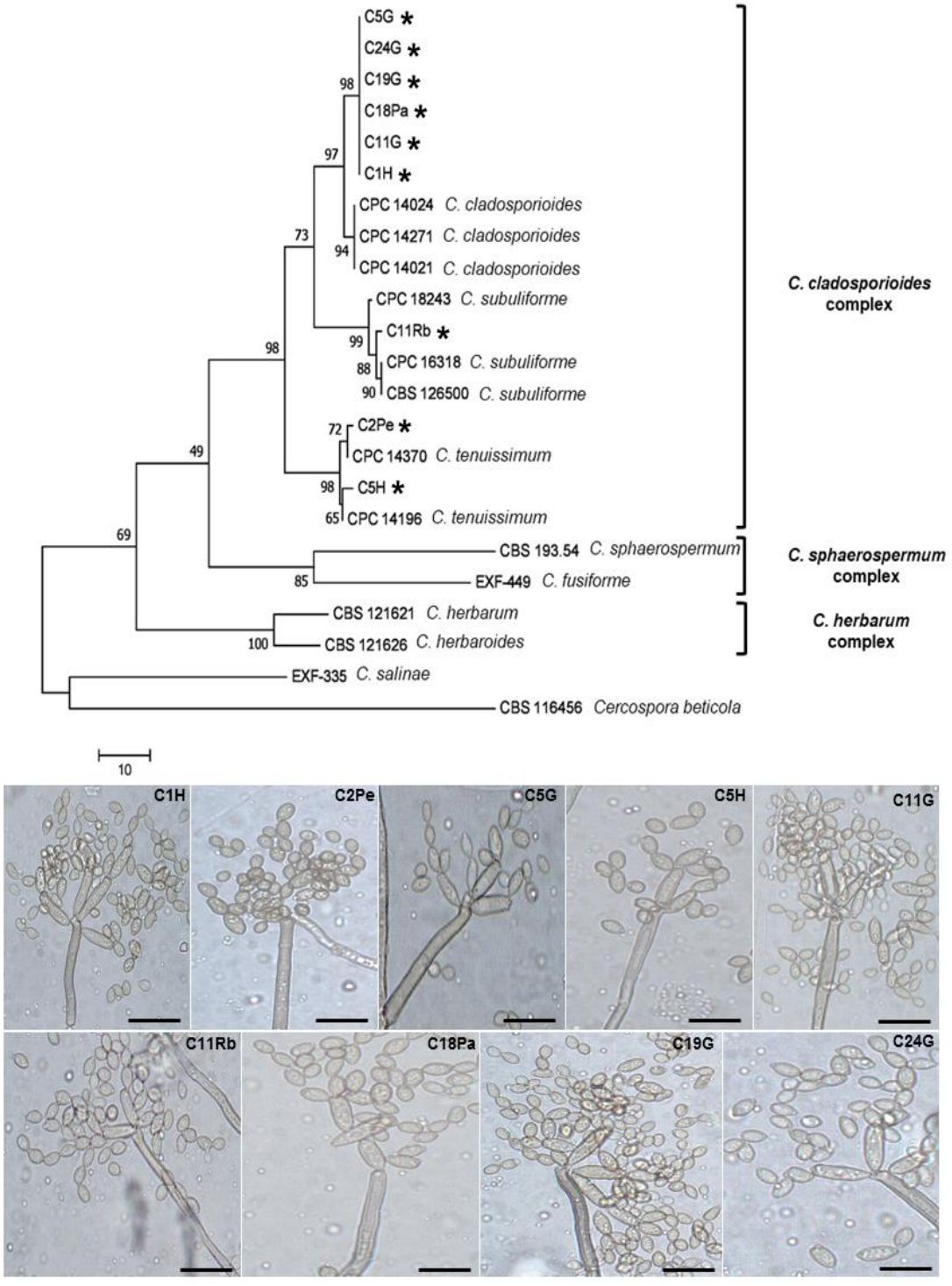

Fig. 1 The first of 9 most parsimonious trees were obtained using the Tree-Bisection-Regrafting (TBR) algorithm involved 23 nucleotide sequences. A most parsimonious tree obtained using the combined sequences of ACT, EF and ITS, with nine *Cladosporium* spp. isolates this work (marked with *) illustrated below the tree with their codes (C1H, C2Pe, C5G, C5H, C11G, C11Rb, C18Pa, C19G e C24G) isolated from rice leaves with other accessions of *Cladosporium* spp. obtained in GenBank. The values at branches are percentages from 1,000 bootstrap replications and are shown at the nodes and the scale bar shows 10 changes. The major species complexes of *Cladosporium* spp. are indicated separately in the tree. Scale bars in the illustrated: 10 μm

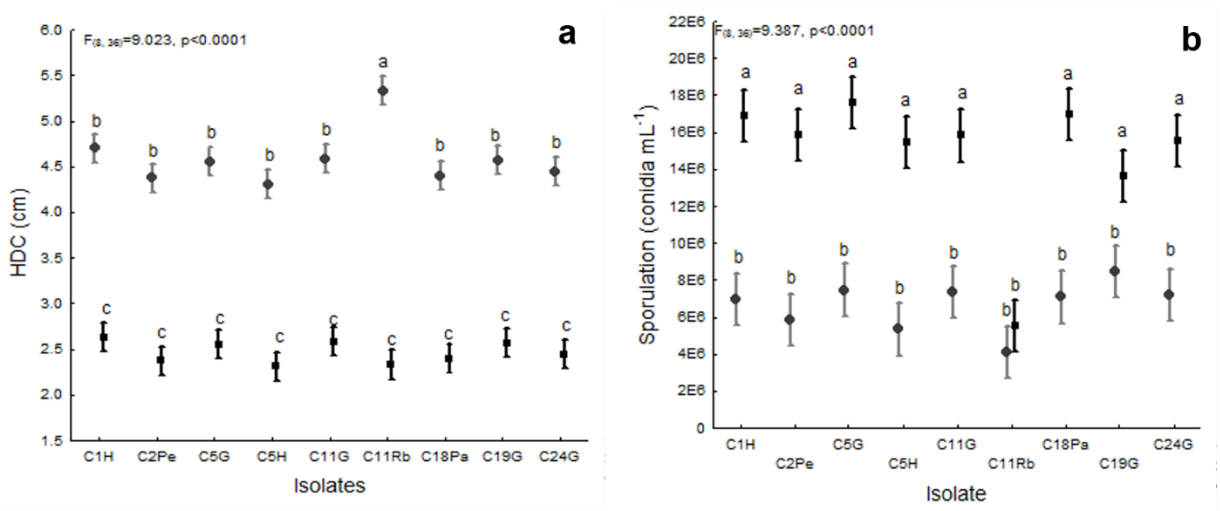

Fig. 2 Horizontal diameter of the colonies (HDC) of *Cladosporium* spp. isolates (C1H, C2Pe, C5G, C5H, C11G, C11Rb, C18Pa, C19G e C24G) under dark (black bars) and light conditions (grey bars) **(a)** and sporulation of *Cladosporium* spp. isolates (C1H, C2Pe, C5G, C5H, C11G, C11Rb, C18Pa, C19G e C24G) under dark (black bars) and light conditions (grey bars) **(b)**. Means followed by the same letters were not significantly different from each other according to Tukey's test ($P < 0.05$).

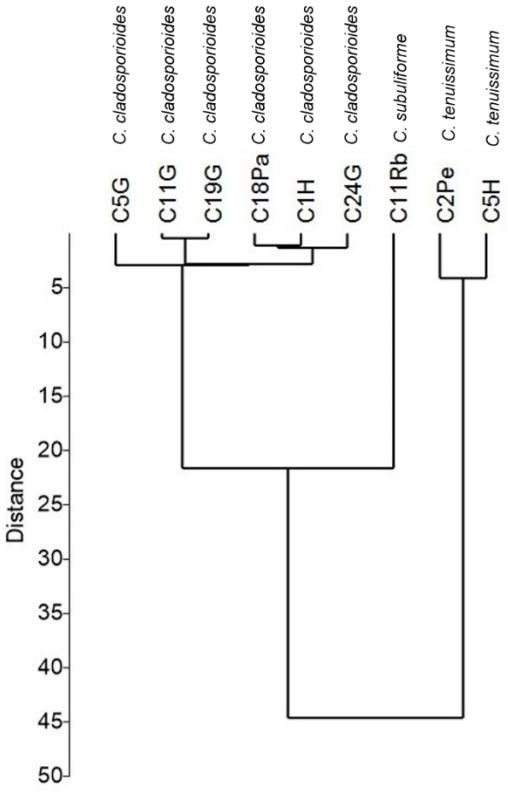

Fig. 3 Clustering of *Cladosporium* spp. isolates (C1H, C2Pe, C5G, C5H, C11G, C11Rb, C18Pa, C19G e C24G) according quantitative morphological data by the UPGMA (Unweighted Pair Group Method using Arithmetic Averages) method based on Euclidian distance matrix.

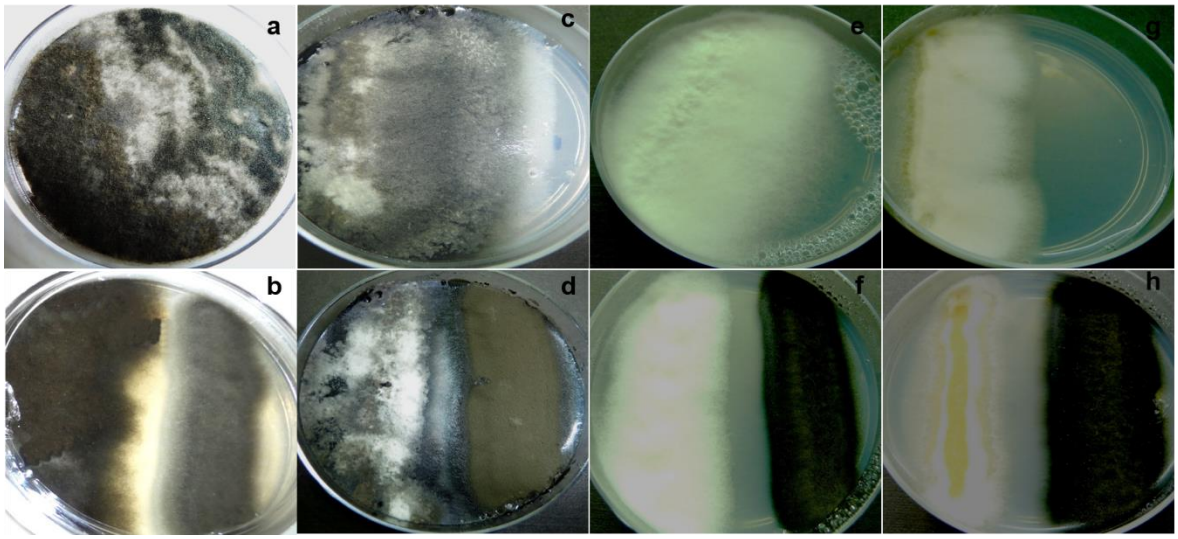

Fig. 4 Antagonism of *Cladosporium* spp. against rice pathogens. *Cochliobolus miyabeanus* **(a)**; *Cochliobolus miyabeanus* + C5G **(b)**; *Magnaporthe oryzae* **(c)**; *Magnaporthe oryzae* + C11G **(d)**; *Monographella albescens* **(e)**; *Monographella albescens* + C24G **(f)**; *Sarocladium oryzae* **(g)***; Sarocladium oryzae* + C1H **(h)**

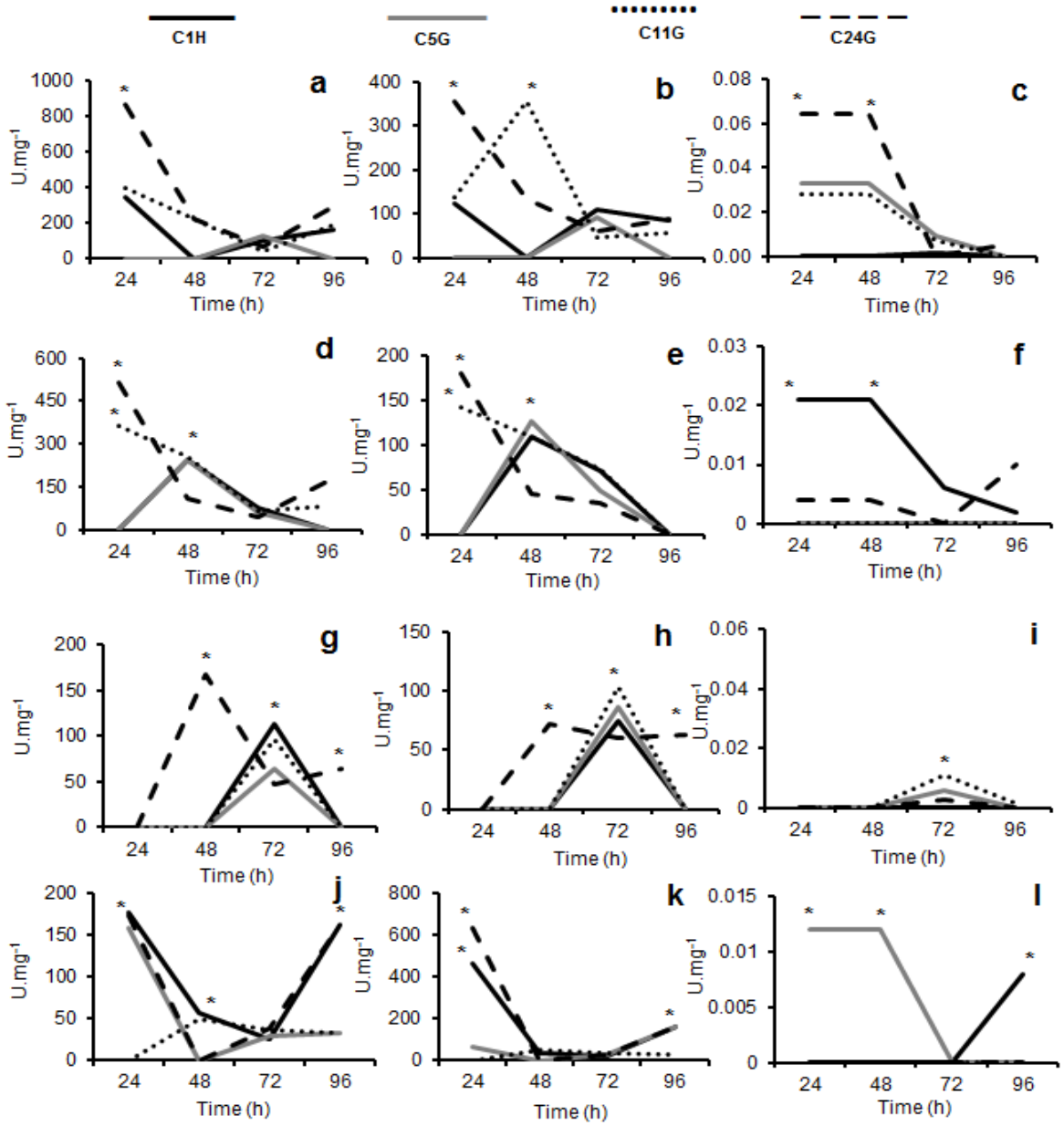

Fig. 5 Activity of β-1,3-glucanase **(a, d, g and j)**, chitinase **(b, e, h and k)** and protease **(c, f, i and l)** when cultivated in minimum medium enriched with cell walls of pathogens. *Cochliobolus miyabeanus* **(a, b and c)**; *Magnaporthe oryzae* **(d, e and f)**; *Monographella albescens* **(g, h and i)** and *Sarocladium oryzae* **(j, k and l)** after 24, 48, 72 e 96 hours. Isolate C1H (black lines); isolate C5G (grey lines); isolate C11G (dots lines) and isolate C24G (stripeds lines).

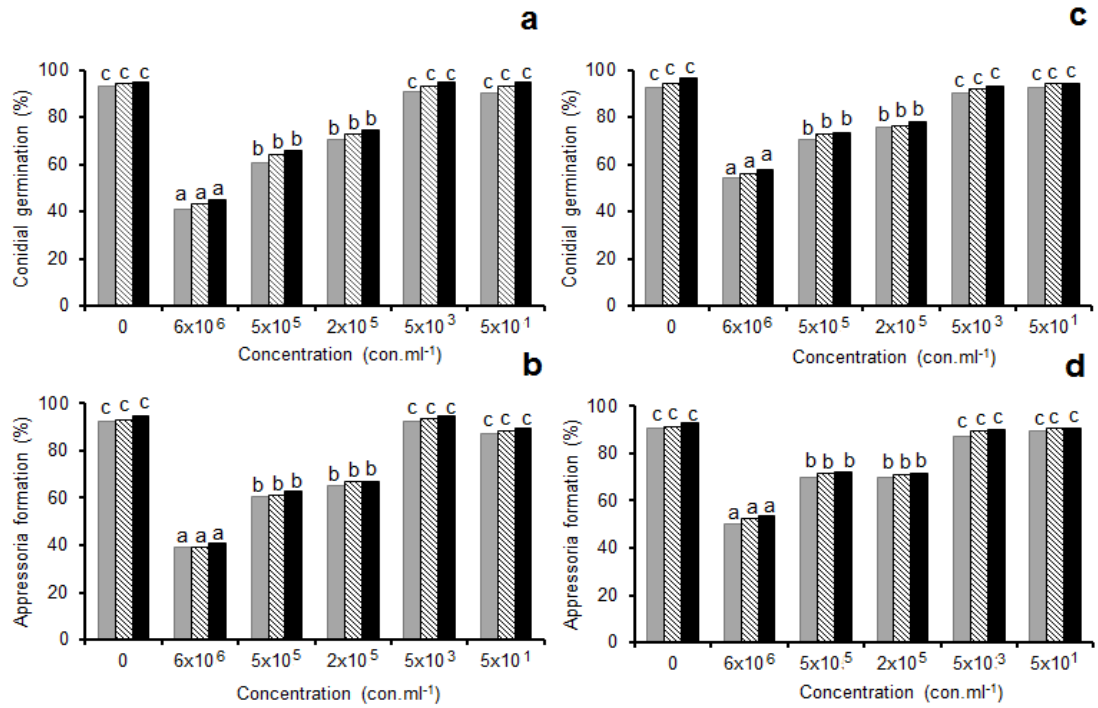

Fig. 6 Effect of C24G suspensions on conidial germination **(a, c)** and appressorium formation **(b, d)** on *M. oryzae* and *C. miyabeanus*, respectively. Four hours after conidia deposition on the surface (grey bars); six hours (stripeds bars) and 24 hours (black bars). Means followed by the same letters were not significantly different from each other according to Tukey's test ($P < 0.05$).

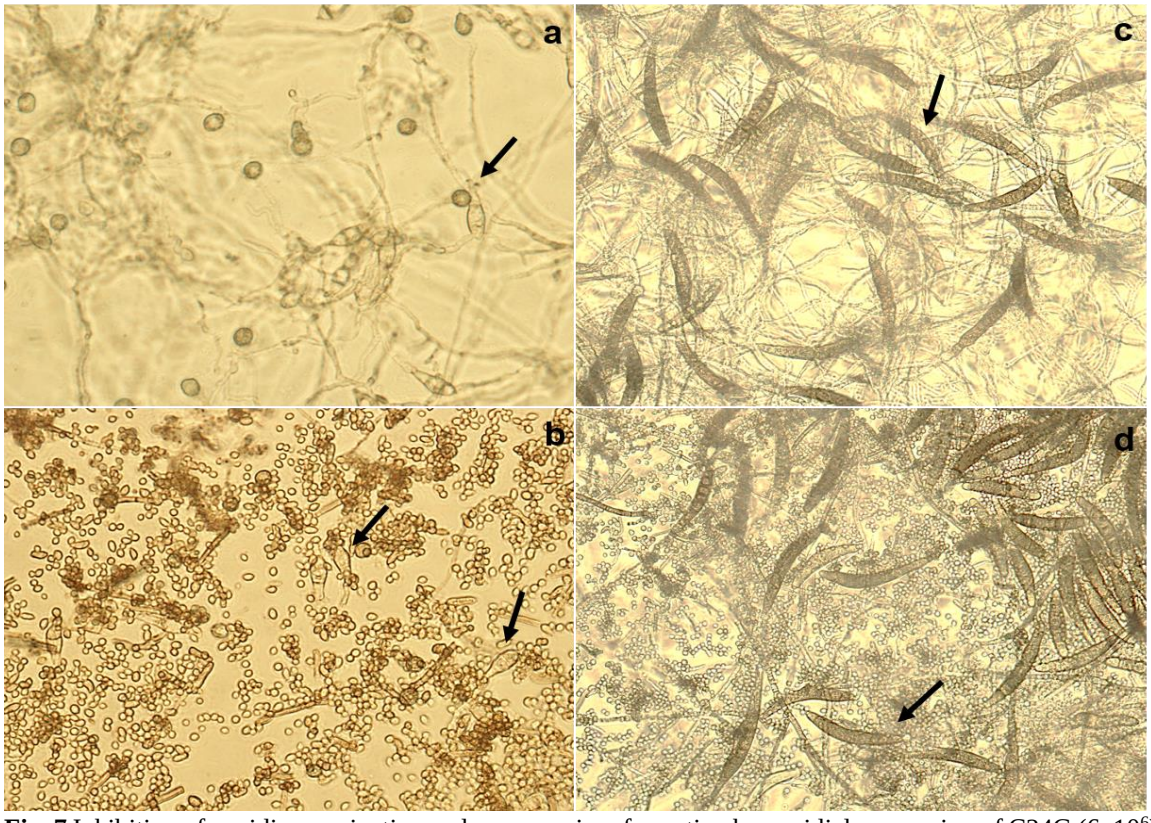

Fig. 7 Inhibition of conidia germination and appressorium formation by conidial suspension of C24G ($6x10^6$) of *M. oryzae* and *C. miyabeanus*. *M. oryzae* control **(a)**; *M. oryzae* + C24G **(b)**; *C. miyabeanus* **(c)**; *C. miyabeanus* + C24G **(d)**.

CAPÍTULO 2

Cladosporium cladosporioides C24G modulates gene expression and enzymatic activity during leaf blast suppression in rice plants.

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ABSTRACT

The inclusion of biological control in the integrated management of rice blast (*Magnaporthe oryzae*) is an alternative to reduce pesticides application. C24G, classified, as *Cladosporium cladosporioides* was isolated from the phylloplane of the rice plant, therefore, adapted to natural conditions to the original habitat. Experiments with the objective of identify the best method and moment for C24G application were performed under greenhouse conditions. It was investigated application in the seed (microbiolization), soil (incorporating and drenching) and foliar spraying pulverization, before (preventive) and after (curative) challenging inoculation with the pathogen (*M. oryzae*). The best-identified treatment was the foliar spray pulverization of C24G as preventive applications. This treatment was further investigated for enzymatic activity and relative gene expression, by Real-time PCR. It suppressed 83.78% leaf blast severity, increased relative expression of the *JIOsPR10*, *LOX-RLL* and *PR1b* genes and increased Chitinase, β-1,3-Glucanase, Lipoxygenase and Phenylalanine ammonia-lyase activity. We conclude that C24G should be tested under field conditions to prove its potential as a component of sustainable blast management, reducing the application of fungicides, generating greater rentability to producer and environment and rice grains free of contaminations.

Keywords: biocontrol, *Oryza sativa*, induced resistance, Real-time PCR, PR proteins

1. Introduction

Rice is a very important cereal crop consumed by more than half the world's population (Wang et al., 2017). Rice blast disease, caused by the fungus *Magnaporthe oryzae*, is a major disease that affects this crop and causes losses in rice cultivation areas of up to 100% (Prabhu et al., 2009).

Cultural practices that minimize the risk of disease development are crop rotation, balanced fertilization, correct date choice and plant spacing (Pooja and Katoch, 2014). In addition to these

practices, the use of fungicides and genetically resistant cultivars are also widely adopted (Pagliaccia et al., 2017; Selisana et al., 2017). However, due to the highly variable nature of the pathogen, the resistance durability of genetically improved cultivars is unstable; therefore, fungicide applications have become the most effective measure in disease control. Thus, the misuse of chemical control is common among producers, leading to the development of resistance to the fungicides by pathogens (Pooja and Katoch, 2014; Castroagudín et al., 2015) or to damage to the environment (Pooja and Katoch, 2014). Similar to these issues, the cost of applications to mitigate blast outbreaks can reach more than \$70 ha^{-1} (Nalley et al., 2016).

One method that can be inserted into integrated blast management to strengthen management tactics is biological control. This approach consists of the application of a beneficial organismal population to reduce a pathogenic population on a given host (Lenteren et al., 2018). Biological agents can act through direct and indirect mechanisms. Direct mechanisms include the following: antibiosis, or producing substances that have deleterious effects; parasitism, through the secretion of lytic enzymes to provide the release of nutrients; and competition, whether by space and/or nutrients, such as sugars, vitamins and minerals vital to the biological agent. An example of an indirect mechanism is the induction of resistance (Francesco et al., 2016). This process occurs in the host after contact between the biological agent and the plant and consists of stimulating the plant immune system with elicitors, with a natural molecule or with a range of molecules secreted by living organisms (Burketová et al., 2015). Both direct and indirect mechanisms are irreplaceable alternatives to blast management, but because the efficiency of biological agents is independent of the pathogen population, their range of effects can, rarely, be supplanted by *M. oryzae* variability.

Cladosporium spp. is a cosmopolitan genus inhabiting several niches, such as the surface of several plants species (Bensch et al., 2010). The *C. cladosporioides* isolate C24G was isolated from rice phylloplane and was previously characterized as a potential biological control agent (Chaibub et al., 2016). Many studies have investigated *Cladosporium* spp. as biological agents for various pathossystems (Zhan et al., 2014; Kohl et al., 2015 and Torres et al., 2017); however, there are still gaps regarding the mechanisms of action, application methods, formulations and host ranges.

Therefore, the objective of this work was to determine the best method of application of the biological agent *C. cladosporioides* C24G in rice plants to maximize its beneficial effects, to compare the enzymatic activity between different application methods and to verify if C24G is able to increase the expression of defense genes in rice plants.

2. Material and methods

2.1. Planting

Rice seeds of BRS Primavera cultivar microbiolized or not were sown in eight plastic tray grooves with 3 kg of soil kg of soil fertilized with NPK (5 g of 5-30-15 + Zn and 3 g of ammonium sulfate). Cover fertilization with 3 g of ammonium sulfate per tray was carried out at eighteen days after sowing.

2.2. Challenge inoculation with M. oryzae

Challenging inoculation of all assays was performed with *M. oryzae* isolate BRM 10900. The isolate was grown in PDA culture medium for seven days. Segments of the colony were transferred to oat-agar medium (oat-dextrose-agar) and incubated under continuous light, at 25 °C for ten days. The colonies aerial mycelium was removed to induce conidiogenesis and the plates were again exposed to continuous light, for 48 hours in high humidity. The conidia were removed with a brush and sterile distilled water and the concentration adjusted to $3x10^5$ con.mL^{-1}. The rice plants in the third leaf issue stage (21 days after planting) were sprayed with the conidial suspension of *M. oryzae*.

After inoculation, the plants were incubated in a humid chamber, inside cages coated with clear plastic, for 24 hours at a temperature of 24 to 26 °C. Then, the inoculated plants were submitted to temperatures varying from 27 to 30 ºC, and high humidity in a greenhouse, favoring the development of the infection. Leaf blast severity evaluations were performed eight days after inoculation using the percentage of leaf area affected by the disease in the first open leaf, using a ten-degree scale according to Notteghem (1981). The AUDPC (area under the disease progress curve) was evaluated beginning at the onset of the first symptoms four times at 2-day intervals. The AUDPC was calculated according to Shaner and Finney (1977). The reduction in rice leaf blast severity was calculated relative to the severity in the inoculated control (100 − (severity of the treatment × 100 / severity of the control)).

2.3. Assays description for application methods screening

Different methods of application were compared in 4 assays, all of them in designed in completely randomized with 3 replicates:

2.3.1 Assay 1: C. cladosporioides (C24G) applied to seeds and soil

The treatments were as follows: 1=seeds microbiolized with a $6x10^6$ con.mL^{-1} suspension of C24G; 2=seeds microbiolized with $2x10^6$ con.mL^{-1}; 3=seeds microbiolized with $5x10^5$

con.mL^{-1}; 4=seeds microbiolized with 5x10^{3} con.mL^{-1}; 5=seeds microbiolized with 5x10^{1} con.mL^{-1}; 6=seeds microbiolized with C24G in powder; 7=incorporation of 2.5 g mycelium mass in the soil; 8=incorporation of 5.0 g mycelium mass in the soil; 9=incorporation of 10.0 g of mycelium mass in the soil; 10=only *M. oryzae* (control) and 11=only water (control).

2.3.2. Assay 2: C. cladosporioides (C24G) soil drenching

C24G was cultivated in PDA culture medium for ten days in an incubation chamber under dark conditions. The conidia were removed utilizing a brush and sterile distilled water. The suspension was filtered with a sterile tissue, and concentrations were adjusted to 6x10^{6}, 2x10^{6} and 5x10^{5} con.mL^{-1}.

In a factorial scheme, the trays containing rice plants were drenched with 100 mL of the 3 different concentrations of C24G suspension (6x10^{6}, 2x10^{6} or 5x10^{5} con.mL^{-1}) at 7, 14 and 19 days after planting (DAP). The assay consisted of 18 treatments, six for each suspension: 1=drenching with suspension 7 DAP, 2=drenching with suspension 14 DAP, 3=drenching with suspension 19 DAP, and 4=drenching with suspension 7, 14 and 19 DAP, 5=only *M. oryzae* (control) and 6=water (control).

2.3.3. Assay 3: preventive foliar spraying of C. cladosporioides (C24G)

C24G was cultivated in PDA culture medium for ten days in an incubation chamber under dark conditions. The conidia were removed utilizing a brush and sterile distilled water. The suspension was filtered with a sterile tissue, and concentrations were adjusted to 6x10^{6}, 2x10^{6} and 5x10^{5} con.mL^{-1}.

In a factorial scheme, the three different concentrations were sprayed onto the rice plants with a suspension of C24G (6x10^{6}, 2x10^{6} or 5x10^{5} con.mL^{-1}) at 7 days, 48 h or 24 h before challenge inoculation with *M. oryzae* (BCI). The assay consisted of 18 treatments, six for each suspension: 1=suspensions sprayed at 7 days BCI; 2=suspensions sprayed at 48 h BCI; 3= suspensions sprayed at 24 h BCI*;* 4=suspensions sprayed at 7 days, 48 h and 24 h BCI; 5=only *M. oryzae* (control) and 6=water (control).

2.3.4. Assay 4: curative foliar spraying of C. cladosporioides (C24G)

C24G was cultivated in PDA culture medium for ten days in an incubation chamber under dark conditions. The conidia were removed with a brush and sterile distilled water. The suspension was filtered with a sterile tissue, and concentrations were adjusted to 5x10^{5} con.mL^{-1}. Twenty one-day rice plants were challenge inoculated by spraying a conidial suspension of *M.*

oryzae ($3x10^5$ con.mL^{-1}). C24G suspension was sprayed onto rice leaves after the first symptoms started (72 hours after inoculation), on 3 different days, totaling 10 treatments: 1=C24G pulverization 3 days after challenge inoculation (DACI); 2=C24G pulverization 4 DACI; 3=C24G pulverization 5 DACI; 4=C24G pulverization 6 DACI; 5=C24G pulverization 3 and 4 DACI; 6=C24G pulverization 4 and 5 DACI; 7=C24G pulverization 5 and 6 DACI; 8=C24G pulverization 3, 4, 5 and 6 DACI; 9=only *M. oryzae* (control) and 10=water (control).

2.3.5. Assay 5: The most efficient application method

The best treatment of each of the 4 assays comprised the fifth, with treatments: 1=seeds microbiolized by C24G in powder; 2=preventive foliar spraying 24 h BCI with *M. oryzae*; 3= preventive foliar spraying 48 h BCI with *M. oryzae*; 4=curative foliar spraying at 3, 4, 5 and 6 h ACI; 5=only *M. oryzae* (control) and 6=water (control).

2.3.6. Assay 6: AUDPC and collection of leaves

The assay was carried out to evaluate AUDPC and plant collection for enzymatic activity and gene expression, with treatments: 1=seeds microbiolized by C24G in powder, 2=preventive foliar spraying 24 h BCI with *M. oryzae*, 3=preventive foliar spraying 48 h BCI with *M. oryzae*, 4=only C24G, 5=only *M. oryzae* (control) and 6=water (control).

2.4. Scanning electron microscopy

Leaf segments of the curative foliar spraying treatment in assay 5 were collected for visualization in scanning electron microscopy (SEM) according to Sena et al. (2013).

2.5. Quantification of enzymatic activities

The activity quantification of the enzymes Chitinase (EC 3.2.1.14), β-1,3-Glucanase (GLU) (EC 3.2.1.6), Lipoxygenase (LOX) (EC 1.13.11.12), Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) and Peroxidase (POX) (EC 1.11.1.7) was performed for the following treatments described in assay 6.

The samples were collected from the third leaf of 15 plants of each treatment according to Chaibub et al. (2016). The assays were performed in triplicate and the collections were performed 24 and 48 h ACI with *M. oryzae*. The specific activity (U mg^{-1}) was calculated by determining the relationship between the enzymatic activity (enzyme units, U) and the quantified protein concentration in each sample (mg). The activity of Chitinase (CHI), β-1,3-Glucanase (GLU),

Lipoxygenase (LOX), Phenylalanine ammonia-lyase PAL and Peroxidase (POX) was determined according Chaibub et al., 2016.

2.6. RT-qPCR

Leaf segments were collected for analysis of the gene expression of the best treatment and the controls, consisting of: 1=preventive foliar spraying 24 h BCI with *M. oryzae*, 2=only C24G, 3=only *M. oryzae* (control) and 4=water (control).

The assay was conducted in 3 replicates. Ten plants of each treatment were collected from each replicate, totaling 12 samples, at 12 h ACI (after challenge inoculation) with *M. oryzae*. Samples were frozen in liquid nitrogen and stored at -80 °C.

The total RNA was extracted by samples maceration in liquid nitrogen and following the instructions of RNeasy Mini Kit (Qiagen®) extraction kit. RNA was quantified and quality analyzed on NanoVue™ (GE Healthcare Life Sciences) and subsequently stored at -80 °C.

The cDNA was obtained with GoScript Transcription System kit (Promega®) according to the manufacturer's instructions. The cDNA was quantified by fluorescence using the Qubit 2.0 Fluorometer kit and the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific) for standardized with ultrapure water at 1 ng/µl concentration.

Five pairs of primers were used, for four defense genes: *Gns1* (F: 5'-GGCGTCGAGCAGAATTGG-3' and R: 5'-CGGAATGCATCAGAAGCTGAT-3'), *JIOsPR10* (F: 5'-GCAGCGTCAGGCAGTTCAA-3' and R: 5'-GAACTCCAGCCTCTCCTTCATG-3'), *LOX-RLL* (F: 5'-AGATGAGGCGCGTGATGAC-3' and R: 5'-CATGGAAGTCGAGCATGAACA-3'), *PR1b* (F: 5'-GGTGTCGGAGAAGCAGTGGTA-3' and R: 5'-GCGAGTAGTTGCAGGTGATGAAG-3'), and as a housekeeping gene *actin* (F: 5'-GAGCTACGAGCTTCCTGATGGA-3' and R: 5'-CCTCAGGGCAGCGGAAA-3') according to Hao et al. (2009).

The RT-qPCR reactions were performed with the PowerUp™ SYBR™ Green Master Mix kit (Thermo Fisher Scientific) following the protocol indicated by the manufacturer. The reactions were prepared to a final volume of 10 µL, containing 5 µL of 2U PowerUp SYBR Green Master Mix, 1 µL of each primer forward and reverse at 5 µM, 2 µL of cDNA at 1 ng/µL (totaling 2 ng of cDNA) and ultrapure water to complete the final volume.

The amplification program consisted of an initial incubation at 70 °C for 15 min for deactivation of the reverse transcriptase enzyme, followed by an incubation at 50 °C for 2 min, for UDG activation. Initial denaturation occurred at 95 °C for 2 min, followed by 40 cycles of

denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 1 min. At the end of the reactions a melting curve (95 °C 15 s, 60 °C 1 min and 95 °C 15 s) was made.

Reactions were performed in three replicates of each treatment (12 samples totaling three biological replicates of each treatment) and in duplicate technique in the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific).

For the analysis of gene expression, the value of $2^{-\Delta\Delta CT}$ was used to estimate the relative level of expression of analyzed genes. Estimation were performed in relation to the control plant (plants treated with water alone) and the data were normalized using housekeeping gene (actin).

To classify the genes according to their function, the Gene Ontology (Gene Ontology Consortium, 2001) term was used to perform a classification analysis. The classification of Gene Ontology (GO) was verified in Rice Genome Annotation (Kawahara et al., 2013) and the categories of GOs were verified on the pathways of interaction of the genes in the amiGO (Carbon et al., 2009).

2.7. Statistical Analysis

For all greenhouse assays, enzymatic activity and RT-qPCR, analyzes of variance were performed in the Statistical Package for the Social Sciences (SPSS), version 18.0 and the means were compared by the Tukey's test ($p<0.05$).

3. RESULTS

3.1. Application methods screening

All the assays of the different methods of application of isolate C24G presented significant differences for the treatments, except assay 2 (Table 1). In assay 3 (preventive foliar spraying), there was no significant difference between the concentrations; all of them behaved in the same way for the treatments tested (Table 1).

Assay 1: treatment 6 (seed microbiolization with C24G in powder) presented the smallest infected leaf area (14.29%) and was the only one that differed from the control and the other treatments (Fig. 1A);

Assay 2: there was no difference among the treatments (Fig. 1B);

Assay 3: all the treatments differed from the control, and treatments 2 (48 h BCI), 3 (24 h BCI) and 4 (7 days, 48 h and 24 h BCI) presented the lowest means for leaf blast severity, with means of 4.84%, 4.40% and 4.46%, respectively (Fig. 1C).

Assay 4: treatments 1 (3 days ACI), 5 (3 and 4 DACI) and 8 (3, 4, 5 and 6 DACI) differed from the control, presenting infected leaf areas of 15.72%, 15.27% and 11.52%, respectively. (Fig. 1D).

3.2. Assay 5: The most efficient application method

All the treatments suppressed leaf blast when compared to the control, and treatments 2 and 3 of the preventive foliar spraying differed from the others (seed microbiolization with C24G in powder and curative foliar spraying). The treatments presented infected leaf areas of 13.95% (treatment 1: microbiolized with powder), 4.38% (treatment 2: 24 h BCI), 5.05% (treatment 3: 48 h BCI) and 10.47% (treatment 4: 3, 4, 5 and 6 DACI), while the control presented with 25.55% (Fig. 2).

In the control, the lesions were very sporulating, with coalescent lesions (treatment 5 in Fig. 2). Seed microbiolization with C24G in powder and curative foliar spraying resulted in sporulating lesions in smaller numbers which did not coalesce (treatments 1 and 4 in Fig. 2). In contrast, preventive foliar spraying 48 h and 24 h BCI resulted in only small, brown pinhead-type lesions (treatments 2 and 3 in Fig. 2).

3.3. Assay 6: AUDPC and collection of leaves

The seed microbiolization with C24G in powder resulted in a diseased leaf area of 16.44% and an AUDPC of 35.58. The preventive foliar spraying at 48 h and 24 h BCI resulted in diseased leaf areas of 5.20% and 4.18% and AUDPC values of 13.16 and 11.31, respectively. The control resulted in a diseased leaf area of 25.77% and an AUDPC of 47.52. Thus, isolate C24G was able to suppress leaf blast in seed microbiolization with C24G powder as well as in preventive applications of 48 h and 24 h BCI by 36.21%, 79.83% and 83.78%, respectively (Fig. 3).

3.4. Scanning electron microscopy

A well-developed leaf lesion caused by the pathogen (Fig. 4A; 4B) and colonized by *C. cladosporioides* (C24G) (Fig. 4C; 4D) was observed by scanning electron microscopy in a plant treated by curative foliar spraying in assay 5.

3.5. Quantification of enzymatic activities

CHI: significantly increased in treatment 2, at 48 haci (hours after challenge inoculation) (Table 2).

GLU: significantly increased in treatments 4 and 2 at 24 haci and increased only for treatment 2 at 48 haci (Table 2).

LOX: significantly increased in treatment 4 followed by treatment 2, at 24 haci and 48 haci (Table 2).

PAL: significantly increased in treatment 4 at 48 haci (Table 2).

POX: there was no difference among the treatments regarding the activity of this enzyme (Table 2).

3.6. RT-qPCR

Treatment with only C24G (*C. cladosporioides*) significantly altered the expression of *JIOsPR10* (Fig. 5B), *LOX-RLL* (Fig. 5C) and *PR1b* (Fig. 5D) when compared to the *M. oryzae*-only control and to the 24 h BCI treatment with *M. oryzae*. The expression of the *Gns1* gene was altered significantly in the *M. oryzae*-only control. (Fig. 5A).

The *GNS1* gene is categorized with 3 GOs: GO:0003674 (molecular function), GO:0016020 (cellular component) and GO:0008150 (biological process); as is *JIOsPR10*: GO:0003674 (molecular function), GO:0005515 (molecular function) and GO:0008150 (biological process).

The *LOX-RLL* gene is categorized with 4 GOs: GO:0005506 (iron ion binding), GO:0016165 (Lipoxygenase activity), GO:0031408 (oxylipin biosynthetic process) and GO:0055114 (oxidation reduction).

The *PR1b* gene is categorized with 7 GOs: GO:0003674 (molecular function), GO:0005618 and GO:0005576 (cellular component), GO:0006950, GO:0009719, GO:0009607 and GO:0009991 (biological process).

We note that the four genes studied are potentially involved in different physiologic processes, according to the Gene Ontology database (Fig. 6), and in different signaling pathways that are interconnected.

4. DISCUSSION

Determining the best application method, such as drenching the soil, seed microbiolization or aerial spray pulverization, for a biological agent is a very important step. The most effective method determines the success of the biological control exerted by an agent for a particular disease. In addition, this approach provides a basis for important field-testing decisions (Law et al., 2017).

According to the results of the tests for the different methods of application, for all the treatments where C24G was drenched into the soil or microbiolized on the seed, no efficiency was observed in the suppression of the leaf blast, except when seeds were treated with the C24G powder with a glue solution, allowing adherence to the seed. Filippi et al. (2011) showed that the efficiency of a biological agent is strongly correlated to its habitat of origin. The appropriate method of application contributes significantly to the success of the biocontrol agents in field trials (Suprapta, 2012). *Cladosporium cladosporioides* was isolated from rice phylloplane and thus may not be suited to soil conditions or to microbiolized suspension without any adhesive solution to help it stick to the seed.

When C24G was applied preventively and curatively, the suppression of leaf blast was positive. Leaf blast suppression reached 83.78% when C24G was applied 24 h or 48 h before *M. oryzae*. In previous studies, Chaibub et al. (2016) showed the potential for the C24G preventive application on activating rice defense responses.

However, curative spraying was effective when applied at the beginning of symptoms (brown pinhead-type lesions). In curative foliar spraying, there is a direct mechanism of the bioagent against the pathogen, which can either exercise the mechanism of competition for space and nutrients present on the leaf surface or of parasitism from the production of lytic enzymes that degrade the cell wall of the pathogen, the pathogen being a nutritional source for the bioagent. By scanning electron microscopy, we observed C24G colonizing the lesion caused by *M. oryzae*. Corroborating our results, Zhan et al. (2014) using scanning electron microscopy also verified that *C. cladosporioides* colonizes the lesions and urediniospores of *Puccinia striiformis* f. sp. *tritici*, the causal agent of wheat rust. However, when C24G was applied by the moment blast, lesions were in an advanced stage; thus, the curative application did not show the same effect. Therefore, there is a need to monitor the appearance of symptoms in field conditions since the decision making for the applications to be performed at the right time is crucial for the efficiency of biological control.

Other studies, corroborating ours, also demonstrate microorganisms that exert different control mechanisms (Sena et al., 2013; Brunner et al., 2005). Chung et al. (2015) reported a new endophytic bacterium isolated from the roots of rice plants with multifunctional activities in rice: direct inhibition of fungi and pathogenic bacteria, induction of systemic resistance and promotion of plant growth.

Among the forms of application of biological agents inducing resistance in the control of rice leaf diseases, the most used have been soil application and microbiolized seeds (Sperandio

et al., 2017; Levy et al., 2015). However, in this study, for *C. cladosporioides*, we consider the best form of application to be the foliar application.

In this study, it seems that *C. cladosporioides* can also elicit host defense responses. We observed the relative expression of the defense genes *Gns1*, *JIOsPR10*, *LOX-RLL* and *PR1b*. *Gns1* encodes β-1,3-Glucanase (Nishizawa et al., 2002) and may be associated with the jasmonic acid (JA) pathway (Zhang et al., 2015) which, similar to chitinase, acts directly on the cell wall degradation of pathogens. Simmons et al. (1992) found higher levels of *Gns1* expression in plants treated with ethylene (ET), cytokinin, salicylic acid (SA) and fungal elicitors derived from *Sclerotium oryzae* and *Saccharomyces cereviseae*. In our study, we verified that the treatment with *M. oryzae* alone presented higher levels of expression of this gene than in plants treated with C24G.

In previous results (data not shown), we verified that C24G produces β-1,3-glucanase. The presence of this enzyme, once recognized by the plant, may suppress the expression of this gene by the plant.

Our results show that plants treated only with C24G had increased expression levels of *JIOsPR10*, *LOX-RLL* and *PR1b*. The *JIOsPR10* gene is related to defense responses in rice plants by encoding PR10 (Jwa et al., 2001) and is regulated by JA, by AS or by pathogenic infection.

LOX-RLL is associated with the JA pathway and encodes lipoxygenase, the enzyme that initiates JA biosynthesis, which is associated with plant physiological processes and stress-defense responses.

PR1b is a defense gene, generally induced 24 h after a plant-pathogen interaction (Ponciano et al., 2006), which encodes a PRP that inhibits the growth and reproduction of pathogens in plants (Duan et al., 2014). This gene is related to the JA and SA pathways (Zhang et al., 2015; Agrawal et al., 2000), as well as to ET (Huang and Vallad, 2018).

According to Pieterse et al. (2014), the induced state of resistance is characterized by the activation of latent defense mechanisms that are expressed after a subsequent challenge by a pathogen or a beneficial microorganism and is regulated by a network with interconnected signaling pathways. Even when studying a few genes, we can verify based on gene ontology (GO) that the genes chosen for this study confirm that these signaling pathways are interconnected, showing that the genes are categorized in molecular functions, cellular components and biological processes as well as, within these, in subcategories involving defense responses.

There are reports in the literature about high levels of expression of these genes when the plant is under different stress situations. Duan et al. (2014) identified higher levels of expression

of *PR1b, LOX* and other defense genes after small brown planthoppers (SBPH *Laodelphax striatellus* Fàllen) fed on rice plants. *L. striatellus* is the vector for rice viral diseases such as rice stripe virus (RSV) and black-streaked rice virus (RBSDV). Huang and Vallad (2018) reported increased levels of *PR1a* and *PR1b* expression after ASM (Acibenzolar-S-methyl) application to rice leaves and soil. Zhang et al. (2015) reported that plant stresses, such as diseases, salinity, and water deficiency or a combination of all these stresses, modulated *PR4*, *PAL*, *Cht-1* and *LOX-RLL* gene expression.

The fact that plants treated only with C24G present higher levels of expression of the genes *JIOsPR10*, *LOX-RLL* and *PR1b* shows that C24G activated these defense responses, reprogramming gene expression (Shoresh et al., 2010) and thus preparing the plant for pathogen attack.

We observed that, in plants treated with C24G and those later challenged with *M. oryzae*, the expression levels of these genes were lower. Thus, we provide hypotheses based on two fronts.

The first: when the pathogen interacted with the rice plant, C24G had already induced the defense responses; thus, there was no need to maintain higher expression of these genes.

The second: at the time of sample collection, in plants treated only with C24G, this solo interaction lasted 36 h. However, plants treated with C24G and those later challenged with *M. oryzae* only interacted with C24G alone for 24 h and then 12 h in the presence of the pathogen.

Thus, the period of interaction between the plants and C24G alone was different between treatments. The reason for this finding may be that a later sample collection might have detected higher expression of these genes in the challenge treatment (C24G + M.o). In addition, in the presence of the pathogen, several pathways may have converged.

We must also consider that other genes are involved in defense responses. The activity of the enzymes determined at 24 h was also higher in plants treated with C24G alone (not challenged) and in plants treated with C24G and challenged with *M. oryzae* when compared to plants only challenged. The activity of CHI, GLU and LOX was increased at 24 h and 48 h after challenge, and PAL activity increased at 48 h. The increased activity of these enzymes shows us that other genes not studied (such as genes involved in PAL activity) may be involved in the plant defense pathway.

In the literature, it is described that systemic acquired resistance (SAR) can be triggered in the plant after a local infection caused by a pathogen and is dependent on SA and its analogues, responsible for activating genes related to pathogenesis genes, which are expressed through pathogenesis-related proteins (PRPs) (Vlot et al., 2009). However, induced systemic resistance

(ISR) is characterized by being triggered by beneficial microorganisms (bacteria and fungi) and is regulated by JA- and ET-dependent signaling pathways (Pieterse et al., 2014). However, it is noted that beneficial microorganisms can trigger both pathways (SAR and ISR) and can regulate a complex network of signals involving SA, JA and ET as well as the crosstalk among them, demonstrating how the pathways are interconnected for defense responses (Vitti et al., 2015; Hermosa et al., 2012; Salas-Marina et al., 2011).

With the ability of isolate C24G (*C. cladosporioides*) to inhabit the rice phylloplane, inducing defense responses and thus modulating the expression of defense genes in rice plants as well as exerting antagonism towards *M. oryzae* (Chaibub et al., 2016), we can infer that the reduction of the leaf blast severity observed in the greenhouse was the result of the interaction among the rice plant, *C. cladosporioides* and *M. oryzae*. This interaction should be tested under field conditions as a concrete alternative for the integrated management of rice diseases.

Several studies have been carried out with *Cladosporium* spp. as a biological agent (Kohl et al., 2015 and Torres et al., 2017). Here, we verified that the best method of application for the control of leaf blast was by the activation of host defense responses. Biological control fits well with the concept of sustainable agriculture because it exploits natural cycles and reduces environmental impact (Spadaro and Gullino, 2004). Aiming at the sustainable management of rice diseases, we must explore the association between *C. cladosporioides* and other bioagents that act in the rhizosphere, such as *Trichoderma* spp. and rhizobacteria, as well as between *C. cladosporioides* and fungicides.

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TABLES

Table 1
Significance of greenhouse assays results in different methods of application of the biological agent *C. cladosporioides* C24G.

Assays	*p* value
1: Soil and microbiolized seeds	
Treatments	0.007
2: Soil drenched	
Treatments	0.200
Concentration	0.978
Treatments*Concentration	0.998
3: Preventive foliar spraying	
Treatments	0.000
Concentration	0.931
Treatments*Concentration	0.999
4: Curative foliar spraying	
Treatments	0.000

Table 2
Enzymatic activity of Chitinase (CHI), β-1,3-glucanase (GLU), Lipoxygenase (LOX), Phenylalanine ammonia-lyase (PAL) and Peroxidase (POX) in rice plants that were treated with different methods of applying C24G and then collected at 24 and 48 h after challenge inoculation with *M. oryzae* are shown.

Enzyme	Treatments	Hours after challenge inoculation		*p* value
		24 hours	48 hours	
CHI ($U.mg^{-1}$)	Microbiolized seed (powder)	0.217 bc	0.211 cd	0.558
	Preventive spraying (24 h before *M. oryzae*)	0.240 b	0.290 a*	0.002
	Preventive spraying (48 h before *M. oryzae*)	0.225 b	0.273 b*	0.009
	C. cladosporioides C24G	0.271 a	0.264 b	0.626
	M. oryzae (control)	0.191 c	0.225 d*	0.049
	Water	0.219 b	0.200 c	0.091
GLU ($U.mg^{-1}$)	Microbiolized seed (powder)	0.596 c	0.787 d*	0.001
	Preventive spraying (24 h before *M. oryzae*)	0.826 a	1.095 a*	0.002
	Preventive spraying (48 h before *M. oryzae*)	0.779 b	1.034 b*	0.002
	C. cladosporioides C24G	0.807 ab	0.956 c*	0.003
	M. oryzae (control)	0.590 c	0.784 d*	0.000
	Water	0.595 c	0.779 d*	0.001
LOX (10^{-5} $U.mg^{-1}$)	Microbiolized seed (powder)	4.054 d*	2.110 e	0.000
	Preventive spraying (24 h before *M. oryzae*)	4.589 b*	4.511 b	0.008
	Preventive spraying (48 h before *M. oryzae*)	4.081 d	4.255 c*	0.005
	C. cladosporioides C24G	4.287 c	4.557 a*	0.000
	M. oryzae (control)	5.993 a*	3.326 d	0.000
	Water	4.024 d*	2.118 e	0.000
PAL (10^{-5} $U.mg^{-1}$)	Microbiolized seed (powder)	2.010 c*	1.110 c	0.000
	Preventive spraying (24 h before *M. oryzae*)	2.566 a	2.483 a	0.074
	Preventive spraying (48 h before *M. oryzae*)	2.290 b*	1.880 b	0.000
	C. cladosporioides C24G	2.306 b	2.566 a*	0.010
	M. oryzae (control)	3.306 a*	1.796 b	0.006
	Water	1.970 c*	1.090 c	0.007
POX ($U.mg^{-1}$)	Microbiolized seed (powder)	0.057 c	0.068 b	0.134
	Preventive spraying (24 h before *M. oryzae*)	0.212 a*	0.115 a	0.004
	Preventive spraying (48 h before *M. oryzae*)	0.197 a*	0.112 a	0.000
	C. cladosporioides C24G	0.088 b	0.111 a*	0.012
	M. oryzae (control)	0.204 a*	0.119 a	0.012
	Water	0.054 c	0.067 b*	0.036

The letters compare between treatments and the asterisk (*) shows a significant difference between collection times, according to the *p value* (24 and 48 h). Means followed by the same letters in a column were not significantly different according to Tukey's test ($P<0.05$).

FIGURES / FIGURE CAPTIONS

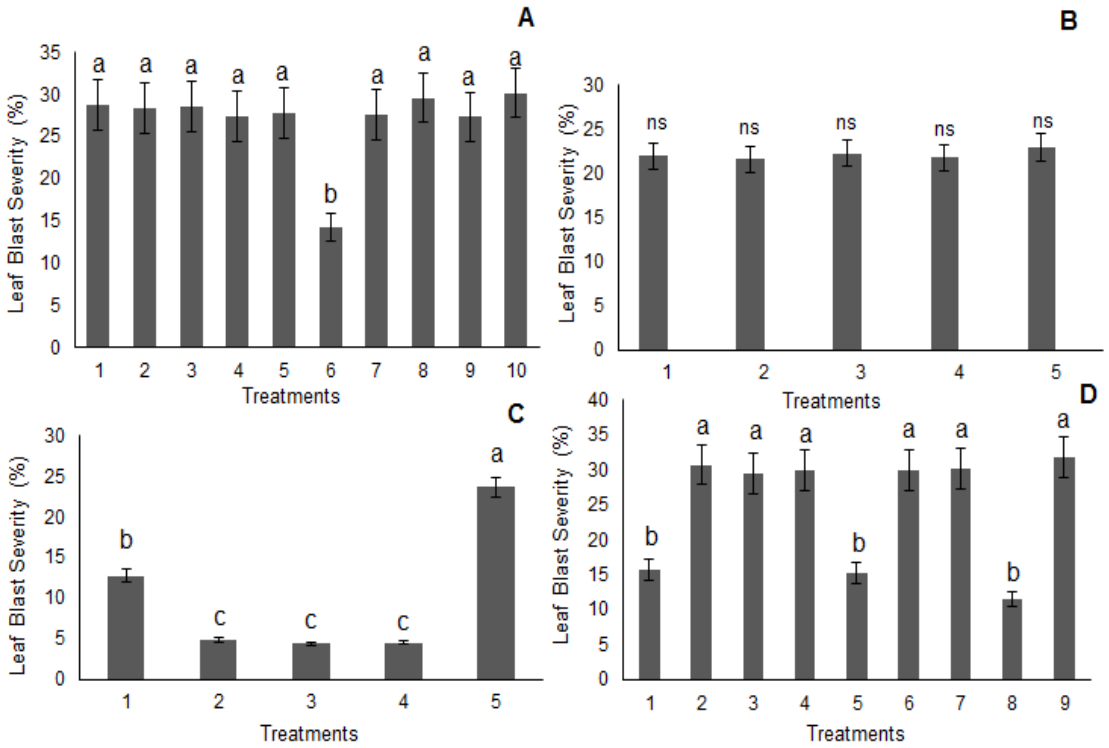

Fig. 1. The severity of rice leaf blast (%) when different methods of applying C24G. **A:** C24G applied by seed and soil: 1=seeds microbiolized with $6x10^6$ con.mL^{-1} suspension of C24G; 2=$2x10^6$ con.mL^{-1}; 3=$5x10^5$ con.mL^{-1}; 4=$5x10^3$ con.mL^{-1}; 5=$5x10^1$ con.mL^{-1}; 6=C24G in powder; 7=2.5 g of mycelium mass in the soil; 8=5.0 g of mycelium; 9=10.0 g of mycelium; 10=only *M. oryzae* (control). **B:** C24G applied by drenching the soil ($6x10^6$, $2x10^6$ and $5x10^5$ con.mL^{-1}) at 7, 14 and 19 days after planting (DAP): 1=drenching with suspension 7 DAP; 2=14 DAP; 3=19 DAP; 4=7, 14 and 19 DAP and 5=only *M. oryzae* (control). **C:** C24G applied by preventive foliar spraying at $6x10^6$, $2x10^6$ and $5x10^5$ con.mL^{-1} at 7 days, 48 and 24 h before challenge inoculation with *M. oryzae* (BCI): 1=suspensions sprayed at 7 days BCI; 2=48h BCI; 3=24 h BCI; 4=7 days, 48 and 24 h BCI and 5=only *M. oryzae* (control). **D:** C24G applied by curative foliar spraying at $5x10^5$ con.mL^{-1} after the first symptoms started (72 hours after inoculation): 1=C24G pulverization 3 days after challenged inoculated (DACI); 2=4 DACI; 3=5 DACI; 4=6 DACI; 5=3 and 4 DACI; 6=4 and 5 DACI; 7=5 and 6 DACI; 8=3, 4, 5 and 6 DACI and 9=only *M. oryzae* (control). Means followed by the same letters were not significantly different according to Tukey's test ($P<0.05$). The bars indicate the mean standard error.

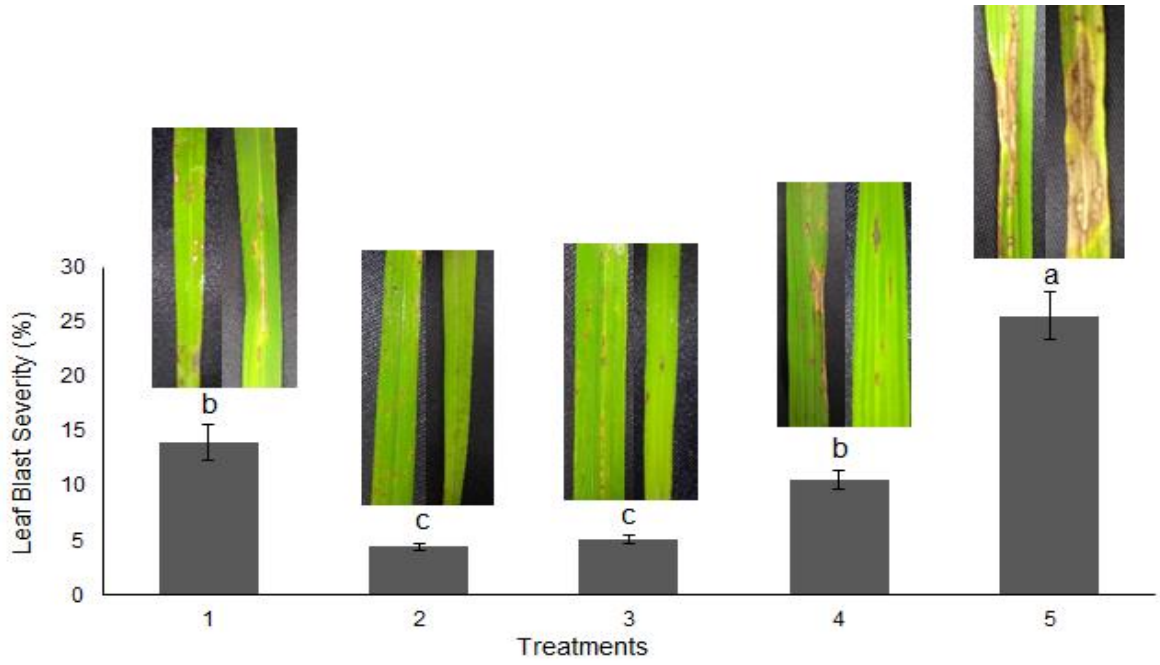

Fig. 2. The best treatment of each of the 4 assays comprised the fifth, with treatments: 1 = seeds microbiolization by C24G in powder; 2 = preventive foliar spraying 24 h BCI (before challenge inoculation) with *M. oryzae*; 3 = preventive foliar spraying 48 h BCI with *M. oryzae*; 4 = curative foliar spraying at 3, 4, 5 and 6 h ACI (after challenge inoculation) and 5 = only *M. oryzae* (control). Means followed by the same letters were not significantly different according to Tukey's test (P<0.05). The bars indicate the mean standard error.

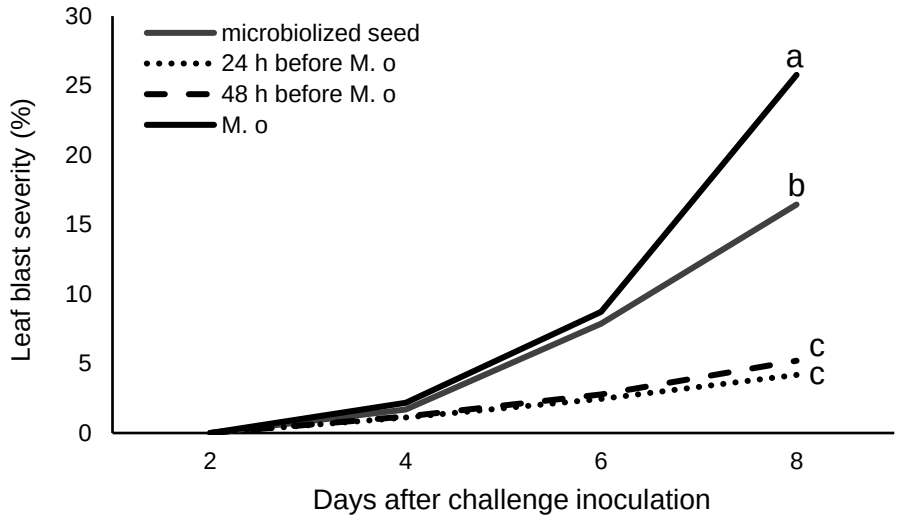

Fig. 3. Leaf blast disease progress (as AUDPC) was evaluated at 2, 4, 6 and 8 days after challenge inoculation with *M. oryzae* under greenhouse conditions. The treatments corresponds to with only *M. oryzae* (M. o) (black line); microbiolized seeds (gray line); preventive foliar spraying 48 h before M. o (striped line) and preventive foliar spraying 24 h before M. o. (dot line). Means followed by the same letters were not significantly different according to Tukey's test (P<0.05). The bars indicate the mean standard error.

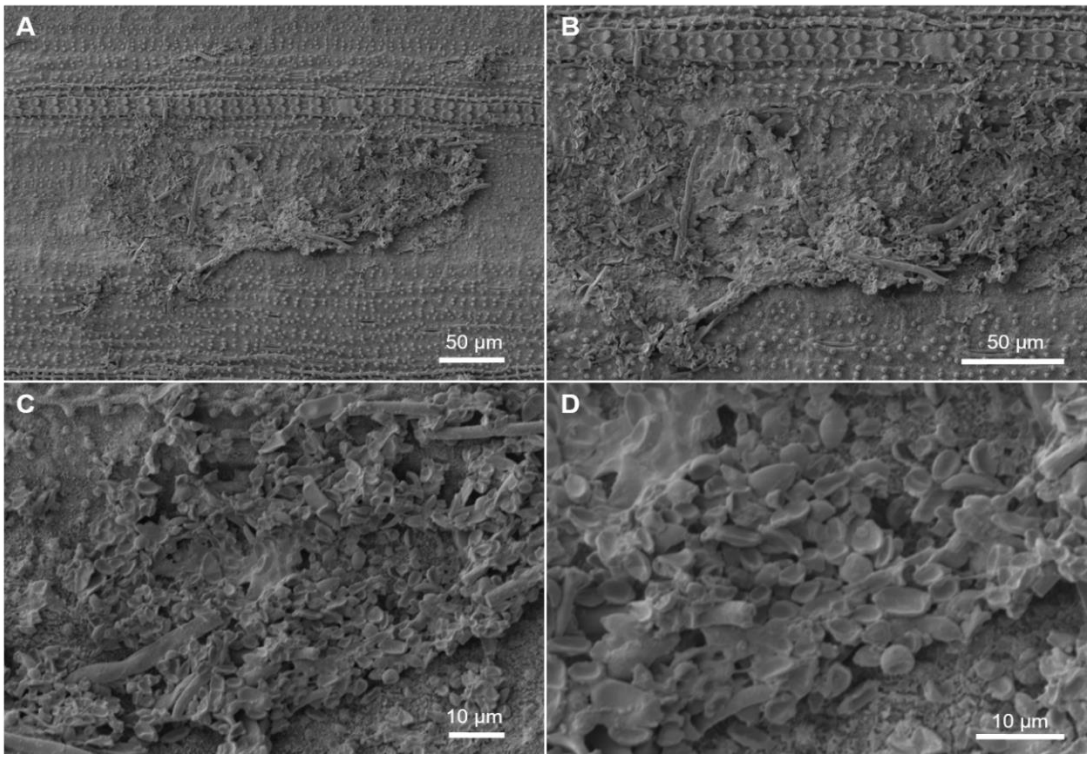

Fig. 4. Scanning electron microscopic analysis of rice leaves. A and B - leaf lesion caused by the pathogen; C and D - approximation within the leaf lesion colonized by *C. cladosporioides* (C24G).

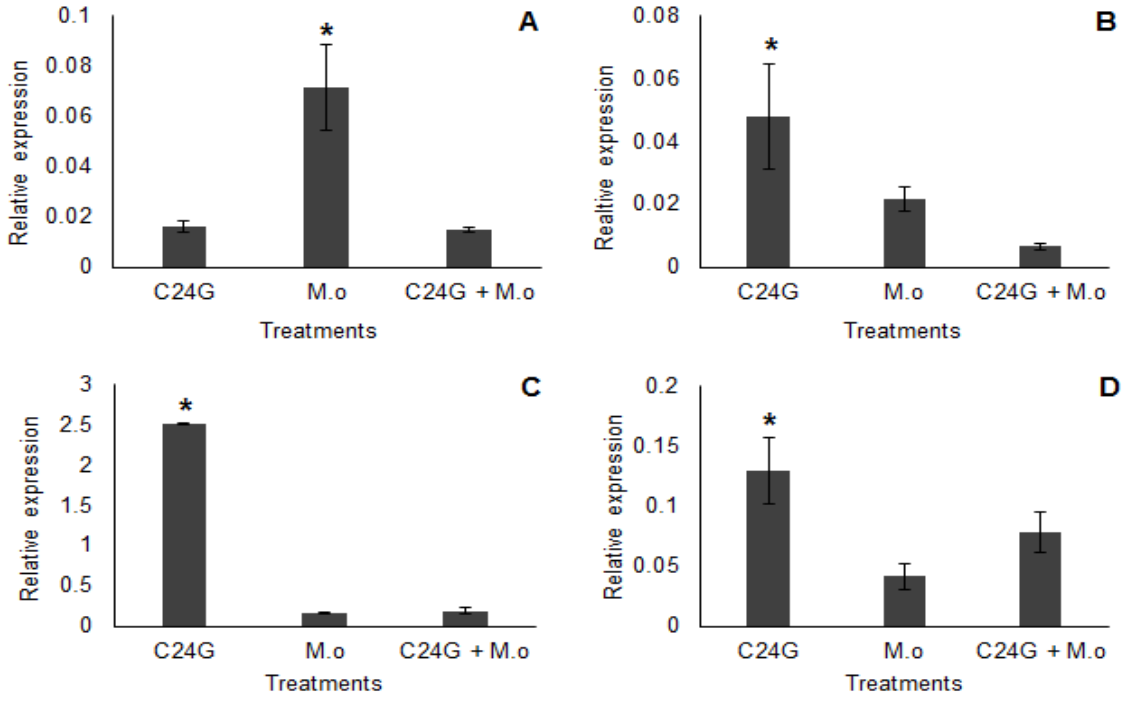

Fig. 5. Effect of C24G on relative expression of defense genes: A - *Gns1*; B - *JIOsPR10*; C - *LOX-RLL* and D - *PR1b* in rice plants treated with only C24G (*C. cladosporioides*); only *M. oryzae* (M. o) and treated with C24G + M. o. Means followed by (*) is significantly different according to Tukey's test ($P<0.05$). The bars indicate the standard deviation (± SD).

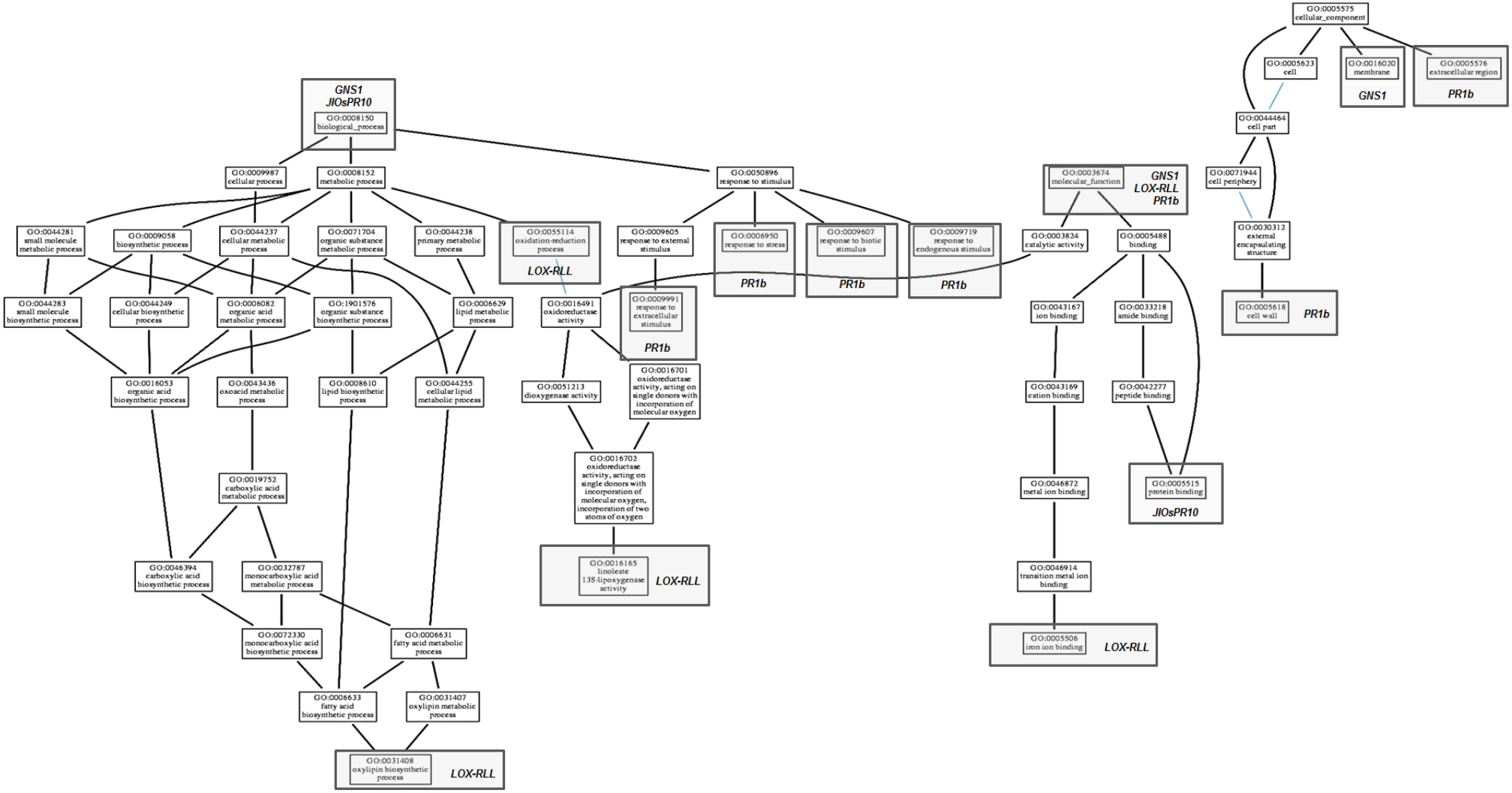

Fig. 6. Schematic overview of a hierarchy of Gene Ontology (GO) categories and pathways of interaction of the genes *Gns1*, *JIOsPR10*, *LOX-RLL* and *PR1b*

CAPÍTULO 3

Insertion of *Cladosporium cladosporioides* C24G as biological control agent in upland rice in agroecological systems.

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Insertion of *Cladosporium cladosporioides* C24G as biological control agent in upland rice in agroecological systems

ABSTRACT

Rice is a crop of great importance because it is the food base of a significant part of the world population. The occurrence of diseases, mainly rice blast (*Magnaporthe oryzae*) is the main cause of yield reduction, and it has been controlled with abusive use of fungicides, in conventional production system. In order to produce rice in an agroecological system, some measures, such as biological control, facilitates the transition from one system to another. The biological agent C24G classified as *Cladosporium cladosporioides* has been isolated from the phylloplane of rice plants and has been shown to be a potential antagonist to rice pathogens. The objective of this work was to verify the efficiency of *Cladosporium cladosporioides* C24G under field conditions, in an agroecological system. During two consecutive growing seasons, *C. cladosporioides* was tested in a randomized block design in 4 replicates. The treatments consisted of: control, microbiolized rice seeds with *C. cladosporioides*, plant sprayed (2 applications), microbiolized rice seeds with *C. cladosporioides* + plant sprayed (2 applications), plant sprayed (8 applications) and microbiolized rice seeds with *C. cladosporioides* + plant sprayed (8 applications) and control (no microbiolization and no spray). We evaluated leaf and panicle blast severity, physiological parameters associated to gas exchange and grain yield. Blast suppression was up to 85.58% and 79.63% in leaves and panicles, respectively; photosynthetic rate (*A*) increased up to 70.99 and 53.52% in vegetative and reproductive stage, during growing season 2016/17 and 29.71% in reproductive stage, during growing season 2017/28; transpiration (*E*) increased up to 22.98% in vegetative and 59.73% in reproductive stage; stomatal conductance (*gs*) increased up to 126.66% in reproductive stage and water use efficiency (*WUE*) was up to 55.29% in vegetative stage, during growing season 2016/17, as well as an increased up to 34.56 and 89.40% in biomass in two growing season and a yield increased in up to 51.30 and 34.19% in both growing season. We conclude that treatments microbiolized rice seeds or plant sprayed allows the insertion of *C. cladosporioides* into the agroecological system, facilitating the agroecological transition with considerable yield of grains and leading to a final product without residues.

Keywords: Oryza sativa, agroecology, rice blast, bioagent, sustainable development.

1. Introduction

Rice is one of the world's most important cereals (Wang et al., 2017), a food that makes up the diet of more than half the global population, and its supply needs to double by 2050 to keep up with the demand for food by the growing population (FAO, 2009).

Rice Blast (*Magnaporthe oryzae*) is the most destructive disease in rice crop-growing areas (Wang et al., 2017) and is a major concern for food insecurity, as the disease accounts for approximately 30% of rice grain loss, the equivalent of feeding 60 million people (Nalley et al., 2016). These losses increase the price of rice production costs, so any reduction in the blast incidence and severity would have consumer benefits (Nalley et al., 2016). Studies have shown that US rice producers would earn $ 69.34 million annually and increase rice supplies to more than one million consumers globally if the blast were eliminated in the South of USA (Nalley et al., 2016).

Even with the occurrence of diseases, world rice production has been increasing (FAO, 2016) annually since the Green Revolution and is related to a set of technological initiatives, including the use of fertilizers and pesticides that favored the increase of harvested area (Alves et al., 2017). Land use intensification has also led to loss of organic matter and soil biodiversity, resulting in greater sensitivity to extreme climatic effects, pest and disease outbreaks (Schrama et al., 2018). Considerable losses are still caused by plant pathogens, which are difficult to control even with synthetic chemical pesticides (Bonanomi et al., 2018), mainly due to the emergence of molecule-resistant isolates (Hayashi et al., 2017).

Increasing public awareness of sustainable production has led to low input farm management practices, with a growing demand for organic, pesticide-free, farmed foods (Pieniak et al., 2010). Therefore, one of the major challenges for the sustainable intensification of agriculture is to produce increasing volumes of food with minimal loss of biodiversity and nutrient leaching. In this scenario, organic farming, as well as agroecological based agriculture is considered the most sustainable, however, often less productive than conventional agriculture (Schrama et al., 2018).

Organic agriculture aims to change the chemical inputs during the production process, by methods that respect the natural resources that adapt to the local reality, facilitating the production of organic products (Zanon et al., 2015). However, in order to carry out the transition from conventional to agroecological farming systems, some tools, such as biological control, which once aided to the process, helps to reduce the incidence of pests and diseases, until the equilibrium of the system is reached.

Biological control has been used for centuries, with the first report of success in the late 1880s, with the introduction of the parasitic fly, *Cryptochaetum iceryae* Williston (Diptera: Cryptochaetidae) and the beetle *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) for the control of *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) in citrus orchards in California (Barrat et al., 2018). However, from the 1940s onwards, the growth and success of the pesticide industry caused the disappearance of the use of biological control until the publication of Rachael Carson's book "Silent Spring", which denounced the use of pesticides emphasizing the damage to life and the environment (Barrat et al., 2018). From then on, alternatives to pesticides were sought and opened up a greater opportunity for the application of biological control (Barratt et al., 2018).

The development of more sophisticated techniques for the isolation and characterization of microorganisms allowed the identification of a variety of bacteria and fungi capable of acting beneficially when they proliferate in the soil to control diseases and promote the growth of plants. Species reported in the 1970s included the genera *Agrobacterium*, *Pseudomonas*, *Bacillus*, *Streptomyces* and *Trichoderma*. The next step (from the mid-1980s to the 1990s) was to study and select active strains to convert soil or growth substrates from conductive to suppressive. This has led to a wide range of plant protection products, officially registered in more than 100 countries worldwide (Bonanomi et al., 2018), especially with *Trichoderma* strains (Woo et al., 2014). It took approximately 10 more years to recognize that often the same species that are useful for pathogen control can also act as growth promoters capable of increasing yield and improving quality in organic farming.

In the literature, the genus *Cladosporium* spp. has few explanatory and effective reports regarding its biocontrol mechanisms, when compared with the genus *Trichoderma* spp. or bacteria such as *Bacillus* spp., in which interactions with several pathogens and hosts have already been studied and described with results at transcriptomic levels (Steindorff et al., 2014; Chacon et al., 2007). The main study with *C. cladosporioides* as a biocontrol agent was carried out by Kohl et al. (2015), who tested and proved the effective control of *Venturia inaequalis*, under field conditions, during two years by *C. cladosporioides* in organic and conventional apple orchards. With rice, this is the first report in which *C. cladosporioides* been tested under field conditions, and in which disease suppression, as well as physiological parameters and yield of grains were verified during two years.

Organic rice represents approximately 3 million hectares and is becoming increasingly popular in China (Huang et al., 2016). However, due to its late onset, there is a lack of research and technology for organic rice production (Huang et al., 2016). In Brazil, organic rice

production is still low and is concentrated in the agrarian reform settlements at Rio Grande do Sul, although it is still the largest in Latin America. The area cultivated with rice within the agroecological principles totaled 5 thousand hectares in the growing season 2014/15. In all, 502 settled farmers cultivate agroecological rice in Rio Grande do Sul, equivalent to 4.5% of the 11,000 rice producers (Gonçalves et al., 2017).

The organic rice production, in the agroecological molds allows a sustainable rural development and the expansion of the production chain of the organic rice because it has lower production cost than the conventional system. It ensures high economic returns due to superior marketing prices (Zanon et al., 2015). However, it is required daily dedication to maintain all agricultural activities (Zanon et al., 2015).

Therefore, the objective of this work was to verify the efficiency of *Cladosporium cladosporioides* C24G under field conditions in different application forms, as well as to investigate changes in rice physiological parameters and grain yield.

2. Material and methods

2.1. Pathogenicity test of C. cladosporioides

2.1.1. Planting

Seeds of rice (*Oryza sativa*), bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), maize (*Zea mays*), soybean (*Glycine max*), *Crotalaria juncea*, *Crotalaria spectabilis*, jack bean (*Canavalia ensiformis*), pearl millet (*Pennisetum glaucum*) and pigeon pea (*Cajanus cajan*) were sown in 7-kg pots were completely filled with the soil that was fertilized with NPK (5 g of 5-30-15 + Zn and 3 g of ammonium sulphate). The seeds were disinfected using 70% alcohol and sodium hypochlorite. Ten seeds were sown per pot and, only 5 plants per pot were subsequently maintained. The assay was conducted under greenhouse conditions.

2.1.2. Seed microbiolization and plant pulverization

Five disks of the C24G isolate, grown on potato-dextrose-agar (PDA) medium for 7 days, were transferred to Erlenmeyer containing 250 g of peeled and autoclaved rice grains. Erlenmeyer were kept during seven days under dark conditions and homogenized every other day. The powder was obtained by grinding grain rice colonized by the C24G. Subsequently, disinfested seeds were wetted with a 5% glue solution (20.0 mL kg^{-1} of seeds) and treated with powder until the seeds are homogeneously covered (30.0 g kg^{-1} of seeds).

For plant pulverization, C24G was cultivated in PDA culture medium for ten days in incubation chamber under dark conditions. The conidia were removed utilizing a brush and

sterile distilled water. The suspension was filtered with a sterile tissue and concentration adjusted to $5x10^5$ con.mL^{-1}.

The sprays pulverization were done 20 days after planting (DAP) and the plants were covered during 24 hours with plastic bags (McKemy et al., 1993), and kept under high humidity conditions for disease development (Nam et al. al., 2015). After the installation of each trial and application of treatments, the development of the plants was monitored daily to identify the occurrence of some abnormalities, up to 30 dap (10 days after spraying with *C. cladosporioides*)

2.1.3. Experimental design and treatments

The experimental design was completely randomized, with three replications. The treatments consisted of: 1=control; 2=plant pulverization (sprayed); 3=seed microbiolized and 4=seed microbiolized + plant pulverization (sprayed).

2.1.4. Evaluation

At 30 days after planting (dap) the seedlings were collected (three plants per pot) and shoot length (SL) and root length (RL) were determined.

2.2. Site description

The experiments were conducted at the Experimental Station for Agroecology of Embrapa Rice and Beans, located at Santo Antônio de Goiás, GO, Brazil (16º29'00"S, 49º17'00"W coordinates and 823 m of elevation). The soil is classified as a Dystrophic Red Latosol, clay loam (410 g sand kg^{-1} soil, 270 g silt kg^{-1} soil and 320 g clay kg^{-1}) with 3.37% organic matter, 2.7 $mg.dm^{-3}$ P, 101.0 $mg.dm^{-3}$ K, 2.5 $cmolc.dm^{-3}$ Ca, 0.8 $cmolc.dm^{-3}$ Mg and a pH (1:2.5, soil/water) equal to 5.4 in the surface 0.20 m of soil.

The climate is Tropical Savanna and is considered Aw (tropical with wet summer and dry winter) according to Köppen classification. Therefore, there are two well-defined seasons: a usually dry season from May to September (autumn/winter) and a wet season from October to April (spring/summer). The average annual rainfall is between 1500 and 1700 mm, and the average annual temperature is 22.7 °C, ranging annually from 14.2 °C to 34.8 °C.

Experimental Station for Agroecology now has 20 ha managed since 2004 according to agroecological principles, such as succession and crop rotation, including green manuring; cultivation of attractive plants of natural enemies; formation of ecological corridors with native species; organic fertilization and complete elimination of the use of chemical pesticides and all type of synthetic product.

After an initial transition of approximately three years, the area now has an ecological balance at a level that guarantees a good yield of common bean, rice and corn. In addition, currently the soils are found with high fertility and good physical and microbiological quality.

2.3. Experimental design and treatments

Trials were conducted in rainfed conditions using rice seeds of BRS Primavera cultivar arranged in a randomized block in a factorial scheme, with four replications, during two growing seasons: 2016/17 (seeded on February 10 of 2017) and 2017/18 (20 December 2018).

The dimensions of the plots consisted of 5 m long and 1.4 m wide, totalizing 4 crop rows with 0.5 m spacing between the lines. It was considered as useful area 6.65 m^2. The treatments consisted of: 1=control, 2=microbiolized rice seeds with *C. cladosporioides*, 3=plant sprayed (2 applications), 4=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (2 applications), 5=plant sprayed (8 applications) and 6=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (8 applications), totalizing of 24 plots.

The fertilization was performed with organic fertilizers (2 L per plot) at 30 and 60 days after planting (DAP). To produce 200 L of organic fertilizer, 40 kg of bovine manure, 4 kg of brown sugar and 5 L of milk were used, filling the volume with water and mixing daily for oxygenation. After 20 days the fertilizer is ready for use, was diluted in water (4 L of fertilizer in 16 L of water).

The application of *C. cladosporioides* in plants was performed at 32 and 62 DAP (in treatments with 2 applications) and started 32 DAP and extended weekly until the beginning of flowering (in treatments with 8 applications).

2.4. Seed microbiolization and plant pulverization

The microbiolization was performed as described above.

Foliar applications were performed by spraying 2 L of C24G suspension per plot. C24G suspension was prepared by diluting 200 g of powder, obtained by crushing rice grains colonized by C24G in 1 L of water. Concentration was adjusted to $5x10^5$ con. mL^{-1}.

2.5. Leaf gas exchange and biomass

Gas exchange performed on rice plants in the vegetative and reproductive stages (45 and 70 DAP). The assessments were taken in the middle third of the upper leaves (completely expanded and with good exposure to the sun), as following: photosynthesis rate - *A* (μmol CO_2 m^{-2} s^{-1}), transpiration rate - *E* (mmol H_2O m^{-2} s^{-1}), stomatal conductance - *GS* (mol H_2O m^{-2} s^{-}

[1]), internal CO_2 concentration - Ci (vpm) and leaf temperature - Tleaf (ºC). The water use efficiency (WUE) (μmol CO_2 mol^{-1} H_2O) was expressed as the ratio between A and E (Doni et al., 2014).

The gas exchange measures were determined by the portable IRGA (infrared gas analyzer LCpro+, ADC BioScientific) in the period from 8:00 to 10:00 in the morning for two consecutive years. The equipment has been configured to use 370-400 mol mol^{-1} CO_2 in the air in air, which is the reference condition used in the IRGA photosynthesis chamber. The photon flux density photosynthetic active (PPFD) used was 1200 μmol [quanta] m^{-2} s^{-1}. The minimum equilibration time set for performing the reading was 2 minutes.

The biomass was performed with a sampled of 50 cm from each of the rows 45 days after planting. The plants were conditioned in paper bags and then dried in an oven with forced air circulation at 65 ºC to dry the aerial part.

2.6. Severity of leaf and panicle blast

Estimates of the area under disease progress curve (AUDPC) of leaf and panicle were performed at four dates in plants previously randomly labeled with the same physiological age, totaling 20 plants for leaf and panicle blast in plants and was calculated according to method of Shaner and Finney (1977).

Leaf blast (LF) evaluations in growing season 2016/17 were carried out 57-LF0, 59-LF1, 61-LF2 and 64-LF3 DAP and in growing season 2017/18 were performed in the 42-BF0, 45-BF1, 47-BF2 and 49-BF3 DAP.

Panicle blast (BP) evaluations were carried out for the growing season 2016/17 at 102-PB0, 105-PB1, 108-PB2 and 112-PB3 DAP and for the growing season 2017/18 to 105-PB0, 107-PB1, 110-PB2 and 112-PB3 DAP, used a six note scale (0: no disease observed, 1: less than 5%, 3: 5-10%, 5: 11-25%, 7: 26-50% and 9: more than 50%) according to Standard Evaluation System (SES) for Rice (IRRI, 2013).

In growing seasons 2016/17 and 2017/18 at 115 DAP the previously marked panicles were collected and evaluated according to the six-grade scale (0%, 5%, 25%, 50%, 75% and 100% infected spikelets/panicle). The mean percentage of panicle blast severity (PBS) was calculated based on 20 panicles per treatment using the formula: PBS (%) = Σ (class value x class frequency)/total number of panicles of the sample (Prabhu et al., 2003).

2.7. Grain Yield

Rice harvest was carried out by precision harvester after physiological maturity of the grains in the usable area of each plot (6.65 m²). The harvested grains were dried in the shade and then shaken, to be weighed and thus to calculate the productivity (Kg ha^{-1}). For the evaluation of the mass of 1000 grains (1000M), a sample was collected for each repetition of all treatments, and the count of one thousand grains was performed and then weighed on a precision scale, corrected to 13% water.

2.8. Statistical analysis

The data were analyzed by ANOVA using software Statistical Package for the Social Sciences (SPSS), version 18.0. Means were compared using Tukey's test, and $P<0.1$ was defined as indicating significance.

3. RESULTS

3.1. Pathogenicity test of C. cladosporioides

No type of symptom was observed in any of the plant species tested after the application of all the treatments. On the contrary, a positive effect, quantified with the shoot length (SL) and root length (RL). In the microbiolized treatments, there was a significant increase in SL and RL in the species *C. juncea, C. spectabilis*, pearl millet and pigeon pea, when compared to the control and treatments sprayed with the C24G suspension (Table 1; Fig. 1).

3.2. Leaf gas exchange and biomass

The analysis of variance (growing seasons 2016/17 and 2017/18) revealed changes in the parameters of gas exchange in rice plants for each physiological stage (vegetative and reproductive) evaluated and for dry matter (Table 2).

Growing season 2016/17: The photosynthetic rate (*A*) for both physiological stages, vegetative and reproductive, plants treated with *C. cladosporioides*, regardless the application form and frequency, was higher than the control (Table 2); the transpiration rate (*E*) was higher than control for seed treatment during vegetative stage, and during reproductive stage the highest rate was observed on seed treatment followed by 8 sprayed applications; stomatal conductance (*GS*): there was no difference among the treatments in the vegetative stage. Although all treatments were higher than control and, among treatments, seed + plant 2 ap. and seed + plant 8 ap. were higher than the others, in the reproductive stage; water use efficiency (*WUE*): treatments plant 2 ap., plant 8 ap. and seed + plant 8 ap. were higher than control, during vegetative stage. Although there was no difference among treatments during

reproductive stage; biomass: treatment plant 8 ap. was statistically different from control (Table 2).

Growing season 2017/18: there was no statistical difference for all evaluated parameters, during vegetative stage. During reproductive stage, photosynthetic rate (*A*) and biomass were higher for all evaluated treatment, when compared to control (Table 2).

3.3. Severity of leaf blast

In both seasons (Growing season 2016/17 and 2017/18), there was statistical difference between treatments and control for leaf blast severity and Area Under Disease Progress Curve (AUDPC) (Table 3). For two Growing seasons, all the treatments suppressed leaves blast, independent of number and mode of application (Table 3).

In Growing season 2016/17 treatments suppressed 46.43% (seed), 77.62% (plant 2 ap.), 82.15% (seed + plant 2 ap.), 83.38% (plant 8 ap.) and 85.58% (seed + plant 8 ap.). In Growing season 2017/18 treatments suppressed 48.32% (seed), 67.39% (plant 2 ap.), 83.06% (seed + plant 2 ap.), 82.66% (plant 8 ap.) and 76.71% (seed + plant 8 ap.).

3.4. Severity of panicle blast

In both seasons (Growing season 2016/17 and 2017/18), there was statistical difference between treatments and control for panicle blast severity and Area Under Disease Progress Curve (AUDPC) (Table 4). For two Growing seasons, all the treatments suppressed panicle blast, and treatments with foliar spray pulverization were statistically higher (Table 4).

In Growing season 2016/17 treatments suppressed 34.30% (seed), 64.48% (plant 2 ap.), 64.15% (seed + plant 2 ap.), 79.63% (plant 8 ap.) and 78.57% (seed + plant 8 ap.). In Growing season 2017/18 treatments suppressed 27.32% (seed), 58.02% (plant 2 ap.), 61.46% (seed + plant 2 ap.), 73.88% (plant 8 ap.) and 74.98% (seed + plant 8 ap.).

In the last panicle blast evaluation, performed before the assay harvest, all treatments reduced panicle blast severity and incidence, in both Growing seasons, especially for the treatments with higher applications number (8 ap.) (Table 5).

3.5. Grain Yield and severity and incidence of panicle blast

The analysis of variance (growing season 2016/17 and 2017/18) was significant for the severity, incidence, grain yield and mass of 1000 grains (1000M).

Treatments seed + plant 8 ap. and plant 2 ap. presented higher grain yield and differed statistically from control. The best results for 1000M was observed on treatments seed and seed + plant 8 ap, in growing season 2016/17 (Table 5).

The best results for grain yield was observed on treatments plant 2 ap., plant 8 ap. and seed + plant 8 ap., which differ from control, in growing season 2017/18. There was no statistical difference for 1000M among treatments (Table 5).

4. DISCUSSION

The monoculture system is considered an artificial system that requires constant human intervention. In most cases, this intervention leads to the use of pesticides, which, while increasing or guaranteeing productivity, entail environmental costs.

On the other hand, agroecology represents a system capable of achieving changes in the food system (Gliessman, 2011), redefining structures that govern our food systems (Molina, 2012), from the search for scientific bases to allow the transition from traditional agriculture to a more sustainable agriculture, promoting the development, from an agriculture less harmful to the environment and of better social and economic conditions to the farmers.

But the transition to the agroecological system is not an easy task and depends on the change in several aspects of the cultivation system. Thus, the use of beneficial microorganisms can bring promising results to reduce diseases severity that may occur and facilitates the plant disease management, after abandoning the use of pesticides, helping to reach a balance and to achieve adequate crop production.

Biological control has been used for centuries, but the green revolution and the success of the pesticide industry have led to the decline of biological control.

However, with the appearance of pathogens resistant to fungal molecules, added to the excesses that cause damage to the environment, to the high cost of pesticides, which in 2015 was $ 58.46 billion (ResearchandMarkets, 2016) and the growing demand for products without residues, biological control now represents an important demand as an alternative to pesticides (Barratt et al., 2018; Lenteren et al., 2018).

Several organisms are used in the biological control of plant pests and diseases and the microorganisms are among the majority of them. Lenteren et al. (2018) listed the majority of biological control agents registered worldwide, although the authors state that the list is not complete, it already provides information on 209 microbial strains of 94 different species commercially available for pest and disease control. Among the most listed are well known and

exploited bacteria and fungi such as various species of *Bacillus* spp., *Streptomyces* spp., *Pseudomonas* spp. and *Trichoderma* spp.

Our results show the innovation of using *C. cladosporioides* as a biological agent in the agroecological system in upland rice. There are few studies with *C. cladosporioides* as a biological agent (Torres et al., 2004; Nasini et al., 2004; Zhan et al., 2014) and among those few who clarify their mechanisms of action and test their effectiveness in field conditions.

Kohl et al. (2015) presented pioneering results in which *C. cladosporioides* were tested in field conditions and obtained promising results in eight trials for two years in orchards located in Hungary, Poland, Germany and the Netherlands. Plants of different cultivars were used in organic and conventional systems, comparing the efficiency of *C. cladosporioides* with the fungicides indicated for the control of *Venturia inaequalis*, and they showed that in both systems, both treatments reached the control levels.

Here we investigated the possibility of using the bioagent *C. cladosporioides* in an agroecological system for upland rice cultivation and verified the suppression of leaf blast and panicle, the positive changes in the physiological parameters related to gas exchange, as well as an increase in biomass and grain yield. We also verified that *C. cladosporioides* is not pathogenic to crops of economic importance such as soybeans, beans, corn and millet and also to crops used as green manure for agroecological system. Thus, we showed that *C. cladosporioides*, isolated C24G is not pathogenic and promotes the growth of plant species *C. juncea*, *C. spectabilis*, pearl millet and pigeon pea), which should be further investigated in the future. To promote growth of plants used as green manure is very important because besides increasing the soil organic matter it can controls of pathogens transmitted by the soil (Larkin and Griffin, 2007).

Cladosporium spp. are not known as plant growth promoters, but some studies corroborate our findings. Paul and Park (2013) reported that *C. cladosporioides* significantly promoted *in vitro* the growth of tobacco seedlings when co-cultivated without physical contact and identified Volatile Organic Compounds (VOCs), such as α-pinene, (-)-*trans*-caryophyllene, tetra-hydro-2,2,5,5-tetramethylfuran, dehydroaromadendrene and (+)-sativene. Hamayun et al. (2009) and Hamayun et al. (2010) identified *Cladosporium sphaerospermum* and *Cladosporium* sp. as gibberellin producers, which promoted soybean and cucumber plants growth, respectively.

The establishment of interaction between microrganisms benefits and plants can promote increase of the biomass and improve the gains in the productivity, suggesting that the use of the microorganisms mitigates damages caused by biotic stresses. Several authors

registered rice blast suppressed by PGPR, such as *Bacillus* spp. and *Streptomyces* spp. under field conditions (Lucas et al., 2009; Rai et al., 2018; Xu et al., 2016). But there are no records of foliar and panicle suppression by *C. cladosporioides*, under field conditions.

In addition to the suppressing of leaf and panicle blast up to 85.58% and 79.63%, respectively, we noticed increases of 34.56 and 89.40% in biomass, during both growing season and positive alterations in physiological parameters such as photosynthetic rate (*A*) in 70.99%, transpiration (*E*) in 59.73%, stomatal conductance (*gs*) in 126.66% and water use efficiency (*WUE*) in 55.29%. Understanding the physiological changes that occur in plants treated with bioagents can help to predict their effects on crops and grain yield (Nascente et al., 2017a).

Several studies on the beneficial effects of microorganisms on plants show that biomass increase occurs due to the higher photosynthetic rates (Sousa et al., 2018; Nascente et al., 2017a; Poupin et al., 2013; Naveed et al., 2014; Doni et al., 2014). The positive influence exercised by microrganisms on stomata's opening and closing directly affects the gas exchange process and contributes to increase biomass accumulation (Nascente et al., 2017b). We also observed that, in addition to promoting the increase of the photosynthetic rate, *C. cladosporioides* also promoted the increase of WUE, at the vegetative stage, during the Growing season 2016/17. The increase of WUE rates, in upland rice plants, represents an incalculable gain, since upland rice cultivation occurs in total dependence on rainfall during the summer (Sousa et al., 2018). Corroborating with ours findings, Andrade et al. (2015) registered that mycorrhizal rice plants showed greater accumulation of biomass, water use efficiency, stomatal conductance and transpiration rates. As well as Doni et al. (2014), who found that isolates of *Trichoderma* spp. were able to increase several physiological processes in rice, including photosynthetic rate, stomatal conductance, transpiration, internal CO_2 concentration and water use efficiency.

The increase of the photosynthetic rate (*A*), accumulation of biomass and higher yield of grains can be explained mainly by the reduction of disease severity provided by *C. cladosporioides*. This bioagent is a direct antagonistic (*Magnaporthe oryzae*) and to other rice pathogens, besides inducing resistance in the host, by activating routes that mainly involve genes encoding defense enzymes.

We can also hypothesize that the increase of the physiological parameters rates, biomass and consequently the grains yield can be explained by the fact that some microorganisms are able to solubilize phosphate and increases P and N absorption. Singh and Kapoor (1998) reported higher uptake of P and N in mungbean (*Vigna radiata*) after combined treatment with *Bacillus circulans*, *Cladosporium herbarum* and *Glomus fasciculatum*. These possibilities

should be investigated in the future to explain the benefits obtained in the rice plant and other plants (*C. juncea*, *C. spectabilis*, pearl millet and pigeon pea).

Finally, we verified that all the treatments reduced the severity and incidence of the blast, with the lowests leaves and panicles of incidence severity values were observed in treatments with 8 sprays, associated or not with seed treatment. But, we must consider that the gain in productivity obtained in this treatment, when compared to the cost of the 8 applications, probably is not compensatory. On the other hand, even with the highest disease severity, seed treatment showed greater changes in gas exchange and biomass increase, besides the greater weight of 1000M grains, during growing season 2016/17 and a very small difference in relation to yield of grains, when compared to the highest grain yield. We also elucidate that the association between seed treatment and spray pulverization does not result in significant gains for both blast suppression and grain yield. This association of two application forms of becomes costly for the producer, especially in a farming system that aims at lower cost and lower demand for technology. Thus, perhaps only one form of application would be more advantageous for rice production in an agroecological system, reducing blast severity and in the same time contributing to develop healthier plant, which reflects in its productive potential, facilitating the transition from the conventional crop to the agroecological system.

We conclude that *C. cladosporioides* is a multifunctional microorganism, which changes the physiology of the plant in contact with the seed, even this form of application showing no greater suppression of the disease and still little explored. And, although it does not promote rice plant growth when cultivated in pots, we hypothesized that *C. cladosporioides* stimulates responses to the stresses since this microorganism modulates hormonal routes involved in the induction of resistance, with the changes in the gas exchanges.

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TABLES

Table 1
Shoot (SL) and root length (RL) of different seedlings species in response to *C. cladosporioides* treatments in the seeds (microbiolized) and plants (sprayed).

Treatments	Rice		Bean		Cowpea		Maize		Soybean	
	SL	RL	SL	RL	SL	RL	SL	RL	SL	RL
Water	44.44 a	18.64 a	24.78 a	22.55 a	43.13 a	34.54 a	72.93 a	28.24 a	22.21 ab	32.47 a
Sprayed	44.46 a	18.54 a	24.75 a	22.46 a	43.07 a	34.71 a	72.75 a	28.33 a	22.73 a	32.92 a
Seed-microbiolized	44.47 a	18.66 a	24.77 a	22.63 a	43.28 a	34.71 a	72.82 a	28.32 a	21.84 b	32.91 a
Seed-microbiolized + sprayed	44.08 a	18.71 a	24.67 a	22.53 a	43.05 a	34.58 a	72.84 a	28.23 a	21.96 b	32.84 a
	C. juncea		*C. spectabilis*		Jack bean		Pearl millet		Pigeon pea	
	SL	RL	SL	RL	SL	RL	SL	RL	SL	RL
Water	49.86 b	21.76 b	24.94 b	18.82 b	33.96 a	22.93 a	51.84 b	21.06 b	28.77 b	23.05 b
Sprayed	49.51 b	21.90 b	24.92 b	18.72 b	33.81 a	21.66 a	52.06 b	21.27 b	28.40 b	22.90 b
Seed-microbiolized	62.05 a	34.98 a	29.33 a	27.95 a	34.10 a	22.41 a	65.06 a	35.02 a	36.20 a	33.91 a
Seed-microbiolized + sprayed	62.10 a	35.12 a	29.44 a	27.95 a	34.30 a	21.78 a	64.72 a	35.12 a	36.51 a	33.75 a

Means followed by the same letters in a column were not significantly different according to Tukey's test ($P<0.05$)

Table 2

Physiological parameters: photosynthesis rate - *A* (μmol CO_2 m^{-2} s^{-1}), transpiration rate - *E* (mmol H_2O m^{-2} s^{-1}), stomatal conductance - *GS* (mol H_2O m^{-2} s^{-1}), water use efficiency - *WUE* (μmol CO_2 mol^{-1} H_2O) and biomass (g) during Growing season 2016/17 and 2017/18.

	Growing Season 2016/17								
	Vegetative				Reprodutive				
Treatments	*A*	*E*	*GS*	WUE	*A*	*E*	*GS*	WUE	Biomass
Control	19.86 b	6.70 b	0.96 a	3.02 b	10.20 c	4.57 b	0.15 c	2.25 a	46.84 b
Seed	33.96 a	8.24 a	2.45 a	4.12 ab	13.46 b	6.28 ab	0.26 b	2.14 a	52.04 ab
Plant 2 ap.	33.90 a	7.23 ab	3.23 a	4.68 a	11.60 c	6.00 b	0.22 b	1.93 a	53.06 ab
Seed + Plant 2 ap.	31.33 a	7.72 ab	2.11 a	4.10 ab	14.25 ab	6.83 ab	0.34 a	2.09 a	56.26 ab
Plant 8 ap.	33.01 a	7.30 ab	3.88 a	4.52 a	13.81 b	6.57 ab	0.26 b	2.10 a	63.03 a
Seed + Plant 8 ap.	36.39 a	7.74 ab	4.02 a	4.69 a	15.66 a	7.30 a	0.32 a	2.14 a	55.62 ab
	Growing Season 2017/18								
Control	14.49 a	5.33 a	0.35 a	2.79 a	30.15 b	9.23 a	0.61 a	3.33 a	41.52 b
Seed	15.19 a	5.23 a	0.36 a	2.90 a	39.11 a	10.65 a	0.70 a	3.67 a	78.64 a
Plant 2 ap.	15.63 a	5.18 a	0.36 a	3.06 a	39.11 a	10.29 a	0.72 a	3.92 a	76.47 a
Seed + Plant 2 ap.	15.09 a	5.20 a	0.35 a	2.93 a	38.98 a	11.22 a	0.75 a	3.49 a	78.61 a
Plant 8 ap.	16.17 a	5.20 a	0.38 a	3.14 a	37.59 a	11.20 a	0.69 a	3.51 a	74.76 a
Seed + Plant 8 ap.	16.17 a	5.95 a	0.37 a	2.72 a	38.68 a	10.73 a	0.73 a	3.69 a	74.12 a
Factors	**ANOVA (*F* probability)**								
Growing season	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Treatments	0.000	0.000	0.120	0.008	0.007	0.078	0.000	0.988	0.002
Growing season * Treatments	0.000	0.070	0.227	0.044	0.912	0.086	0.633	0.732	0.099

Analysis of variance combined for Physiological parameters according treatments: 1=control, 2=microbiolized rice seeds with *C. cladosporioides*, 3=plant sprayed (2 applications), 4=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (2 applications), 5=plant sprayed (8 applications) and 6=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (8 applications). Means followed by the same letters in a column were not significantly different according to Tukey's test ($P<0.05$).

Table 3
Leaf Blast Severity during growing seasons 2016/17 and 2017/18.

	Growing Season 2016/17			
Treatments	**LB1 (%)**	**LB2 (%)**	**LB3 (%)**	**AUDPC**
Control	11.11 a	22.95 a	35.78 a	175.48 a
Seed	4.43 b	8.78 b	19.17 b	83.96 b
Plant 2 ap.	2.33 c	4.81 bc	8.01 c	38.35 c
Seed + Plant 2 ap.	2.53 bc	4.77 bc	6.39 c	33.80 c
Plant 8 ap.	2.29 c	4.46 c	5.95 c	31.39 c
Seed + Plant 8 ap.	2.01 c	4.17 c	5.16 c	27.89 c
	Growing Season 2017/18			
Control	1.23 a	8.12 a	24.90 a	93.42 a
Seed	0.55 b	4.85 b	12.87 b	49.42 b
Plant 2 ap.	0.67 ab	4.25 b	8.12 c	34.23 c
Seed + Plant 2 ap.	0.77 ab	4.16 b	4.22 c	22.55 c
Plant 8 ap.	0.59 b	3.03 b	4.32 c	20.23 c
Seed + Plant 8 ap.	0.54 b	2.90 b	5.80 c	24.30 c
Factors	**ANOVA (*F* probability)**			
Growing season	0.000	0.000	0.000	0.000
Treatments	0.000	0.000	0.000	0.000
Growing season * Treatments	0.000	0.000	0.000	0.000

Analysis of variance combined for Leaf Blast (LB) and AUDPC (Area Under Disease Progress Curve) according treatments: 1=control, 2=microbiolized rice seeds with *C. cladosporioides*, 3=plant sprayed (2 applications), 4=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (2 applications), 5=plant sprayed (8 applications) and 6=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (8 applications). Means followed by the same letters in a column were not significantly different according to Tukey's test ($P<0.05$).

Table 4
Panicle blast Severity during growing seasons 2016/17 and 2017/18.

	Growing Season 2016/17			
Treatments	**PB1%**	**PB2%**	**PB3%**	**AUDPC**
Control	8.26 a	17.99 a	39.80 a	237.98 a
Seed	3.61 b	10.38 b	26.15 b	146.58 b
Plant 2 ap.	2.18 bc	6.08 c	14.14 c	81.38 c
Seed + Plant 2 ap.	1.71 c	6.15 c	14.27 c	80.70 c
Plant 8 ap.	1.50 c	3.03 d	8.11 d	46.04 d
Seed + Plant 8 ap.	1.53 c	4.03 cd	8.53 d	50.81 d
	Growing Season 2017/18			
Control	3.68 a	9.65 a	22.70 a	81.75 a
Seed	3.11 a	8.09 a	16.50 b	64.05 b
Plant 2 ap.	0.81 b	4.38 b	9.53 c	33.83 c
Seed + Plant 2 ap.	0.56 b	3.75 b	8.75 cd	29.88 cd
Plant 8 ap.	0.68 c	3.09 b	5.93 d	22.53 cd
Seed + Plant 8 ap.	0.62 c	2.65 b	5.68 d	20.59 d
Factors	**ANOVA (*F* probability)**			
Growing season	0.000	0.000	0.000	0.000
Treatments	0.000	0.000	0.000	0.000
Growing season * Treatments	0.000	0.000	0.000	0.000

Analysis of variance combined for Panicle Blast (PB) and AUDPC (Area Under Disease Progress Curve) according treatments: 1=control, 2=microbiolized rice seeds with *C. cladosporioides*, 3=plant sprayed (2 applications), 4=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (2 applications), 5=plant sprayed (8 applications) and 6=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (8 applications). Means followed by the same letters in a column were not significantly different according to Tukey's test ($P<0.05$).

Table 5
Panicle rice blast severity and incidence, grain yield and mass of 1000 grains (1000M) during Growing season 2016/17 and 2017/18.

	Growing Season 2016/17			
Treatments	**Severity**	**Incidence**	**Grain Yield**	**1000M**
Control	56.90 a	71.00 a	1394.99 b	21.64 b
Seed	28.70 b	37.00 b	2017.12 ab	27.25 a
Plant 2 ap.	15.45 c	22.00 c	2043.51 a	24.99 ab
Seed + Plant 2 ap.	15.45 c	26.00 bc	1974.12 ab	25.84 ab
Plant 8 ap.	9.85 d	23.00 c	1993.88 ab	26.02 ab
Seed + Plant 8 ap.	9.75 d	19.75 c	2110.67 a	27.81 a
	Growing Season 2017/18			
Control	25.35 a	48.00 a	2469.92 b	22.89 a
Seed	17.85 b	36.00 ab	3030.86 ab	22.93 a
Plant 2 ap.	10.25 c	32.00 b	3314.51 a	23.78 a
Seed + Plant 2 ap.	8.90 c	30.00 b	2824.43 ab	23.39 a
Plant 8 ap.	5.45 d	11.75 c	3174.04 a	24.29 a
Seed + Plant 8 ap.	5.15 d	13.00 c	3180.00 a	24.16 a
Factors	**ANOVA (*F* probability)**			
Growing season	0.000	0.018	0.000	0.001
Treatments	0.000	0.000	0.001	0.008
Growing season * Treatments	0.000	0.000	0.863	0.064

Analysis of variance combined for Panicle rice blast severity and incidence, grain yield and mass of 1000 grains (1000M) according treatments: 1=control, 2=microbiolized rice seeds with *C. cladosporioides*, 3=plant sprayed (2 applications), 4=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (2 applications), 5=plant sprayed (8 applications) and 6=microbiolized rice seeds with *C. cladosporioides* + plant sprayed (8 applications). Means followed by the same letters in a column were not significantly different according to Tukey's test ($P<0.05$).

FIGURE/FIGURE CAPTION

Fig. 1. Shoot and root length in different seedlings species, in response to treatments with *C. cladosporioides* in seed (microbiolized) and water (control). Plants marked with (*) represent seed-microbiolized. a) Pigeon pea; b) Pearl millet (shoot); c) Pearl millet (root); d) *C. spectabilis*; e) *C. juncea* (shoot); f) *C. juncea* (root).

CONSIDERAÇÕES FINAIS

Este trabalho trouxe informações inéditas, como o primeiro relato de identificação a nível de espécie de isolados de *Cladosporium* spp. habitantes do filoplano do arroz, além de desvendar alguns dos seus mecanismos de ação como agente de controle biológico em seu habitat original.

Verificamos que além de exercer antagonismo direto, este bioagente, altera a expressão gênica e a atividade enzimática de plantas tratadas, assim como os parâmetros fisiológicos relacionados a trocas gasosas como a taxa fotossintética, transpiração, condutância estomática e eficiência do uso da água. Conclui-se que com essas alterações obtivemos menor severidade de brusone foliar e da panícula, maior biomassa das plantas tratadas e maior rendimento de grãos.

Podemos afirmar, que *C. cladosporioides* é um microrganismo multifuncional, que altera a fisiologia da planta e estimula respostas aos estresses a partir da modulação das vias hormonais.