UNIVERSIDADE FEDERAL DO PARANÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE GENÉTICA

ALAN DE OLIVEIRA SILVA

IDENTIFICAÇÃO DE ESPÉCIES DE *Colletotrichum* ASSOCIADAS COM A QUEDA PREMATURA DE FRUTOS CÍTRICOS NO BRASIL E INFLUÊNCIA DO GENE *SNF1* NA VIRULÊNCIA DE *Colletotrichum graminicola*

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Orientadora: Profª Drª Chirlei Glienke

Co-orientador: Prof. Dr. Holger B. Deising

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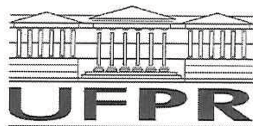

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de ALAN DE OLIVEIRA SILVA intitulada: Identificação de espécies de Colletotrichum associadas com a queda prematura de frutos cítricos no Brasil e influência do gene SNF1 na virulência de Colletotrichum graminicola., após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

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Curitiba, 18 de Outubro de 2017.

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Dedico

A todos os problemas e dificuldades que me fizeram parar, pensar, estudar e me reestruturar. Eles fazem parte do que eu sou hoje.

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“A man will be imprisoned in a room with a door that’s unlocked and opens inwards; as long as it does not occur to him to pull rather than push”

Ludwig Wittgestein

RESUMO

O gênero *Colletotrichum* representa um grupo de fungos conhecidos mundialmente como fitopatógenos, responsáveis por perdas expressivas em inúmeras culturas vegetais de importância econômica. No Brasil, as espécies *Colletotrichum abscissum* e *Colletotrichum graminicola* causam prejuízos às culturas de citros e milho, respectivamente, e sendo culturas que destacam o país no cenário agrícola mundial, estimulam uma série de pesquisas que buscam a melhor compreensão de suas doenças. O primeiro passo para o entendimento de um patosistema é a correta identificação do seu agente causal e então, estudos de interação patógeno-hospedeiro podem trazer dados relevantes sobre aspectos epidemiológicos da doença e as bases genéticas da patogenicidade. Em citros, a doença Queda Prematura de Frutos Cítricos (QPFC) é conhecida, mas seu agente causal sofreu uma reclassificação e a nova espécie *C. abscissum* foi recentemente descrita, porém em um estudo com poucos isolados, necessitando de informações sobre sua incidência e também abrindo uma lacuna para a revisão de estudos anteriores e métodos diagnósticos. Neste trabalho foi proposto um estudo etiológico envolvendo 227 isolados de flores sintomáticas de citros de diferentes pomares no estado de São Paulo, Brasil. Com base na análise da região intrônica GAPDH, foi observada uma prevalência da espécie *C. abscissum* causando a doença nos pomares, e a análise multilocus de diferentes isolados revelou ser uma população clonal. A espécie *C. gloeosporioides* também foi associada com a doença, em uma frequência maior que a observada anteriormente e embora seja reconhecidamente sensível a fungicidas do grupo dos benzimidazóis, a maioria dos isolados exibiu resistência ao fungicida benomyl. Foram então propostos *primers* para uma reação de PCR multiplex, que podem ser utilizados na distinção das espécies associadas com a QPFC no Brasil, *C. abscissum* e *C. gloeosporioides*. Em milho, a espécie *C. graminicola* causa a antracnose foliar e representa um patosistema mais bem estudado, que dispõe de vários estudos de interação apontando genes importantes para a patogenicidade. Durante a infecção, fungos fitopatogênicos normalmente secretam enzimas degradadoras de parede para penetrar no hospedeiro e absorver seus nutrientes. Em leveduras, o gene regulatório *SNF1* controla o uso de fontes alternativas de carbono, num processo conhecido como repressão por glicose, e devido à redundância de função de genes codificadores para enzimas individuais, o estudo de genes regulatórios pode ser uma alternativa mais eficiente na busca de fatores de virulência em fungos fitopatógenos. Neste estudo buscou-se e deletou-se o gene ortólogo *SNF1* de *C. graminicola*, resultando em mutantes que exibiram redução no crescimento vegetativo e esporulação, assim como baixa taxa de crescimento em diferentes fontes de carbono além de sacarose. Observações microscópicas exibiram um atraso na penetração e colonização dos tecidos do hospedeiro, confirmando a expressão de sintomas reduzidos observada em nível macroscópico. Estes dados foram confirmados por PCR quantitativo, que revelou uma redução acentuada da biomassa do fungo em folhas de milho, sugerindo a associação do gene *CgSNF1* com a virulência de *Colletotrichum graminicola*. Os dados apresentados neste trabalho contribuem de forma significativa com o estudo de dois importantes patosistemas, trazendo novos conhecimentos sobre a epidemiologia e interação fungo-planta dentro do gênero *Colletotrichum*.

Palavras-chave: *Colletotrichum abscissum*, *Colletotrichum gloeosporioides*, Queda Prematura de Frutos Cítricos, Antracnose, Citros, Milho, Identificação, Interação Planta-Patógeno, *GAPDH*, *SNF1*.

ABSTRACT

The genus *Colletotrichum* is a group of plant pathogens well known for causing yield losses in a wide variety of crops worldwide. In Brazil, the species *Colletotrichum abscissum* and *Colletotrichum graminicola* affect the citrus and maize cultures, respectively, being important diseases that stimulate many researches into *Colletotrichum* genetics, pathology and host-parasite interactions. Species differentiation is a critical step for understanding a pathosystem and then, studies about the plant-pathogen interaction can contribute with new aspects of the disease, such as epidemiology and the genetic bases of pathogenicity. In citrus, Postbloom Fruit Drop (PFD) is a known disease that causes petal necrosis and retention of flower calyces. The pathogen associated with PFD was recently reclassified, and the new species *C. abscissum* was described in a short communication using a small number of isolates, so that the incidence of the pathogen in the main citrus producing area is still unclear, and studies involving epidemiology and diagnostic on this pathosystem need to be revised. In this study, it was performed an etiological study with 227 isolates of symptomatic citrus flowers from different regions of São Paulo state, Brazil. Based on GAPDH intron region, the prevalence of *C. abscissum* causing the disease was observed, and a multilocus sequence analysis using different isolates revealed a clonal population. The species *C. gloeosporioides* was also found associated with the disease, with a higher frequency than observed in previous study and showing resistance to the benzimidazole-based fungicide benomyl, for which the species is known to be highly sensitive. Based on the efficiency of the GAPDH region in discriminating both species, primers for a single multiplex PCR reaction were proposed, which successfully distinguished the species associated with PFD in Brazil, *C. abscissum* and *C. gloeosporioides*. In maize, the species *C. graminicola* causes Leaf Blight Anthracnose, representing a better studied pathosystem, in which a wide range of interaction studies have been implemented. During infection, plant pathogenic fungi usually are nutrient deprived until they access the host tissue, where they secrete cell wall degrading enzymes (CWDE) for nutrient acquisition, and the expression of genes for CWDEs are repressed in the presence of glucose, in a process called carbon catabolite repression. In yeasts, the process involves the regulatory gene *SNF1*, which controls the expression of genes for alternative carbon sources usage, including CWDE genes. Due to the redundancy of function of single CWDEs, the study of regulatory genes could be a better alternative to find virulence factors in plant pathogenic fungi. In this study, the ortholog of the *SNF1* gene was found and deleted from *C. graminicola*, resulting in mutants with reduced vegetative growth rate, especially in complex carbohydrates such as pectin and CMC. Microscopic observations exhibited a delay in penetration and spread through the host leaf tissue, confirming the impaired virulence symptoms showed in macroscopic inoculation assays. qPCR data confirmed these observations, by showing a significant reduced fungal biomass *in planta*, suggesting the association between the *SNF1* gene with virulence in *Colletotrichum graminicola*. The present study provides new findings about two important pathosystems, which contributes to knowledge into epidemiology and plant-host interaction in the genus *Colletotrichum*.

Keywords: *Colletotrichum abscissum*, *Colletotrichum gloeosporioides*, Postbloom Fruit Drop, Anthracnose, Citrus, Maize, Identification, Plant-Pathogen Interaction, *GAPDH*, *SNF1*.

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1. INTRODUÇÃO

O gênero *Colletotrichum* compreende um grupo de fungos de importância mundial, patógenos de monocotiledôneas e dicotiledôneas com vasta distribuição geográfica, trazendo perdas expressivas na produção de várias culturas anualmente (PERES et al., 2005). No Brasil, espécies do gênero causam prejuízos às culturas de citros e milho. A espécie *Colletotrichum abscissum* causa a queda prematura dos frutos cítricos (QPFC), que acomete flores, provocando a abscisão de frutos jovens e formação de cálices persistentes (TIMMER et al., 1994; FEICHTENBERGER et al., 2005), enquanto a espécie *Colletotrichum graminicola* causa a antracnose foliar do milho, responsável por necrose no limbo foliar até podridão do colmo, causando perdas estimadas de até 40% em cultivares suscetíveis no Brasil (COTA et al., 2009) e até um bilhão de dólares anuais nos EUA (FREY et al., 2011). Sendo doenças com importância econômica a nível global, o gênero *Colletotrichum* estimula grande número de pesquisas tanto sobre a genética, quanto epidemiologia e interação patógeno-hospedeiro (CANNON et al., 2012).

Em citros, isolados de QPFC eram associados com as espécies *Colletotrichum acutatum* e *Colletotrichum gloeosporioides*. A dificuldade na identificação de isolados dessas espécies em diversos hospedeiros levou a uma confusão taxonômica e à proposição da existência de infra grupos, que resultou na reorganização de *C. acutatum* e *C. gloeosporioides* em complexos de espécies contendo 31 e 23 espécies, respectivamente (DAMM et al., 2012; WEIR et al., 2012), porém sem a inclusão de isolados de citros do Brasil. Em 2015 a nova espécie *C. abscissum* foi proposta por Pinho e colaboradores como o agente causal da QPFC, mas deixando uma lacuna sobre a incidência da espécie e sua variabilidade genética em pomares brasileiros. Ainda, a partir do desdobramento do complexo *C. acutatum* em diferentes espécies, trabalhos prévios sobre a genética da interação planta-patógeno neste patosistema necessitam ser revistos, uma vez que podem ter incluído espécies do complexo *C. acutatum* que não o real agente causal da QPFC (*C. abscissum*). Para que tais estudos sejam realizados, inicialmente se faz necessário um levantamento de quais as espécies de *Colletotrichum* são associadas com a doença QPFC no Brasil.

Em milho, a antracnose foliar e seu patógeno *C. graminicola* representam um patosistema mais bem estudado. A presença alternativa de conídios ovais diferenciados com paredes celulares finas (PANNACCIONE et al., 1989) permitiram o estabelecimento de um sistema eficiente de transformação genética sítio-dirigida nessa espécie

(VAILLANCOURT; HANAU, 1994), sendo considerada um bom modelo de estudo da interação fungo-planta dentro do gênero. Desde então, vários genes associados com patogenicidade já foram descritos e caracterizados (WERNER et al. 2007, KRIJGER et al. 2008, OLIVEIRA-GARCIA e DEISING, 2013) . Fungos filamentosos fitopatogênicos secretam enzimas extracelulares para degradação e utilização de polímeros da parede celular do hospedeiro (HERBERT et al., 2002), e a expressão de muitas dessas enzimas é controlada pela disponibilidade de glicose ou outro açúcar simples em um processo metabólico conhecido como repressão por glicose (RUIJTER; VISSER, 1997). Em leveduras, genes glicose-reprimidos possuem em seus promotores um sítio conservado de ligação para a proteína repressora Mig1p, codificada pelo gene *MIG1*. Na ausência de glicose, um outro gene, *SNF1*, codifica a proteína Snf1p, que fosforila Mig1p, permitindo a expressão de genes que codificam enzimas para a degradação de fontes alternativas de carbono (TREITEL,1998). Um gene ortólogo ao *SNF1* foi descrito inicialmente em fungos filamentosos no estudo de Tonukari et. al (2000) com a espécie *Cochliobolus carbonum*. Desde então, outros trabalhos descreveram a relação do gene *SNF1* com a patogênese em outros fungos filamentosos fitopatogênicos como *Fusarium oxysporum* (OSPINA-GIRALDO et al. 2003), *Magnaporthe oryzae* (YI et al. 2008), *Verticillium dahlia* (TZIMA et al. 2011), *Penicillium digitatum* (ZHANG et al. 2013) e *Leptosphaeria maculans* (FENG et al. 2014). Embora seu mecanismo de ação ainda não esteja bem elucidado, este gene pode representar um importante alvo para estudos de fatores de virulência no gênero *Colletotrichum*.

Diante do exposto, este estudo propõe a caracterização molecular de espécies associadas à QPFC em diferentes regiões do estado de São Paulo, Brasil, com a proposição de *primers* espécie-específicos para a distinção e um melhor diagnóstico das espécies *C. abscissum* e *C. gloeosporioides*. Também é proposto o estudo do gene *SNF1* e sua caracterização como fator de virulência no patógeno de milho *Colletotrichum graminicola*.

2. OBJETIVOS

Capítulo I

- Identificar e caracterizar as espécies de *Colletotrichum* associadas à doença QPFC em diferentes regiões do estado de São Paulo, Brasil;
- Desenvolver PCR multiplex para o diagnóstico molecular e distinção de espécies de *Colletotrichum* associadas a QPFC no Brasil;

Capítulo II

- Identificar e caracterizar funcionalmente o gene *SNF1* em *Colletotrichum graminicola* por meio de deleção sítio dirigida.

3. REVISÃO BIBLIOGRÁFICA

O gênero *Colletotrichum* compreende cerca de 600 espécies de fungos mundialmente reconhecidos como fitopatógenos, podendo causar doenças em até 3200 espécies de plantas (O'CONNELL et al. 2012). Este grupo de fungos tem maior importância nos trópicos, onde causam perdas expressivas na produção de várias culturas, causando sintomas desde podridões em frutos a lesões em ramos, folhas e flores (PERES et al., 2005). No Brasil, pode-se destacar duas espécies de *Colletotrichum* com grande importância econômica, *Colletotrichum abscissum* Pinho & O.L. Pereira, sp. nov. (anteriormente denominada *Colletotrichum acutatum* lato sensu), que causa a queda prematura de frutos cítricos, e a espécie *Colletotrichum graminicola* (Ces.) G.W. Wilson, responsável pela antracnose foliar do milho.

O agronegócio representa considerável parte da estrutura socioeconômica brasileira, no qual cultivos como citros e milho possuem grande relevância. A citricultura brasileira é responsável por mais da metade da produção mundial de suco de laranja, sendo que 98% da produção de citros do Brasil é destinada à exportação (NEVES, 2010). Este cultivo gera empregos diretos e indiretos e responde por notável parte do PIB nacional, contribuindo de forma definitiva com o desenvolvimento do país. Igualmente importante para o Brasil é a produção de milho, cuja produção no país representa a terceira maior em escala mundial (MAPA, 2015), com safra de 75 milhões de toneladas em 2013/2014 (CONAB, 2014). Dentre os esforços para o aumento da produção destes cultivos, grande empenho está reunido no combate a pragas e doenças, evitando assim perdas e o aumento nos custos de produção.

3.1 Queda prematura de frutos cítricos e identificação dos patógenos associados

Entre as doenças que acometem as plantas de citros, destaca-se a Queda Prematura de Frutos Cítricos (QPFC), também conhecida como Podridão Floral do Citros (PFC) ou *Postblom Fruit Drop*, causada pelo fungo *C. abscissum*, que infecta as flores de citros, causando a abscisão de frutos jovens e retenção de cálices florais nos ramos (FEICHTENBERGER et al., 2005). O uso de caracteres morfológicos e associação com hospedeiro costumavam ser os únicos critérios de identificação de espécies do gênero

(JOHNSTON et al., 2005), mas a alta variabilidade morfológica e baixa especificidade de hospedeiro nesses fungos dificultou a correta identificação das espécies.

Isolados de citros com sintomas de QPFC foram inicialmente classificados como pertencentes à espécie *C. gloeosporioides* (FAGAN, 1979), mas a variabilidade intraespecífica nesses isolados sugeriu a existência de diferentes tipos morfológicos da espécie causando doenças em citros. O uso de dados moleculares na identificação de espécies revelou que a linhagem causadora da QPFC tratava-se da espécie *C. acutatum* (BROWN, 1996), mas ainda havia uma confusão taxonômica quanto a presença de diferentes linhagens de *C. acutatum* causando doença em vários hospedeiros, sendo então proposta a existência de infragrupos. Diante dessa inconsistência, foi então proposta a reclassificação do grupo, com o desmembramento de *C. acutatum* em um complexo que hoje abriga 34 espécies. Isolados de citros do Brasil não foram inclusos neste estudo, o que conduziu à descrição da nova espécie *C. abscissum* a partir de isolados provenientes de flores de citros no Brasil com sintomas de QPFC (PINHO et al., 2015). Este trabalho baseou-se em três isolados, deixando ainda uma lacuna sobre a distribuição do patógeno ao longo da principal região produtora de citros do país, bem como a existência de variabilidade genética nos isolados.

Em 2011 a espécie *C. gloeosporioides* foi novamente associada com a QPFC no Brasil por Lima e colaboradores, que propuseram *primers* específicos para identificar ambas as espécies associadas com a doença no Brasil, porém utilizando uma combinação de *primers* universais para os complexos *C. acutatum* e *C. gloeosporioides*, e não para as espécies, pois essas ainda não haviam sido elucidadas. Dessa forma, não havia um método diagnóstico rápido e preciso para a identificação dos patógenos associados a doença QPFC, necessitando de análise filogenética multigênica, demandando maiores custos, tempo e análise criteriosa. Ainda assim, é importante confirmar se *C. abscissum* é a única espécie do complexo *C. acutatum* associada com a doença no Brasil, bem como avaliar a existência de variabilidade genética entre os isolados, permitindo validar trabalhos anteriores que basearam-se em caracteres gerais do complexo e não no real agente causal da QPFC (*C. abscissum*).

Experimentos visando a compreensão da interação e fatores envolvidos nos processos de infecção e colonização do fungo *C. abscissum* em citros são de extrema importância. Autores como Marques e cols. (2012) não observaram estágio biotrófico em flores de citros, sugerindo que o fungo entraria imediatamente no estado necrotrófico,

mas em folhas assumiria uma forma subcuticular latente, sendo então classificado como necrotrófico. Ainda existe essa lacuna sobre a interação com seu hospedeiro, mas o esclarecimento do estilo de vida deste patógeno através de um estudo epidemiológico ainda é o primeiro passo para o entendimento do ciclo da doença.

3.2 *Colletotrichum graminicola* e estudos de interação planta-patógeno

A espécie *C. graminicola* causa a Antracnose foliar do milho, caracterizada por lesões e necrose no limbo foliar (Panaccione, 1988), podendo reduzir a produção deste cultivo em até 40% (Parreira et al., 2014). A antracnose foliar do milho ocorre em todas as principais regiões produtoras de milho do Brasil, seja em plantios de período normal ou tardios (CRUZ et al., 1996; FERNANDES & BALMER, 1990). Evidenciando ainda mais a problemática desta doença, segundo Bergstrom e Nicholson (1999), as lesões foliares podem servir como fonte de inóculo para infecções no colmo que podem acarretar no tombamento da planta e que, como consequência, reduz a produtividade da cultura entre 18% e 50% (SMITH, 1976; CARSON & HOOKER, 1981; CALLAWAY et al., 1992; BERGSTROM & NICHOLSON, 1999; PINTO, 2003; CASELA 2008; COTA et al., 2009). Em milho, a espécie *C. graminicola* exibe comportamento hemibiotrófico, assumindo um estágio biotrófico inicial curto, seguido por um estágio necrotrófico destrutivo de patogenicidade (KRIJGER et al., 2008), e este comportamento fornece bons modelos para estudos sobre as bases da patogenicidade em fungos.

Um meio efetivo de estudar os mecanismos de patogenicidade em fungos é promovendo a deleção ou disrupção de seus genes, e então isolar mutantes exibindo virulência alterada (MULLINGS & KANG, 2001). Alguns genes têm expressão constitutiva e não podem ser deletados, enquanto outros são recrutados apenas durante estágios específicos de interação com o hospedeiro, podendo trazer dados úteis sobre as bases genéticas da patogenicidade. Vários genes associados com patogenicidade já foram descritos e caracterizados, e estão ligados a diversas funções. Alguns genes atuam no metabolismo de compostos nitrogenados, como a enzima *allantoicase*, importante na reciclagem e reutilização do nitrogênio presente em compostos da célula hospedeira (MÜNCH et al., 2011; LEE et al., 2013). Outros genes participam da síntese da parede celular fúngica, cuja composição e conformação são fundamentais para o crescimento vegetativo e para o sucesso no processo infeccioso, como a quitina, sintetizada por

complexos de enzimas *chitin synthases* (MIYAZAKI & OOTAKI, 1997; MUNRO & GOW, 2001; WERNER et al., 2007), os beta-glucanos, sintetizados a partir de beta-*glucan synthases* (MOUYNA et al., 2004; BOWMAN & FREE, 2006; HA et al., 2006; OLIVEIRA-GARCIA & DEISING, 2013) e a melanina, sintetizada por complexos enzimáticos que envolvem *polyketyde sinthases* (LUDWIG et al., 2004).

Em fungos filamentosos fitopatogênicos, a secreção de enzimas extracelulares, como pectinases, xilanases, glucanases, arabinosidases, celulases, entre outras, promove a degradação de polímeros da parede celular do hospedeiro (HERBERT et al., 2002). Embora muitas destas enzimas tenham sido caracterizadas, suas funções podem estar associadas a múltiplos genes, e mutantes para apenas um gene em particular podem acarretar em uma atividade enzimática reduzida ou até inalterada, dificultando assim sua caracterização. Ao contrário da deleção de genes codificadores, genes regulatórios podem ser uma alternativa para o estudo de fatores de virulência, de modo que mutantes para esses genes resultariam em perda total ou redução acentuada da atividade de múltiplas enzimas (TONUKARI et al., 2000). Assim, efetivamente, se um mutante com ausência da produção de enzimas degradadoras de parede ainda for patogênico, colocará em dúvida o papel de tais enzimas na patogenicidade (WALTON, 1994).

A glicose é o açúcar preferencialmente utilizado pela maioria dos organismos, e a expressão de genes envolvidos no metabolismo de fontes alternativas de carbono é inativada na presença deste açúcar (HARDIE et al., 1998). Este fenômeno é conhecido como repressão por glicose, e foi descrito em leveduras após a obtenção de mutantes para o *locus SNF1* em *Saccharomyces cerevisiae*, que perdiam a habilidade de utilização de sacarose (mutantes *sucrose non fermenting*), assim como outras fontes de carbono fermentáveis e não fermentáveis (CARLSON et al., 1981). O gene *SNF1* de leveduras codifica a proteína Snf1, que possui similaridade com proteínas do tipo *serine/threonine protein kinase* de inúmeras espécies (CELENZA; CARLSON, 1986). Para assumir sua forma ativa, a proteína quinase Snf1p precisa formar um complexo quinase heterotrimérico, pela ligação com a subunidade ativadora Snf4 (codificada pelo gene *SNF4*) e com um terceiro domínio de interação, Sip1, Sip2 ou Gal83, que são adaptadores que medeiam a interação do complexo quinase com alvos intracelulares específicos (JIANG; CARLSON, 1997).

Sabe-se que uma das funções do complexo SNF1 em leveduras é a fosforilação do repressor Mig1, uma proteína de ligação ao DNA que se liga ao promotor de genes

glicose reprimidos (TREITEL et al., 1998). Mig1 é uma proteína do tipo *zinc finger* C_2H_2 que é rapidamente translocada para o citoplasma na presença de glicose, e que na ausência de *SNF1* torna-se constitutivamente localizada no núcleo (DEVIT et al., 1997). Ambos os genes *SNF1* e *MIG1* possuem ortólogos em outras espécies, inclusive em fungos filamentosos, onde o gene *MIG1* é conhecido como *CREA*.

O gene *SNF1* foi inicialmente estudado em fungos filamentosos no patógeno de milho *Cochliobolus carbonum* (TONUKARI et al., 2000), onde foi descrita sua relação com a patogenicidade nessa espécie, e desde então, outros trabalhos buscaram a relação do gene regulatório *SNF1* com patogenicidade ou virulência em fungos filamentosos fitopatogênicos. Sabe-se que a expressão de muitas enzimas degradadoras de parede na maioria dos fungos também sofre repressão por glicose (RUIJTER & VISSER, 1997), de modo que mutantes para o gene *SNF1* podem ser úteis para avaliar a importância de enzimas degradadoras de parede como fatores de virulência em fungos patogênicos (TONUKARI et al., 2000). O estudo desta proteína como fator transcricional na expressão de genes codificantes para enzimas degradadoras de parede pode representar uma nova etapa no entendimento da interação planta-patógeno em fungos do gênero *Colletotrichum*. Além disso, o conhecimento sobre vias específicas associadas a patogenicidade e virulência em fitopatógenos pode auxiliar na identificação de alvos para controle, como o uso de fungicidas com ação sítio específica ou controle mediante técnicas modernas utilizando RNA de interferência, garantindo maior eficiência, menos impacto ambiental e à saúde e reduzindo assim o custo de produção.

4. CAPÍTULO I

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Identification of *Colletotrichum* species associated with postbloom fruit drop in Brazil through GAPDH sequencing analysis and multiplex PCR

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Abstract The genus *Colletotrichum* comprises a group of important fungal pathogens that can infect a wide variety of host plants worldwide. Postbloom fruit drop (PFD) of citrus plants is responsible for extensive crop losses annually, and is particularly detrimental to Brazilian citrus production. The disease was first associated with *Colletotrichum gloeosporioides* and subsequently linked to *Colletotrichum acutatum*. However, a new species, *C. abscissum*, was described in 2015 as the causative agent of PFD in Brazil. The species description used a small number of strains and the distribution of the pathogen remains unclear. The proportion of PFD caused by this species is also unclear, because both *C. abscissum* and *C. gloeosporioides* are associated with the disease as well. By analyzing sequences of the GAPDH intron region, we identified 227 isolates of *Colletotrichum* associated with PFD in orchards of São Paulo state, 172 isolates were identified as *C. abscissum* and 55 as *C. gloeosporioides*. Morphological characters and multilocus sequencing confirmed species *C. abscissum* was the only species in the *C. acutatum* complex associated with PFD disease in Brazil. Although described as sensitive to benzimidazole-based fungicides, 20% of *C. gloeosporioides* isolates were found in regions with high use of this class of fungicide. Evaluated strains exhibited resistance to this fungicide in vitro. Because previously described primers differentiate between *C. acutatum* and *C. gloeosporioides* complexes, but not the particular species associated with PFD, we proposed and validated primers for a single multiplex PCR that specifically distinguished the *C. abscissum* and *C. gloeosporioides* sensu stricto.

Keywords *Colletotrichum abscissum* · *Colletotrichum gloeosporioides* · Postbloom fruit drop · Citrus · Identification · GAPDH · Multiplex PCR · Specific primers

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Introduction

The genus *Colletotrichum* comprises approximately 600 species of fungi that devastate many crop plants worldwide (O'Connell et al. 2012). Some are also asymptomatic endophytes (Lima et al. 2012). The most common symptoms caused by *Colletotrichum* species are the sunken necrotic lesions called anthracnose (Freeman et al. 1998). In citrus, besides the key lime anthracnose (KLA), another two important diseases are associated with species of *Colletotrichum*, postbloom fuit drop (PFD) and postharvest anthracnose (Lahey et al. 2004). The postbloom fruit drop is a condition that

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affects citrus crops at blossom stage, causing petal lesions and abnormal abscission of young fruits, resulting in persistent calyces (Fagan 1979). Species belonging to *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* species complexes have been commonly associated with such diseases in citrus plants.

Before the mid-20th century, many *Colletotrichum* species were described solely based on their host association (Johnston et al. 2005). Because the genus exhibits highly variable morphology and biology, many studies failed to clarify relationships within this genus because they examined substrate and mycelia or conidia morphology, without using DNA sequence analyses. Agostini et al. (1992) described three morphological types of *C. gloeosporioides* in Florida, United States, associated with citrus diseases: the slow-growing orange (SGO), the fast-growing grey (FGG), and the key lime anthracnose (KLA). From these, the SGO isolates were determined to be the direct cause of the PFD disease.

Based on internal transcribed spacer (ITS) sequence data, Brown et al. (1996) suggested the classification of the SGO and KLA isolates as *C. acutatum* and the FGG isolates as typical forms of *C. gloeosporioides.* Comparing ITS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences, Peres et al. (2008) recognized SGO and KLA of *C. acutatum* as two distinct phylogenetic strains. Using ITS, GAPDH, and GS sequences, MacKenzie et al. (2009) compared different isolates of *C. acutatum* sensu lato and grouped strains affecting the same hosts, and high pathogenicity was observed only to their hosts of origin. Other studies used multilocus analysis to propose the existence of infraspecific groups, leading to the assumption that these are species complexes, each containing a large number of constituent taxa (Lardner et al. 1999; Guerber et al. 2003; Sreenivasaprasad and Talhinhas 2005; Whitelaw-Weckert et al. 2007).

In light of taxonomic inconsistency in the *Colletotrichum* genus, Damm et al. (2012) and Weir et al. (2012) proposed a revision of the species *C. acutatum* and *C. gloeosporioides*, recognizing them as two complexes divided into 31 and 23 species, respectively. These studies used multilocus sequence analysis combined with morphological and cultural characters, including a great number of strains from different countries and hosts, but they did not include isolates of citrus plants from Brazil nor the SGO strain from USA associated with PFD in sweet orange (*Citrus sinensis*). In order to characterize these isolates, Pinho et al. (2015) proposed the new species, *Colletotrichum abscissum*, as the causal agent of PFD disease in Brazil, by analyzing three isolates from symptomatic flowers in São Paulo state, Brazil, and in Florida, by comparing ITS and GAPDH sequences of a PFD isolate previously identified as *C. acutatum* by Peres et al. (2008). Likewise, Bragança et al. (2016) grouped two PFD isolates from Peres et al. (2008) with the type strain *C. abscissum* COAD 1877 from Pinho et al. (2015) and also included a new strain isolated from *Psidium guajava*, which was found to be a new host for this species. Nevertheless, there is no information about *Colletotrichum abscissum* distribution and genetic variability in Brazil or whether only other species in the *C. acutatum* complex can be associated with PFD in Brazil.

Despite the causal agent of PFD being described as *C. abscissum*, the species *C. gloeosporioides* was again associated with PFD disease in Brazil by Lima et al. (2011) and in Bermuda by McGovern et al. (2012). However, there is a lack of an efficient method to identify these species, mainly because prior the description of *C. abscissum*, Lima et al. (2011) proposed the identification of *Colletotrichum* species associated with PFD in Brazil through species-specific PCR, but they used a combination of universal primers for *C. acutatum* and *C. gloeosporioides* species complexes.

In this study, we performed molecular and morphological characterization of the PFD-associated pathogens from different regions of São Paulo State, Brazil, and proposed species-specific PCR primers to distinguish the phytopathogens *C. abscissum* and *C. gloeosporioides* for a more reliable and accurate diagnosis.

Material and methods

Isolates

A total of 227 strains were isolated from flowers of *Citrus sinensis* exhibiting petal necrosis, located in different orchards in São Paulo State, Brazil (Table 1). Petals were surface-sterilized according to the protocol described by Petrini et al. (1992), fragmented, and incubated on potato dextrose agar medium (PDA) (Sigma-Aldrich) at 25 °C. Single monosporic isolates were obtained from each culture, then stored on PDA medium (Sigma-Aldrich) at 25 °C for further analysis. The isolates were deposited in the Culture Collection of

Table 1 *Colletotrichum* isolates investigated in this study

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
Colletotrichum abscissum	LGMF1257[a]						KM897676	KM897680	KM897674	KM651843	KM245576	KM897678
	LGMF1258[b]						KM897677	KM897681	KM897675	KM897682	KM897683	KM897679
	LGMF1268	Barretos	20°33′26″S 48°34′04″W	2008	Low	Pera	KX059038					
	LGMF1269						KX059039	KX059263	KX059276	KX059289	KX059302	KX059315
	LGMF1270						KX059040					
	LGMF1271						KX059041					
	LGMF1272						KX059042					
	LGMF1273						KX059043					
	LGMF1274						KX059044					
	LGMF1275						KX059045					
	LGMF1276						KX059046					
	LGMF1277						KX059047					
	LGMF1278						KX059048					
	LGMF1279						KX059049					
	LGMF1280						KX059050					
	LGMF1281						KX059051	KX059264	KX059277	KX059290	KX059303	KX059316
	LGMF1282						KX059052					
	LGMF1283						KX059053					
	LGMF1284						KX059054					
	LGMF1285						KX059055					
	LGMF1286						KX059056					
	LGMF1287	Pedranópolis	20°14′42.5″S 50°06′42″W	2008	Low	Pera	KX059057					
	LGMF1288						KX059058					
	LGMF1289						KX059059					
	LGMF1290						KX059060					
	LGMF1291						KX059061					
	LGMF1292						KX059062					
	LGMF1293						KX059063					
	LGMF1294	Gavião Peixoto	21°83′71″ S 48°49′61″ W	2008	Moderate	Pera	KX059064	KX059265	KX059278	KX059291	KX059304	KX059317
	LGMF1295						KX059065					
	LGMF1296						KX059066					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1298						KX059068					
	LGMF1299						KX059069					
	LGMF1300						KX059070					
	LGMF1301						KX059071					
	LGMF1302						KX059072					
	LGMF1303						KX059073					
	LGMF1304	Mogi Guaçu	22°18′23.9″S 46°55′51.4″W	2008	Moderate	Valência	KX059074					
	LGMF1305						KX059075					
	LGMF1306						KX059076					
	LGMF1307						KX059077	KX059266	KX059279	KX059292	KX059305	KX059318
	LGMF1308						KX059078					
	LGMF1309						KX059079					
	LGMF1310						KX059080	KX059267	KX059280	KX059293	KX059306	KX059319
	LGMF1311						KX059081					
	LGMF1312						KX059082					
	LGMF1313						KX059083					
	LGMF1314						KX059084					
	LGMF1315						KX059085					
	LGMF1316						KX059086					
	LGMF1317						KX059087					
	LGMF1318						KX059088					
	LGMF1319	Taquarituba	23°36′53.9″S 49°14′17.4″W	2008	High	Pera	KX059089					
	LGMF1320						KX059090					
	LGMF1321						KX059091					
	LGMF1322						KX059092					
	LGMF1323						KX059093					
	LGMF1324						KX059094					
	LGMF1325						KX059095					
	LGMF1326						KX059096					
	LGMF1327						KX059097					
	LGMF1328						KX059098					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1320						KX059100					
	LGMF1321						KX059101					
	LGMF1332	Santa Cruz do Rio Pardo	22°49′43.6″S 49°20′20.8″W	2008	High	Pera	KX059102	KX059268	KX059281	KX059294	KX059307	KX059320
	LGMF1333						KX059103					
	LGMF1334						KX059104					
	LGMF1335						KX059105					
	LGMF1336						KX059106					
	LGMF1337						KX059107					
	LGMF1338						KX059108					
	LGMF1339						KX059109					
	LGMF1340						KX059110					
	LGMF1341						KX059111					
	LGMF1342						KX059112					
	LGMF1343						KX059113					
	LGMF1344						KX059114					
	LGMF1345						KX059115					
	LGMF1346						KX059116					
	LGMF1347						KX059117					
	LGMF1348	Santa Cruz do Rio Pardo	22°49′33.7″S 49°22′01.4″W	2009	High	Valência	KX059118					
	LGMF1349						KX059119					
	LGMF1350						KX059120					
	LGMF1351						KX059121					
	LGMF1352						KX059122					
	LGMF1353						KX059123					
	LGMF1354						KX059124					
	LGMF1355						KX059125					
	LGMF1356						KX059126					
	LGMF1357						KX059127					
	LGMF1358						KX059128					
	LGMF1359						KX059129					
	LGMF1360						KX059130					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1362						KX059132					
	LGMF1363						KX059133					
	LGMF1364						KX059134					
	LGMF1365	Piracicaba	22°43′3.6″S 47°37′07″W	2011	Low	Valência	KX059135					
	LGMF1366						KX059136					
	LGMF1367						KX059137					
	LGMF1368						KX059138					
	LGMF1369						KX059139					
	LGMF1370						KX059140					
	LGMF1371						KX059141					
	LGMF1372	Américo Brasiliense	21°43′32.04″S 48°06′4.10″W	2011	Moderate	Natal	KX059142					
	LGMF1373						KX059143	KX059269	KX059282	KX059295	KX059308	KX059321
	LGMF1374						KX059144					
	LGMF1375						KX059145					
	LGMF1376						KX059146					
	LGMF1377	Engenheiro Coelho	22°28′35.4″S 47°12′54″W	2011	Moderate	Valência	KX059147					
	LGMF1378						KX059148					
	LGMF1379						KX059149					
	LGMF1380						KX059150	KX059270	KX059283	KX059296	KX059309	KX059322
	LGMF1381						KX059151					
	LGMF1382						KX059152					
	LGMF1383						KX059153					
	LGMF1384						KX059154					
	LGMF1385						KX059155					
	LGMF1386	Santa Cruz do Rio Pardo	22°49′19.4″S 49°20′59.3″W	2011	High	Pera	KX059156					
	LGMF1387						KX059157					
	LGMF1388						KX059158					
	LGMF1389						KX059159					
	LGMF1390						KX059160					
	LGMF1391						KX059161					
	LGMF1392						KX059162					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1394						KX059164	KX059271	KX059284	KX059297	KX059310	KX059323
	LGMF1395						KX059165					
	LGMF1396						KX059166					
	LGMF1397						KX059167					
	LGMF1398						KX059168					
	LGMF1399	Pardinho	23°05′2.76″S 48°22′37.39″W	2011	High	Valência	KX059169					
	LGMF1400						KX059170					
	LGMF1401						KX059171					
	LGMF1402						KX059172					
	LGMF1403						KX059173					
	LGMF1404						KX059174					
	LGMF1405						KX059175					
	LGMF1406						KX059176					
	LGMF1407						KX059177					
	LGMF1408						KX059178					
	LGMF1409						KX059179					
	LGMF1410						KX059180					
	LGMF1411						KX059181					
	LGMF1412						KX059182					
	LGMF1413						KX059183					
	LGMF1414						KX059184					
	LGMF1415						KX059185					
	LGMF1416						KX059186					
	LGMF1417						KX059187					
	LGMF1418						KX059188	KX059272	KX059285	KX059298	KX059311	KX059324
	LGMF1419						KX059189					
	LGMF1420						KX059190	KX059273	KX059286	KX059299	KX059312	KX059325
	LGMF1421	Santa Cruz do Rio Pardo	22°49′43.6″S 49°20′20.9″W	2011	High	Pera	KX059191	KX059274	KX059287	KX059300	KX059313	KX059326
	LGMF1422						KX059192					
	LGMF1423						KX059193					
	LGMF1424						KX059194					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1425						KX059195					
	LGMF1426						KX059196					
	LGMF1427						KX059197					
	LGMF1428						KX059198					
	LGMF1429						KX059199					
	LGMF1430						KX059200					
	LGMF1431						KX059201					
	LGMF1432						KX059202					
	LGMF1433						KX059203					
	LGMF1434						KX059204					
	LGMF1435						KX059205					
	LGMF1436						KX059206	KX059275	KX059288	KX059301	KX059314	KX059327
	LGMF1437						KX059207					
Colletotrichum gloeosporioides	LGMF1438	Santa Cruz do Rio Pardo	22°49′43.6″S 49°20′20.8″W	2008	High	Pera	KX059208					
	LGMF1439						KX059209					
	LGMF1440	Santa Cruz do Rio Pardo	22°49′33.7″S 49°22′01.4″W	2009	High	Valência	KX059210					
	LGMF1441	Piracicaba	22°43′3.6″S 47°37′07″W	2011	Low	Valência	KX059211					
	LGMF1442						KX059212					
	LGMF1443						KX059213					
	LGMF1444						KX059214					
	LGMF1445						KX059215					
	LGMF1446						KX059216					
	LGMF1447						KX059217					
	LGMF1448						KX059218					
	LGMF1449						KX059219					
	LGMF1450						KX059220					
	LGMF1451	Américo Brasiliense	21°43′32.04″S 48°06′4.10″W	2011	Moderate	Natal	KX059221					
	LGMF1452						KX059222					
	LGMF1453						KX059223					
	LGMF1454						KX059224					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1456						KX059226					
	LGMF1457						KX059227					
	LGMF1458						KX059228					
	LGMF1459						KX059229					
	LGMF1460						KX059230					
	LGMF1461						KX059231					
	LGMF1462						KX059232					
	LGMF1463						KX059233					
	LGMF1464						KX059234					
	LGMF1465						KX059235					
	LGMF1466						KX059236					
	LGMF1467						KX059237					
	LGMF1468						KX059238					
	LGMF1469						KX059239					
	LGMF1470						KX059240					
	LGMF1471						KX059241					
	LGMF1472						KX059242					
	LGMF1473						KX059243					
	LGMF1474						KX059244					
	LGMF1475						KX059245					
	LGMF1476						KX059246					
	LGMF1477						KX059247					
	LGMF1478	Engenheiro Coelho	22°28′35.4″S 47°12′54″W	2011	Moderate	Valência	KX059248					
	LGMF1479						KX059249					
	LGMF1480						KX059250					
	LGMF1481						KX059251					
	LGMF1482						KX059252					
	LGMF1483						KX059253					
	LGMF1484						KX059254					
	LGMF1485	Santa Cruz do Rio Pardo	22°49′19.4″S 49°20′59.3″W	2011	High	Pera	KX059255					
	LGMF1486						KX059256					

Table 1 (continued)

Species	Strain number	County of origin	Coordinate	Year of collection	Fungicide use	Orange variety	GenBank access number					
							GAPDH	ACT	CHS-1	HIS3	ITS	TUB2
	LGMF1487						KX059257					
	LGMF1488						KX059258					
	LGMF1489						KX059259					
	LGMF1490	Santa Cruz do Rio Pardo	22°49'43.6"S 49°20'20.9"W	2011	High	Pera	KX059260					
	LGMF1491						KX059261					
	LGMF1492						KX059262					

[a] The isolate LGMF1257 is the strain Ca142, obtained from the Fundecitrus Fungi Collection

[b] The isolate LGMF1258 is the strain IAC42, obtained from the APTA Citrus Culture Collection, and used for Morphological Characterization

Laboratory of Genetic of Microorganism (Federal University of Paraná/Brazil, http://www.labgem.ufpr.br). The isolates LGMF1257 and LGMF1258, previously isolated from *Citrus sinensis* and shown to cause PFD, were also included in this study (Kupper et al. 2003; de Menezes et al. 2014; Marques et al. 2014).

Morphological study

A morphological analysis was performed with the isolate LGMF1258 collected in Engenheiro Coelho City, São Paulo State, Brazil (22° 28 40. 59 S, 47° 10 57. 43 W) to compare its morphological characteristics with the type strain of *C. abscissum* described by Pinho et al. (2015), which was suspected as the causative agent of PFD in Brazil. Cultures were grown for 5 days on PDA plates at 25 °C, and then, 5-mm diameter discs were plated on fresh PDA plates. The culture diameter and appearance were analyzed in triplicate after 10 d growth at 25 °C in a 12 h light/12 h dark photoperiod. The hyphae and apressorium were evaluated using the slide culture technique (Johnston and Jones 1997) after 5 days and the characteristic of 50 appressoria were recorded via Zeiss Axioskop 2 microscopy with the digital camera MRc 3 (Germany) and Axiovision software for Zeiss.

Molecular analysis

DNA extraction

Genomic DNA was extracted from mycelium grown on cellophane membrane over PDA medium for three days at 28 °C. Mycelium was harvested and ground in liquid nitrogen, then submitted to the DNA extraction protocol of Raeder and Broda (1985). The DNA was resuspended in 50 μL of Tris–HCl, pH 7.6, 10 mM, and treated with 0.5 μL RNAse (20 mg mL^{-1}). The quality and concentration of DNA was evaluated by 1% agarose gel electrophoresis, stained with GelRed (Life Technologies, USA) and compared under UV light with a Lambda DNA/Hind III marker (Invitrogen) of known concentration.

GAPDH sequencing

The 200-bp intron of GAPDH was selected for species identification because this gene was the most informative for species recognition of *C. gloeosporioides* and

C. acutatum complex (Damm et al. 2012). This region was amplified and sequenced for all 227 isolates, using primers GDF (5′-GCCGTCAACGACCCCTTCATTGA-3′) and GDR (5′-GGGTGGAGT CGTACTTGAGCATGT-3′) (Templeton et al. 1992).

PCRs were performed in a total volume of 12.5 μL, using the Top Taq PCR Master Mix (Qiagen), following the manufacturer's instructions. Conditions for PCR constituted an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C, and a final denaturation step of 7 min at 72 °C.

Amplicons were purified using FastAp and Exo1 enzymes (ThermoScientific), and sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The sequencing products were purified using the polymer Sephadex G-50 medium (GE Healthcare) in a Multiscreen Column Loader (Millipore). Next, DNA sequences were obtained on an ABI Prism 3500 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

DNA sequences generated with forward and reverse primers were used to obtain consensus sequences using Bioedit v.7.2.5 (Hall 1999), and then compared with available sequences in the GenBank database of NCBI, using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on this analysis, the strains were characterized as belonging to the *C. gloeosporioides* or *C. acutatum* complex, and sequences of type strains included in each complex were selected for the phylogenetic analysis. A multiple sequence alignment was obtained with ClustalW (Thompson et al. 1994) and manually adjusted with Mega v.6 (Tamura et al. 2013). The species *C. nymphaeae* and *C. boninense* were used as outgroups for the *C. abscissum* and *C. gloeosporioides* phylogenetic analysis, respectively.

Bayesian Inference was computed with MrBayes 3.2.1 (Ronquist et al. 2012), using the permutations needed to reach split frequencies of less than or equal to 0.01 and discarding the first 25% generated trees. The HKY + G model was selected and incorporated in the Bayesian analysis, the same model used by Damm et al. (2012) and Weir et al. (2012). GAPDH sequences were deposited in GenBank.

Multilocus sequence analysis

Fifteen *C. abscissum* strains from different regions were selected to perform a multilocus analysis to confirm the ability of the GAPDH region to identify species, as well as to verify the intraspecific variability in *C. abscissum* isolates. The multilocus analysis was performed using the genome regions proposed by Damm et al. (2012) to resolve the *C. acutatum* complex. These regions were: the 5.8S nuclear ribosomal gene with two flanking ITSs, the 200-bp intron of GAPDH, a partial sequence of chitin synthase 1 (CHS-1), histone 3 (HIS3), actin (ACT), and beta-tubulin (TUB2). The PCRs were performed as described by Damm et al. (2012) for all regions except ITS, which followed the conditions described by Woudenberg et al. (2009). The primers used in this study are described in Table 2. Bayesian Inference was computed as previously described, incorporating the K2 model for analysis. The sequences obtained were deposited in GenBank.

Fungicide sensitivity of *C. gloeosporioides*

Because of the isolation of *C. gloeosporioides* from regions with high fungicide application, we selected 15 isolates to perform the fungicide sensitivity evaluation. This species is commonly known for being inhibited by low concentrations of benzimidazole-based fungicides (Peres et al. 2004), and our goal was to investigate the sensitivity of isolates derived from regions with different levels of fungicide use. For this, 5-mm diameter PDA disks of mycelium were transferred to PDA media supplemented with carbendazim at 0.1 $\mu g\ mL^{-1}$ and a control without fungicide. The experiment was performed with three repetitions, incubated at 25 °C in the dark for 7 days, at which time the diameter of colonies was measured and compared with that of the control plates.

Primers for species-specific PCR

To design specific primers for both species associated with PFD in Brazil, the GAPDH sequences of *C. abscissum* and *C. gloeosporioides* were aligned with sequences of type strains from the *C. acutatum* and *C. gloeosporioides* complexes using the software Mega v.6 (Tamura et al. 2013). Region of 17–20 bp specific to each species, with no homology to other species of *Colletotrichum*, were selected and used to design the primers. The primers CaF (5′-GACTAGCACTCTCGACTTTG-3′) and CaR (5′-GTCAGCAAGTTTTGTCTCG-3′) were design to identify *C. abscissum*, whereas the primers CgF (5′-GTGAGTATCACCCCACC-3′) and CgR (5′-AAATGTGACAGATGGGGC-3′)

Table 2 Primers used in this study for PCR and sequencing

Gene	Product	Primer	Direction	Sequence (5′–3′)	Reference
ACT	Actin	ACT-512 F	Forward	ATG TGC AAG GCC GGT TTC GC	Carbone and Kohn (1999)
		ACT-783R	Reverse	TAC GAGTCC TTC TGG CCC AT	Carbone and Kohn (1999)
CHS-1	Chitin synthase	CHS-79 F	Forward	TGG GGC AAG GAT GCT TGG AAG AAG	Carbone and Kohn (1999)
		CHS-345R	Reverse	TGG AAG AAC CAT CTG TGA GAG TTG	Carbone and Kohn (1999)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GDF	Forward	GCC GTC AAC GAC CCC TTC ATT GA	Templeton et al. (1992)
		GDR	Reverse	GGG TGG AGT CGT ACT TGA GCA TGT	Templeton et al. (1992)
HIS3	Histone 3	CYLH3F	Forward	AGG TCC ACT GGT GGC AAG	Crous et al. (2004)
		CYLH3R	Reverse	AGC TGG ATG TCC TTG GAC TG	Crous et al. (2004)
ITS	Internal transcribed spacer	ITS-1	Forward	CTT GGT CAT TTA GAG GAA GTA A	Gardes and Bruns (1993)
		V9G	Forward	TTA CGT CCC TGC CCT TTG TA	de Hoog and Gerrits van den Ende (1998)
		ITS-4	Reverse	TCC TCC GCT TAT TGA TAT GC	White et al. (1990)
TUB2	β-tubulin	T1	Forward	AAC ATG CGT GAG ATT GTA AGT	Woudenberg et al. (2009)
		Bt2b	Reverse	ACC CTC AGT GTA GTG ACC CTT GGC	Woudenberg et al. (2009)

were designed to identify *C. gloeosporioides* sensu stricto. The propensity of primers to make homo or hetero duplexes was analyzed by the OligoIDT Analyzer (https://www.idtdna.com/calc/analyzer). Designed primers were also screened against nucleotide database in GenBank by BLASTN (Altschul et al. 1997) for similarity with *Colletotrichum* species closely related to *C. abscissum* and *C. gloeosporioides*. The annealing temperature was calculated with the NEB Tm Calculator (http://tmcalculator.neb.com).

The multiplex PCR contained the two primer pairs designed to produce amplicons sufficiently different in size and migration rate to identify both species. The reaction was performed using the Top Taq PCR Master Mix (Qiagen), following the manufacturer's instructions. Reaction conditions were 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C, and a final extension of 7 min at 72 °C. The PCR products were electrophoresed in a 1% agarose gel stained with GelRed (Life Technologies, USA) and compared under UV light with the 100 bp DNA Ladder (Invitrogen).

Results

Morphological analysis

The morphology of the isolate LGMF1258 exhibited the same characters described by Pinho et al. (2015) for *C. abscissum*. Both asexual structures and culture characteristics agreed with the type strain description. The complete description is detailed in the supplementary data (Fig. S1).

Molecular analysis

DNA sequences of the GAPDH region obtained for the 227 isolates in this study produced an alignment of 246 nucleotides including gaps. By comparison to available data in the GenBank database, the sequences obtained were split into two groups, one with similarity to species in the *C. acutatum* complex Clade 1 (Damm et al. 2012), and the other with similarity to species in the *C. gloeosporioides* complex. Two alignments were then performed, including sequences downloaded from GenBank, in order to generate trees with the two sets of sequences obtained in this study. The Bayesian inference tree for Clade 1 of the *C. acutatum* complex (Fig. 1) showed a single branch group of isolates (LGMF1257, LGMF1258, and LGMF1268-LGMF1437) from this study with the type strain of *C. abscissum* COAD 1877 described by Pinho et al. (2015), having a Bayesian posterior probability value of 0.99 and 65% of the bootstrap for the ML tree (Fig. 1 and Sup. Inf. Fig. S4). The phylogenetic tree for the *C. gloeosporioides* complex grouped isolates LGMF1438 to LGMF1492 to the type strain *C. gloeosporioides* sensu stricto IMI 356878, having a

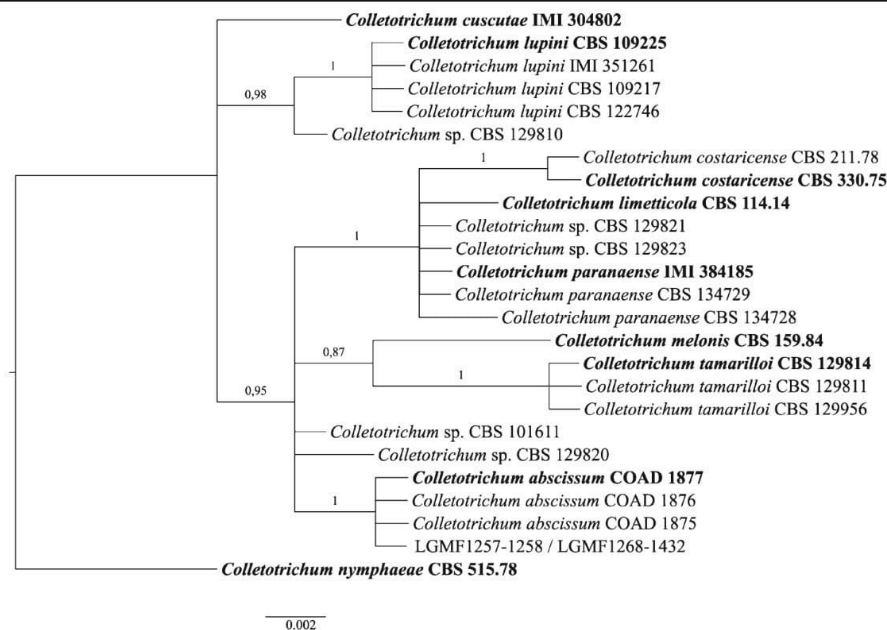

Fig. 1 Bayesian inference tree based on GAPDH sequences of 172 isolates of this study and species from Clade 1 of the *Colletotrichum acutatum* species complex (Damm et al. 2012; Bragança et al. 2016). Values on the nodes indicate the Bayesian posterior probability. The species *Colletotrichum nymphaeae* is used as outgroup. Type material sequences are indicated in bold

Bayesian posterior probability support value of 1.00 and 100% of the bootstrap value for the ML tree (Fig. 2 and Sup. Inf. Fig. S5). This analysis revealed a subgroup of 16 isolates (LGMF1439, LGMF1442, LGMF1446, LGMF1448-1449, LGMF1452, LGMF1457, LGMF1459, LGMF1460, LGMF1463, LGMF1468, LGMF1474, and LGMF1487-1490) that were represented by a transversion from T to A nucleotides. The other 39 *C. gloeosporioides* isolates (LGMF1438, LGMF1440-1441, LGMF1443-1445, LGMF1447, LGMF1450-1451, LGMF1453-1456, LGMF1458, LGMF1461-1462, LGMF1464-1467, LGMF1469-1473, LGMF1475-1486, and LGMF1491-1492) were 100% similar to the *C. gloeosporioides* type strain IMI 356878.

The concatenated alignment of six loci for 15 isolates of *C. abscissum* and related species from Clade 1 of the *C. acutatum* complex (Damm et al. 2012), including the outgroup *C. nymphaeae* CBS 515.78, produced 1,879 characters (including gaps), of which 127 were phylogenetically informative. The consensus tree obtained for the Bayesian analysis had the same tree topology obtained by Damm et al. (2012) and Pinho et al. (2015). Multilocus analysis did not reveal intraspecific variability among the 20 isolates investigated in this study, wherein sequences showed 100% similarity for the six genes (Sup. Inf. Fig. S2).

Fungicide sensitivity of *C. gloeosporioides*

Different isolates of *C. gloeosporioides* showed a variable response to carbendazim fungicide in vitro (Fig. 3). Mean values of colony diameter ranged from 19.5 to 49.5 mm, which represented a growth of 33 to 96%, respectively, compared to the control plates without fungicide. In addition, the highest growth rates were observed in isolates from areas with high fungicide use. Isolates were considered resistant when the colony diameter was greater than 50% of that of the control. Concentrations of 0.1 $\mu g\ mL^{-1}$ of benzimidazole-based fungicides have been previously characterized as highly inhibitory to *C. gloeosporioides* sensitive isolates (Peres et al. 2004).

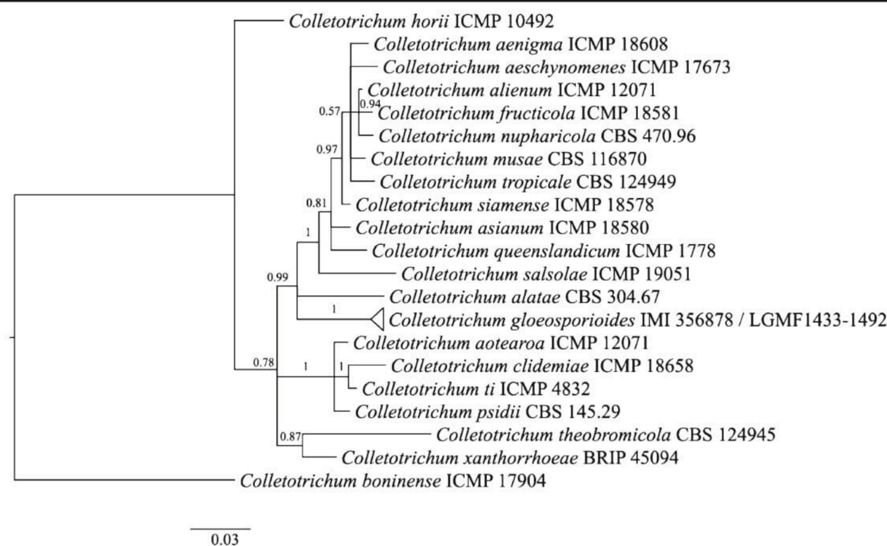

Fig. 2 Bayesian inference tree based on GAPDH sequences of 55 isolates from PFD symptoms in sweet orange, and the available sequences from the *Colletotrichum gloeosporioides* species complex (Weir et al. 2012). The tree was rooted to sequence of *Colletotrichum boninense* (CBS 123755). All sequences used in the alignment are from type material

Primers for species-specific PCR

The primer sets CaF/CaR and CgF/CgR developed in this study did not show similarity to the GAPDH region from other species of the *C. acutatum* and *C. gloeosporioides* complexes. Primers also did not show propensity to make homo or hetero duplexes. For multiplex PCR, both primer sets were combined in a single tube and the obtained band sizes were of 100 bp for *C. abscissum* and 190 bp for *C. gloeosporioides* (Fig. 4). All tested isolates were successfully amplified and the validation of PCR multiplex was performed comparing the isolates amplified to sequencing data (Figs. 1, 2 and 3).

Discussion

Postbloom fruit drop is a citrus disease that affects flowers of sweet orange and most other citrus plants. The symptoms include petal necrosis, abscission of developing fruits, and in Brazil, the PFD is associated with large economic losses (de Goes et al. 2008; de Menezes et al. 2014). In view of the economic importance of PFD in Brazil, better management control is necessary, which can be achieved by a precise identification of the pathogenic species and their distribution along the primary citrus-producing regions. Identification of *Colletotrichum* species are performed through multilocus sequencing, which is time and cost consuming, and requires knowledge concerning phylogenetic analysis. Furthermore, diversity within and among species might also influence disease severity, the ability to overcome the resistance of cultivars, and treatment measures (MacKenzie et al. 2006).

In this study, we evaluated 227 strains from different citrus orchards in São Paulo State (Brazil), in which the PFD is present, including regions with different rates of fungicide application, to characterize the species associated with this disease in Brazil. The PFD has been previously associated with *C. acutatum* (Lahey et al. 2004); however, in 2015, Pinho et al. suggested the new species *C. abscissum* as the causal agent of PFD in Brazil, although the authors only analyzed three isolates. Therefore, we proposed an epidemiologic study with a larger number of isolates recovered from symptomatic flowers, in an attempt to analyze the distribution of the pathogens throughout São Paulo State, Brazil.

The *Colletotrichum* spp. isolates were identified through GAPDH sequence analysis, and we found the presence of *C. abscissum* and *C. gloeosporioides* with a frequency of 76% (172 isolates) and 24% (55 isolates), respectively. The morphological and cultural characters of the *C. abscissum* LGMF1258 isolate analyzed were

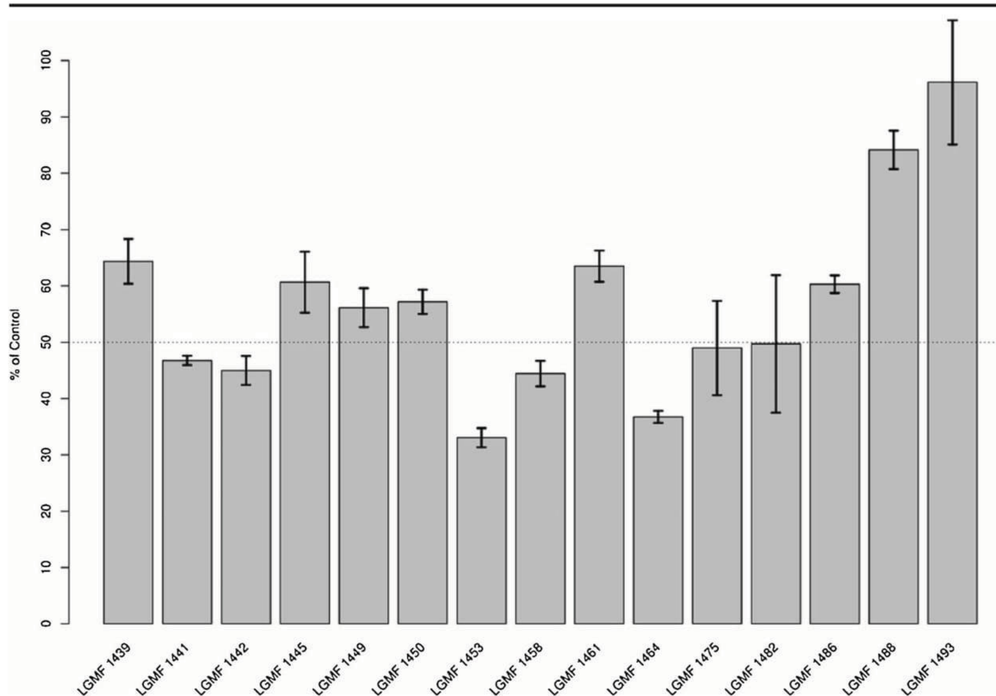

Fig. 3 Effect of carbendazim on the colony area of different isolates of *Colletotrichum gloeosporioides* in potato dextrose agar amended with 0.1 μg mL^{-1} of carbendazim

highly similar to the type strain COAD 1877 described by Pinho et al. (2015).

GAPDH sequences from 172 isolates showed 100% similarity to the *C.abscissum* type strain. The Bayesian inference phylogenetic analysis for this gene revealed most branches were well supported by high probability values, and exhibited the same topology shown by Damm et al. (2012) for species in the *C. acutatum* complex and the species *C. citri* (Huang et al. 2013). We also performed a multilocus sequence analysis with 15 isolates to confirm the resolution of GAPDH sequences and to verify the existence of intraspecific variability among *C. abscissum* isolates (Sup. Inf. Fig. S2). Wherein, the isolates had 100% of nucleotide identity in all sequences analyzed, and the multilocus sequence tree topology maintained similarity to the GAPDH analysis, with congruence in most branches (Fig. 1 and Sup. Inf. Fig. S2). This result suggests the

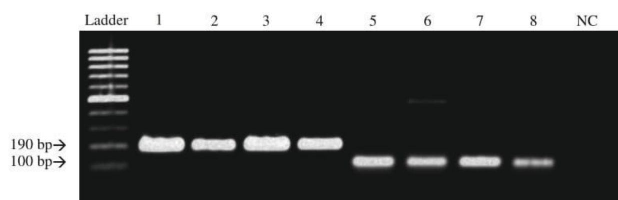

Fig. 4 Multiplex PCR amplification of a specific fragment from *Colletotrichum gloeosporioides* (190 bp) and *Colletotrichum abscissum* (100 bp). Agarose gel (1%) stained with GelRed™ and visualized under UV light. Samples 1 to 8 represent the isolates LGMF1463, LGMF1467, LGMF1469, LGMF1447, LGMF1303, LGMF1292, LGMF1293, and LGMF1353, respectively. NC represents the negative control without DNA. The marker is Ladder 100 bp (Ludwig Biotec™)

GAPDH intron as an effective marker for species identification in the *C. acutatum* complex belonging to Clade 1 (Damm et al. 2012).

The remaining 55 isolates were clustered with the *C. gloeosporioides* type strain IMI 356878 in the GAPDH phylogenetic analysis (Fig. 2). Of these, 39 showed 100% similarity to the type strain and 16 isolates exhibited a single transversion (T to A substitution) (Sup. Inf. Fig. S3). However, this substitution occurred in an intron region – in which a nucleotide changes might not represent modifications in morphology or physiological behavior – and there was no correlation between this transversion with the host, year, region, or fungicide application (Table 1). As in the *C. acutatum* complex tree, the GAPDH tree for *C. gloeosporioides* complex likewise showed a good resolution, high posterior probability values and ability to discriminate species, with similar topology to the multilocus phylogenetic analysis performed by Weir et al. (2012).

The multilocus analysis revealed no genetic variability within isolates of *C. abscissum* and also within isolates of *C. gloeosporioides*, showing a clonal population on both species in the area studied, even though the isolates were obtained from three different cultivars of *C. sinensis* (Table 1). We suggest that the absence of intraspecific variability observed could be explained by the establishment of a single genotype spread through the citrus producer regions. However, the host specificity of *C. abscissum* cannot be assumed, since the species was recently described and we analyzed only isolates from PFD symptoms in sweet orange. Further studies are necessary to clarify this relationship, including data from populations affecting different hosts as well as studies on the interaction and pathogenic effects (Cannon et al. 2012). Nonetheless *C. abscissum* was previously isolated from fruits of *Psidium guajava* with anthracnose symptoms, and showed the potential to cause PFD symptoms in citrus flowers (Bragança et al. 2016), what may suggest the absence of host specificity for this species. The lack of host specificity was also observed for species like *Colletotrichum nymphaeae*, *C. fiorinae* and *C. godetiae*, which are associated to the anthracnose of strawberry (Baroncelli et al. 2015) but also can be found causing anthracnose in different other hosts like olive (Talhinhas et al. 2015). Unlike what we observed, some studies have demonstrated host specificity of *Colletotrichum* species infecting fruits, as observed for *Colletotrichum tamarilloi* which was only found associated to the anthracnose pathosystem of tamarillo (Afanador-Kafuri et al. 2003) as well as *Colletotrichum lupini*, which exhibits preference for its lupin host (Talhilhas et al. 2016).

The percentage of *C. gloeosporioides* isolated from flower lesions in our study (24%) was higher than that observed by Lima et al. (2011), who found 17.3% of *C. gloeosporioides* in São Paulo orchards. Nevertheless, 83% of Lima et al. (2011) isolates derived from one variety of acid lime (*Citrus latifolia*) cultivated in a region with no fungicide application. Our study found 24% of *C. gloeosporioides* from *Citrus sinensis*, all isolates derived from regions with fungicide use, with percentages of 18.2, 61.8, and 20% from regions with low, moderate, and high fungicide application, respectively. We tested the carbendazim sensitivity from 15 of these *C. gloeosporioides* isolates, and colony growth ranged from 33 to 96% of that of the control plate (no fungicide) at 0.1 $\mu g\ mL^{-1}$ of carbendazim. The highest growth rates were observed for isolates from regions with high fungicide use. As expected most part of them (10 isolates) grew to more than 50% of that of the control plate, characterizing these strains as resistant to this fungicide. Peres et al. (2004) found that the concentration of 0.1 $\mu g\ mL^{-1}$ can reduce the growth of benomyl-sensitive *C. gloeosporioides* to 8% of the control, whereas benomyl-resistant isolates are unaffected by concentrations lower than 10 $\mu g\ mL^{-1}$. The observed resistance of *C. gloeosporioides* can justify the increased isolation rate from *C. sinensis*, wherein *C. gloeosporioides* species may be contributing to the exacerbation of the PFD disease.

As both species, *C. abscissum* and *C. gloeosporioides*, are associated with PFD disease in Brazil, a rapid and robust method to differentiate them is required. A technique broadly used for species identification is multiplex PCR (Potrykus et al. 2014; Chen et al. 2015; Cho et al. 2016). Species-specific PCR was previously used for *C. acutatum* and *C. gloeosporioides* identification (Lima et al. 2011); however, the primers described amplified the species belonging to the complex and not the specific species associated with the PFD (*C. abscissum* and *C. gloeosporioides*). As such, we designed new primers to identify these species and to be used in a multiplex PCR. Ninety-one isolates were evaluated and the multiplex PCR indicated 100% congruence with the molecular analysis, confirming this protocol as a reliable tool to discriminate the species *C. abscissum* and *C. gloeosporioides* isolated from citrus flower lesions.

In this study, we identified through molecular analysis – GAPDH sequence – the species associated with PFD in São Paulo State, the highest citrus producing region in

Brazil. The predominant species associated with PFD was the recently described species *C. abscissum*; however, the presence of *C. gloeosporioides* was higher than observed in a previous study (Lima et al. 2011). This finding may be associated with the increase of resistance to the fungicide carbendazim observed in vitro and by the isolation of these strains from orchards with low to high fungicide application. A multilocus analysis was performed for 15 isolates using six concatenated genes, wherein the topology was similar to the GAPDH analysis, suggesting that this region is a reliable source for identification of species belonging to the *C. gloeosporioides* and *C. acutatum* complexes. We also proposed species-specific primers for multiplex PCR to easily identify the species *C. abscissum* and *C. gloeosporioides*, which can be used in species identification and epidemiological studies.

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Supplementary Information

Identification of *Colletotrichum* species associated with Postbloom Fruit Drop in Brazil through GAPDH sequencing analysis and Multiplex PCR

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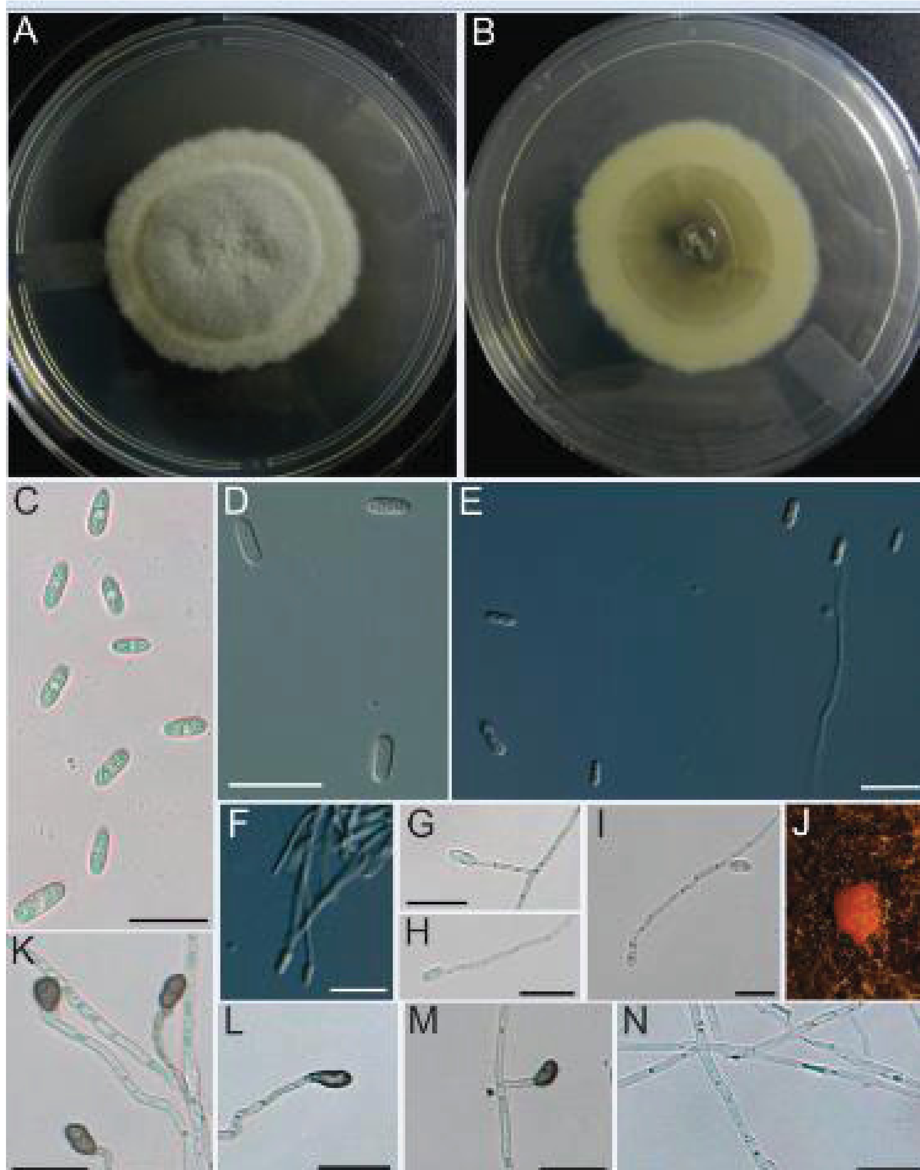
Morphological analysis of strain LGMF1258 – *Colletotrichum abscissum*

Asexual morph on PDA: The vegetative hyphae in PDA medium has 2-5 μm diameter, hyaline, smooth-walled, septate, branched. Conidiophores formed directly on vegetative hyphae. Setae not observed. Conidiophores hyaline, smooth-walled, mostly simple, sometimes septate and branched, to 25 μm long. Conidiogenous cells hyaline, smooth-walled, cylindrical to slightly inflated. Conidia hyaline, smooth-walled, aseptate, straight, cylindrical and rounded ends, (6,5 -) 8,3 – 11,2 (- 12,5) x (2,3 -) 2,6 – 3,7 (- 4,6) um, mean +- SD = 9,7 +- 1,5 x 3,2 +- 0,5 μm, L/W ratio = 3,1. Appressoria solitary, medium brown, smooth-walled, ellipsoidal to obovate, entire edge, sometimes undulate, (7 –) 7,9 – 10,7 (– 13) x (4 –) 4,3 – 5,9 (– 7) um, mean +- SD = 9,3 +- 1,4 x 5,1 +- 0,8 um, L/W ratio = 1,82.

Culture characteristics: Colonies on PDA flat with entire margin; surface buff, rosy buff, salmon to peach due to sporulation, with olivaceous sectors in the centre, partly covered by white floccose aerial mycelium, reverse rosy buff to flesh, smoke grey to olivaceous grey in the centre; growth rate 33 – 35 mm in 7 d (53.5 – 55.5 mm in 10 d). Conidia in mass saffron to orange



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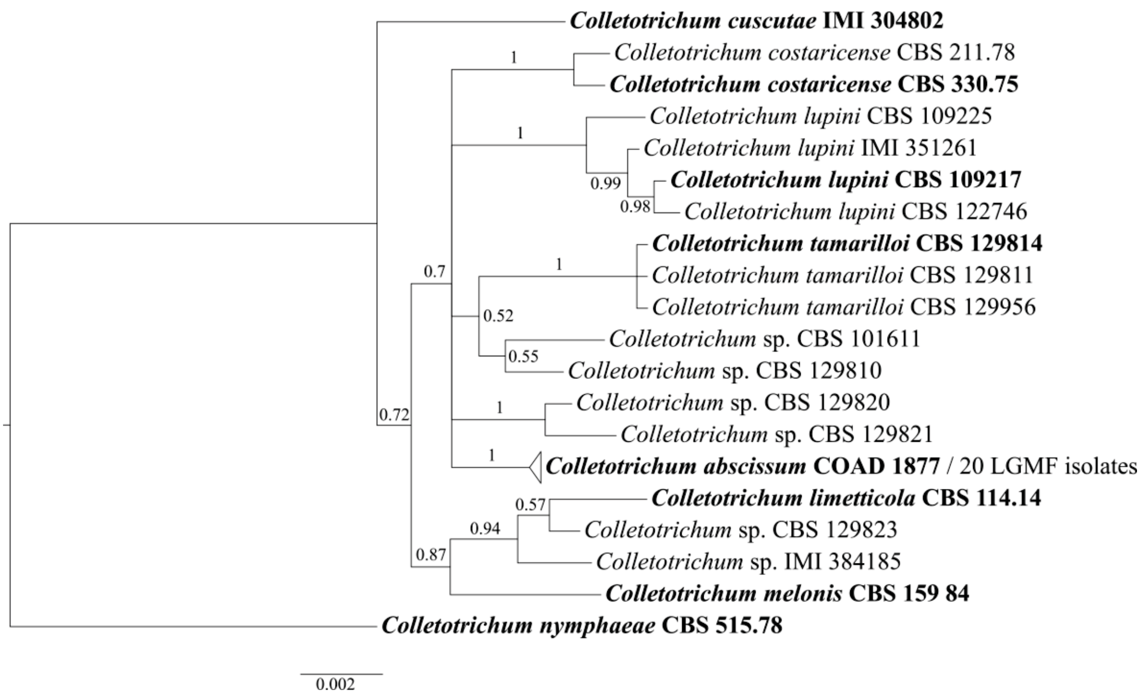


Figure S2 – Bayesian Inference tree using ITS, GAPDH, CHS-1, HIS3, ACT and TUB2 concatenated sequences. Values on the nodes indicate Bayesian posterior probability values. The species *Colletotrichum nymphaeae* was used as outgroup. Type material are indicated in bold.

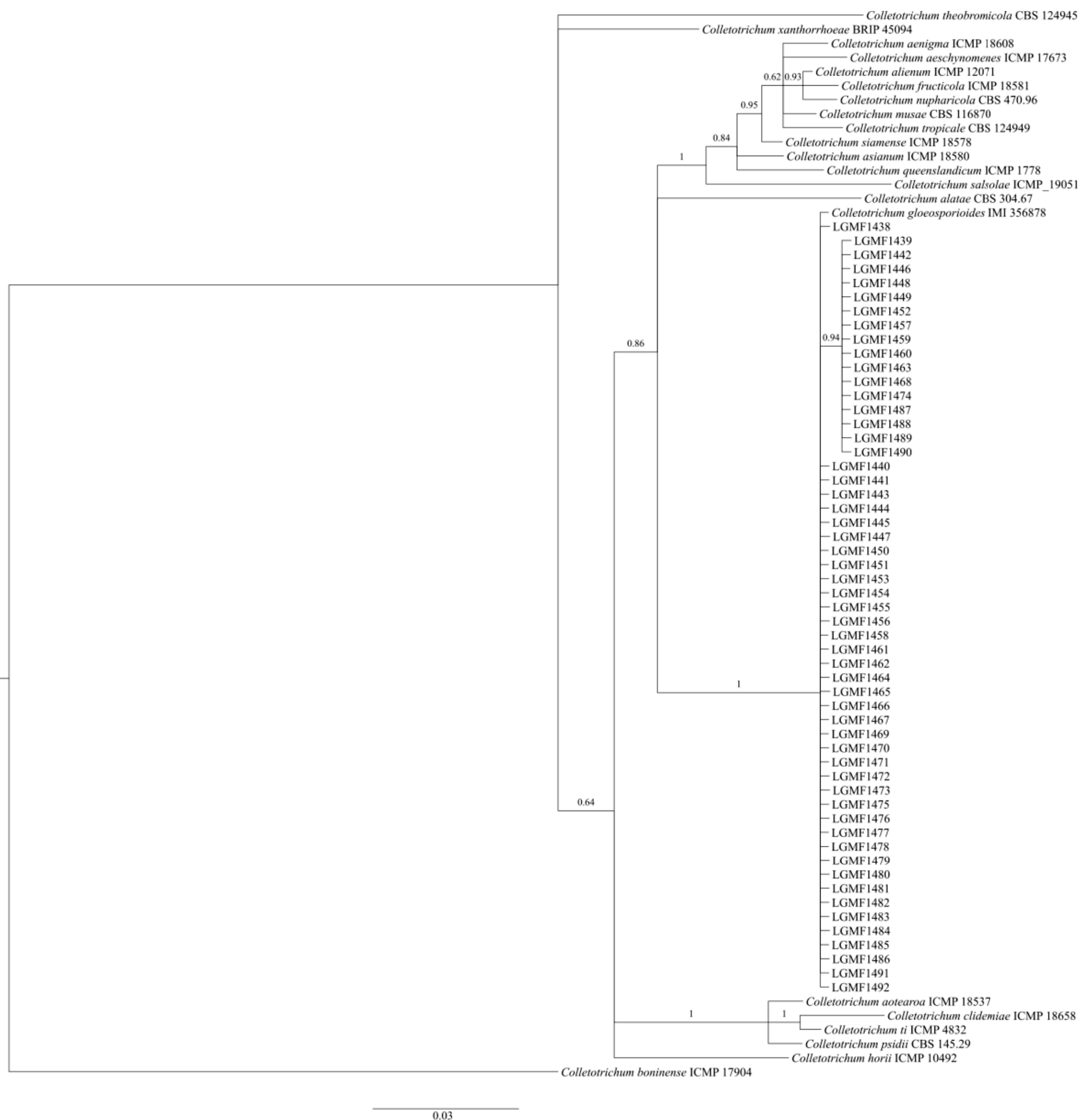
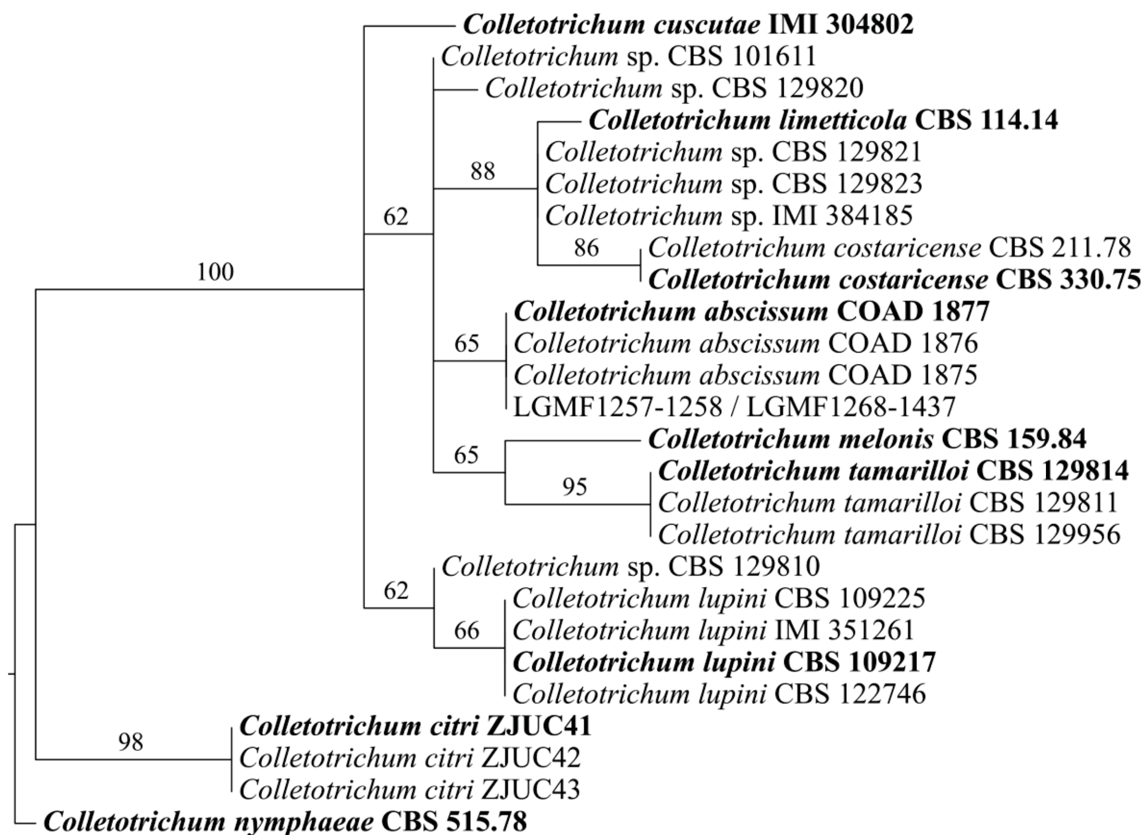


Figure S3 – Bayesian Inference tree based on GAPDH sequences of 55 isolates of this study and species from *Colletotrichum gloeosporioides* species complex (Weir *et al*., 2012). Values on the nodes indicate Bayesian posterior probability values. The species *Colletotrichum boninense* was used as outgroup. All sequences downloaded from GenBank are from type material.



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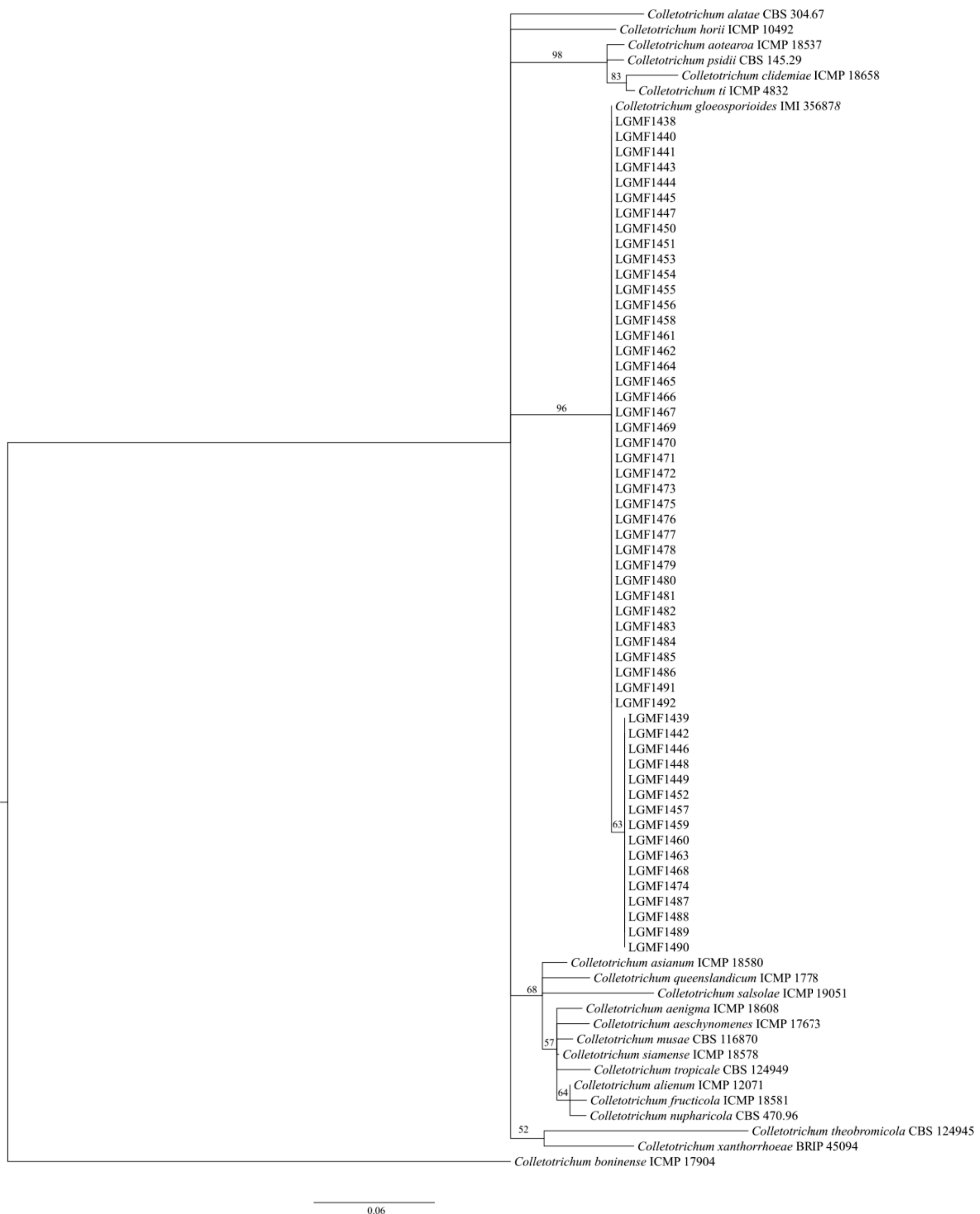

Figure S5 – Maximum Likelihood tree based on GAPDH sequences of 55 isolates from this study and species from *Colletotrichum gloeosporioides* species complex (Weir *et al*., 2012). Values on the nodes indicate bootstrap values. The species *Colletotrichum boninense* was used as outgroup. All sequences downloaded from GenBank are from type material.

5. CAPÍTULO II

The serine-threonine protein kinase Snf1 is required for full virulence of the maize pathogen *Colletotrichum graminicola*

Alternative: Cell wall-degrading enzymes support penetration of melanized appressoria of the maize pathogen *Colletotrichum graminicola* and colonization of the host tissue

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Abstract

Colletotrichum graminicola causes leaf anthracnose and stalk rot in maize, leading to severe yield losses in maize-growing regions world-wide. The pathogen differentiates an infection cell called an appressorium in order to invade the epidermal maize cell in a turgor pressure-driven fashion, possibly supported by secreted cell wall-degrading enzymes (CWDEs). After establishment within the first epidermal cell, biotrophic and necrotrophic hyphae successively form to colonize the host tissue. CWDEs have been associated with virulence in different filamentous fungi. However, due to their enormous number and functional redundancy, targeted deletion of genes encoding CWDEs rarely caused a virulence phenotype, and their role of these enzymes at different stages of the fungal infection process remains poorly understood. As an alternative, targeted deletion of genes simultaneously controlling the expression of many genes

encoding CWDEs may represent an alternative to improve understanding the function of these enzymes during fungal pathogenesis. The *SNF1* gene, identified in Sucrose Non-Fermenting yeast mutants, encodes a serine-threonine protein kinase required for de-repression of genes subject to catabolite repression, including many genes encoding CWDEs. *SNF1* is highly conserved among species, allowing to investigate the role of CWDEs in fungal plant pathogenesis. In this study we identified and functionally characterized the *SNF1* homolog of *C. graminicola* (*CgSNF1*). Δ*snf1* mutants showed reduced vegetative growth rates on polymeric carbon sources and reduced sporulation on rich media such as oat meal agar. Microscopic observations revealed a clear delay in turgor-driven penetration of cuticle and cell wall, spread through maize leaves and occurrence of anthracnose disease symptom. The data presented indicate that *SNF1* of *C. graminicola* is required for expression of CWDEs and identifies *SNF1* as a novel virulence factor.

Keywords: *Colletotrichum graminicola*, maize, *SNF1*, CWDEs, appressorial penetration, de-repression of catabolite-repressed genes, anthracnose disease.

Introduction

The plant cell wall is a complex structure primarily composed of polymeric polysaccharides such as cellulose, hemicellulose and pectin, associated with proteins and lignin to determine shape of the cell, confer rigidity and to protect it against adverse environmental factors such as plant pathogens (Aro et al., 2004). In order to invade its host and to spread throughout their host tissues, a fungal plant pathogen must overcome a set of complex plant cell wall structures. Fungal plant pathogens use different strategies

to penetrate their host, such as employing melanized appressoria for turgor-driven penetration of the first cell, but also produce extracellular enzymes that can degrade cell wall polymers (Tonukari et al. 2000).

Hydrolytic enzymes are thought to be important in pathogenesis in various fungal species (Kubicek et al., 2014). However, considering the various fungal lifestyles, mode of infection and functional redundancy, it has been difficult to characterize the role of single/individual CWDEs in pathogenesis (Tzima et al., 2011). The expression of many CWDEs in most fungi is subject to a process called carbon catabolite repression (Ruijter and Visser 1997). In yeasts, the gene *SNF1* is considered a key regulator of the glucose repression pathway, by encoding the protein kinase Snf1p, necessary for the expression of glucose-repressed genes when glucose is limited (Celenza & Carlson, 1986). The gene *MIG1* encodes the promoter-binding protein Mig1p. Mig1-binding sites exist in several genes encoding CWDEs, and Mig1-binding to the promoters thus mediates transcriptional repressor of these genes. The gene *SNF1* encodes the serine-threonine protein kinase Snf1, which is required to de-repression of catabolite-repressed genes. Snf1 is activated by auto-phosphorylation, and subsequently phosphorylates Mig1p, leading to a conformational change and release of the promoter of Mig1-controlled genes (Treitel et al. 1998).

Importantly, orthologs of *SNF1* have been identified in several filamentous fungi, including plant pathogens such as *Cochliobolus carbonum* (Tonukari et al. 2000), *Fusarium oxysporum* (Ospina-Giraldo et al. 2003), *Magnaporthe oryzae* (Yi et al. 2008), *Verticillium dahlia* (Tzima et al. 2011), *Penicillium digitatum* (Zhang et al. 2013) and *Leptosphaeria maculans* (Feng et al. 2014). *SNF1*-deficient mutants showed reduction of virulence, but this reduction differend considerably. In addition, also the growth defects on different carbon sources differed considerably, suggesting that CWDEs are

differentially controlled and play differential roles in fungal virulence, depending on the lifestyle of the pathogen.

Colletotrichum graminicola is the causal agent of stalk rot and anthracnose leaf spots in maize, being an important disease in a global level that brings a further body of research into *Colletotrichum* genetics, pathology and host-parasite interactions (Cannon et al. 2012). Enormous appressorial turgor, unclear which role CWDEs play in primary penetration. Hemibiotroph, so that generation of cell wall polymer fragments (DAMPs) may represent a disadvantage during biotrophy, as the host may activate defense responses. The role of CWDEs in pathogenesis of *C. graminicola* is unknown. Thus, functional characterization of *CgSNF1* may strongly support understanding the role of CWDEs in pathogenesis of this hemibiotroph. Therefore, we deleted the 2371bp *CgSNF1* gene by homologous recombination and evaluated the role of this key regulator of the carbon catabolite repression pathway in virulence of the maize pathogen *C. graminicola*.

Methods

Fungal Strains and Culture Conditions

The wild type strain M1.001 of *Colletotrichum graminicola* was used to obtain the *SNF1* deleted strains (Δ*CgSNF1*) generated in this study. Media for fungal cultivation included potato dextrose (Difco Laboratories) with 1.5% agar and 100µg/mL hygromycin for transformants cultivation, oatmeal agar (OMA; Werner et. al., 2007) to induce sporulation, complete medium [1g $Ca(NO_3)_2$, 0.2g KH_2PO_3, 0.25g $MgSO_4$, 0.054g NaCl, 10g glucose, 1g yeast extract and 1g peptone per liter] for fungal DNA extraction and YES medium (171g sucrose and 1g yeast extract per liter) for microspores production and transformation. On solidified agar plates, strains were grown at 23 ^{o}C under continuous black light, and in liquid media, strains were cultivated in a shaker at 23 ^{o}C and 90rpm.

Targeted Deletion of *SNF1* gene of *Colletotrichum graminicola*

For targeted deletion of the 2371bp *SNF1* gene of *C. graminicola*, an intergenic region of approximately 1kb length was chosen for targeted homologous recombination and substitution of the *SNF1* gene for a deletion cassette containing the hygromycin resistance gene (*hph*). These flanking regions should not overlap with coding sequences of neighbor genes. Left (900bp) and right (1037bp) flanks of the *SNF1* gene were amplified from M1.001 genomic DNA using the primer pairs SNF1CgF5-SNF1CgR5 and SNF1CgF3-SNF1CgR3, and the hygromycin phosphotransferase (*hph*) gene of *Escherichia coli* was amplified from plasmid pAN7.1 using the primer pair UniHygF-UniHygR. The products contained overhang ends were fused by double-joint PCR (DJ-PCR; Yu et. al., 2004), and nested primers SNF1CgNestF-SNF1CgNestR were used to amplify the 3979bp cassette assembly. The cassette was then inserted into pJET1.2 vector (Thermo Scientific), cloned into thermo-competent *E. coli* strain and the purified plasmids were used as template for nested amplification of the linear cassette, which was transformed into *C. graminicola* conidial protoplasts (Werner et. al., 2007). For cassette assembly, all PCR reactions were performed using the Phusion High Fidelity DNA Polymerase (Thermo Scientific) following manufacturer's instructions. All primers used in this study are described in Supplementary table S1.

The correct integration of a single copy of the deletion cassete into the genome of *C. graminicola* transformants was verified by PCR and Southern blot analysis. Strains grown for seven days in complete medium at 23°C and 90rpm were filtered, dried and grounded into fine powder with liquid nitrogen using a mortar and pestle. DNA extraction was performed according to Sugui and Deising (2002).

Tests for homologous integration of the deletion cassette were done with primer pairs SNF1CgOutFdel-SNF1CgOutRev. The PCR reactions were conducted with 0.3 U/TopTaq Master Mix reaction (QIAGEN), 0.2 µM of each primer in a final volume of 12.5 µL and 50 ng of genomic DNA. The amplification conditions were initial denaturation at 94°C for 3 minutes, followed by 30 cycles with denaturation at 94°C for 30 seconds, 30 seconds at the manufacturer's annealing temperature (60°C) and extension at 72°C for 1 min/kb, followed by a final extension at 72°C for 10 minutes. PCR products were visualized on 1% agarose gel electrophoresis with GelRed (Biotium, USA) under UV-light.

To analyze the transformants strains for the copy number of integrations, 10µg of *Xho*I-digested genomic DNA was used in electrophoresis on 0.7 % agarose gel. The depurination and transfer onto nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Freiburg im Breisgau, Germany) followed standard protocols (Brown, 1999; Sambrook et al., 1989).

The digoxygenin-labeled probes (Roche Diagnostics) were amplified from wild-type genomic DNA for the *SNF1* gene (513bp) using the primer pair SNF1CgProbeF-SNF1CgProbeR and from the plasmid pAN7.1 for the *hph* gene (474bp) using the primer pair HygProbeF-HygProbeR. In a total volume of 20 µL, the PCR mixture consisted in 1 x reaction buffer containing 1.5 mM MgCl2, 0.025 U/mL Taq DNA Polymerase (Amersham Pharmacia Biotech), 0.2 mM deoxynucleoside triphosphate (dNTP), 0.7 mM digoxygenin-labeled probes (Roche Diagnostics), 0.5 mM each of primers and 10 pg of plasmid DNA. Hybridization and visualization of the hybridized Dig-labelled probe were performed as suggested by the manufacturer (Roche, Mannheim, Germany).

Mycelial growth and sporulation assays

In order to compare mycelium growth rates and conidiation among strains, OMA plates were inoculated with 2μL conidia suspension (10^6 conidia/mL) of each strain, incubated at 23°C in the dark, and then colonies diameters were measured at 3, 4, 6, 7 and 8 days post inoculation (dpi). Conidiation was evaluated 15dpi, by washing the plates with 10mL 0.02% Tween 20 for 10min on shaker at 50rpm, then diluting the solution at 1:10 proportion and counting conidia under a light microscope using a hemocytometer. All experiments were performed in triplicate.

Growth assays in different carbon sources

The wild type, one deleted (Δ3.1) and one ectopic (Ec2.1) strains were tested for their ability to use different carbohydrates as carbon sources. The minimum media [1g $Ca(NO_3)_2$, 0.2g KH_2PO_3, 0.25g $MgSO_4$, 0.054g NaCl, 0,01g yeast extract, 15g agarose] was supplemented with 2% of glucose, xylose, sucrose, cellulose, pectin or CMC. Each plate was inoculated with 2μL droplets (10^6 conidia/mL) of each strain, in triplicate, then incubated in the dark at 23°C. Colonies diameters were measured 10dpi.

Plant Material and Infection Assays

The variety of maize (*Zea mays* L.) used in this study was Mikado (KWS Saat AG, Einbeck, Germany), cultivated in growth chambers as described by Behr et al. (2010). For inoculation assays, third leaves of 14-days-old plants were harvested and their central segments (~10cm) were placed onto plastic boxes of 12 x 12cm containing a moistened filter paper. Leaves were inoculated on the midrib with 10μL droplets of fungal conidia suspension produced by washing 15-day OMA plates with 0.02% (v/v) Tween 20, then incubated in darkness at 23 °C.

To compare development of macroscopic symptoms, leaves inoculated with 10^4 conidia were photographed every 24h for five days. In order to microscopically observe and count infection structures, inoculum spots with 10^3 conidia were extracted, bleached for 24h in ethanol-acetic acid (3:1), washed in water, stained in 0.01% aniline blue, visualized under a Nikon Eclipse 600 microscope, photographed with a CCD-camera Digital Sight DS-Fi1 (Nikon) and the pictures were analyzed with the software NIS-Elements D (Nikon, version 2.30).

For fungal biomass quantification, samples comprised a pool of 16 inoculation spots each containing 10^4 conidia. Droplets were carefully dried with filter paper and inoculation spots were extracted with a cork borer with 8mm diameter, placed in 2mL microtubes containing 2 steel balls, frozen in liquid nitrogen and stored at -80°C. Samples were then macerated in a Tissue Lyzer at 30Hz for 30s and further DNA extraction was employed with the peqGOLD Plant DNA Mini Kit (PeqLab), following the manufacturer's instructions, with a final elution in 100µL. All experiments were conducted in triplicates.

qPCR

qPCR was performed according to Weihmann et al. (2016), using the same plasmid pUC18 as an external standard control, added to the grounded material prior to DNA extraction to serve as a correction factor for fungal DNA. Total DNA from samples was measured in Nano Drop 1000 spectrophotometer (Thermo Scientific), diluted to a concentration of 10ng/µL and 2 µL of diluted DNA was used for qPCR reaction, using three technical repetitions per sample. C_T values were adjusted based on the threshold values described by the author and then used to calculate the amount of target DNA in

each reaction. After correction using pUC18 values, the absolute fungal DNA of each strain *in planta* was calculated.

Results

Targeted deletion of the *SNF1* gene of *Colletotrichum graminicola*

BLAST searches using *SNF1* genes and proteins of different fungi (*Saccharomyces cerevisiae*, *Cochliobolus carbonum*, *Magnaporthe oryzae*, *Verticillium dahliae*) as querie sequences were performed to identify the *SNF1* homolog in the *C. graminicola* genome (https://www.ncbi.nlm.nih.gov/genome/genomes/2138?). A single copy gene of 2371 nucleotides was found, with 4 exons, 3 introns and a predicted protein of 729 amino acids (aa) (Fig. 1). Fungal Snf1 proteins showed sizes ranging from 576 (SSP2 of *Schizosaccharomyces pombe*) to 880aa (*SNF1* of *Cochliobolus carbonum*). *SNF1* is a serine-threonine protein kinase. *C. graminicola* shares 562, 538 and 511 amino acids (77, 74 and 70%) with the homologous protein from the filamentous fungi *Verticillium dahliae*, *Fusarium oxysporum* and *Neurospora crassa*, respectively. The kinase domain of the protein has a higher conserved region, being similar to the species above by 83, 93 and 92%, respectively. The activation segment of the kinase domain is represented by a 30aa region, which is even more conserved, being 100% similar among many filamentous fungi and 86.7 % similar to the same domain of the human AAPK1 protein. Phylogenetic inference tree using the amino acid sequence of the active kinase domain showed separate clades grouping filamentous fungi and yeasts (Fig. 1A), and the same was observed for the complete *SNF1* amino acid sequence (Sup. Fig. S1)

To delete the *SNF1* gene of *C. graminicola* we employed double-joint PCR (Yu et al. 2004) to construct a deletion cassette consisting of 877 bp of the 5’-flank of the *CgSNF1* gene, the 2055 bp hygromycin phosphotransferase (*hph*) gene of *Escherichia*

coli (amplified from the plasmid pAN7.1), and 1007 bp of the 3'-flank of *CgSNF1* (Fig. 1B). Sequencing of the *SNF1* deletion cassette confirmed the correct assembly. The construct was transformed into *C. graminicola* wild-type strain CgM2. Transformants growing on Hygromycin were first screened for the presence of 474bp *hph* fragment and the absence of a 513bp *SNF1* fragment by PCR (Fig. 1C). *Xho*I-digested genomic DNA isolated from the WT and selected transformant strains was then analyzed by Southern blot hybridization, using digoxygenin-labeled probes for *CgSNF1* and *hph* genes (Fig. 1B, C). Using the *SNF1* probe only the WT strain showed a 3.6 Kb band. Using the *hph* probe, only strains 2.3, 2.5 and 3.1 exhibited a single 5.9 Kb band, as expected for deletion mutants harboring a single deletion cassette insertion. Strain 2.1 showed a 5 Kb band, resulting from an ectopic integration of the deletion cassette, as well as 3.6 Kb band indicative of the *SNF1* gene (Fig. 1C). Thus, the transformation experiments yielded the three independent deletion strains Δ*snf1-2.3*; Δ*snf1-2.5*, and Δ*snf1-3.1*, as well as the transformation harboring an ectopic transformation of the deletion cassette, designated Ec2.1.

SNF1 of _C. graminicola_ is required for asexual sporulation growth on different polymeric carbon sources

Compared to wild-type and Ec2.1 strains, all Δ*snf1* mutants exhibited reduced growth rates on OMA plates. Supplementing OMA with 2% sucrose significantly increased growth rate of the mutants (Fig. 2A). On OMA plates, conidiation rates of the deletion mutants were reduced by 73-87%, as compared to the WT strain (Fig. 2B). However, the conidia and hyphae did not show morphology alterations. When different carbohydrates were used as carbon sources, media containing sucrose did not show significant differences in growth rate among wild-type, ectopic and the Δ*snf1* mutant.

However, in the simple sugars glucose and xylose and when using complex polysaccharides (cellulose, pectin and CMC) the growth rate of deleted mutant was impaired, with an average growth reduction of 27, 46, 64, 43 and 34%, respectively, compared to wild-type strain (Fig. 2C).

SNF1* gene is required for full virulence of *C. graminicola

Infection assays performed on maize leaf segments clearly revealed reduced virulence of the Δ*snf1* strains. The WT and the Ec2.1 strains produced first macroscopically visible symptoms at 48 hpi (hours post inoculation), and typical soaked lesions occurred at 72 hpi. At this time point the deletion mutants exhibited first visible symptoms. At 120 hpi, Δ*snf1* strains produced minor necrotic lesions, compared to wild-type and Ec2.1 strains (Fig. 3A). In order to confirm the differences observed in symptom severity, fungal development was assessed by quantitative PCR at 72 hpi. Measurements of the amount of fungal DNA of the WT and Ec2.1 strains were significantly higher than the mass of DNA of the Δ*snf1* mutants (Fig. 3B), clearly confirming the virulence defects of the mutants observed macroscopically.

To confirm delay in penetration and spread, infection structures were observed on a microscopic level, by counting first and second plant cell penetration from 100 appressoria at 48 hpi. Δ*snf1* strains showed an average rate of 11 and 3 % penetration of first and second cells, respectively, compared to 63 and 25 % for the wild type (Fig. 2E). Most of the primary hyphae observed from Δ*snf1* mutants at this time point showed a reduced length compared the wild-type and Ec2.1 strains. Infection structures formed by individual appressoria at 72 hpi could not be counted, due to massive colonization of the plant tissue. However, cell wall penetration and spreading of Δ*snf1* mutants through neighboring cells was clearly observed (Sup. Fig. S4).

Discussion

Carbon catabolite repression is an important regulatory metabolic process allowing a fungus, irrespective of its lifestyle, to switch from consumption of simple monomeric sugars to depolymerizing and consuming complex carbohydrate polymers as present in plant cell walls. Thus, for pathogenic fungi catabolite de-repression may be indispensable to invade and spread within a host plant (Ruijter & Visser, 1997).

We have identified the *SNF1* ortholog of *C. graminicola*, a gene encoding a protein that shows high similarity with Snf1 proteins of other filamentous fungi, especially in the serine/threonine protein kinase domain, and also confirmed the evolutionarily conserved amino acid sequence from the yeast *SNF1* gene to its homologs counterparts in plants (SnRK1) and even human (AMPK) (Beri et al., 1994; Takano et al., 1998). Phylogenetic analysis both for the active kinase domain or the whole protein showed a clear separation of clades grouping filamentous fungi and yeasts, and the tree topology proved them to be ortholog genes.

By applying a targeted gene deletion approach, we successfully generated Δ*snf1* strains of the maize pathogen *C. graminicola* and observed that vegetative growth rates of Δ*snf1* strains were reduced in OMA medium as well as on plates containing single different polymeric carbohydrates. Interestingly, growth rates of *C. graminicola* clearly differed from those of the necrotroph *C. carbonum* (Tonukari et al., 2000). Conidiation of *CgSNF1* mutants was also reduced. but maybe due to the reduced growth rate, since mutant strains restored significantly the growth rate when the media was supplemented with sucrose.

In the presence of complex carbohydrates such as pectin, CMC and cellulose, the Δ*snf1* strains showed impaired radial growth. Likewise, on pectin, growth was seriously affected in *snf1* mutants of *C. carbonum* (Tonukari et al., 2000) and *P. digitatum* (Zhang

et al., 2012), but only partially affected *V. dahlia* (Tzima et al., 2011) and *L. maculans* (Feng et al., 2014), and did not affect *F. oxysporum* (Ospina-Giraldo et al., 2003), what might be associated with specific carbon utilization of different species in association with their hosts cell components.

The SNF1 gene was originally isolated in *S. cerevisiae* after isolating mutants unable to ferment sucrose (Sucrose-Non-Fermenting) (Carlson et al., 1981). However, while invertase genes are catabolite-repressed in *S. cerevisiae*, they may not be under catabolite repression in filamentous fungi, as shown in the Δ*snf1* strains of *C. graminicola*, with normal growth on sucrose, and in *SNF1* mutants of *C. carbonum* (Tonukari et al., 2000) and *V. dahlia* (Tzima et al., 2011). In contrast, *SNF1* mutants of *F. graminearum* (Lee et al., 2009) and *P. digitatum* (Zhang et al., 2013) showed less efficient utilization of and growth on sucrose, indicating that the regulation of invertase – and of CWDEs – may vary on the species level.

On maize leaves and on epidermis cells of the alternative host onion (*Allium cepa*), conidia of both the WT and Δ*snf1* strains germinated and differentiated appressoria at comparable rates. However, while the WT strain efficiently penetrated through the intact leaf surface on both hosts tested, penetration and spread of Δ*snf1* strains in the host tissue occurred at a reduced rates, resulting in reduced symptom severity and virulence.

C. graminicola requires the melanized appressoria for turgor-driven penetration of the first epidermal host cell (Deising et al., 2000; Ludwig et al., 2014). Likewise, the rice blast fungus produced elaborate melanized appressoria, which generate enormous turgor pressure in order to invade the host. Comparison of the penetration efficiency of the rice epidermal cell wall and of non-biodegradable membranes of comparable hardness suggested that CWDEs support the penetration process even in elaborate infection cells

(Howard et al., 1991). However, direct evidence supporting the requirement for CWDEs in fungi with elaborate infection cells is not available.

Indeed, in species that do not produce appressoria such as *Penicillium digitatum*, *Fusarium graminearum*, *Fusarium oxysporum* and *Leptosphaeria maculans*, *SNF1* deletion abolished the expression of most or all tested CWDE genes (Ospina-Giraldo et al., 2003; Lee et al., 2009; Zhang et al., 2013; Feng et al., 2014). Similar observations were made in species that produce normal appressoria but do not require the structure for causing disease, such as *Verticillium dahliae* and *Cochliobolus carbonum* (Tonukari et al., 2000; Tzima et al., 2011). In contrast, species that produce and require elaborate appressoria, such as *Magnaporthe oryzae* and *Ustilago maydis*, showed low or no effect of *SNF1* on the expression of CWDEs (Yi et al., 2008; Nadal et al., 2010), especially in earlier stages of infection.

Deletion or disruption of *SNF1* genes of plant pathogenic filamentous fungi revealed a wide variety of phenotypes and growth defects, but the common result is the impaired virulence in Δ*snf1* strains. Infection assays performed with Δ*snf1* strains of *C. graminicola* suggest that *SNF1* is an important virulence factor in this species. The different lifestyles of plant pathogenic fungi may explain the different roles of *SNF1* in different pathogen-host interactions. In this study we observed that the absence of *SNF1* caused a reduced growth rate *in vivo* and impaired growth in different carbon sources *in vitro*. Reduced capability of depolimerizing complex polymers may be causal for the delay in penetration and hyphal spread observed at the macroscopic and microscopic levels. The direct mechanism behind the action of *CgSNF1* gene is still unclear and need to be addressed, but our data collectively show that *CgSNF1* plays important roles on vegetative growth, carbon utilization and virulence in the maize anthracnose pathogen.

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Fig. 1

A) Bayesian phylogenetic tree based on serine/threonine active kinase domain of putative SNF1 proteins of *Colletotrichum graminicola* and other species. Values on nodes indicate the posterior probability values. The tree was rooted with the outgroup *Homo sapiens* and *Oryza sativa*.

B) *SNF1* deletion construct transformed into *C. graminicola* through homologous recombination. Bars indicate fragments amplified for PCR screening of mutants and for digoxygenin-labeled probes used in *Xho*I-digested DNA gel blots for sucrose non fermenting 1 (*SNF1*) and hygromycin resistant (*hph*) genes.

C) PCR amplifications and DNA gel blots of *C. graminicola* wild type (wt), ectopic (Ec2.1) and *SNF1* deleted strains (Δ2.3, Δ2.5, Δ3.1).

Fig. 2

A) Morphology and mycelial growth of Wt, Ec2.1 and Δ*SNF1* strains seven days after inoculation in OMA medium and OMA medium supplemented with 2% sucrose.

B) Conidiation rates of Wt, Ec2.1 and Δ*SNF1* strains 15 days after inoculation in OMA medium.

C) Mycelial growth of *C. graminicola* wild type (Wt), Ec2.1 (Ec) and Δ3.1 (Del) strains 10 days after inoculation in minimum medium supplemented with 2% of different carbohydrates as carbon sources.

Fig. 3

A) Disease symptoms in non-wounded maize leaves inoculated with wild type (wt), ectopic (Ec2.1) and *SNF1*-deleted strains (Δ2.3, Δ2.5, Δ3.1), 24, 48, 72 and 120 hours after inoculation. Mock-inoculated treatments were made with 0.02% (v/v) Tween 20.

B) Quantification of infection structures on maize leaves of wild type, Ec2.1 and Δ*SNF1* strains 48 hours post inoculation. For each independent experiments performed, infection structures in first and second cells of maize leaves were counted from 100 appressoria.

C) Quantification of fungal DNA of wild type (wt), ectopic (Ec2.1) and *SNF1*-deleted (Δ2.3, Δ2.5, Δ3.1) strains *in planta* 72hpi. For each strain, four leaf discs from four leaves were pooled, in triplicate, and for qPCR reaction three technical repetitions of each sample were performed. Error bars indicate standard errors and letters indicate significant variation between strains at $p < 0.05$, which was determined by pairwise comparison using the Tukey's test.

Fig. 1

A

Schizosaccharomyces pombe
Saccharomyces cerevisiae
Candida glabrata
Kluyveromyces lactis
Candida tropicalis
Candida albicans
Penicillium digitatum
Leptosphaeria maculans
Cochliobolus carbonum
Sclerotinia sclerotiorum
Neurospora crassa
Magnaporthe oryzae
Colletotrichum graminicola
Verticillium dahliae
Fusarium oxysporum
Hypocrea jecorina
Homo sapiens
Oryza sativa

B

SNF1 probe
5' – flank
SNF1
3' – flank
XhoI
XhoI
XhoI
XhoI
hph probe
5' – flank
hph
3' – flank
XhoI
XhoI
3661 bp
5964 bp

C

hph
SNF1
Ec2.1 Δ2.3 Δ2.5 Δ3.1 wt
Ec2.1 Δ2.3 Δ2.5 Δ3.1 wt
0.5 kb
PCR
6 kb
3 kb
Southern blot

Fig. 2

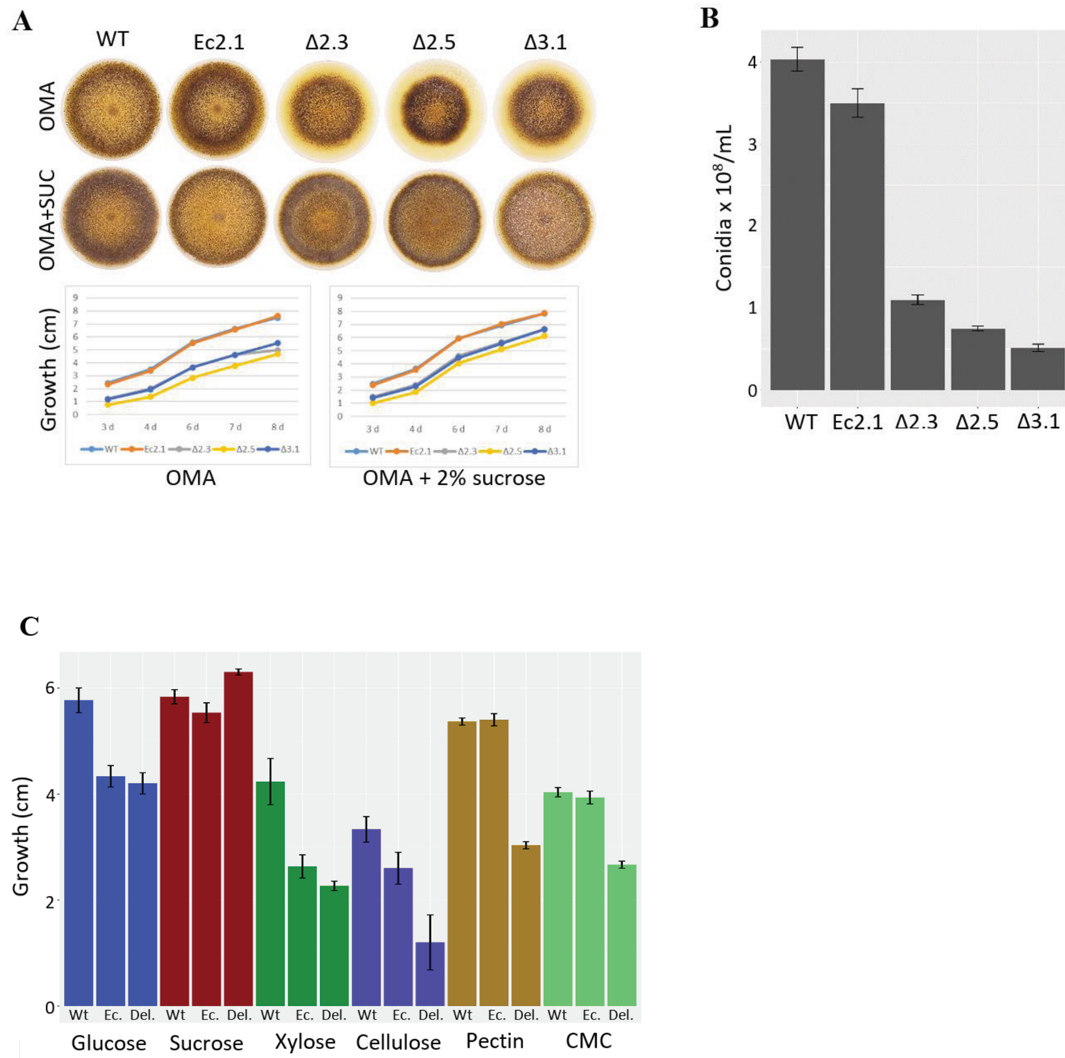
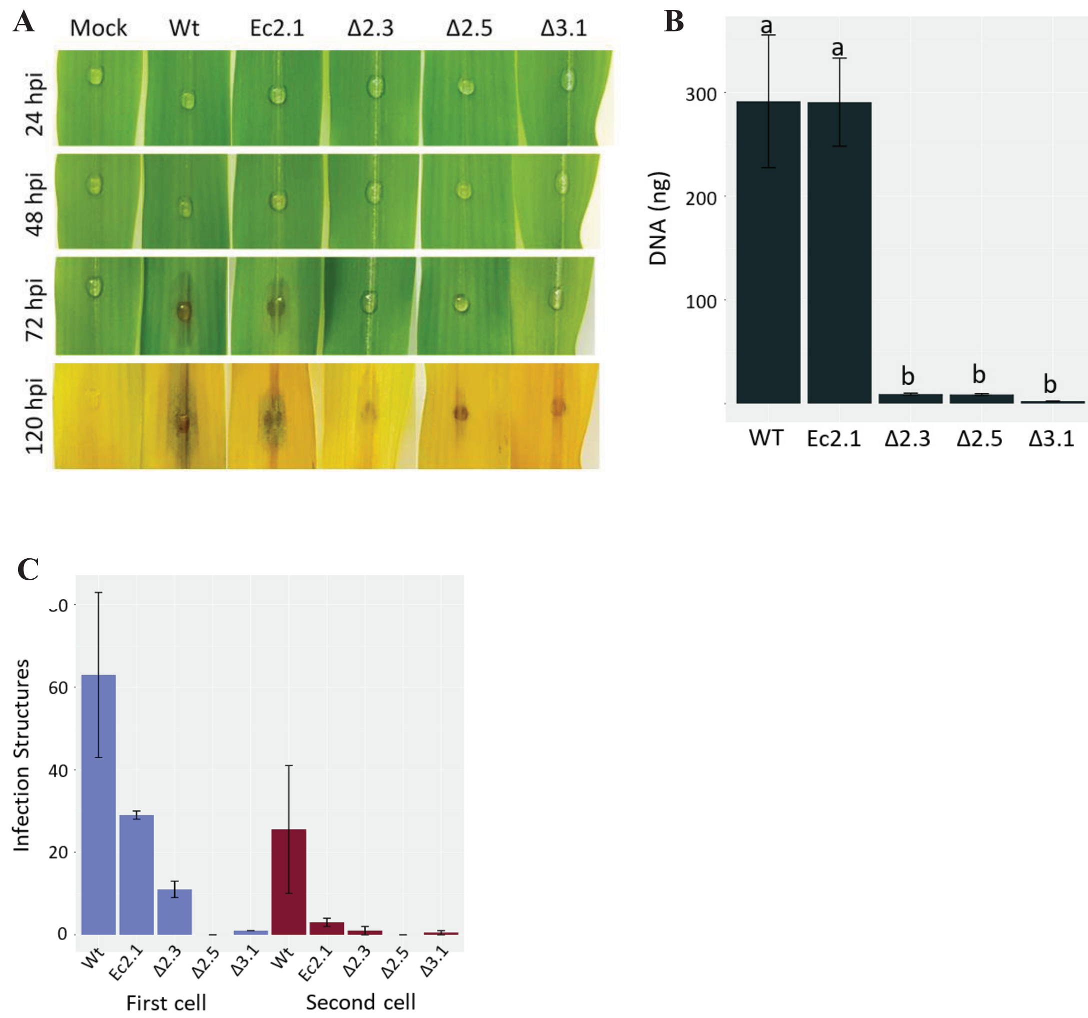


Fig. 3



Supplementary Information

Influence of SNF1 gene in pathogenicity of *Colletotrichum graminicola*

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Table S1 – List of primers used in this study

Primer name	5' – primer sequence – 3'	Target
SNF1CgF5_1	CCACTGGGTCTCTATCTCC	*C. graminicola* M2 gDNA
SNF1CgR5_1	gtgcaactgacagtcgtacaGCATGACGAAGTTGGATG	*C. graminicola* M2 gDNA
SNF1CgF3_1	gtctggagtctcactagcttAGGTCGTTTCTTTGATGTTC	*C. graminicola* M2 gDNA
SNF1CgR3_1	GACGAGGGTCCTATCTGC	*C. graminicola* M2 gDNA
SNF1CgF5nested	TGCCGTCTATTGGTCTG	Targeted cassettes
SNF1CgR5nested	GCTTTGAACAGGTGTATCG	Targeted cassettes
UniHygTF	tgtacgactgtcagttgcacTGACCGGTGCCTGGATCTTC	pAN7.1
UniHygTR	aagctagtgagactccagacGGTCGGCATCTACTCTATTCC	pAN7.1
HygProbeF	GTCCCTGGTAGGCAGCTTTG	pAN7.1
HygProbeR	CCATCGGCGCAGCTATTTAC	pAN7.1
SNF1CgProbeF	ATGGCTTCCGGTTTCGACGAGG	*C. graminicola* M2 gDNA
SNF1CgProbeR	CGGTATACCTAGGGTCATTCGGG	*C. graminicola* M2 gDNA
Cg_ITS2-F1.1	CGTCGTAGGCCCTTAAAGGTAG	*C. graminicola* M2 ITS2
Cg_ITS2-R1	TTACGGCAAGAGTCCCTC	*C. graminicola* M2 ITS2
M13new-For	GTAAAACGACGGCCAGTGC	pUC18
M13new-Rev	CACAGGAAACAGCTATGACC	pUC18

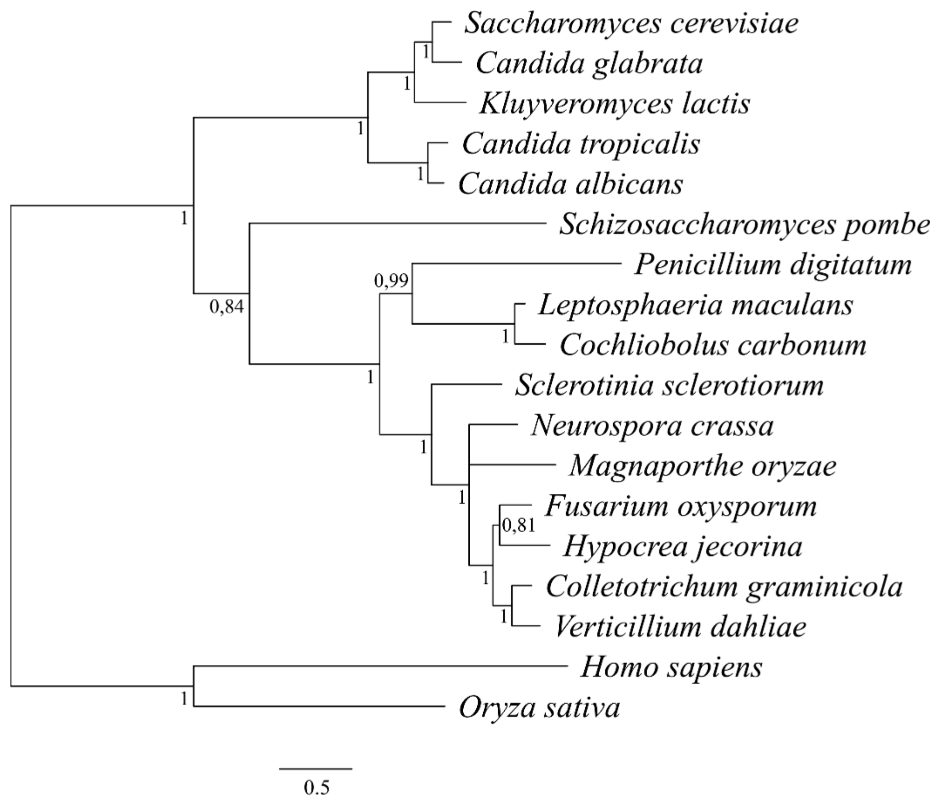

Figure S1 - Bayesian Inference tree using complete SNF1 amino acid sequence of *Colletotrichum graminicola* and other species. Values on the nodes indicate Bayesian posterior probability values. Sequences were obtained from the UniProt Database (http://www.uniprot.org/).

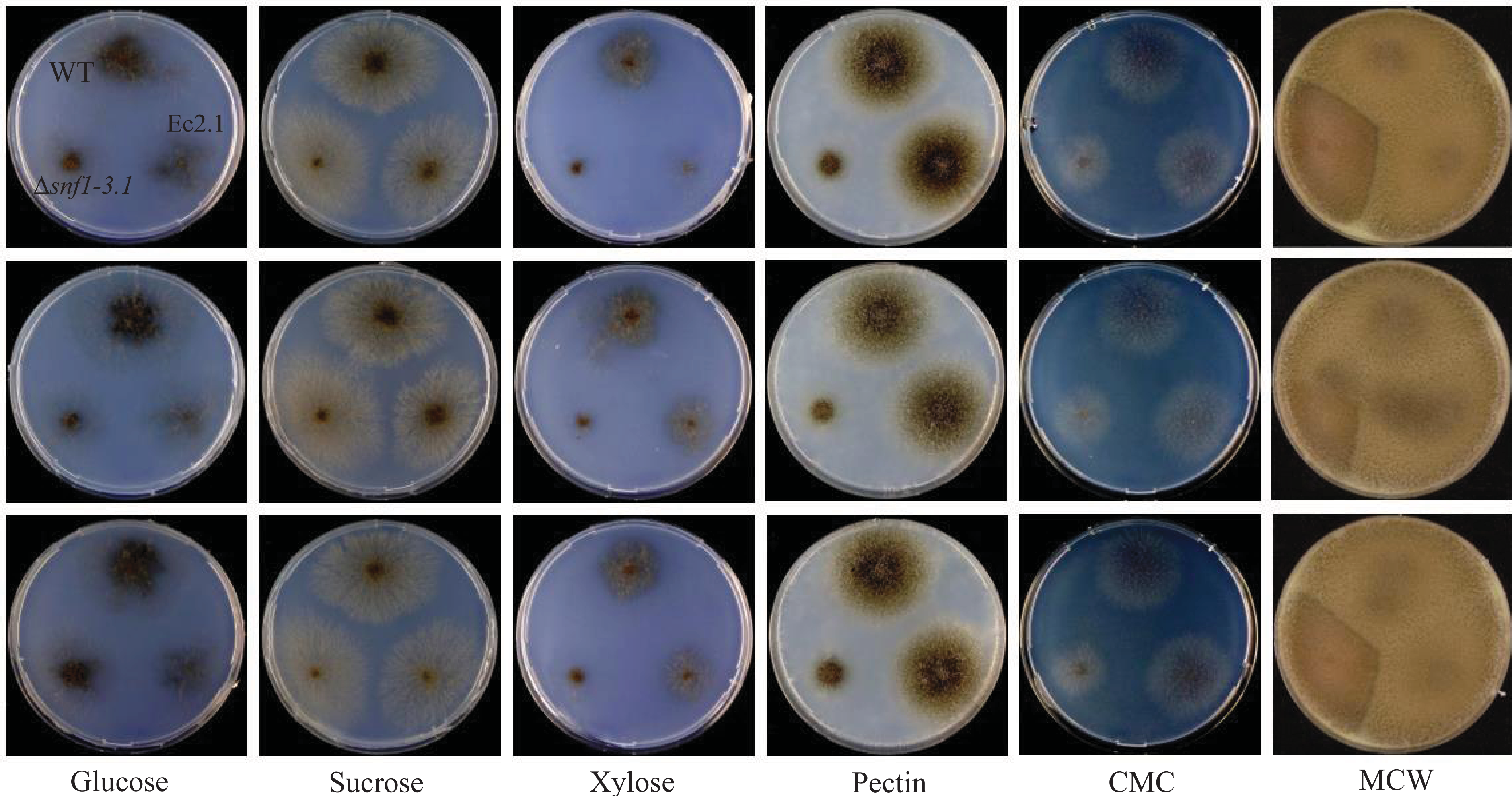

Figure S2 – Growth of *Colletotrichum graminicola* wild-type, Ec2.1 and *Δsnf1-3.1* strains on minimal medium supplemented with 2% of different carbon sources 10 days post inoculation. Inocula consisted of 2μL droplets of conidial suspension of each strain containing 10^6 conidia/mL.

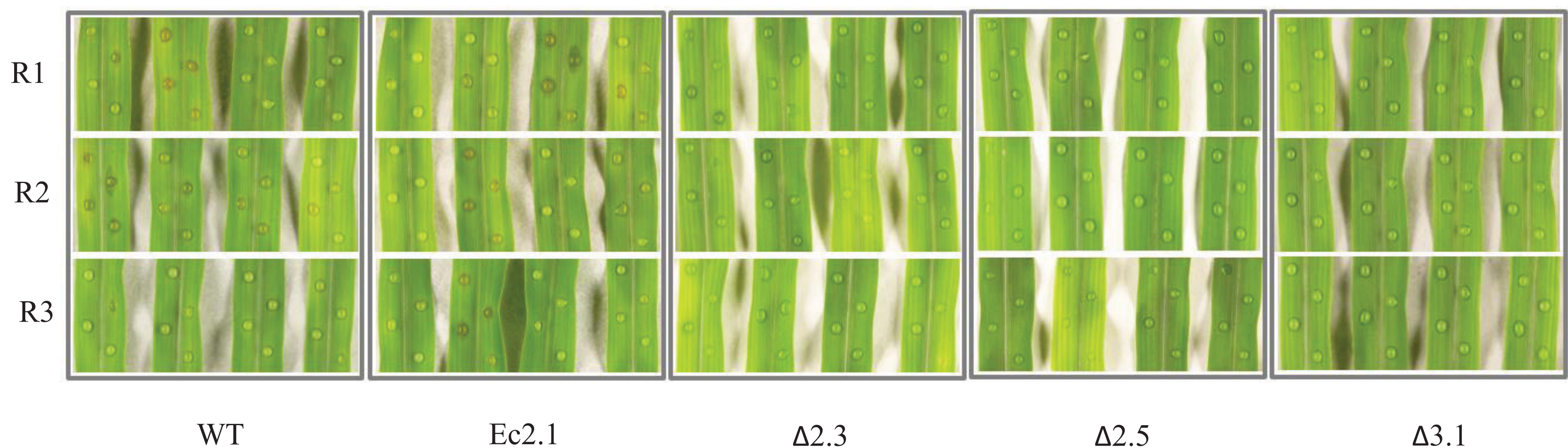

Figure S3 – Leaf spots inoculated and extracted after 72h for qPCR analysis. Each treatment is represented by four leaves each containing four inoculation spots. Experiments were carried in triplicate and each inoculation spot consisted of 10µL of a 10^6 conidia/mL suspension.

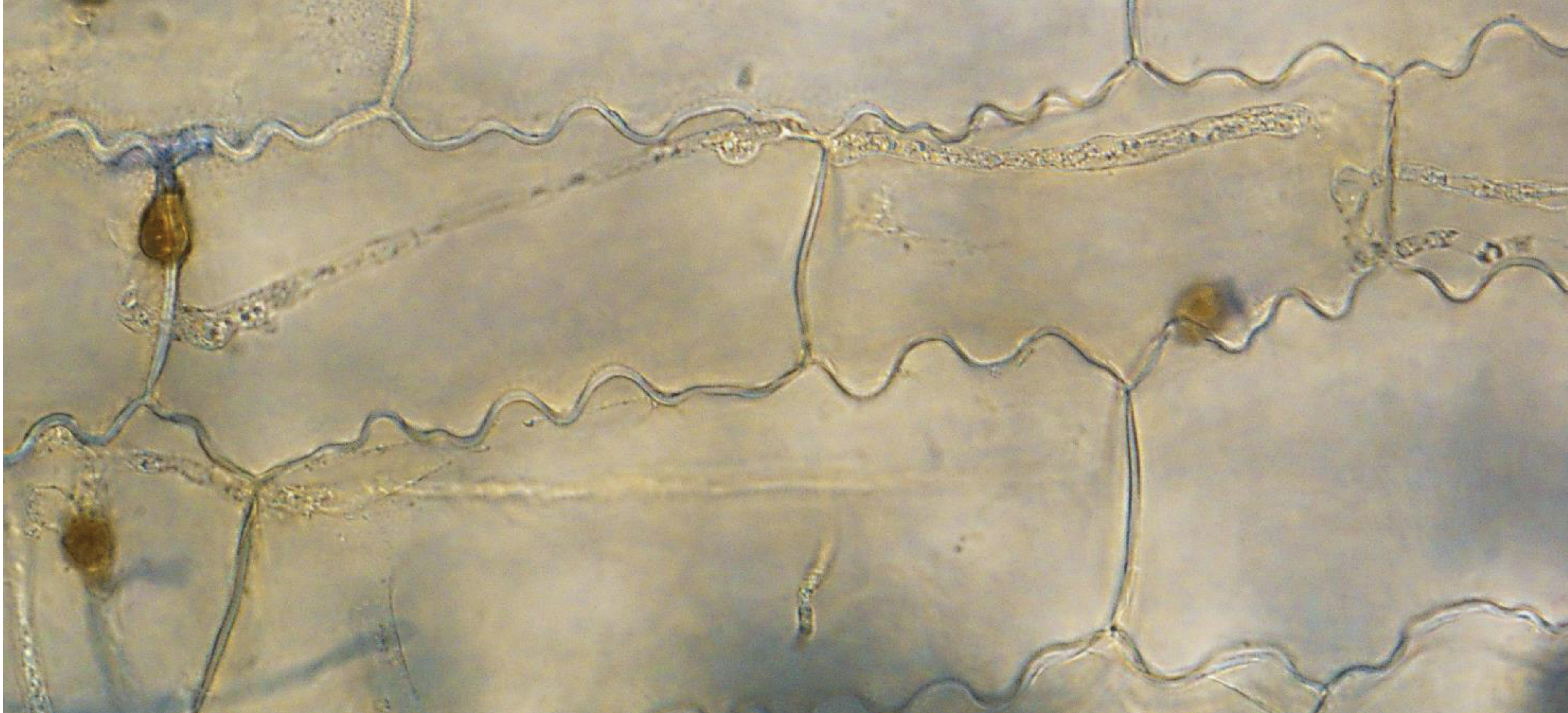

Figure S4 – *SNF1* deleted mutant strain Δ2.3 colonizing maize leaf cells 72 hours post inoculation.

6. DISCUSSÃO GERAL

Decifrar interações entre microrganismos e plantas é sem dúvida um aspecto promissor no entendimento dos benefícios e especialmente dos efeitos patogênicos destas interações na produção agrícola (IMAM et al., 2016). As doenças Queda Prematura de Frutos Cítricos (QPFC) e Antracnose Foliar do Milho representam importantes ameaças à agricultura brasileira, mas também em nível global, e seus estudos fornecem ideias e perspectivas não apenas restritos a esses patosistemas, mas a uma ampla gama de interações, uma vez que as relações patógeno-hospedeiro tendem a apresentar semelhanças em diferentes espécies (TORTO-ALALIBO et al., 2009). Embora seja uma doença conhecida, a QPFC e seu patógeno *C. abscissum* ainda não representam um patosistema bem elucidado e em meio a uma recente reclassificação da espécie, ainda é necessária uma revisão dos trabalhos envolvendo a espécie anteriormente associada com a doença, *C. acutatum* lato senso. Em contrapartida, o ciclo infeccioso da Antracnose Foliar do Milho é bem mais elucidado, o comportamento do seu patógeno *C. graminicola* é bem descrito e já existem inúmeros estudos envolvendo sua manipulação genética para compreensão de fatores de virulência na espécie.

O primeiro importante passo para se conhecer um patosistema é a correta identificação dos patógenos associados, mas a alta variabilidade morfológica existente dentro do gênero *Colletotrichum* sempre dificultou a distinção de espécies com base apenas em morfologia. A exemplo disso, o estudo da doença QPFC passou por muitas etapas até a recente definição do seu principal agente causal, a espécie *C. abscissum*. Vários estudos que ainda definiam seu agente causal como *C. acutatum* equivocadamente compararam seus resultados com estudos envolvendo outras espécies, ainda erroneamente designadas como *C. acutatum*. No caso da QPFC, além da problemática envolvendo a correta definição do agente causal, a recente proposição da espécie *C. gloeosporioides* como também associada com a doença abriu mais uma lacuna sobre a importância de se realizar um estudo mais amplo sobre os patógenos associados, sua distribuição, proporção e a existência de variabilidade genética nesses isolados.

Para compreender melhor a QPFC, neste trabalho foram analisados 227 isolados de flores sintomáticas distribuídos em diferentes pomares do estado de São Paulo, Brasil, que representa o maior produtor de citros do país. Os isolados foram identificados como pertencentes às espécies *C. abscissum* e *C. gloeosporioides*. Embora a descrição de novas espécies necessite da elaboração de uma análise filogenética multigênica, a região GAPDH mostrou-se informativa para a distinção das espécies associadas com a QPFC no Brasil, e a

análise multigênica em uma sub-amostra de isolados de *C. abscissum* não revelou variabilidade intraespecífica entre isolados provenientes de diferentes regiões. Os seis marcadores utilizados foram os mesmos propostos para a resolução da taxonomia no grupo, sendo suficientemente informativos para revelar qualquer variabilidade nestes isolados, que podem então serem considerados uma população clonal. Assim sendo, pode-se considerar que estudos anteriores sobre a QPFC no Brasil associados com a espécie *C. acutatum* lato senso tratavam-se de estudos envolvendo a nova espécie descrita *C. abscissum*. Mesmo assim, não se pode descartar o aumento da incidência da espécie *C. gloeosporioides* associada com a doença, que pode estar contribuindo para o seu agravamento.
O estudo etiológico aqui proposto confirmou a prevalência da espécie *C. abscissum* associada com a QPFC no estado de São Paulo, Brasil, e revelou um aumento na população de *C. gloeosporioides* associada com a doença em comparação com os estudos de Lima et al. (2011). Ainda, todos os isolados foram provenientes de amostras de laranja doce em regiões com diferentes níveis de aplicação de fungicida, ao contrário do estudo prévio que recuperou a maioria dos isolados de *C. gloeosporioides* de lima ácida em regiões de cultivo orgânico (LIMA et al., 2011). Foi observado que a maioria dos isolados de *C. gloeosporioides* testados exibiam resistência a concentrações discriminatórias do fungicida benomil, porém um estudo mais detalhado com maior número de isolados e diferentes concentrações do fungicida é necessário para avaliar o aumento da resistência dessa espécie. Uma vez que ambas as espécies, *C. abscissum* e *C. gloeosporioides*, estão associadas com a doença QPFC no Brasil, foram propostos novos primers para discriminação das espécies em uma reação de PCR multiplex, revelando-se uma ferramenta rápida e confiável na distinção de isolados de QPFC a partir de lesões florais. Esclarecidos alguns pontos centrais sobre a incidência da doença e dos patógenos associados, abre-se margem para o início dos estudos de interação planta-patógeno em *C. abscissum*, especialmente no que diz respeito à manipulação de genes para busca de fatores genéticos associados com patogenicidade na espécie.

Esforços anteriores em entender e manipular genomas de organismos-modelo avançaram imensamente o conhecimento biológico fundamental e as tecnologias para sua obtenção (SPRADLING et al., 2006). Nesse sentido, a espécie *C. graminicola* é um bom modelo de estudo dentro do gênero, visto que possui características que permitiram o estabelecimento prévio de técnicas de manipulação genética (VAILLANCOURT; HANAU, 1994). Embora muitos estudos descreveram genes associados com patogenicidade em

C. graminicola, muitas vezes genes codificadores podem ter funções redundantes, de modo que sua deleção ou disrupção não acarrete alterações significativas.

Por conta disso, no presente trabalho, o gene regulatório *SNF1* foi avaliado. Trata-se de um gene que codifica uma proteína quinase do tipo serina/treonina, com sequência de aminoácidos bastante conservada inclusive entre organismos distantes, especialmente na região que contém o domínio quinase ativo. Este gene possui cópia única em *C. graminicola*, e sua deleção revelou não ser um gene essencial, porém trouxe alterações relevantes na fisiologia e virulência da espécie. O crescimento vegetativo mostrou-se reduzido especialmente em fontes de carbono complexas, assim como foi observada redução da virulência em folhas de milho. Não foi possível confirmar microscopicamente o motivo da virulência reduzida, pois observou-se atraso na penetração tanto da primeira quanto das demais células do hospedeiro, embora os mutantes mostraram massa micelial dramaticamente reduzida, o que confirmou os sintomas reduzidos a nível macroscópico.

Sendo um fungo que produz e necessita do apressório funcional para penetração no hospedeiro, poderia-se esperar atraso apenas na penetração das demais células do hospedeiro, pela possível indisponibilidade de enzimas degradadoras de parede, porém mesmo na presença de apressórios completamente funcionais, o fungo pode necessitar de tais enzimas mesmo durante a fase de penetração apressorial (Howard et al. 1991), ou alternativamente atravessar as demais paredes celulares por meio de plasmodesmos (Kankanala et al. 2007). Assim, o papel das enzimas degradadoras de parede precisa ser investigado em diferentes condições de cultivo e diferentes estágios de infecção no hospedeiro, mediante análise da expressão de genes possivelmente controlados pelo complexo SNF1. Além disso, deve-se avaliar se também excluir a possibilidade de que a ausência do gene *SNF1* influencie a pressão de turgor dos apressórios, dificultando assim a primeira penetração.

Os dados obtidos neste estudo apontam para a importância do gene *SNF1* na patogênese da espécie *C. graminicola*, e abrem possibilidade para novos estudos de associação do gene *SNF1* com os genes glicose-reprimidos em *Colletotrichum graminicola*, como por exemplo genes que codificam enzimas degradadoras de parede. A elucidação dos mecanismos envolvidos na atuação do gene *SNF1* depende de novos experimentos, e dentre eles alguns são aqui sugeridos: a busca e avaliação da expressão de enzimas degradadoras de parede nas linhagens Δ*CgSNF1*; a comparação da taxa de crescimento vegetativo e dentro do hospedeiro por microscopia; a fusão dos promotores dos genes associados com os estágios de biotrofia e necrotrofia com a proteína fluorescente *gfp* (*green fluorescent protein*), para avaliar se existe

influência da ausência do gene *CgSNF1* na taxa de crescimento do fungo nos estágios biotrófico e necrotrófico; a obtenção e caracterização de mutantes para o gene *CREA* (*MIG1* em leveduras), que codifica a proteína repressora dos genes que atuam no uso de fontes alternativas de carbono e que é fosforilada pelo complexo SNF1 na ausência de glicose.

Com relação a essas proposições, já foram selecionados possíveis genes alvo da proteína CreA, com base em dados de transcriptoma na espécie *C. graminicola* e avaliando a existência do sítio comum de ligação desta proteína nos seus promotores. Além disso, a metodologia de RTqPCR já está padronizada na espécie, possibilitando que os primeiros testes sejam conduzidos na sequência. Com relação à avaliação da taxa de crescimento, a metodologia está sendo padronizada e logo os primeiros testes também serão conduzidos. A metodologia de fusão com o gene para a proteína *gfp* também está padronizada em *C. graminicola*, e segue o mesmo princípio utilizado para a deleção de genes. Com relação ao gene *CREA*, este já foi encontrado no genoma de *C. graminicola*, confirmada sua similaridade com o mesmo gene de outras espécies por alinhamento e análise filogenética, os *primers* para montagem dos cassetes de deleção foram sintetizados, e o cassete já montado foi transformado na linhagem selvagem de *C. graminicola*. Atualmente 76 colônias mutantes já foram obtidas para o gene *CREA*, que estão em fase de purificação para obtenção de colônias monospóricas, e que em seguida serão caracterizados. Sendo assim, os novos testes para estudo do gene *SNF1* e *CREA* já estão encaminhados, e seus resultados serão incorporados no artigo aqui proposto para a publicação.

Paralelamente, também foi realizado o estudo dos genes associados com o estágio de biotrofia e necrotrofia em *C. abscissum*, os quais foram encontrados no seu genoma, e o trabalho encontra-se em fase de montagem dos cassetes de fusão com a proteína *gfp*, para elucidar a lacuna que existe sobre a existência do estágio biotrófico em *C. abscissum* em flores de citros. Também se iniciou o estudo do gene *SNF1* na espécie *C. abscissum*, mas a existência de falhas no *draft* do genoma disponível trouxe dificuldade na amplificação dos fragmentos para montagem dos cassetes de deleção. De qualquer forma, as metodologias existentes e as que foram padronizadas neste estudo poderão ser aplicadas para a espécie *C. abscissum*, contribuindo para novos estudos de interação planta-patógeno nessa espécie.

7. CONCLUSÕES GERAIS

Com base nas condições testadas e resultados obtidos, o presente estudo permitiu observar que:

- Nos pomares analisados as espécies *Colletotrichum abscissu*m e *C. gloeosporioides* são associadas à doença Queda Prematura de Frutos Cítricos, sendo a espécie *C. abscissum* encontrada em maior frequência;
- Os isolados de *C. abscissum* analisados pertencem a uma única população genética.
- O uso do fungicida benomil pode estar selecionando isolados de *C. gloeosporioides* resistentes nos pomares analisados;
- O gene *SNF1* está presente no genoma de *C. graminicola* e sua deleção promove a redução nas taxas de crescimento vegetativo, especialmente em fontes de carbono complexos;
- O gene *SNF1* é importante para a completa virulência de *C. graminicola*.
- O exato mecanismo de ação do gene *CgSNF1* permanece ainda não esclarecido.

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