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Análise da influência das fontes de carbono na patogenicidade do *Moniliophthora perniciosa* em *Theobroma cacao*

Este exemplar corresponde à redação final da tese defendida pela candidata Fátima Cerqueira Alvim e aprovada pela Comissão Julgadora

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Genética de Microorganismos.

Orientador: Prof. Dr. Michel George Albert Vincentz

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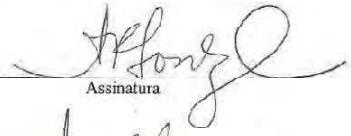

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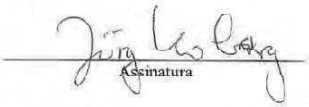

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Ao meu pai, Paulo Alvim,

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RESUMO

O fungo basidiomiceto hemibiotrófico *Moniliophthora perniciosa*, agente causal da doença vassoura-de-bruxa (VB) em *Theobroma cacao*, é o principal patógeno da lavoura cacaueira nas Américas e no Caribe. O presente trabalho apresentou como objetivo primordial identificar proteínas relacionadas com a patogenicidade deste fungo. No primeiro capítulo desta tese foi demonstrado o efeito de diferentes fontes de carbono sobre a morfologia e fisiologia do *M. perniciosa.* O fungo foi crescido em fontes de carbono fermentáveis e/ou não-fermentáveis. Foram observadas diferenças significativas na morfologia do micélio que se correlacionaram com a fonte de carbono utilizada. Foram observadas também diferenças qualitativas e quantitativas marcantes para as proteínas secretadas (secretoma) pelo micélio. O ensaio biológico efetuado em folhas de *Nicotiana tabacum* revelou que a capacidade do secretoma em induzir necrose nos tecidos vegetais também diferia em função da fonte de carbono utilizada pelo fungo durante o seu crescimento. O glicerol, como fonte única de carbono, foi identificado como o composto que mais induziu a atividade necrótica do secretoma do patógeno. O nível do chaperone molecular BiP (Binding Protein) em meristema de cacau aumentou em resposta à infiltração do secretoma de *M. perniciosa*, indicando uma resposta fisiológica do hospedeiro às proteínas secretadas *in vitro* pelo patógeno. Foi sugerido então que o metabolismo energético de *M. perniciosa*, dependendo das fontes de carbono aplicadas, resulta em alterações fisiológicas na expressão e secreção de proteínas e que esses efeitos atuam, não apenas afetando o crescimento do fungo, mas também, na capacidade de expressar proteínas de patogenicidade. No segundo capítulo investigamos mais detalhadamente as modificações induzidas por glicerol em *M. perniciosa.* Inicialmente demonstramos que glicerol induz a secreção de proteínas relacionadas com indução de morte celular especificamente no *M. perniciosa* dicariótico,

tipo celular característico da fase necrotrófica do patógeno. O secretoma induzido por glicerol foi o único capaz de induzir necrose em meristemas de cacau do genótipo resistente a *M. perniciosa*. Simultaneamente, o glicerol induziu alterações na composição/estrutura da parede celular do micélio tornando-a mais resistente a agentes indutores de estresse em parede celular, como o SDS e vermelho congo. Adicionalmente, o micélio apresentou maior nível de transcritos de catalases, indicando uma maior resistência a estresse oxidativo. Análises em géis bi-dimensionais e fracionamento das amostras em HPLC de alta capacidade, ligados a espectrometria de massas, resultaram na identificação de proteínas secretadas que acumularam especificamente em resposta ao glicerol. Proteases e lipases, enzimas hidrolíticas relacionadas com a patogenicidade em outros fungos hemibiotróficos, também apresentaram aumento de atividade em resposta a glicerol. Essas alterações correlacionaram com um aumento no nível de transcritos do gene *Rho1p*-g*ef*, um elemento chave da rota RHO1p GTPase, a qual sabidamente está relacionada a virulência de fungos fitopatogênicos. Análises conduzidas com isolados de *M. perniciosa,* que apresentaram diferentes graus de patogenicidade em ensaios conduzidos em casa de vegetação, demonstraram que a sensibilidade a glicerol é maior nos genótipos mais patogênicos. Esse estudo ressalta a importância do glicerol como uma molécula chave na interação cacau: *M. perniciosa*.

ABSTRACT

The basidiomycete hemibiotrophic fungus *Moniliophthora perniciosa*, the causal agent of witches' broom disease in *Theobroma cacao,* is the major cacao disease pathogen present in the Americas and the Caribbean. A compatible interaction of this fungus with its host comprehends a series of concerted biochemical and molecular events. In the first chapter of the thesis, we performed analyses where quantitative and qualitative relationships were found between secreted proteins and their activity, and the hyphal morphology of *Moniliophthora perniciosa*. This fungus was grown on fermentable and non-fermentable carbon sources; significant differences in mycelial morphology were observed and correlated with the carbon source. A biological assay performed with *Nicotiana tabacum* leaves revealed that the necrosis-related activity of extracellular fungal proteins also differed with carbon source. There were clear differences in the type and quantity of the secreted proteins. In addition, the expression of the cacao molecular chaperone BiP (HSP 70) increased after treatment with secreted proteins, suggesting a physiological response to the fungus secretome. We suggest that the carbon source-dependent energy metabolism of *M. perniciosa* results in physiological alterations in protein expression and secretion; these may affect not only *M. perniciosa* growth, but also its ability to express pathogenicity proteins. In the second chapter, we showed that glycerol efficiently triggers the production of secreted pathogenicity proteins by *M. perniciosa*, as revealed by the increased ability of the secreted proteins (secretome) in promoting cell death on *Nicotiana benthamiana* cell suspensions, *N. tabacum* leaves and meristems of a resistant cacao genotype, when compared to other carbon sources such as glucose. Simultaneously, glycerol induces cell wall modifications turning hyphae more resistant to inducers of cell wall stress and

increasing the resistance of the fungus to oxidative stress. These modifications correlated with the up-regulation of *Rho1p-gef*, a key element of the RHO1p GTPase pathway that is known to be related to fungal virulence in plants. Two-dimensional gel electrophoresis and high throughput HPLC coupled with mass spectrometry resulted in the identification of secreted proteins that specifically accumulated in response to glycerol. This study highlights the importance of glycerol as a key molecule modulating the fungus-induced pathogenicity.

INTRODUÇÃO

1. O patossistema *Theobroma cacao*: *Moniliophthora perniciosa*

A lavoura do cacau sempre foi a maior fonte de renda da região sul da Bahia. Contudo, no final da década de 1980 essa atividade agrícola foi grandemente afetada principalmente pela introdução do agente etiológico da doença vassoura de bruxa do cacaueiro (*Theobroma cacao* L.): o fungo *Moniliophthora (=Crinipellis) perniciosa* (Stahel) Aime & Phillips-Mora. Esse é um patógeno destrutivo da cultura do cacau em toda América Latina e Caribe (Griffith et al., 2003).

O *M. perniciosa* é um basideomiceto hemibiotrófico pertencente à ordem Agaricales, família Tricholomataceae e abrange espécies com basideomas pileados, estipitados, lignícolas e capazes de reviver após secamento quando são umedecidos (Pegler, 1978). Até recentemente, o conhecimento disponível sobre o *M. perniciosa* era baseado em estudos de patogenicidade, testes bioquímicos e de incompatibilidade do patógeno no estágio saprofítico e uso de marcadores do tipo RAPD (Random Amplified Polymorphic DNA) (Andebrhan et al., 1998). Com base na gama de hospedeiros foram discriminados quatro biótipos de *M. perniciosa*: 1) biótipo C, que infecta, predominantemente, *Theobroma* e *Herrania spp.*; 2) biótipo S, que infecta membros da família das solanáceas; 3) biótipo L, predominantemente saprofítico e que coloniza diversos substratos; e 4) biótipo B, quer infecta *Bixa orellana* (Andebrhan & Furtek, 1994). O padrão cromossomal e de microsatélites é variável para os diferentes biótipos (Rincones et al., 2006).

Um alto grau de variabilidade genética e cromossomal são encontrados entre isolados do biótipo C da Bahia em comparação com os da Amazônia (Rincones et al., 2006). As análises genéticas indicam também que os isolados da Bahia, provavelmente

foram originados de dois genótipos somente (Andebrhan et al., 1999; Rincones et al., 2006), mas apesar da recente chegada do fungo na região (1989) já ocorre ampla variabilidade genética entre os isolados (Rincones et al., 2006; Gramacho et al., 2007).

Em cacau, os basidiosporos do *M. perniciosa* infectam preferencialmente regiões meristemáticas e induzem uma gama de sintomas nos ramos vegetativos, almofadas florais, flores e birros. Essa diversidade de sintoma é dependente da região da planta infectada, da constituição genética do hospedeiro (resistência ou suscetibilidade) bem como da patogenicidade do isolado, além dos efeitos de fatores abióticos em um ou mais dos fatores citados. Compreender a natureza dessas modificações vem sendo um desafio para os pesquisadores. Recentemente algumas das modificações bioquímicas do hospedeiro em resposta ao *M. perniciosa* foram reveladas por Scarpari e colaboradores (2005). Além disso, a possibilidade de manter o fungo na fase biotrófica ou necrotrófica em condições laboratoriais (Meinhardt et al., 2007; Pungartnik et al., 2009) vem possibilitando o estudo mais detalhado das necessidades bioquímicas e fisiológicas de cada fase do ciclo de vida do patógeno (Pires et al., 2009; Pungartnik et al., 2009).

Considera-se que a vassoura de bruxa é a doença mais séria do cacaueiro nos países onde ela existe, sendo que o grau de severidade varia em função das condições climáticas e dos tratos culturais da lavoura. Uma estimativa precisa a respeito das perdas provocadas unicamente pela doença não é simples de obter visto que o fungo reduz a produtividade não apenas diretamente, mas também de uma maneira indireta devido ao estado debilitado das plantas. Esta doença não causa a morte da árvore, a menos que infecte plantas jovens que podem morrer em conseqüência da destruição das regiões meristemáticas. Evans (1981) atribuiu que a queda na produção agrícola fique compreendida entre 50 e 90% dependendo dos tratos culturais aplicados a lavoura. No Brasil, particularmente, a produção anual de

cacau caiu de 400.000 para 120.000 toneladas em conseqüência da disseminação da vassoura de bruxa nas regiões produtoras (Bowers et al., 2001)

1.1.O ciclo de vida do *M. perniciosa*

Como patógeno hemibiotrófico o *M. perniciosa* apresenta uma fase biotrófica e uma fase necrotrófica. Em conjunto, essas provocam alterações histológicas, morfológicas, fisiológicas e temporais nos tecidos do cacaueiro (Frias et al., 1991; Orchard et al., 1994; Scarpari et al., 2005; Ceita et al., 2007). A infecção do cacau começa quando os tubos germinativos dos basidiosporos penetram em tecidos jóvens via estômatos, epiderme ou tricomas (Sreenivasan, 1989). Esta hifa primária possui aproximadamente de 5 a 20 μm de largura (Kilaru & Hasenstein, 2005), não apresenta grampo de conexão (Evans, 1980), se desenvolve no espaço intercelular da planta e coloniza, prefencialmente, os tecidos do córtex e da medula do caule (Silva & Matsuoka, 1999). As células são mononucleadas e por este motivo o micélio primário é também denominado de monocariótico (Evans, 1980). Com o progresso da doença, a hifa primária/monocariótica sofre uma transição para a secundária. Esta é mais fina (1,5 a 3,0 μm de largura), bi ou multinucleda, contem grampo de conexão e apresenta crescimento inter e intracelular (Kilaru & Hasenstein, 2005). Eventualmente, após a alternância de períodos de alta e baixa umidade, a hifa secundária produz, no ramo seco de cacau, basidiocarpos e conseqüentemente basidiósporos, completando o ciclo da doença (Figura 1).

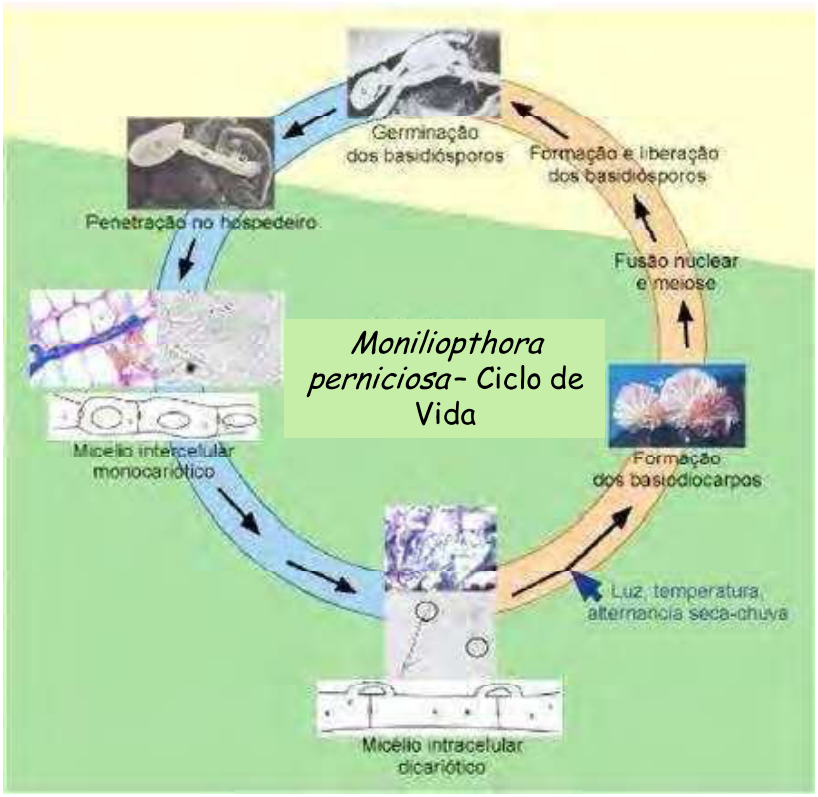

Figura 1- Ciclo de vida do *M. perniciosa* - Adaptado de Lopes, 2005.

A resposta do hospedeiro, localizada a partir do ponto de infecção, resulta na hipertrofia, hiperplasia e quebra de dominância apical dos ramos infectados, levando a um considerável aumento de tecidos e desenvolvimento de ramos laterais (Orchard et al., 1994, Ceita et al., 2007). Em conjunto, estes sintomas conferem ao ramo vegetativo do cacau a aparência de vassoura e a este ramo dá-se o nome de vassoura verde. O tempo estimado para se visualizar estes sintomas nas plantas infectadas varia, mas usualmente demora entre 5 e 6 semanas. As vassouras permanecem verdes por pouco período de tempo. O ramo começa a modificar a cor e após cinco semanas tornam-se marrons. A partir de então vai

progressivamente secando, a partir do ápice, até ficarem completamente secos, quando passam a ser denominados de vassoura seca. (Figura 2). A relação das fases miceliais de *M. perniciosa* com a morte dos tecidos infectados de cacau ainda não está bem esclarecida (Purdy & Schmidt, 1996). O que se observa na planta é a predominância do micélio monocariótico nas vassouras verdes e do micélio dicariótico nas vassouras secas (Evans e Bastos, 1980; Frias et al., 1992; Silva e Hasentein, 1999). ~~Por este motivo o micélio monocariótico é referido como o tipo celular padrão da fase biotrófica, e o micélio dicariótico o tipo celular representativo da fase necrotrófica, do *M. perniciosa* (Evans e Bastos, 1980; Meinhardt et al., 2007). Recentemente, foi demonstrado que o *M. perniciosa* produz hormônios e altera a concentração de auxinas e ácido salicílico endógeno em folhas de cacau infectadas (Kilaru *et al.*, 2007). Diversas modificações bioquímicas também ocorrem nos ramos infectados de cacau até o aparecimento da vassoura seca(Scarpari et al., 2005).

Desvendar os mecanismos moleculares envolvidos na interação cacau: *M. perniciosa* é imprescindível para compreender as peculiaridades do patossitema VB. Neste sentido, as informações geradas com seqüenciamento genômico (www.lge.ibi.unicamp/vassoura) e de bibliotecas de cDNA representativas das diferentes fases do ciclo de vida do *M. perniciosa* trouxeram informações relevantes para a compreensão das bases moleculares do desenvolvimento do patógeno (Rincones et al., 2008; Mondego et al., 2009; Pires et al., 2009). Essas informações, aliadas as geradas com o seqüenciamento de cDNAs representativos da interação compatível e incompatível de cacau: *M. perniciosa* (Gesteira et al., 2007) e outras pesquisas focando o patossistema VB, vem gerando informações importantes para a compreensão dos mecanismos moleculares envolvidos especificamente na interação cacau: *M. perniciosa* (Scarpari et al., 2005; Ceita

et al., 2007; Pirovani et al., 2008; Pungartnik et al., 2009; Pungartnik et al., 2009). Recentemente, Pungartnik e colaboradores (2009) sugeriram um modelo comparando os eventos fisiológicos e morfológicos que ocorrem durante o crescimento e desenvolvimento de *M. perniciosa* tanto *in vitro* quanto *in planta*.

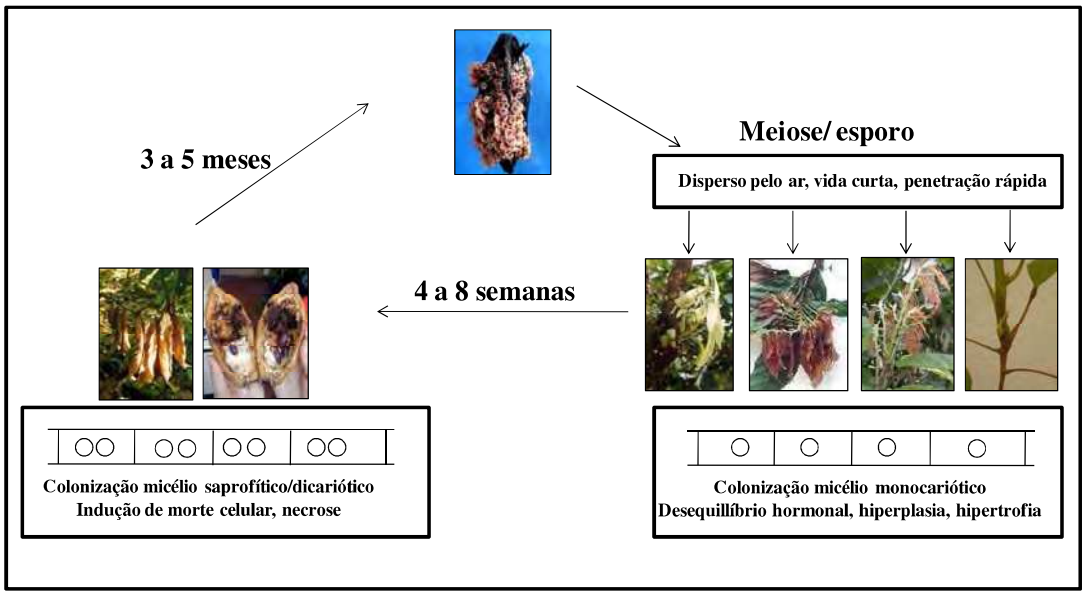

Figura 2. Ciclo de vida do *M.* perniciosa e os sintomas apresentados em ramos de cacau infectados.

Apesar do avanço das pesquisas na área da biologia molecular, os sinais envolvidos na cascata de eventos relacionados com a mudança de fase do *M. perniciosa* ainda não foram identificados. Contudo, o fato da dicariotização do micélio começar a ocorrer em regiões adjacentes às células apoptóticas do hospedeiro (Ceita et al., 2007), sugere que o aumento na disponibilidade de nutrientes para as células do patógeno (Kilaru & Hasentain, 2004; Meinhardt et al., 2007), ou o stress oxidativo (Pungartnik et al., 2009) podem ser

alguns dos sinais envolvidos na transição de fases. Os fungos hemibiotróficos começam a induzir a morte celular do hospedeiro a partir da transição da fase biotrófica para necrotrófica (Torto et al., 2003; Garcia et al., 2007; Kamoun, 2007). Portanto, identificar os sinais, bem como as rotas bioquímicas desencadeadas pelos mesmos, será importante para desenvolver novos programas de controle do patógeno e impedir a morte celular do hospedeiro (Monge et al., 2006).

2. Bases moleculares na interação planta:patógeno

Define-se como patogenicidade a capacidade absoluta de um microrganismo em promover o desenvolvimento da doença nos hospedeiros susceptíveis; e virulência como a quantidade relativa de doença provocada (Nelson et al., 1970).

A patogenicidade é resultado de um complexo processo definido pelo arsenal de mecanismos que possibilita o patógeno a invadir e adquirir nutrientes do hospedeiro. Até recentemente o conhecimento a cerca da patogenicidade de fungos estava limitado a estudos de estruturas especializadas de infecção, como os haustórios, a secreção de enzimas hidrolíticas e a produção de toxinas para os hospedeiros (Kamoun et al., 2007). Com o progresso das pesquisas, muito acentuada com a ampla utilização de ferramentas da genômica e proteômica, novos aspectos foram associados à patogenicidade, como composição da parede celular e resistência a estresse oxidativo (Zhao et al., 2005; Martínez-Rocha et al., 2008; Chi et al., 2009; Kamoun et al., 2009; Lu et al., 2009). Atualmente, sabe-se que os fungos fitopatogênicos atuam como manipuladores das atividades das células vegetais mediante a secreção de um arsenal de proteínas e pequenas moléculas denominadas de efetores. Os efetores atuam modulando a estrutura e função de células do hospedeiro de maneira a facilitar a invasão e colonização pelo patógeno (fatores

de virulência e toxinas), ou ativando os mecanismos de defesa da planta (fatores de avirulência e elicitores) (Hogenhout et al., 2009). Apesar de recente, o uso do termo "efetor" vem sendo cada vez mais utilizado pelos fitopatologistas (Hogenhout et al., 2009). Por ser um termo geral e neutro não atribui uma característica negativa ou positiva à molécula secretada pelo patógeno, como sugerido pelos termos virulência/avirulência, toxina e elicitor. O uso de um termo geral e neutro é também importante em face da observação de que uma mesma proteína pode ser de avirulência em determinado hospedeiro e de virulência em outro. Similarmente pode atuar como toxina, ao ser secretada em maior quantidade, ou elicitor, ao ter a sua secreção diminuída.

Geralmente, os efetores produzidos por microrganismos fitopatogênicos são secretados e liberados nos espaços inter- ou intracelular do hospedeiro e, dependendo das características genéticas dos organismos, promovem a infecção ou ativam o sistema de defesa da planta (Kjemtrup et al., 2000; Torto et al. 2003; Kamoun, 2006; Kamoun, 2007; Hogenhout et al., 2009). A interação entre *Cladosporium fulvum* e tomate ilustra bem o modelo mais aceito de interação planta-patógeno. *C. fulvum* secreta Avr2, um inibidor de protease que interage especificamente com $RcR3^{pim}$, uma cisteino protease presente no fluido apoplástico de tomate. Em tomates que possuem a proteína de resistência cf2, Avr2 não induz a doença, pois ativa os mecanismos de defesa do hospedeiro. Contudo, em plantas que não contêm cf2, Avr2 promove a infecção (Song et al., 2009). O balanço entre as atividades de proteases e seus inibidores, tanto do fungo quanto do hospedeiro, é importante também no estabelecimento de outros patossistemas (Shabab et al., 2008; Shindo & Van der Hoorn, 2008; Song et al., 2009). O uso de indutores químicos, como o acibenzolar-S-metil (ASM) (Resende et al.,2002), ou a colonização dos hospedeiros por fungos endofíticos (Arnold et al., 2003), são outros fatores que podem ativar o sistema de

defesa da planta e limitar os danos provocados pelo patógeno. Essas observações demonstram que o estabelecimento de um patossistema, seja ele compatível ou incompatível, implica em interações complexas entre as moléculas efetoras secretadas pelo patógeno e as de resposta produzidas pelo hospedeiro (Song et al., 2009; Yoshida et al., 2009).

Em ambos os organismos a expressão diferencial de genes deve ocorrer a partir da tentativa de invasão do patógeno. Por este motivo, o isolamento de cDNAs, representando genes de expressão induzida durante a interação patógeno-hospedeiro, auxilia na identificação de proteínas envolvidas no processo, facilitando o estudo de seus modos de ação. Esta estratégia já foi utilizada com sucesso na identificação de genes de resistência (*r*) de plantas e de virulência (*vir*) ou avirulência (*avr*) do patógeno, colaborando com a hipótese estabelecida por Flor, 1971 (Staskawicz et al., 1995; Song et al., 2009; Yoshida et al., 2009). Esta interação acarreta em uma resposta hipersensitiva (HR) que culmina com a morte rápida das células localizadas ao redor da infecção, o que impede a disseminação do patógeno (Dangl et al., 1996). Assim, quando ambos os genes, *r* e *avr*, estão presentes o que se observa é a resistência do hospedeiro ao microrganismo (Song et al., 2009). Com base nesse conhecimento, parte dos programas de melhoramento vegetal, conduzidos visando à resistência das plantas à patógenos, apresenta como meta a transferência de genes *r* para plantas com características agronômicas interessantes. Contudo, nem todos os processos de defesa do hospedeiro são intermediados por genes *r* (Van der Hoorn & Kamoun, 2008). A tradução de proteínas relacionadas com defesa (Heath, 2000), a formação de barreiras físicas (Walton, 1994) e a produção de metabólicos secundários e outros produtos naturais (Dixon, 2001) são alguns dos mecanismos que são independentes da presença de genes *r* e que ilustram a complexidade dos mecanismos de resposta em

patossistemas. Outro fator que desfavorece o controle de fitopatógenos baseado na presença de genes *r* é que devido à especificidade da interação *r: avr*, toda vez que ocorre a modificação na população genética do patógeno, ocorre também a quebra da resistência apresentada pelo hospedeiro (Scholthof, 2001). Assim, os controles mais eficientes de fitopatógenos têm como estratégia debelar o patógeno com base em suas características moleculares. Neste sentido, a identificação de genes que codificam para proteínas relacionadas à patogenicidade dos microrganismos é uma estratégia cada vez mais utilizada visando definir métodos mais eficientes de controle da doença (Torto et al., 2003; Monge et al., 2006; Ellis et al., 2007; Kamoun et al., 2007; Yoshida et al., 2009).

A genômica, ao disponibilizar grandes quantidades de seqüências gênicas, gerou a oportunidade de desenvolver e testar programas de bioinformática que visam identificar proteínas secretadas relacionadas com a patogenicidade de microrganismos. Nesta área, um dos trabalhos pioneiros foi o realizado por Torto e colaboradores (2003) ao desenvolverem um programa denominado pexfinder (*Phytophtora* extracellular proteins) objetivando identificar proteínas secretadas por *Phytophtora infestans* relacionadas com o desenvolvimento da doença. Nesse estudo foram identificadas duas novas proteínas indutoras de necrose, denominadas de *crn1* e *crn2*. Utilizando essa mesma estratégia, Bos e colaboradores (2003) identificaram um gene candidato a ser de avirulência denominado de *scr91*. A bioinformática vem também desenvolvendo programas que visam comparar genomas de patógenos e hospedeiros com o intuito de identificar novos genes *r* e *avr*. Recentemente, a "associação genética" identificou três genes de avirulência de *M. oryzae* (Yoshida et al., 2009). Nesse sentido, o projeto genoma do *M. perniciosa* bem como o

genoma funcional do *M. perniciosa* vem contribuindo fortemente por seqüenciar genes do patógeno disponibilizando-os para posteriores estudos funcionais.

Os avanços na área da genômica vêm permitindo a identificação massiva de proteínas alvo utilizando-se metodologias de alto desempenho, tal qual a análise via espectrometria de massas de amostras complexas (Tan et al, 2009). As análises proteômicas apresentam a vantagem de poderem ser também utilizadas em estudos de microrganismos que possuem pouca ou nenhuma informação genômica disponível (Medina et al., 2005). De maneira geral, as análises incluem a separação e o isolamento de proteínas em géis de poliacrilamida bidimensionais seguido do seqüenciamento dos peptídeos isolados via espectrometria de massa (Hooshdaran et al., 2005). Contudo, proteínas com PI extremos, de baixo peso molecular, pouco abundante ou não solúveis são dificilmente detectadas em géis 2-DE. Como resultado, técnicas como o MudPIT, que perpassam a separação das amostras em géis bidimensionais, foram desenvolvidas e vem sendo amplamente utilizadas. Esta técnica se baseia na digestão tríptica de amostras complexas de proteínas, seguida da separação dos peptídeos em LC e subseqüente análise em MS (Washburn et al., 2001). A proteômica vem sendo utilizada em estudos que objetivam elucidar mecanismos moleculares complexos envolvidos na patogenicidade, na morfogênese de fungos fitopatogênicos, nas respostas dos patógenos durante a interação com o hospedeiro e nos seus processos de resistência (Solomon et al., 2004; Srinivasa et al., 2004; Lee et al., 2009; Tan et al., 2009). A proteômica, ao permitir a identificação de proteínas diferencialmente expressas em resposta a presença ou ausência de determinado composto, vem auxiliando na elucidação das rotas de sinalização genética, principalmente as envolvidas nos processos de patogenicidade e resistência (Bro et al., 2003; Tan et al., 2009).

Identificar as cascatas de eventos induzidas durante a interação planta patógeno mostra-se fundamental para a compreensão dos mecanismos envolvidos tanto com a patogenicidade como com a resistência. Até o momento diversas rotas bioquímicas já foram ligadas a patogenicidade de fungos hemibiotróficos. Entre essas, pode-se citar a HOG (high osmolarity glycerol) e as MAP-kinases (mitogen-activation-protein kinase, Xu & Hamer,1996; Zhao et al., 2005) dentre as quais a *MKC*1 (mitogen-activated-protein kinase 1), também conhecida como Rho-cell wall integrity (CWI, Martínez-Rocha et al., 2008). Essas rotas de sinalização podem atuar individualmente ou apresentam pontos de interligação, o que aumenta a complexidade do processo e dificulta a identificação de elementos específicos de cada rota (Monge te al., 2006). Por exemplo, elementos que participam da rota HOG, que é desencadeada por modificações no turgor celular, podem também desencadear a rota CWI, em situações onde a estrutura da parede celular seja afetada devido a um estresse hídrico intenso (García-Rodriges et al., 2005, Monge et al., 2006). As mudanças fenotípicas desencadeadas por estas rotas muitas vezes são também similares. Recentemente, um estudo conduzido com promotores isolados de quatro genes de uma família multigênica de quitina sintase (*ch1, ch2, ch3 e* ch4) revelou que todos os genes tem a expressão aumentada em resposta a estresses induzidos na parede celular e são controlados pela ação coordenada das rotas CWI e HOG (Lenardon et al., 2009). Essa aparente sobreposição de ações figura na realidade como um elaborado mecanismo de defesa do patógeno, visto que a ação coordenada das rotas apresenta um efeito sinergístico para o microrganismo (Monge et al., 2006; Lenardon et al., 2007). Alguns dos sinais que desencadeiam as rotas bioquímicas HOG e MAPK já foram identificados. Entre eles pode-se citar o estresse oxidativo, geralmente aumento na concentração de peróxido de hidrogênio, mudanças na temperatura, fitohormônios, como auxinas, etileno e ácido

ascórbico, estresse osmótico e fontes de carbono (Monge et al., 2006). Todos esses compostos tem a concentração modificada durante a interação cacau: *M. perniciosa* (Scarpari et al., 2005; Ceita et al., 2007; Kilaru et al, 2007) e portanto podem servir de sinalizadores na VB.

Pesquisas utilizando fungos mutantes vêm auxiliando na identificação de proteínas envolvidas na sinalização em cascata da HOG e PKC, como receptores de membrana plasmática, quinases e fatores de transcrição (Hogmann, 2002; Monge et al., 2006; Lenardon et al., 2009). Em organismos modelo, o bloqueio das rotas de sinalização HOG e PKC demonstrou interferir na virulência do patógeno (Monge et al., 2006). Este conhecimento abre novas possibilidades para os programas de controle do patógeno. A identificação e validação, mediante mutação ou mesmo silenciamento via RNAi (Caribé dos Santos, et al., 2009), desses novos genes alvo abre novas perspectivas para o desenvolvimento ou identificação de moléculas com potencial fungicida. Essa estratégia difere das anteriores, onde se buscavam moléculas para interagir com proteínas de virulência tais quais a própria MpNep (Garcia et al., 2007) do fungo *M. perniciosa*. Esta proteína está envolvida no desencadeamento do processo de morte celular programada em cacau (Ceita et al., 2007), processo essencial para a progressão da doença visto que o *M. perniciosa* é um fungo hemibiotrófico.

OBJETIVOS

No presente trabalho propôs-se o uso simultâneo de análises proteômicas e genômicas visando identificar proteínas extracelulares e sinalizadores relacionados com a patogenicidade do *M. perniciosa*. A identificação de novas proteínas de patogenicidade de *M. perniciosa* pode auxiliar os programas de combate ao fitopatógeno, seja por contribuir na compreensão das bases moleculares da interação planta:patógeno ou por identificar possíveis alvos para o desenvolvimento de drogas sintéticas.

RESULTADOS

Capítulo 1 – Artigo publicado

Carbon source-induced changes in the physiology of the cacao pathogen *Moniliophthora perniciosa* (Basideomycetes) affect mycelia morphology and secretion of necrosis-inducing proteins.

Capítulo 2 – Artigo submetido

Glycerol triggers RHO/CWI pathway and efficiently promotes secretion of pathogenicity proteins in *Moniliophthora perniciosa*, the pathogen of cacao Witches' Broom disease

Capítulo 1 – Artigo publicado

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EVOLUTION AND TECHNOLOGY

Carbon source-induced changes in the physiology of the cacao pathogen *Moniliophthora perniciosa* (Basidiomycetes) affect mycelial morphology and secretion of necrosis-inducing proteins

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ABSTRACT. Quantitative and qualitative relationships were found between secreted proteins and their activity, and the hyphal morphology of *Moniliophthora perniciosa*, the causal agent of witches' broom disease in *Theobroma cacao*. This fungus was grown on fermentable and non-fermentable carbon sources; significant differences in mycelial morphology were observed and correlated with the carbon source. A biological assay performed with *Nicotiana tabacum* leaves revealed that the necrosis-related activity of extracellular fungal proteins also differed with carbon source. There were clear differences in the type and quantity of the secreted proteins. In addition, the expression of the cacao molecular chaperone BiP increased after treatment with secreted proteins, suggesting a physiological response to the fungus secretome.

We suggest that the carbon source-dependent energy metabolism of *M. perniciosa* results in physiological alterations in protein expression and secretion; these may affect not only *M. perniciosa* growth, but also its ability to express pathogenicity proteins.

Key words: Protein secretion; Virulence; Enzyme activity; Hyphal morphology; Molecular chaperones

INTRODUCTION

Fungal secreted proteins (secretome) are a known key feature during the plant-microbe interaction, since they manipulate host signaling and metabolic pathways in order to provide a suitable environment for completion of the pathogen's life cycle (Hegedus and Rimmer, 2005; Kamoun, 2007). The broad spectrum of secreted proteins in a fungus-plant interaction ranges from structural proteins to enzymes, the majority being hydrolytic enzymes that enable the fungus to penetrate and infect host tissue (Knogge, 1996; Hegedous and Rimmer, 2005). Also, these secreted proteins can act as elicitors of host defense or, depending on the stage of the fungal life cycle, can be involved in the acquisition of nutrients (Feng et al., 2005; Kamoun, 2007).

Nutrient utilization pathways increase metabolic versatility and enable fungi to use a variety of complex compounds as nutrient sources (Keller and Hohn, 1997; Medina et al., 2004). During infection, the nutritional offer of the plant to a phytopathogenic fungus may change, e.g., during *Moniliophthora perniciosa* x *Theobroma cacao* interaction there is a clear correlation between fungal differentiation and plant responses (Evans and Prior, 1987; Frias et al., 1991), confirmed by biochemical and nutritional alterations that occur during *in vivo* infection (Orchard et al., 1994; Scarpari et al., 2005; Kilaru and Hasenstein, 2005; Kilaru et al., 2007).

The phytopathogen *Moniliophthora* (=*Crinipellis*) *perniciosa* (Stahel) Aime & Phillips-Mora is a hemibiotrophic basidiomycete that infects cacao (*Theobroma cacao* L.) in all producing countries of the Americas and Caribbean Islands, causing witches' broom disease (WBD). Interactions in this pathosystem are complex and include sequential alterations or differentiation in the fungus (germination, penetration, colonization, dikaryotization, and fructification; Silva et al., 2002; Kilaru and Hasenstein, 2005) and in the plant (hypertrophy, hyperplasia, branching, activation of defense responses, and cell death; Orchard et al., 1994; Scarpari et al., 2005; Ceita et al., 2007). Recently, in-depth studies of this fungus have been published: *M. perniciosa* was found to produce hormones and alter endogenous auxin and salicylic acid levels in infected cocoa leaves (Kilaru et al., 2007), as well as necrosis-inducing proteins during infection, i.e., *Mp*-NeP1 and *Mp*-NeP2, which are differentially secreted during the course of infection *in planta* (Garcia et al., 2007); Rincones et al. (2008) found that genes expressed in the biotrophic phase of *M. perniciosa* are under catabolite and nitrogen repression. Glycerol seems to play several roles in *M. perniciosa* biology; Santos et al. (2008) showed that *M. perniciosa's* resistance to stress depends on glycerol, and Pungartnik et al. (2009) showed that small amounts of hydrogen peroxide in glycerol medium induce the formation of clamp connections *in vitro* and that basidiospores are the most resistant life form of this fungus when compared to mono- and dikaryotic cells.

In this study, we evaluated the relationship between the general carbon metabolism of the dikaryotic life form of *M. perniciosa* with fungal morphology, the composition of its

secretome and the secretome's ability to induce plant cell death. While mycelial morphology seemed to be related to general energy metabolism, the secretory activity and secretome composition varied depending on the specific carbon source provided. Moreover, there was enhanced cell death in the plant by secretome activity when mycelia had been grown in glycerol.

MATERIAL AND METHODS

Moniliophthora perniciosa culture and growth conditions

Moniliophthora perniciosa isolate 553 was obtained from the CEPLAC/CEPEC (Cacao Research Center, Ilhéus, BA, Brazil) culture collection. Dikaryotic mycelia were grown on solid and in liquid mineral media (0.1% ammonium phosphate, 0.02% potassium chloride, 0.02% magnesium sulfate, 0.5% yeast extract, 0.01% copper sulfate, and 0.01% zinc sulfate) supplemented with 20 mM of one of the following carbon sources: glucose (GLU), glycerol (GLY), mannitol, galactose, fructose, mannose, or sucrose. We also tested the addition of two different carbon sources to the mineral medium (GLU + GLY, GLU + galactose, GLU + mannitol, GLU + sucrose; 10 mM of each carbon source) and mineral medium with no addition of carbon source. Mycelia were grown in the dark, at 25°C for 4, 7, 10, or 14 days, with no shaking.

Monitoring growth of *M. perniciosa* and microscopic analysis

Fungal growth on solid media was determined as the diameter of the mycelium derived from one mycelium disc of 1 cm, after 10 days. The biomass of mycelium grown in liquid was determined by isolation via filtration; the mycelium was freeze-dried (Labconco) for 48 h and then weighed. Results are reported as dry weight.

Light microscope analyses were performed of apical tips from 4- or 7-day-old *M. perniciosa* cultures. Photographs were taken of either fresh hyphae (liquid culture) visualized with a Leica microscope (DM RA2, Germany, 100X) or apical tips (solid medium) with an Olympus microscope (DX40, phase contrast, 40X).

Protein quantification of secretome and SDS-PAGE

The secretome (30 mL) was separated from 10-day-old mycelium of *M. perniciosa* liquid cultures by filtration (0.45 µm, Millipore), freeze-dried (Labconco, USA) for 48 h, and resuspended in 5 mL phosphate buffer (PB, 10 mM, pH 5.5). Protein quantification was performed with a 2-D quantification kit, according to manufacturer recommendations (GE Healthcare). Proteins were resolved on SDS-gels (12.5%) and visualized after staining with Colloidal Coomassie G-250 solution (Neuhoff et al., 1988).

Biological activity assay of secretome

Peroxidase activity

From each secretome of *M. perniciosa*, 2.5 µg extracellular proteins was used. Samples were added to an ELISA plate and mixed (1:1, v/v) with a peroxidase activity buffer (50 mM

acetate buffer, H_2O_2 and guaiacol). Plates were immediately read on a kinetic dosage program (4 reads with 30-s intervals), at 570 nm, on a microplate reader (Molecular Device). Peroxidase activity was indicated by the consumed guaiacol/min (nmol guaiacol·mg protein^{-1}·min^{-1}).

Infiltration in tobacco leaves

Secretome from a 14-day-old *M. perniciosa* culture was desalted in a PD-10 Sephadex™ G-25M column (GE Healthcare), and proteins were recovered in PB (10 mM, pH 6.0), quantified using the Bradford assay (Bradford, 1976), and adjusted to a final concentration of 0.5 mg/mL (w/v). One hundred microliters of each protein suspension obtained was allowed to infiltrate *N. tabacum* (variety Havana) leaves. Controls were infiltrated with PB and also with sterile medium. The plants were maintained in a greenhouse and visually evaluated daily until necrotic symptoms were detected (up to 7 days). Images were acquired with a loupe, at 10X magnification (EZ4 D, Leica, Germany).

T. cacao meristem infiltration and induction of binding protein

Total proteins were isolated from a mix of 20 *T. cacao* meristems, 3 days after infiltration with either a GLY-grown *M. perniciosa* secretome, *Mp*-NeP2 (*M. perniciosa* necrosis-inducing protein; Garcia et al., 2007), PB (10 mM, pH 5.5) or a suspension of *M. perniciosa* basidiospores. The meristems were frozen in liquid nitrogen and immediately submitted to protein extraction according to Pirovani et al. (2008). Precipitated protein was resuspended in sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM dithiothreitol). Protein concentration was determined using a 2-D quantification kit according to manufacturer recommendations (GE Healthcare).

Equivalent amounts of *T. cacao* meristematic proteins (20 µg) were resolved with SDS-PAGE (12.5%) and transferred to nitrocellulose membranes using the Mini-Protean blot apparatus II XI cell (Bio-Rad) according to manufacturer recommendations. BiP (binding protein) was detected with a polyclonal antibody at a 1:1000 dilution (Figueiredo et al., 1997) followed by an anti-rabbit IgG antibody conjugated to alkaline phosphatase at a 1:5000 dilution (Promega). The activity of alkaline phosphatase was assayed using 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium (Promega) as recommended by the manufacturer.

Statistical analyses

All results are the means of at least 3 independent experiments and error bars represent standard deviation; when necessary, data were analyzed using two-way ANOVA and the Tukey test for analysis of variance as calculated by the Instat program® and set at $P < 0.01$.

RESULTS

M. perniciosa growth, determined as the mycelial radial growth on solid media, was impaired when fungi grew with mannitol as carbon source or in the absence of carbon. In comparison, GLY, mannose and fructose accelerated *M. perniciosa* growth. The highest growth rate was observed when *M. perniciosa* was grown with galactose, GLU or a mixture of GLU

and GLY (Figure 1A). Biomass production of *M. perniciosa*, determined as mycelium dry weight, was smaller in all non-fermentable, in comparison to fermentable carbon sources (Figure 1B). Growth on medium with no carbon was lowest and growth on GLU, fructose and mannose produced the highest *M. perniciosa* biomass. Moreover, growth on a mixture of GLU and GLY resulted in *M. perniciosa* biomass increment, in comparison to GLY alone.

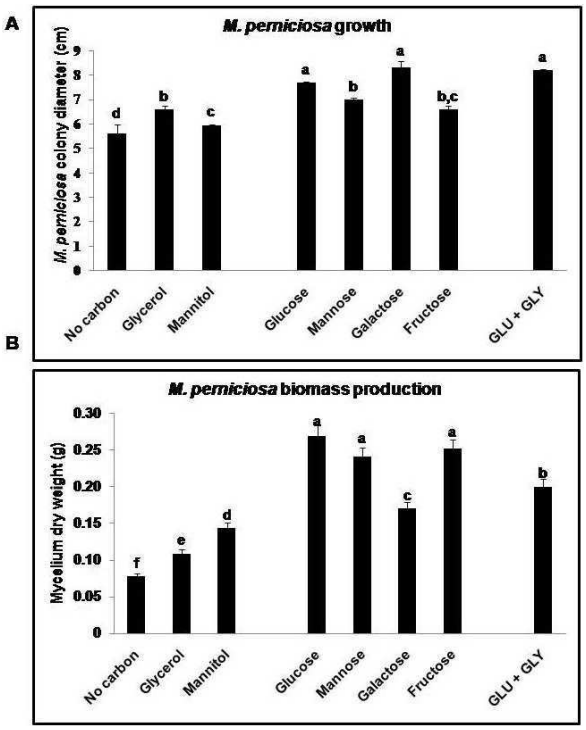


Figure 1. *Moniliophthora perniciosa* growth and biomass production is modified depending on the carbon source. Growth on a non-fermentable or with no carbon source impairs *M. perniciosa* growth (A) and biomass production (B) in comparison to growth on fermentable carbon source. Graphs are representative of 3 biological experiments, 10 days of growth. GLU = glucose; GLY = glycerol. Same letters do not differ statistically.

Growth on solid media on Petri dishes revealed an initial difference in *M. perniciosa* mycelial phenotype that persisted throughout the period of observation (Figure 2). Typically, growth with fermentable carbon sources, such as GLU, mannose and fructose, induced a more compact (C)-mycelium, in comparison to growth on non-fermentable carbon sources. C-mycelium is dense, shows high adherence between hyphae and has low amount of aerial hyphae. In contrast, growth on a non-fermentable carbon source, such as GLY or mannitol, or without added carbon source induced a flocculent (FC)-mycelium phenotype. Characteristically, hyphae were more dispersed as they did not have much adherence to each other or to the solid culture medium. Besides that, aerial hyphae were abundant in this growth type. Galactose and sucrose were the only fermentable carbon sources tested that induced the FC-mycelium phenotype (Table 1). Moreover, *M. perniciosa* grown in the presence of a mixture of GLU

and GLY, as well as GLU and galactose, GLU and mannitol or GLU and sucrose, showed a C-mycelium phenotype, the same observed when growth was in GLU alone (Table 1).

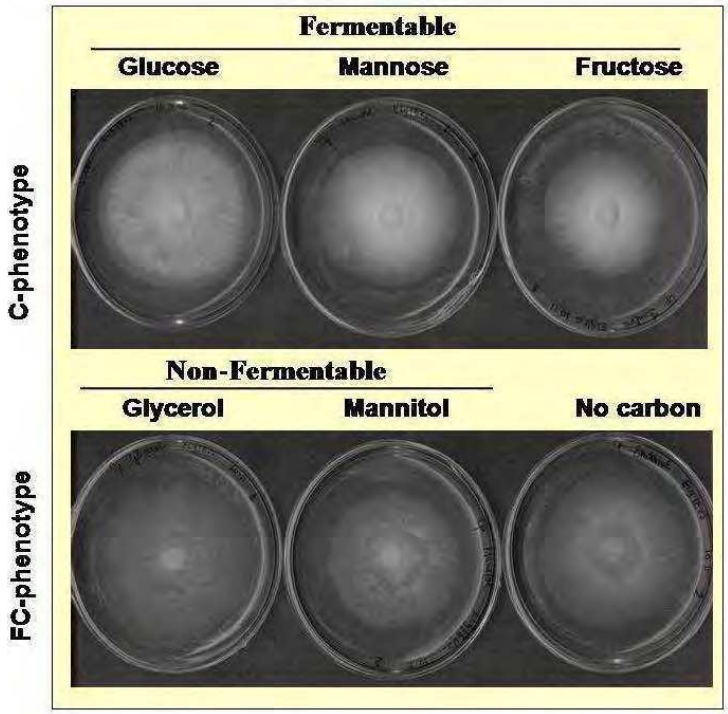


Figure 2. Carbon source affects *Moniliophthora perniciosa* mycelial phenotype. Fermentable carbon source induced a more compact *M. perniciosa* growth pattern (C-phenotype) whereas non-fermentable or no additional carbon source (No carbon) induced a less compact and more aerial (flocculent) mycelial phenotype (FC-phenotype). Photographs are representative of 10 replications, 10 days of growth.

Table 1. Carbon sources induce differences in *Moniliophthora perniciosa* mycelium phenotype.

Carbon source	C	FC
No carbon		+
Non-fermentable		
GLY		+
Mannitol		+
Fermentable		
GLU	+	
Mannose	+	
Galactose		+
Fructose	+	
Sucrose		+
Fermentable + non-fermentable		
GLU + GLY	+	
GLU + mannitol	+	
Fermentable + fermentable		
GLU + galactose	+	
GLU + sucrose	+	

Depending on the carbon source provided in culture medium, *M. perniciosa* developed a compact mycelium phenotype (C) or a flocculent mycelium phenotype (FC). GLY = glycerol; GLU = glucose.

Microscopic evaluation revealed that *M. perniciosa* FC-mycelium phenotype has thin and branched hyphae (Figure 3A, C, and E). In comparison, C-mycelium showed thicker and less branched hyphae (Figure 3B, D, F, and H). However, the fermentable carbon source galactose that induced the FC-mycelium produced thick hyphae (but less dense) like those observed for the C-mycelium phenotype (Figure 3G). A microscopic evaluation of *M. perniciosa* mycelium apical tips revealed that there is translocation of cell components from older to younger hyphae in all non-fermentable carbon source phenotypes, including galactose (Figure 4).

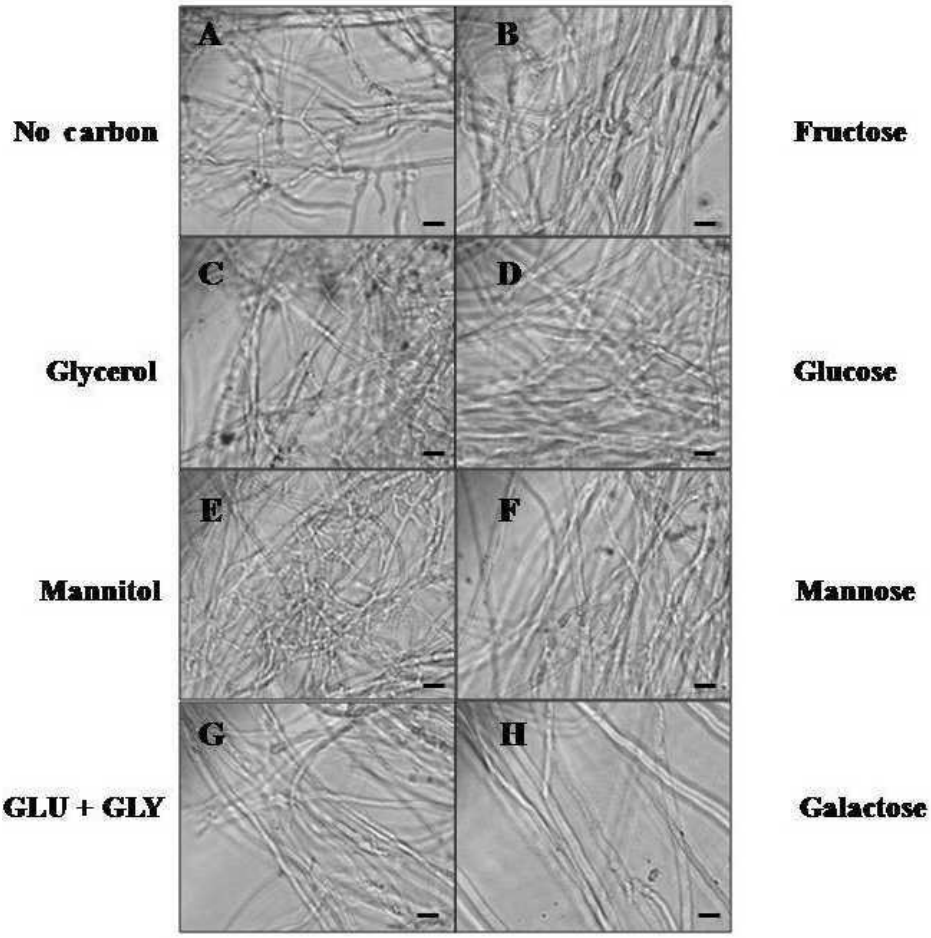


Figure 3. Hyphae of *Moniliophthora perniciosa* on non-fermentable carbon sources were thinner and highly branched. *M. perniciosa* grown with no additional carbon source (A) or non-fermentable carbon (C, E) showed thinner and more branched hyphae. Growth on fermentable carbon source (B, D, F) induced a thicker and more compact mycelium. In the presence of a fermentable + non-fermentable (G) carbon source, morphology was similar to the observed on fermentable growth. Galactose (H) induced a thick but less dense mycelium. Photographs are representative of 3 biological replications, 4 days of growth. GLU = glucose; GLY = glycerol. Bars = 10 µm.

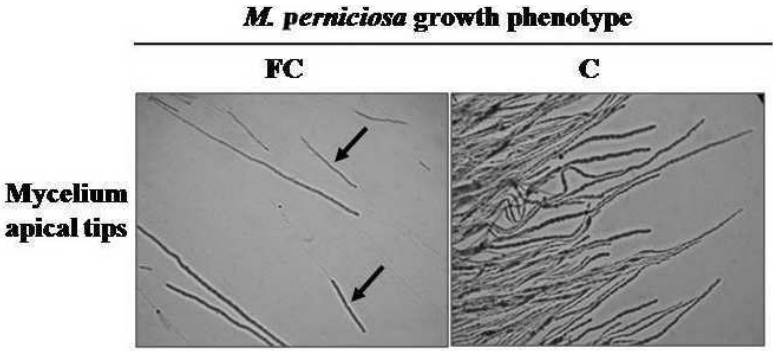


Figure 4. Remobilization of hyphal cellular components. Microscopic evaluation of *Moniliophthora perniciosa* mycelium revealed a translocation of cellular components from older to apical hyphae in flocculent (FC)-mycelium phenotype (arrows on left photograph). Compact (C)-mycelium were all filled (right). Photographs are representative of 3 independent biological replications, 4 days after inoculation. 40X magnification.

The offered carbon source also influenced *M. perniciosa* secretory activity, judged by the amount of secreted protein per gram of dry mycelium. Growth on medium with no carbon source other than yeast extract (no carbon) induced the highest secretory activity, in comparison to medium with the addition of a carbon source. Of all carbon sources tested, mannitol was the most efficient in inducing secretory activity (Figure 5A). The

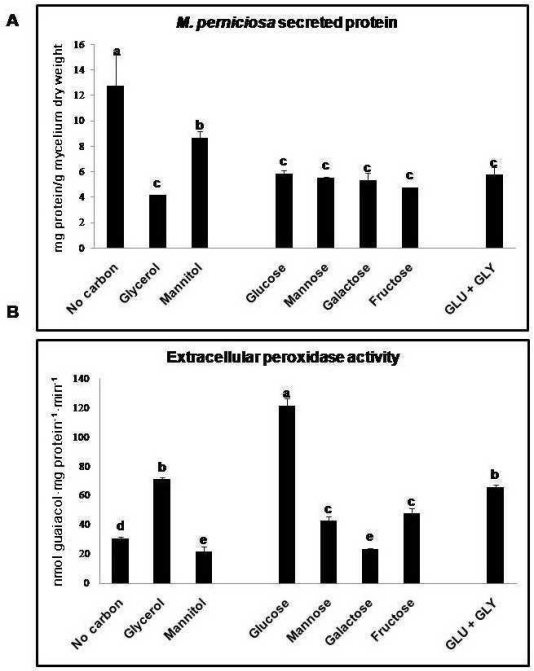


Figure 5. Carbon source affects *Moniliophthora perniciosa* secretory activity and secretome composition. Ten days after inoculation, *M. perniciosa* secretome was analyzed for extracellular protein (A) and peroxidase activity (B). Graphs are representative of 3 independent biological replications. GLU = glucose; GLY = glycerol. Same letters do not differ statistically.

addition of GLU to the mannitol carbon supplement resulted in the same lower secretory activity as observed when GLU was the only carbon source (data not shown). In contrast to secretome induction by mannitol, all other carbon sources resulted in a lower *M. perniciosa* secretory activity with no significant difference between them.

Extracellular proteins of *M. perniciosa* were resolved on SDS-PAGE gels in order to investigate possible changes in the composition of secretory proteins derived from growth in different carbon sources. Multiple protein bands were visualized in all secretomes derived from different growth conditions. The size of most of the proteins ranged from 60 to 20 kDa. We could identify changes in many protein bands according to the carbon source provided in the culture medium (Figure 6). We further conducted an activity assay of secreted enzymes to establish that modifications in the composition of the secretome's crude extracellular protein extract depend on the carbon source of the growth media. Extracellular peroxidase activity was quantified and indeed showed significant carbon source-dependent variation. Among the samples, secretome from GLU-grown *M. perniciosa* mycelia had the highest extracellular peroxidase activity, whereas the peroxidase activities from galactose- and mannitol-grown mycelia were the lowest. Secretome isolated from GLU + GLY-grown hyphae showed the same peroxidase activity as the one isolated from GLY-grown hyphae.

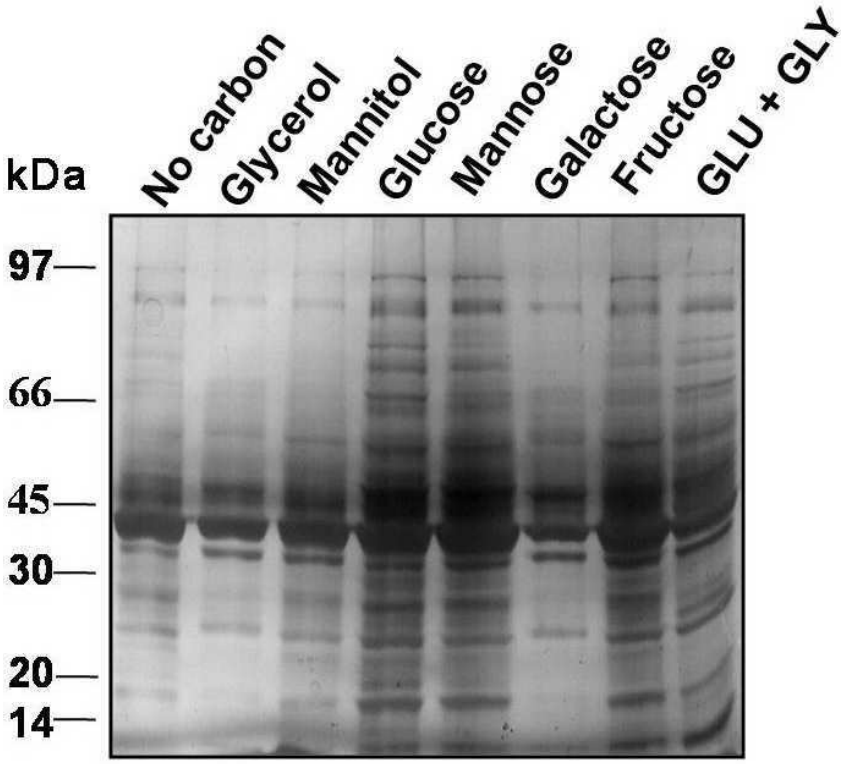


Figure 6. Different carbon sources affect *Moniliophthora perniciosa* extracellular protein profile. *M. perniciosa* secreted proteins were resolved on SDS-PAGE (12.5%) and visualized after Coomassie blue staining. Molecular weight marker (kDa) and carbon sources are indicated. GLU = glucose; GLY = glycerol.

We also evaluated *M. perniciosa* secretome ability to promote cell death in a biological activity assay in *Nicotiana tabacum*. All secretomes, independent of the carbon source used during fungal growth, were capable of inducing leaf necrosis. However, secretome of GLY-grown *M. perniciosa* was the most potent, as it caused the appearance of necrosis within five days (Figure 7), whereas all other secretomes required ten days.

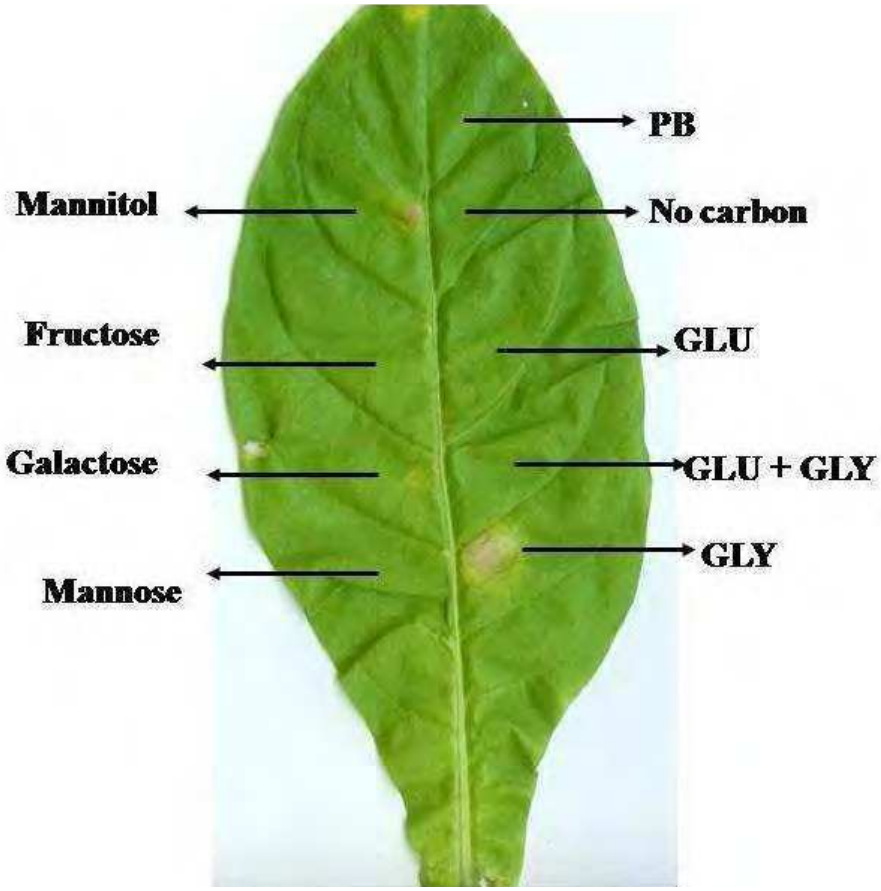
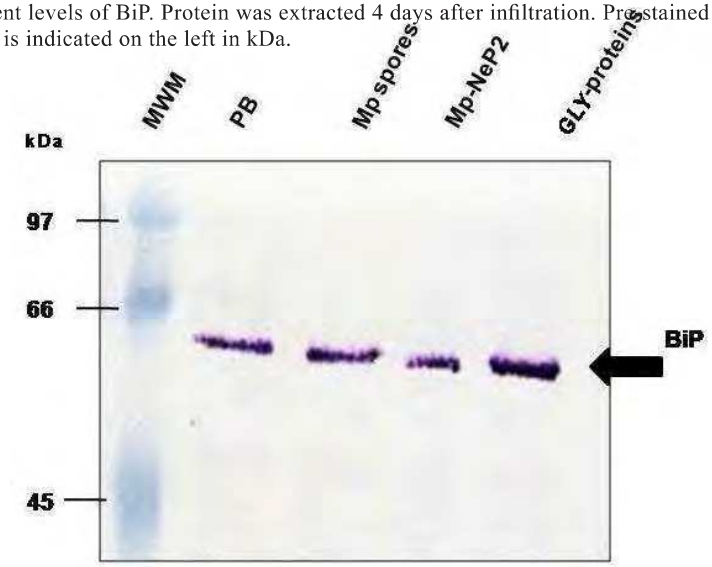


Figure 7. Necrosis symptoms induced by *Moniliophthora perniciosa* secreted proteins in *Nicotiana tabacum*. *M. perniciosa* was grown for 14 days with different carbon sources. Secreted proteins were desalted and recovered in phosphate buffer (PB). Same amounts of proteins were placed on *N. tabacum* leaves. Appearance of necrosis was observed daily. Photographs were taken 7 days after infiltration. GLU = glucose; GLY = glycerol.

An immunoblot assay performed with equal amounts of cacao proteins revealed that extracellular proteins of GLY-grown *M. perniciosa* secretome could up-regulate the expression of BiP in infiltrated cacao meristem (Figure 8). BiP levels also increased in response to *Mp*Nep2 (Garcia et al., 2007) infiltration and *M. perniciosa* spore germination. However, the latter two applications were less effective as compared to the proteins from the secretome of GLY-grown fungus (Figure 8).

Figure 8. Induction of binding protein (BiP) in infiltrated meristems of *Theobroma cacao* by glycerol-grown *Moniliophthora perniciosa* secretome. Equivalent amount of total protein (20 µg per lane) extracted from *T. cacao* (TSH 1188) meristems infiltrated with 10 mM phosphate buffer (PB), *M. perniciosa* spores (Mp spores), *Mp*-NeP2 and glycerol-grown *M. perniciosa* extracellular proteins (GLY-protein) were fractionated by 12.5% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-soybean BiP antibody (1:5000), revealing different levels of BiP. Protein was extracted 4 days after infiltration. Pre-stained molecular weight marker (MWM) is indicated on the left in kDa.



DISCUSSION

Carbon source induces differences in *M. perniciosa* growth, mycelium phenotype and hyphal morphology.

Microorganisms are often exposed to a broad range of variations, especially regarding the quality and availability of nutrients, which influence the progression of their life cycle, alter physiological and morphological patterns, and may influence the profile of secreted proteins (Thevelein et al., 2000; Oliver et al., 2002; Medina et al., 2004; Vanoni et al., 2005; Bouws et al., 2008). In yeast, the correlation of nutrient deprivation and these responses is well described. Usually, growth with GLU, or a related rapidly fermentable sugar, stimulates rapid growth and the development of larger cells (Thevelein et al., 2000; Vanoni et al., 2005). In the filamentous fungus *M. perniciosa*, rapid growth judged by biomass production was observed when mycelia grew in the presence of GLU or similar fermentable sugars, e.g., fructose and mannose, which enter the glycolytic pathway directly (Figure 1B). Fermentable carbon sources that are not promptly used by the cell, such as galactose, a non-cellulosic polysaccharide that is part of the plant cell wall structure, hindered fungal biomass production (Figure 1B). Besides growth, the facility in metabolizing different carbon sources also seems to affect *M. perniciosa* morphology. Growth in the absence of a carbon source, with non-fermentable carbon sources

or with galactose, all representing nutritional limitations in comparison to the addition of GLU, induced the development of branched and thinner mycelia; it also showed the translocation of components between hyphae (Figures 2, 3, and 4), a characteristic already reported for other fungi grown under nutritional limitation (Shoji et al., 2006). This phenotype correlates with a smaller biomass production and, with the exception of galactose, is related to a slower radial growth of mycelia (Figures 1A,B and 2). Probably, it is the intensive branching of mycelia induced by non-fermentable carbon sources that led to the observed slower growth (Figures 1A, and 2). The addition of GLU to the medium containing GLY (Figure 3), mannitol, sucrose or galactose (Table 1) reversed the mycelial phenotype, indicating that GLU is the preferentially used carbon source of this filamentous fungus. Since only 10 mM of this sugar was sufficient to reverse the mycelial phenotype, GLU may also act as a signal molecule.

The differences in *M. perniciosa* morphology were not related to intense nutritional limitation stress, as yeast extract, present in all culture media, contains many components including carbohydrates. In addition, growth with sucrose produced almost the same biomass as that with GLU (data not shown) but induced the non-fermentable carbon source phenotype (Table 1). Taken together, these data reinforce the hypothesis that mycelial morphology is not directly related to a nutritional stress situation but more likely to a cell sensing machinery that triggers pathways related to morphological changes. Indeed, in *S. cerevisiae*, GLU and galactose are sensed differently (Brown et al., 2009). Moreover, galactose inhibited the expression of genes that are commonly induced by GLU, mannose and fructose (e.g., hexose transporters; Brown et al., 2009). Carbon sources are well-known biochemical signals in other fungi (Gupta and Kaur, 2005), and special attention has been given to GLU, usually a preferred metabolized sugar (Verstrepen et al., 2004; Thevelein et al., 2005; Vanoni et al., 2005; Gancedo, 2008). The correlation of GLU with known signal cascades involved in fungal morphology and pathogenicity has been previously described for yeast (Vanoni et al., 2005; Santangelo, 2006; Cipollina et al., 2008) and for filamentous fungi (Oliver et al., 2002; Lee et al., 2003). In WBD, carbohydrate content undergoes alterations in *T. cacao* (Scarpari et al., 2005); hence, this may influence the life cycle and pathogenesis of *M. perniciosa*.

We further determined if the carbon source induces changes in *M. perniciosa* secretome composition. This has been described for other fungi that especially modify the set of secreted enzymes to adapt their metabolism to varying carbon sources (Bouws et al., 2008). In a first attempt, we analyzed the secreted protein profile on SDS-PAGE and detected some differences in specific protein bands (Figure 6). To better characterize and quantify the modification induced by different carbon sources in extracellular protein composition we performed a peroxidase activity assay. The production of this enzyme in *Coprinus* species is known to be affected by GLU concentration (Ikehata et al., 2004). The GLU-grown secretome of *M. perniciosa* exhibited the highest peroxidase activity (Figure 5B), and this activity declined with the depletion of GLU, as suggested by the results found in GLU + GLY-derived secretome (where GLU was only 10 mM). Taken together, the results reveal that carbon source indeed influenced the extracellular protein composition; however, in the particular case of peroxidase, enzyme activity was not correlated with general energy metabolism but it was influenced by the concentration of GLU in the medium.

Carbon source affects *M. perniciosa* secretory activity, secretome composition and ability to induce plant cell death

Beside protein composition, carbon source also influenced *M. perniciosa* secretory

capacity, as observed by protein quantification (Figure 5A). Nutrient deprivation, such as in the absence of carbon source or presence of mannitol, a hexose that is not produced in the plant and is usually used by fungi as a carbohydrate reserve (Solomon et al., 2007), induced the highest protein secretion in comparison to all other carbon sources tested. This was not related to a low specific growth rate, as in *Trichoderma reesei* (Pakula et al., 2005), or to the general energy metabolism of *M. perniciosa*, as suggested by our results with GLY (Figure 5A). Here, both non-fermentable carbon sources, mannitol and GLY, impaired growth (Figure 1), but only mannitol could induce high secretory activity in *M. perniciosa*. The observation that the addition of GLU to mannitol-supplemented medium reduced *M. perniciosa* secretory activity to the same level as that found for the secretome of GLU-grown hyphae suggests that secretory activity is to some extent related to the presence of GLU in the culture medium. This again reinforces the hypothesis that in *M. perniciosa*, as in other organisms, GLU may act as a signal molecule, apart from its role in nutrition.

The observed carbon source-induced modifications of the morphology of *M. perniciosa* mycelia together with changes in secretome composition could be correlated to the pathogenesis of this fungus, as observed for other microorganisms (Hegedus and Rimmer, 2005; Perez-Martin et al., 2006; Kamoun, 2007; Klosterman et al., 2007). Specifically, for hemibiotrophic and necrotrophic fungi, the induction of host cell death, a characteristic of pathogenesis, is related to the secretion of pathogenesis proteins into the inter- or intra-cellular space (Kamoun, 2007). Indeed, in WBD, the thinner and branched *M. perniciosa* hyphae, such as the ones observed after growth on non-fermentable carbon sources, are more abundant in a phase that slightly precedes necrosis of infected cacao tissues (Ceita et al., 2007). Thus, we speculate that all secretomes isolated from thin and branched *M. perniciosa* mycelia could have enhanced necrosis-inducing activity. However, a biological activity assay performed using *N. tabacum* leaves revealed that this is not the case. The morphology of *M. perniciosa* mycelium was not related to the necrosis-inducing potency of the secretomes, since only GLY growth-derived secretome showed an increment in necrosis activity (Figure 7). This suggests again that in *M. perniciosa*, like in other pathogens, carbon sources behave as signaling molecules in addition to their nutritional role (Oliver et al., 2002; Thevelein et al., 2005; Vanoni et al., 2005). Indeed, GLY was previously related to acquired resistance to paraquat and oxidative stress in *M. perniciosa* (Santos et al., 2008) and to maintenance of the monokaryotic life form of this fungus *in vitro* (Meinhardt et al., 2006).

Glycerol growth-derived extracellular proteins induce unfolded protein response in meristem of *T. cacao*

To further evaluate the differences in the activity of *M. perniciosa* secretomes, we tested the differences of the most effective secretome (GLY growth-derived) on tissues of *T. cacao*. The host response to the GLY growth-induced secretome was biochemically evaluated by analyzing the BiP levels in infiltrated meristems before the onset of necrosis (Figure 8). BiP is a molecular chaperone and resident of the endoplasmic reticulum of eukaryotic cells, which acts as a major regulator of the unfolded protein response (UPR) and whose level has a direct correlation with biotic or abiotic stress sensing (Alvim et al., 2001; Irsigler et al., 2007). Recent studies have connected cell apoptosis with endoplasmic reticulum stress that induces the activation of the UPR pathway (Lai et al., 2007). When

the UPR is perturbed or not sufficient to overcome the stress, it triggers pro-apoptotic pathways (Iwata and Koizumi, 2005). The increased BiP levels in meristems of *T. cacao* (TSH 1188) treated with GLY-grown *M. perniciosa* proteins, before the appearance of necrosis, suggest that the induction of endoplasmic reticulum stress takes place after secretome application (Figure 8) or that the plant secretory machinery is being prepared to secrete proteins involved in defense (Jelitto-Van Dooren et al., 1999). Indeed, levels of cystatine, a family of protease inhibitors that are at the front-line of defense against pathogens (Shen and Bogyo, 2008), increase in response to this GLY growth-induced extracellular protein extract before the appearance of necrosis in the infiltrated cacao meristem (Pirovani CP, personal communication). The higher BiP level in response to the GLY growth-induced secretome sample, in comparison to *Mp*-Nep2 infiltration or to *M. perniciosa* spore germination, indicates a more severe stress caused by this extracellular protein extract.

Taken together, our results demonstrate that *M. perniciosa* morphology and growth rate are regulated by the general energy metabolism. However, the secretory capacity, protein composition and expression of pathogenicity genes are more related to specific carbon sources, which may act as regulatory signals. Moreover, the results indicate that glycerol is involved in the signal cascade related to the expression of pathogenicity genes.

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Capítulo 2 – Artigo a ser re-submetido

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Glycerol triggers RHO/CWI pathway and efficiently promotes secretion of pathogenicity proteins in *Moniliophthora perniciosa*, the pathogen of cacao Witches' Broom disease

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Running head: RHO/CWI pathway induced in *M. perniciosa*

Summary

The hemibiotrophic fungus *Moniliophthora perniciosa* is the major cacao disease pathogen present in the Americas and the Caribbean. A compatible interaction of this fungus with its host comprehends a series of concerted biochemical and molecular events. We showed that glycerol efficiently triggers the production of secreted pathogenicity proteins by *M. perniciosa*, as revealed by the increased ability of the secreted proteins (secretome) in promoting cell death on *Nicotiana benthamiana* cell suspensions, *N. tabacum* leaves and meristems of a resistant cacao genotype, when compared to other carbon sources such as glucose. Simultaneously, glycerol induces cell wall modifications turning hyphae more resistant to inducers of cell wall stress and increasing the resistance of the fungus to oxidative stress. These modifications correlated with the up-regulation of *Rho1p-gef*, a key element of the RHO1p GTPase pathway that is known to be related to fungal virulence in plants. Two-dimensional gel electrophoresis and high throughput HPLC coupled with mass spectrometry resulted in the identification of secreted proteins that specifically accumulated in response to glycerol. This study highlights the importance of glycerol as a key molecule modulating the fungus-induced pathogenicity.

Keywords: *Moniliophthora perniciosa*, Glycerol, secretome, proteomics, cell death, cell wall integrity, guanine exchange factor, *Theobroma cacao*

Introduction

Witches' broom disease (WBD) of cacao, caused by the phytopathogen *Moniliophthora (=Crinipellis) perniciosa* (Stahel) Aime & Phillips-Mora, is present in all cacao (*Theobroma cacao* L.) producing countries of the Americas and the Caribbean

Islands. Currently, it is considered the most serious cacao disease, with severity degrees depending on climate and crop management. *M. perniciosa* is a hemibiotrophic basidiomycete with a parasitic (monokaryotic) and a saprophytic (dikaryotic) phase causing histological, morphological, physiological, and biochemical alterations during the development of the disease in cacao trees (Ceita et al., 2007; Scarpari et al., 2005; Orchard et al., 1994). In both, the pathogen and the host organisms, differential patterns of gene expression occur after pathogen penetration. Generally, elicitor molecules produced by phytopathogenic microorganisms are secreted and released into the inter- and intra-cellular host spaces and depending on the genetic characteristics of the pathosystem, promote infection or activate the plant defense system (Kamoun, 2007). The focus on the study of *M. perniciosa* secreted proteins can be an interesting strategy to discover novel pathogenicity proteins involved on WBD development.

Recently, the *T. cacao*: *M. perniciosa* interaction was analyzed at the genomic level (Gesteira et al., 2007; Leal Junior et al., 2007). There is also a description of the *M. perniciosa* functional (Rincones et al., 2008) and structural genomics (Mondego et al., 2008), cytology, biochemistry (Ceita et al., 2007; Scarpari et al., 2005) and a preliminary proteomic study on cacao interaction with *M. perniciosa* (Pirovani et al., 2008). However, very little is known about the signals involved in regulating the pathogenesis genes expressed during WBD. An important aspect of a hemibiotrophic interaction is related to the transition from the biotrophic (monokaryotic) to the necrotrophic (dikaryotic) phase which includes secretion of necrosis-inducing molecules such as proteins (Garcia et al., 2007; Qutob et al., 2002; Torto et al., 2003) and secondary metabolites such as oxalic acid (Kim et al., 2008). Oxalic acid may induce the accumulation of reactive oxygen species (H_2O_2 and OH^-) in plants, which could act as signal molecules to the fungi (Pungartnik et

al., 2009) ultimately leading to host cell death (Ceita et al., 2007; Shetty et al., 2007). Revealing relevant features of the mechanisms underpinning this phase change would be of great importance to further understand the WBD pathosystem. More specifically, an important aspect would be to identify signals that modulate the necrosis-inducing capacity of the fungus. Since the monokaryotic mycelium that precedes the dikaryotic necrotic phase is mainly growing in the intercellular space of the host (Frias et al., 1991), we assumed that apoplastic metabolic molecules represent good candidates for promoting necrotic activities. We therefore performed systematic experiments using plant metabolites such as carbon sources (Scarpari et al., 2005) and also plant hormones (Kilaru et al., 2007) whose concentration undergoes changes during WBD and that may induce the production of fungal pathogenic molecules. We identified glycerol (GLY) as an important nutrient that somehow enhances the *M. perniciosa* secretome promoting plant cell death (Alvim et al., 2009). Indeed, GLY seems to play several roles on *M. perniciosa* biology, once it is sufficient to maintain fungi cultures in the biotrophic phase (Meinhardt el al., 2007) and enhances *M. perniciosa* tolerance to oxidative stress (Santos et al., 2008).

Glycerol is a metabolite widely distributed within plant tissues whose levels was shown to increase in some hemibiotrophic fungi during plant interaction, in a phase just preceding necrosis in infected tissues (Scarpari et al., 2005; Wei et al., 2004). GLY also plays an important role in fungi osmoprotection favoring hyphal growth in infected tissues (Clark et al., 2003; de Jong et al., 1997; Jobic et al., 2007). During interaction of the hemibiotrophic fungus *Colletotrichum gloeosporioides* with, *Malva pusilla*, GLY is an important nutrient hose metabolism is not only related to the fungal life cycle but also to the pathogenesis process itself (Wei et al., 2004). Recently, GLY metabolism was also related to host defense against plant pathogens (Chanda et al., 2008; Chandra-Sekara et al., 2007;It

appears therefore that GLY and/or its metabolism are involved in both host defense and pathogen virulence processes.

In this study, we provide evidences that in *M. perniciosa* GLY triggers the expression of genes involved in pathogen defense, such as catalases, and promotes an increase in secreted hydrolytic enzymes such as proteases and lipases. Furthermore, GLY induced cell wall structure modification that seems to be related to increased fungal tolerance to stress and may be also partly related to the activation of the Rho1-protein kinase gene; (cell wall integrity pathway. Lengeler et al., 2000; Martínez-Rocha et al., 2008). The relationship between GLY and the up regulation of the pathogenicity pathway may help in elucidating the molecular mechanisms underlying the death of infected *T. cacao* tissues. This is important not only to understand the host/pathogen interaction process during WBD but also to improve disease control programs.

Results

Glycerol enhances the production of necrosis inducing proteins in M. perniciosa dikaryotic mycelia

In order to evaluate the involvement of carbon sources in the induction of necrotic activity from *M. perniciosa*, the same amount of extracellular proteins, which hereafter referred to as secretome, isolated from *M. perniciosa* GLY- or GLU-grown monokaryotic or dikaryotic hyphae, were infiltrated on *N. tabacum* leaves and the generation of lesions was monitored over time. Four days after infiltration (DAI) necrosis was visualized only in response to the GLY-grown dikaryotic secretome and was restricted to the infiltrated area (Fig. 1A and 1B). Infiltrated plants were allowed to grow for a further 15 day period under green house conditions but no necrotic symptoms were observed in response to the GLY-

grown monokaryotic secretome or to the phosphate buffer (PB), indicating that the necrotic activity, in this case, is restricted to the dikaryotic phase. Moreover, since the GLU- or GLU+GLY-grown dikaryotic mycelium were, unlike the GLY-grown mycelium, unable to induce any necrotic lesions 4 DAI (Fig. 1B), it can be deduced that the specific induction of dikaryotic necrotic activities by GLY is counteracted by GLU, which appears to have a dominant effect.

A set of additional approaches have been used to confirm these conclusions. First, necrotic activity was also evaluated on a line of *N. benthamiana* cell suspension expressing *GFP*. As observed for *N. tabacum*, the secretome from GLY-grown dikaryotic *M. perniciosa* was the most effective in inducing *N. benthamiana* cell death as compared to secretomes isolated from other carbohydrate growth conditions. Indeed, all cells appeared to be dead as early as 4 hours after infiltration (HAIF), while GFP fluorescence was still detected 36 h after treatment with protein extracts from other carbon sources (Fig. 1C). Moreover, when GLU and GLY were provided together, the phenotype observed was similar to the GLU-induced response (Fig. 1B and C).

Next, we tested the activity of *M. perniciosa* secretome on *T.* cacao, its natural host. Same protein amounts of GLY-grown monokaryotic and GLY or GLU-dikaryotic *M. perniciosa* induced secretomes were infiltrated in meristems of a cacao resistant (THS 1188, plants naturally present red stems) or susceptible (Catongo, plants present green stems) genotype (Fig. 2). Monokaryotic secretome was unable to induce necrosis on both genotypes even after an extended time period. Secretome from GLU-grown dikaryotic mycelium was able to induce necrosis only on the susceptible clone (catongo) and 7 DAI, while GLY-grown dikaryotic secretome promotes necrosis on the susceptible genotype more efficiently, as soon as 3 DAI. The higher necrotic activity associated with the GLY-induced secretome

was further confirmed by its capacity to induce necrosis on the resistant TSH 1188, 7 DAI. In addition, it was noticed that the symptom induced on the susceptible genotype (catongo) was more intense than the one observed on the resistant clone (TSH 1188) (Fig. 2). Plants were kept in green house for up to 30 days with no further differences on the results. The control used, phosphate buffer (PB), did not induce necrosis. Finally, we tested the capacity of GLY-secretomes to increase the ability of inducing necrosis of four *M. perniciosa* isolates exhibiting different levels of pathogenicity under greenhouse experiments. We performed an activity assay on *N. benthamiana*-GFP cell suspensions with secretome isolated from the less pathogenic genotype 1445 and from the three more pathogenic genotypes 641, 263 and 487, all of them being more pathogenic than the previously tested isolate 553 (Mondego et al., 2008). The three more pathogenic isolates were able to induce cell damage when grown in GLU (Fig. 3A and B) and the damages were clearly higher when the carbon source was GLY (Fig. 3A and B). No significant cell damages were observed by the extracts from the GLU-grown less pathogenic isolate 1445 (Fig. 3A and B, compare results with PB). However, the necrotic activity of this isolate was also enhanced when grown with GLY. Furthermore, our data established a correlation between the GLY-grown induced secretome activities in inducing *N. benthamiana* cell death, with the degree of virulence observed in field trials, where the isolate 487 is the most virulent followed by 263 and 641 (Dr. Karina Gramacho, personal communication).

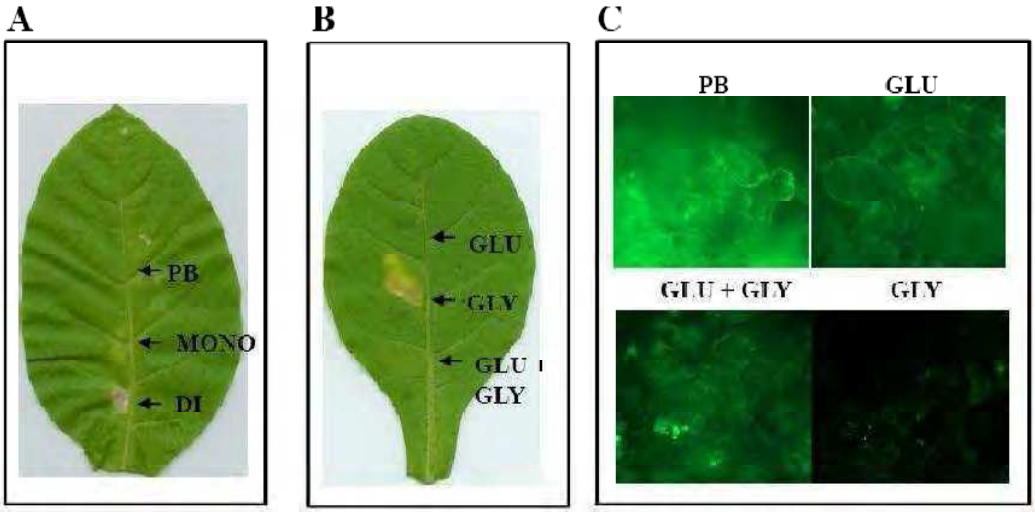

Figure 1 – Necrosis symptoms induced on *N. tabacum* leaves by the *M. perniciosa* secretome - A- same amounts of secretome proteins isolated from monokaryotic (Mono) or dikaryotic (Di) GLY-grown *M. perniciosa*, recovered on PB, were infiltrated on *N. tabacum* leaves. 7 DAI necrosis symptoms were observed only in response to dikaryotic secretome. PB used as control. On B, same amounts of secretome proteins from dikaryotic GLU, GLY or GLU + GLY grown *M. perniciosa* were infiltrated on *N. tabacum* leaves. 7 DAI necrosis symptoms were observed only in response to GLY-secretome. On C, same amounts of proteins from dikaryotic GLU, GLY or GLU + GLY-induced secretome were infiltrated on *N. benthamiana*-GFP cells. Pictures representative of cells at 4 hours after infiltration. PB used as control.

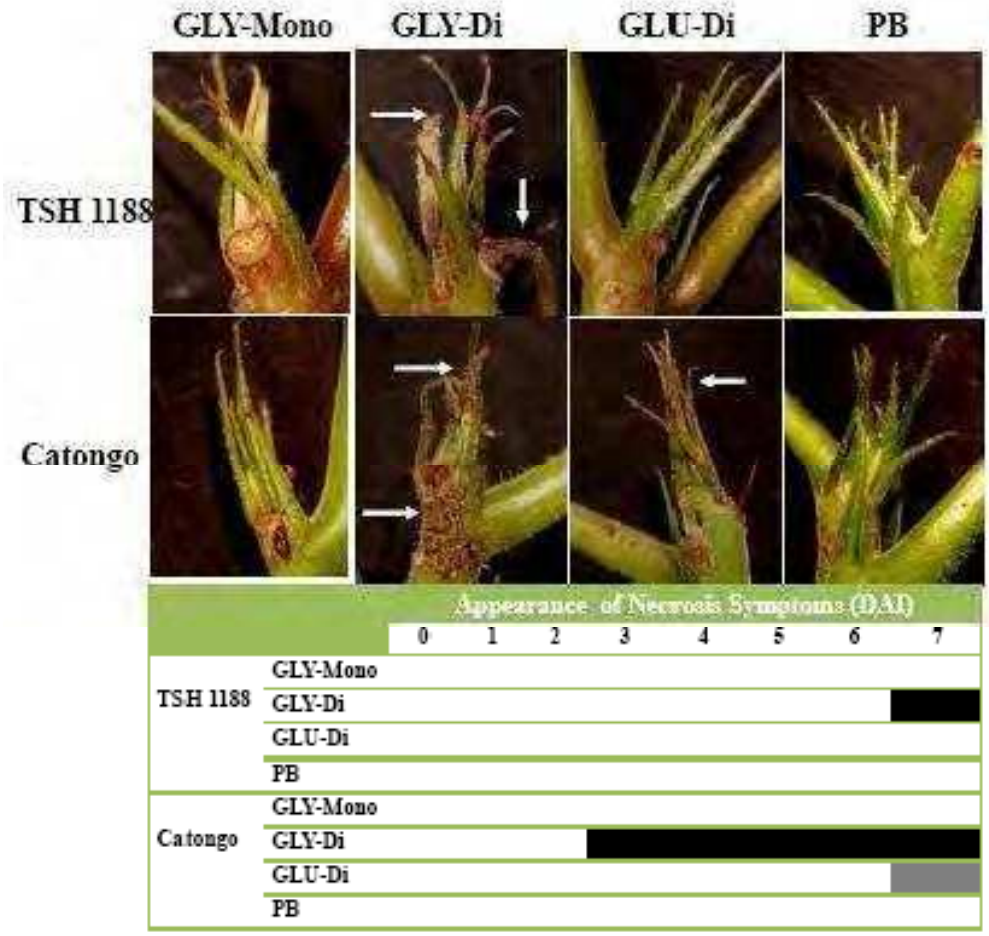

Figure 2 – *T. cacao* response to *M. perniciosa* secretome. A- same amounts of secretome proteins, recovered on PB, isolated from monokaryotic GLY-grown and dikaryotic GLY or GLU- grown *M. perniciosa* was infiltrated on meristems of *T. cacao* resistant (TSH1188) or susceptible (Catongo) to WBD genotype. Plantlets were 15 days-old. Meristems were photographed 14 d post treatment. Arrows indicate necrosis symptom. Pictures representative of 10 plantlets. A table indicates the DAI when necrosis started to be visualized on each treatment. PB used as control.

Our analyses provide clear evidences that under *in vitro* experimental conditions, GLY acts as a potent inducer of necrotic activities on different *M. perniciosa* genotypes. These results, together with the observation that GLY content increase in infected cacao branches during WBD (Scarpari et al., 2006), suggest that GLY can play a role in the *M. perniciosa* pathogenesis process. To be an efficient signaling molecule, GLY would be expected to be abundant in the intercellular space of infected *T. cacao*. To evaluate this assertion, we decided to quantify GLY exclusively in the apoplastic fluid of *T. cacao*. More precisely, two different cacao genotypes, a resistant clone (TSH 1188) and a very susceptible one (Catongo) to *M. perniciosa,* were inoculated with *M. perniciosa* spores or were treated with acilbenzolar-S-methyl (Bion®), a known plant defense activator (Faize et al., 2004). Three days after inoculation when colonization of cacao by *M. perniciosa* was already established, the GLY content was almost undetectable in the apoplastic fluid of the resistant genotype, while in the susceptible Catongo clone, GLY accumulated to significantly higher levels (Figure 4). No accumulation was observed in the apoplasm of both genotypes treated with Bion (Fig. 4). Together, these results indicate that an increase of the GLY content in the cacao apoplast is part of the plant response specifically triggered by the *M. perniciosa* colonization process and that a higher GLY level can be correlated with the susceptibility degree of the plant to *M. perniciosa*. Furthermore, it supports the notion that GLY can play a role on the pathogenesis process since it is a metabolite present in the plant apoplastic fluid where it can interact directly with the fungus (Frias et al., 1991).

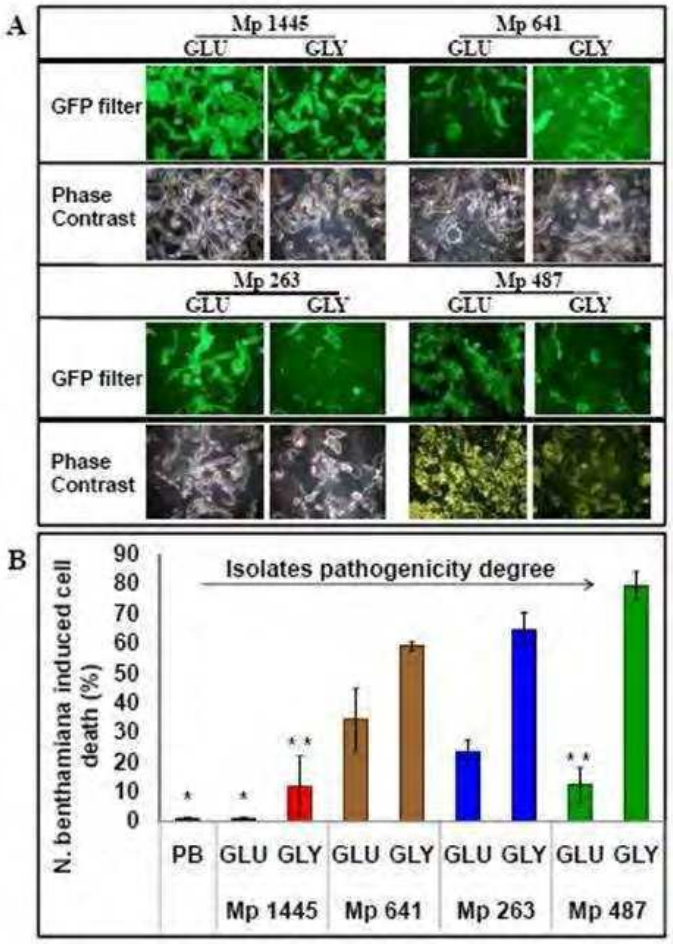

Figure 3 – Cell death induced by secretomes from different *M. perniciosa* isolates in *N. benthamiana*–GFP cell suspension lines - Four *M. perniciosa* isolates (1445, 641, 263 and 487) were grown with GLY or GLU. After 14 days, same amounts of secretome proteins, recovered on PB, were infiltrated on 4 days-old *N. benthamiana*- GFP cell suspension. On A, cell suspensions photographed 4 hours after secretome infiltration, with GFP filter or in phase contrast. Pictures are representative of 3 biological replicas. PB used as control. On B, a quantification of induced *N. benthamiana*-GFP death (GFP fadeout) by GLY and GLU-induced secretomes. Arrow indicates the isolate´s pathogenicity degree under field conditions. Error bars representative of standard deviation from 3 independent experiments.

Statistical difference was determined using Student's test. Asterisks indicate data with no statistical difference, $p < 0,01$.

Identification of glycerol–induced secreted proteins

We then asked whether the higher necrotic activity observed with the GLY secretome can be correlated with a differential protein profile. Therefore, proteomic approaches were used in order to identify proteins specifically induced in GLY-grown *M. perniciosa* dikaryotic mycelium. The tests were conducted with the intermediate pathogenic isolate 553, which corresponds to the sequenced genotype (Mondego et al., 2008). Initially, a two dimensional gel analyzes revealed modifications on the protein profile depending on the carbon source used for growth; GLU or GLY (Figure 5), confirming that these two carbon sources yielded different secretomes. The six more differently expressed spots were isolated from the GLY-extracellular proteins in 2DE gel and the peptides sequences were identified by MALDI-QTOF mass spectrometry.

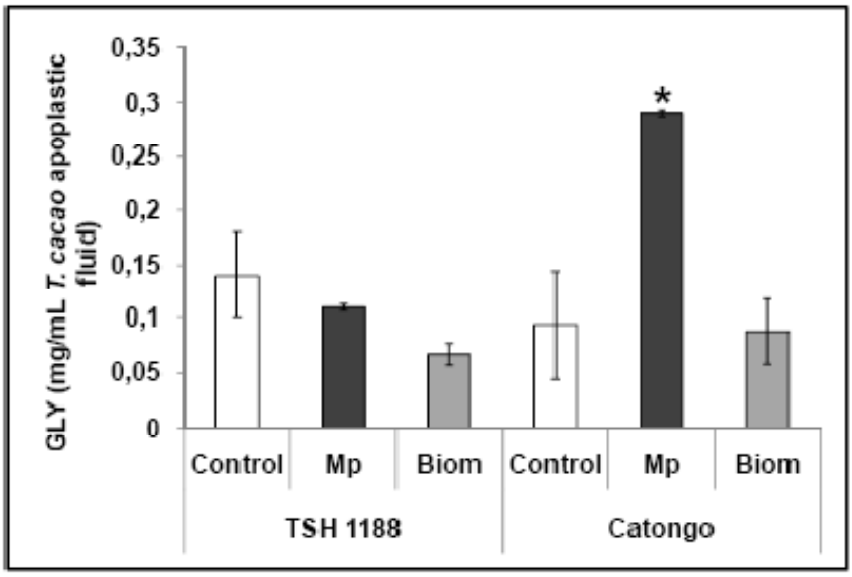

Figure 4 – Glycerol levels on *M. perniciosa* inoculated *T. cacao* plants– Apoplastic fluid GLY content in *T. cacao* 3 DAI with *M. perniciosa* (Mp, spore drop and spray 10^5 mL^{-1} basideospores in sterile water) or acibenzolar-S-methyl (Biom, 60% in steril water). Results from two *T. cacao* genotypes: a resistant, TSH 1188, and a susceptible, Catongo, to WBD. Control plants were inoculated with sterile water. Error bars represent the standard deviation from 3 independent replicas (each replica is composed of apoplastic fluid isolated from 3 different plants). Asterisk indicates statistical difference $p<0,01$.

The comparison of peptides against a fungal genome database allowed us to identify two hypothetical proteins (EAU 88719.1 and XP 757049.1), a glucan synthase (XP 001382588.1), a citrate synthase (BAF 31127.1) and a c6 zinc finger transcriptional factor (XP 001389170.1). A lipase (NP 518560.1) was also found among the identified proteins which prompted us to perform several activity assays for secreted hydrolytic enzymes such as protease and cellulase, that, similarly to lipase, are known to be involved

in hemibiotrophic fungi pathogenicity (Soanes et al., 2008). We first used a colorimetric assay to evaluate secreted lipase and protease activity in the secretome of *M. perniciosa* isolates 487 and 553. As shown in Table 1, both genotypes presented a two-fold increase in lipolytic and proteolytic activities in the GLY- versus GLU-induced secretome. We further analyzed the secreted protease activity, for the four different *M. perniciosa* isolates, in a semi-native SDS-PAGE gel. No protease activity band was visualized in the GLU-induced secretome. However, GLY induced one protease activity which corresponds to a 42 KDa band in all *M. perniciosa* isolates, even in the less pathogenic isolate 1445 (Fig. 6). Moreover, in the more pathogenic isolates, 487 and 641, GLY also induced a second protease activity band around 14.5 KDa (Figure 6), suggesting that the protease activity can be correlated with virulence. Unexpectedly, cellulase activity was not detected in any secretome tested from the four *M. perniciosa* genotypes grown on GLU or GLY-containing medium (data not shown).

Table 1. Enzymatic activities determined in secretome of *Moniliophthora perniciosa* grown on culture medium containing glucose or glycerol.

		***M. perniciosa* isolate 553**		***M. perniciosa* isolate 487**	
		Glucose	**Glycerol**	**Glucose**	**Glycerol**
Enzyme activity	**Protease**	0,7	3,0	1,4	2,9
(U/mg extracelular protein)	**Lipase**	0,4	0,8	4	9

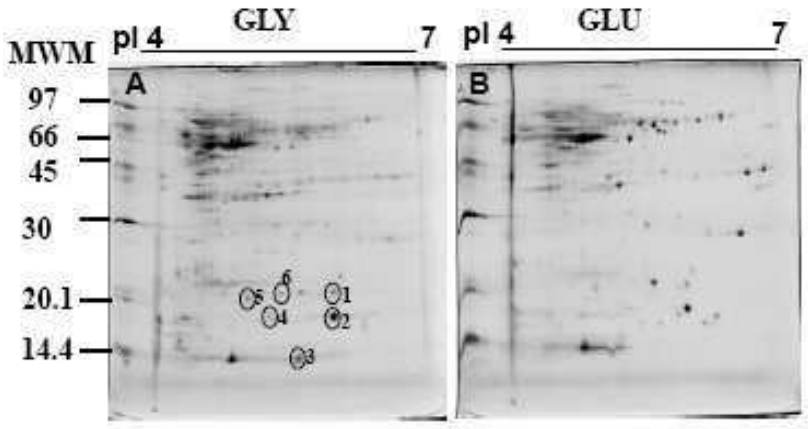

Fig. 5 – *M. perniciosa* secretome protein profile – Two-hundred and fifty micrograms of secretome proteins from GLY or GLU-grown *M. perniciosa* were resolved on 2DE gels (pI 4-7). Proteins were visualized after staining with Colloidal Comassie. Circles indicate spots isolated and further identified by mass spectrometry. The position of Molecular Weight Marker (MWM) bands are represented on the left in kilodaltons.

The second proteomic approach consisted in an ion exchange HPLC fractionation of GLU and GLY-induced secretomes, followed by assaying the necrotic activity of the fractions on *N. benthamiana*-GFP cell suspension as described above. A significant difference in the HPLC chromatogram of GLY- versus GLU- induced proteins was observed (Fig. 7A). Fifty fractions from each GLY- or GLU-induced secretome were obtained and assayed for their cell death-inducing activity. For both treatments, specific fractions effective in promoting cell death with variable efficiency were identified (Fig. 7B to 7D). Since the HPLC fraction 04 resulting from GLY-induced secretome was the most efficient to induce cell death, we reasoned that by comparing the protein content of GLY- and GLU-derived fractions 04, it should be possible to identify new candidates involved in

cell death. These fractions were therefore analyzed by LC nano-ESI MS/MS, leading in each of them, to the identification of the 9 more abundant proteins.

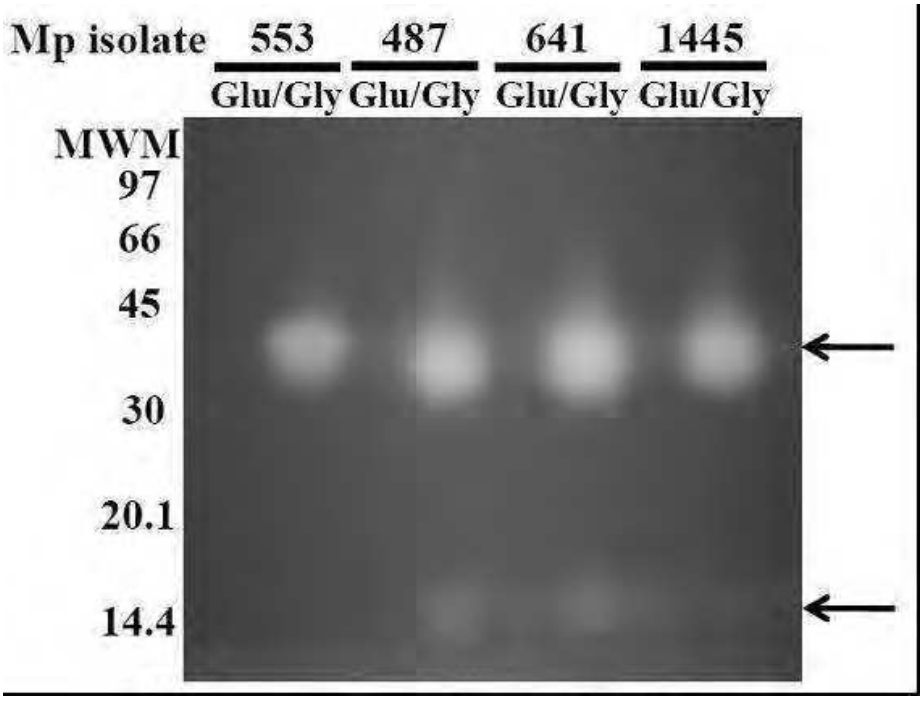

Fig. 6 - GLY induces *M. perniciosa* extracellular proteolytic activity- Same amount of native secretome proteins, isolated from GLY- and GLU- growth *M. perniciosa*, were resolved on semi-native SDS-PAGE gel. Proteins were transferred into a gelatin gel and protease activity bands (arrows) were visualized after staining with Colloidal Comassie. Assay conducted with 4 *M. perniciosa* isolates (553, 487, 641 and 1445). The position of Molecular Weight Marker (MWM) bands are represented on the left in kilodalton.

Most proteins were restricted to the GLY or GLU- induced secretomes. Only one protein, Q4I554 GIBZE, was common to both treatments (Table 2). Part of the proteins identified in the GLU-derived fraction 04, were identified as hypothetical or predicted when compared with other fungi pathogen protein data bases. In This GLU- treatment we also identified an endopolygalacturonase 1, a secreted hydrolytic enzyme whose gene expression is strongly regulated by carbon sources (Qin et al., 2007) and is associated to virulence in *Aspergillus flavus* (Mellon et al., 2007) and *Botrytis cinerea* (Have et al., 1998; Rowe and Kliebenstein, 2007). Unexpectedly, no protein identified in the GLY-induced fraction 04 corresponds to a known cell death inducing protein. The only GLY-induced protein identified corresponded to a deoxyhypusine synthase (T47195), a key regulatory enzyme exclusively required for the translation of a sub-set of mRNAs required exclusively for cell division (Park et al., 1997; Sasaki et al., 1996; Wang et al., 2001). Hence, it is necessary for cell viability and growth. The other 9 proteins were hypothetical or predicted, and already identified on other fungi pathogens (Table 2). Interestingly, the protein MGG 01215 was the only one identified exclusively in a cDNA library from a compatible WBD interaction (Gesteira et al., 2007). The fact that solely 0.2% of the cDNA sequenced on this library were fungal transcripts, together with the observation that the homolog protein MGG 01215 accumulated specifically in response to GLY, reinforces the importance of GLY as a signal molecule able to modulate fungal development during plant colonization, as already suggested in other *in vitro* experiments (Meihardt et al., 2007; Pungartnik et al., 2009; Santos et al., 2008). The high diversity between the proteins identified on the GLU versus the GLY fraction 04 suggests again, that the *M. perniciosa* extracellular protein pattern indeed suffers variation depending on the carbon source provided for growth, therefore playing a role in the differential cell-death activity.

Table 2. Proteins identified in the HPLC fraction 4 by Nano Esi MS/MS.

Fraction 04	Accession	Putative function	Mass (Da)	PI
Glucose	CNAG 05174.1	*Cryptococcus neoformans* hypothetical protein translation	200066	6.1
	Q9MR93 PODAN	*Podospora anserine* reverse transcriptase homologue ND5 i4 grp II protein	88490	9.9
	Q4WFG7 ASPFU	*Aspergillus fumigatus* hypothetical protein	36968	5.7
	Q4I554 GIBZE	*Gibberella zea* hypothetical protein	111381	6.6
	AAT50086	AY657811 synthetic construct	18222	4.5
	Q2PIV8 ASPOR	*Aspergillus oryzae* predicted protein	25129	9.2
	Q4G496 BOTCI	*Botrytis cinerea* endopolygalacturonase 1	37913	8.1
	T39663	*Schizosaccharomyces pombe* probable transcription regulator protein fission yeast	132798	6.4
	Q2HBS6 CHAGB	*Chaetomium globosum* hypothetical protein	36633	5.0
Glycerol	MGG 01215	*Magnaporthe grisea* hypothetical protein	82427	9.8
	Q7S9I7 NEUCR	*Neurospora crassa* predicted protein	43418	5.1
	Q5CMC9 CRYHO	*Cryptosporidium hominis* hypothetical protein	52528	5.7
	Q4IA87 GIBZE	*Gibberella zeae* hypothetical protein	70704	8.3
	Q5AXV6 EMENI	*Emericella nidulans* hypothetical protein	52564	8.4
	Q7SH25 NEUCR	*Neurospora crassa* hypothetical protein	60355	9.3
	Q7SH26 NEUCR	*Neurospora crassa* predicted protein	17319	9.8
	T47195	*Neurospora crassa* deoxyhypusine synthase	38935	5.5
	Q4I554 GIBZE	*Gibberella zeae* hypothetical protein	111381	6.6

PI: isoelectric point; DA, Dalton

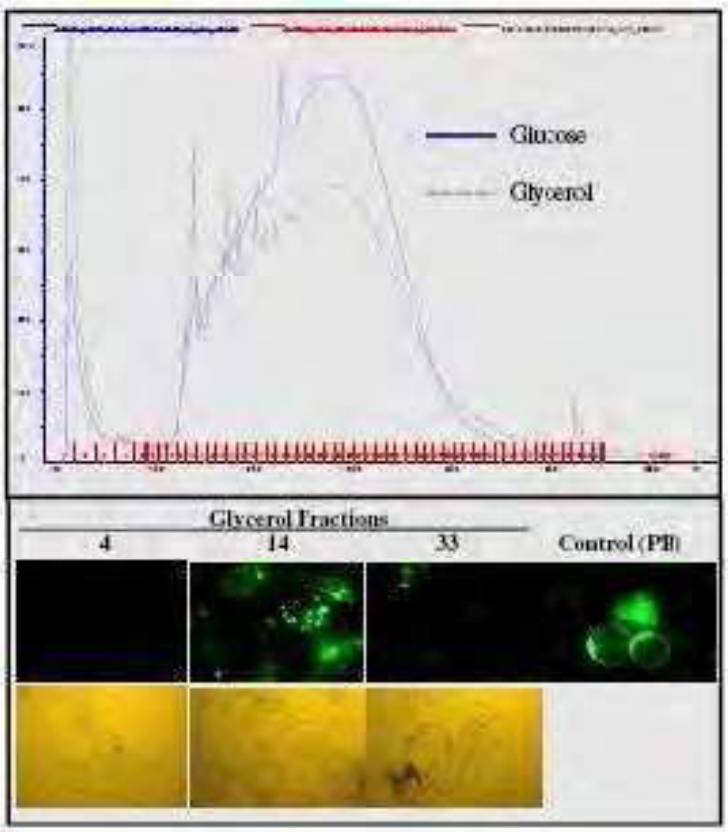

Fig. 7 - Differences in the secretome protein profile revealed by HPLC separation – A: HPLC chromatogram representative of same amounts (0.5mg) of extracellular proteins from *M. perniciosa* GLU (blue line) or GLY (brown line) grown, fractionated on an ion exchange column. B to D: biological assay performed in *N. benthamiana* cells with individual HPLC fractions. The same field was captured with GFP filter (top) or in Phase contrast (bottom). E: cells immersed on PB, 10mM, control. Pictures are representative of 3 replicas, 24 h after treatment with 4 µg of protein on PB.

Glycerol induces changes in M. perniciosa mycelium morphology and increases cell wall resistance

Besides its ability to alter the *M. perniciosa* secretome composition and its increased cell death activity, GLY also induced significant changes of mycelium morphology and cell wall composition. The first evidence for such alteration was obtained by double staining of the dikaryotic mycelia with astra blue and safranine. While GLU-grown mycelia were thicker and stained in blue, the GLY-grown mycelia were thinner, less abundant and stained purple (Fig. 8A).

Differences in cell wall composition can be revealed by the impact of compounds that interfere with cell wall formation. Thus, the influence of congo red and SDS was evaluated on GLY and GLU mycelium. After only 24 hours, the GLY *M. perniciosa* hyphae was still capable of sustaining growth in the presence of either cell wall stress inducer components, while the mycelium grown with GLU already presented clear growth retardation under the same conditions (Fig. 8B).

To better understand the possible differences induced by GLY on the *M. perniciosa* cell wall composition, we evaluated the activity pattern of chitinase; an enzyme whose function is correlated with morphological changes and the chitin content of fungi cell wall (McCreath et al., 1995; Selvaggini et al., 2004; Yamazaki et al., 2008). We showed that the chitinase activity increased in response to GLY (Fig. 8C), further suggesting that GLY may induce changes in the glucan/chitin ratio of the cell wall when compared to GLU growth conditions. Finally, digestion of equal amounts of GLY- and GLU-grown *M. perniciosa* mycelia with Glucanex G200 (Novozyme®); a mix of cell wall hydrolytic enzymes (cellulase, chitinase, protease and glucanase), revealed that GLU-grown mycelium released

4 times more reducing sugars then the GLY-grown mycelium (Fig. 8C). Together, these results showed that *M. perniciosa* cell wall composition/structure can be significantly altered in response to GLU versus GLY and that the GLY-related modifications can confer significant resistance to stress agents whose primary target is the cell wall.

Expression of two catalase genes and a guanine exchange factor for RHO1 GTPase is enhanced by glycerol indicating an involvement of the RHO- signalling pathway

The modifications on the *M. perniciosa* cell wall induced by GLY led us to evaluate the expression pattern of a gene involved in the control of the cell wall integrity pathway (CWI): the guanine exchange factor (GEF) for RHO1 GTPase protein (gene code *RHO*. Pires et al., 2009). In other fungi, the CWI pathway is related to cell wall modification and virulence. The analysis was conducted using transcripts isolated from the highly pathogenic *M. perniciosa* isolate 487 and the mildly pathogenic isolate 553. *RHO* transcripts were up-regulated in both isolates in response to GLY. However, while in the isolate 487 *RHO* transcripts increased 600%, in comparison to GLU growth condition, in the mildly pathogenic isolate 553, the transcripts increased only 50%, in comparison to the GLU-mediated response (Figure 9A). It appears therefore, that GLY induces the expression of *RHO* and that the degree of induction can be correlated with the pathogenicity of the strain.

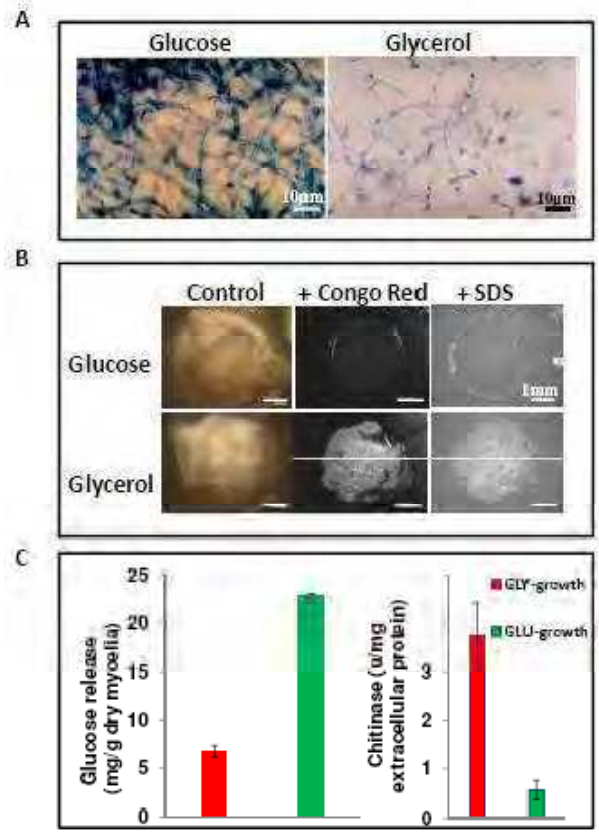

Fig. 8 - Glycerol induces modification on *M. perniciosa* cell wall composition and enhances its resistance to cell wall stress. A, Microscopy of astra blue and safranine-stained apical hyphae of GLY- and GLU-grown *M. perniciosa*. The hyphae were collected from a 7 d old culture. B, Plugs from 3 d old culture of GLU- or GLY-*M. perniciosa* grown for 24 h on same carbon source medium (Control) containing either congo red (0.5%) or SDS (0.1%). Pictures are representative of 10 replicas. C, Glucose release from GLY and GLU grown *M. perniciosa* mycelium treated with glucanex G 200 (0.2 mg/mL). Chitinase activity from GLY- and GLU- grown *M. perniciosa*. Assays conducted with 14 d old *M. perniciosa* culture. Error bars represent standard deviation from 3 replicas. Statistical significance ($p<0.01$) analyzed by Student's test.

Since the CWI pathway usually leads to an enhanced resistance to oxidative stress (Park et al., 2005), the expression of two catalase genes; one peroxissomal (Gene code *CTA*) and one secreted (gene code *CTT*), both of which are likely to be involved in the detoxification of reactive oxygen species (Pungartnik et al., 2009), were evaluated. The results revealed that GLY up regulated the transcription of both catalase genes, in comparison to GLU. Once again, the highly pathogenic isolate 487 presented a higher increase of both *CTA* and *CTT* mRNAs, in comparison to the mildly pathogenic isolate 553 (Figure 9A). We next evaluated the secreted catalase activity on native polyacrylamide gel containing ampholytes (pH range 3.5 to 10.0). No catalase activity band was visualized in GLU-induced secretome. However, GLY-induced secretomes of both isolates, 553 and 487, presented a protein with isoelectric point (iP) around 6.2 with catalase activity. Interestingly, a second catalase isoform, with a higher iP, was detected only in the secretome from GLY-grown highly pathogenic isolate 487 (Fig. 9B), suggesting once again, that the more pathogenic isolate is more responsive to GLY.

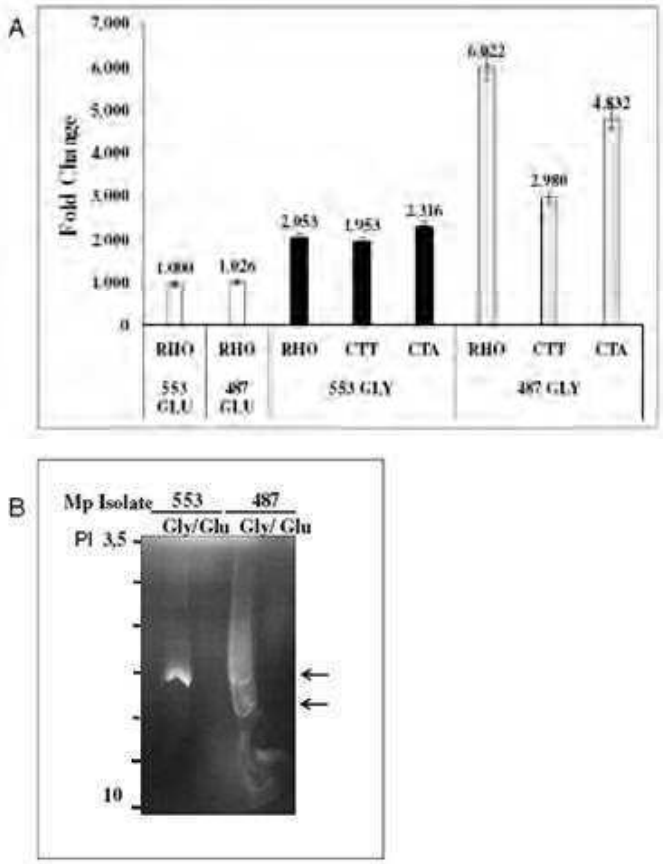

Fig. 9 – Guanine exchange factor for Rho1 GTPase and catalase transcripts are induced by Glycerol. –A, RT-qPCR was performed with cDNAs representative of GLU and GLY-grown dikaryotic *M. perniciosa*, isolate 553 (red column) and 487 (green column). mRNA transcripts for a *Rho-gef* (RHO), a peroxissomal catalase (CTA) and secreted membrane catalase (CTT) were analyzed. The total RNA was isolated from 14 d old culture. Error bars represent standard deviation from 3 replicas. Statistical difference from control (553 GLU) analyzed by student's test ($p<0.01$). Actin was used as a reference gene. B, Same amount of native secretome protein (25 µg), isolated from GLY and GLU grown mycelium, isolates 553 and 487, were resolved on an acrylamide gel containing Bio-lyte 3/10. Catalase activity bands (arrow) were revealed after gel staining with potassium iodide and acetic acid. Isoelectric point indicated on left. Secretome purified from 14 d old culture.

Discussion

We presented evidences that in *M. perniciosa* there is an intrinsic relationship between GLY and secretion of pathogenesis proteins. In several pathosystems, including the *M. perniciosa*-cacao interaction, GLY concentration increases in the host during pathogen colonization (Clark et al., 2003; Jobic et al., 2007; Scarpari et al., 2005; Van Schooten et al., 2006; Wey et al., 2004). During WBD, GLY reaches its highest concentration in a phase that slightly precedes tissues necrosis (Scarpari et al., 2005). In this context, it is possible that the addition of GLY to the growth medium of the *M. perniciosa* dikaryotic mycelium mimics the natural environment that the fungus is submitted to during the necrotrophic phase of the disease, when accentuated host cell death occurs (Ceita et al., 2007; Evans and Bastos, 1980). Indeed, the secretome isolated from the GLY-grown dikaryotic *M. perniciosa* was the most effective in inducing cell death in *N. tabacum* leaves and *N. benthamiana* cell suspension, in comparison to GLU (Figure 1B and C). Further, it was the only secretome capable to trigger necrosis in the meristems of an *M. perniciosa*-resistant cacao clone (Figure 2, clone TSH 1188). Taken together, these results suggest that GLY efficiently activates signal cascades related to necrosis promotion in the host. This GLY-induced response is conserved among all different *M. perniciosa* isolates tested. In addition, the level of the GLY-induced necrotic activity was found to be positively related to the pathogenicity degree of the fungus isolates as observed on field trials (Figure 3A and B). This correlation suggests that the fungus responsiveness to GLY partly determines its pathogenicity, which in turn reflects in increased levels of necrosis-inducing secreted proteins. This effect of GLY is not triggered on monokaryotic/biotrophic *M. perniciosa* mycelium (Evans and Bastos, 1980; Griffith et al., 2003) (Figure 1A), although GLY may be used by the *M. perniciosa* monokaryotic phase in order to sustain growth (Meinhardt et

al., 2006). These observations led to the hypothesis that the ability of GLY to trigger specific signal cascades related to promoting cell death is restricted to the dikaryotic/ necrotrophic mycelium, which is the fungal life form present during the occurrence of cell death in WBD (Ceita et al., 2007; Evans and Bastos, 1980; Griffith et al., 2003; Pungartnik et al., 2009).

Cell death promoted by the GLY-induced *M. perniciosa* secretome was apparently not due to activity of previously identified *M. perniciosa* Necrosis-Inducing Protein (Mp-Nep) (Garcia et al., 2007), since we could neither detect the corresponding mRNA by sqRT-PCR nor DNA laddering, a hallmark of programmed cell death on *N. benthamiana*-GFP cells treated with the GLY secretome (data not shown). In addition, the boiled secretome lost its ability to promote cell death, whereas MpNep2 was found to be heat stable (Garcia et al., 2007). This observation, together with the fact that all secretomes were purified by gel filtration columns (G25) or fractionated on ion exchange columns, still maintained the ability to induce necrosis, supports the concept that proteins present in the secretome extract are the molecules involved in the induction of plant cell death.

GLY induces modification on extracellular protein composition

Protein 2DE-gel revealed that the *M. perniciosa* GLY- and GLU- induced secretomes have different protein profiles (Fig. 5). We identified the six more differentially expressed proteins that accumulated in response to GLY when compared to GLU by MS analysis. Two of these proteins, a citrate synthase and a transcription factor, are not classical secreted proteins, but have been previously identified in plant (Slabas et al., 2004) and fungi secretomes (Soanes et al., 2008). Among the other up-regulated proteins we detected a lipase and a glucan synthase which are proteins known to be involved in pathogenicity in

other pathogens (Feng et al., 2005). The two-fold increment of the lipolytic activity in the GLY-induced secretome, in comparison to GLU (Table 1), is in agreement with the MS identification process. Together, these results establish the first evidence linking GLY with the up-regulation of known pathogenicity proteins for this fungus. Higher protease activity was also detected in the GLY-induced secretome as compared to GLU (Table 1 and Fig. 6). According to the mildly acid buffer pH (6.0) used in the experiments; the detected proteases may be aspartic or cystein proteases, but not serine protease which have a higher enzymatic activity at alkaline pHs (Pirovani et al., 2008). Because protease are known to be involved in PCD, it could be hypothesized that some extent of the necrosis induced by GLY-secretome could be related to the enhanced protease activity (Poussereau et al., 2001; Shindo and Van der Hoorn, 2008). The correlation between the level of protease and lipase activities with the pathogenicity degree of *M. perniciosa* isolates is in agreement with previous results that demonstrated a link between pathogenicity and hydrolytic enzyme activity in other pathosystems (Gácser et al., 2007; Hegedus and Rimmer, 2005; Qin et al., 2007; Voigt et al., 2005) and supports the idea that lipases and proteases play a role in virulence levels of hemibiotrophic fungi.

A comparative analysis by nano-ESI MS/MS was also performed on the HPLC fractions more efficient in inducing necrosis in *N.* benthamiana-GFF cell suspension. This approach allowed us to identify a protein known to be involved with pathogenesis and cell death: endopolygalacturonase I. This protein accumulated on GLU-but not on the GLY-induced secretome suggesting a possible role on the cell death activity of the GLU-related secretome. However, as observed by the GFP cell assay, proteins present in the HPLC fraction of GLY-induced secretome are good candidates to be involved on cell death induction. Together, the data gave support to the differential activity patterns when

comparing the GLY- and GLU-induced secretomes. Although we could not efficiently identify a specific protein possibly involved in inducing cell death from the GLY-induced secretome, the identification of the ten more abundant proteins was important to demonstrate the high diversity between the secretomes.

GLY induces modification on *M. perniciosa* cell wall composition

Typically, the common core of fungi cell walls is formed by the structural polysaccharides chitin and glucan (Latgé, 2007). The fact that glucan synthase (2DE analyses, Fig. 5) presence and chitinase activity (Fig. 8C) were found to be more intense in GLY-grown secretome, suggested that specific differences in the *M. perniciosa* cell wall can be induced according to the carbon source. More precisely, the data supports the idea, already raised previously for some pathogens (Reese et al., 2007; Selvaggini et al., 2004), that chitin loss associated with higher chitinase activity may be compensated by glucan synthesis (higher glucan synthase). Our results indicate that this switch in cell wall composition can be triggered by GLY. A modification on the chitin/glucan content according to the carbon source (GLU and GLY) can explain (i) the different patterns of cell wall staining (Fig. 8A and D), (ii) the differences observed on *M. perniciosa* resistance to compounds targeted to the cell wall such as congo red and SDS (Fig. 8B) and (iii) the difference in sensitivity to cell wall hydrolytic enzymes (Fig.8C). Chitinase activity has also been related with fungus autophagy, which in turn may be partially responsible for the thinner hyphae observed under GLY-growth (Fig. 8A). Indeed, *M. perniciosa* autophagy is induced by GLY (Pungartnik et al., 2009).

The changes in cell wall structure in response to GLY were correlated with the up-regulation of the Rho1 GTPAse-related guanine exchange factor (GEF) gene (Fig. 9A).

GEF is a key regulator that is responsible for sensing cell wall modifications and promoting the switch from the Rho1 GDP inactive form to the active GTP-bound form (Bickle et al., 1998; Schmidt and Hall, 2002). It appears that Rho1 Cell Wall Integrity (CWI) signaling pathway is involved in GLY-associated cell wall characteristics. This conclusion is compatible with the well established cell wall changes resulting from activation of the CWI pathway in response to abiotic stresses in fungi (Jung and Levin, 1999; Levin, 2005; Park et al., 2005; Roh et al., 2002). Moreover, the GLY-induced accumulation of glucan synthase and deoxyhypusine synthase (Fig. 5 and Table 2), the GLY-mediated up-regulation of catalase genes (Fig. 8, gene names CTT and CTA) and catalase activity (Fig. 9B) and the enhanced resistance to cell wall stresses compounds, (Fig. 8B) well-known CWI-related responses (Jung et al., 1999; Martínez-Rocha et al., 2008; Park et al., 2005; Santos et al., 2008), give further support of the participation of the CWI pathway in GLY-induced responses in *M. perniciosa*.

Quite significantly, our results establish a positive correlation between GLY-induced accumulation of Rho-gef and catalase mRNAs, intensity of the secretome-necrotis-inducing activity and pathogenicity degree of the different fungus isolates tested. A plausible explanation for this correlation is that the more virulent the *M. perniciosa* genotypes are, the more responsive they are to GLY. Since catalase may provide a mean for the fungus to efficiently deal with hydrogen peroxide produced by the host as part of its defense strategy (Ceita et al., 2007; Pungartnik et al., 2009; Qin et al., 2007; Shetty et al., 2007), we further propose that higher GLY-responsiveness may confer a selective advantage to the pathogen. Other confirmations for this hypothesis came from the observation that a higher necrotic activity and improved resistance to oxidative stress induced by GLY in the more virulent strains, provides the pathogen an advantage that

ensures the completion of its life cycle, even on more resistant cacao clones such as TSH1188. The molecular aspect underlying the interplay between GLY, the CWI pathway and necrotic activity are unknown and identifying components of the GLY-related signaling pathway would be quite relevant to improve our understanding of the GLY role.

In the complex interaction between *M. perniciosa* and *T. cacao*, the possible relation of GLY or its metabolism on cacao defense mechanisms must be further investigated. Recently, it was demonstrated that plants that accumulate more GLY are more susceptible to the hemibiotrophic fungi *Colletotrichum higgisianum* (Chanda et al, 2008), just as observed on a compatible WBD interaction (Fig. 4). The presence of specific GLY in the cacao apoplastic fluid, where *M. perniciosa* initially grows, may signalize to the dikaryotic/ necrotrophic mycelium to modify the cell wall structure/composition (CWI pathway), secreting necrosis-inducing proteins and defense-related proteins, such as catalases. Based on our data, we propose this new role for GLY on the pathogenesis process of hemibiotrophic fungi.

Experimental Procedures

***Moniliophthora perniciosa* isolate cultures**

Isolates of *M. perniciosa* with high (487, 641 and 263), moderate (553) and low (1445) virulence levels were obtained from the CEPLAC/CEPEC (Cacao research center, Ilhéus-BA, Brazil) culture collection. Isolates were chosen based on ability to cause disease on resistant and susceptible cacao genotypes, referred here as pathogenicity.

Mycelia were grown in 50 mL of liquid mineral medium (ammonium phosphate 0.1%, potassium chloride 0.02%, magnesium sulphate 0.02%, yeast extract 0.5%, copper sulphate 0.01%, zinc sulphate 0.01%) supplemented with 20 mM of glucose, glycerol, 10 mM of each carbon source or no additional carbon source. Mycelia were grown in the dark

at 25°C for 14 d. The supernatants were filtrated through a 0.45 µm Millipore membrane in order to remove any loose cell debris, and lyophilized for 48 h. The mycelium also was collected, washed with DEPC treated water and submitted to lyophilization. Both materials were kept at –80°C for further analysis.

Plant material

The *Nicotiana benthamiana* cell suspension was obtained from transgenic plants (seeds provided by Dr. S. Brommonschenkel), as described by Alvim et al. (2001). The cell culture was maintained in the NT-1 medium [1% MS salts (Murashige and Skoog, 1962), 3% sucrose, potassium phosphate 0.18 g/L, 2,4-diclorophenoxiacetic acid 0.2 mg/mL, inositol 0.1 g/L, thiamine-HCl 1 mg/L, pH 5.7] and sub-cultured every seventh day. The cell suspension was maintained at 25°C, with photoperiod of 16 h of light and 8 h of dark, under 25 RPM agitation. Light irradiation 36 µmoles fotons.m^2.s^{-2}. Four d-old cell suspension was used for the biological assays.

***Theobroma cacao* apoplastic fluid glycerol quantification**

One month old *Theobroma cacao* genotype susceptible (Catongo) or resistant (clone TSH 1188) to *Moniliophthora perniciosa* were grown in sterile substrate in the green house at Mars Center of Cacao Studies (Barro Preto/BA). Apical meristems of 30 plants were inoculated by the spraying method using a 10^5 mL^{-1} basidiospore suspension from a mixture of *M. perniciosa* strains. The *T. cacao* meristems were also inoculated with *M. perniciosa* spore drop (10^5 mL^{-1} in sterile water). After inoculation, plants were acclimated during 24 h at 25 °C in a water-saturated atmosphere to allow *M. perniciosa* spore germination,

penetration and infection (Frias et al., 1995). Fifteen control plants of each *T. cacao* genotype were inoculated with sterile water or sprayed with the plant defence activator acibenzolar-S-methyl (60g of active ingredient/100L water) (Bion ®). Control plants were submitted to the same growing conditions as the inoculated ones. Expression of susceptibility was estimated 4 weeks after inoculation by detection of the Catongo and TSH 1188 plants with disease symptoms.

Tree-days after inoculation 3 apical leaves from infected and non-infected resistant and susceptible *T. cacao* plants were harvested. Leaves were immediately submitted to apoplastic fluid extraction, as described by Pirovani et al., 2008. Glycerol content was determined using a glycerol dosage kit (Novazyme, Germany). We considered a biological sample a complex mixture of leaves isolated from 3 plants. GLY level on each treatment is the average of 3 biological samples (total of 9 plants). Five inoculated *T. cacao* plants were kept in the green house and monitored until the WBD symptoms were observed.

Biological activity assay

Prior to the biological assays, a fresh crude extracellular sample obtained from a 14 day-old *M. perniciosa* culture, was filtered through a Millipore membrane with 0.45 μm mesh and desalted in a PD-10 Sephadex™ G-25M column (GE Healthcare). The proteins were recovered in 10 mM phosphate buffer (PB), pH 6.0 and quantified using the Bradford assay (Bradford, 1976). Protein concentration was adjusted with PB to 0.5 mg/mL. Protein samples were kept on ice throughout the entire process.

Five micrograms of proteins were infiltrated in 1-month-old leaves of *N. tabacum* (variety Havana) or *T. cacao*, Catongo genotype (susceptible to *M. perniciosa*) and TSH

1188 (resistant to *M. perniciosa*) meristems, using an insulin needle and syringe. Controls were infiltrated with 10 mM PB, pH 6.0 and also with fresh culture medium. The plants were maintained in the green house and visually evaluated daily until the necrosis symptoms were detected, usually for a 30 d period. Ten plant clones were used for each experiment. Images were acquired at 10x magnification (model EZ4 D, Leica, Germany).

In an alternative bioassay, 300 µL of four-day *N. benthamiana*-GFP cell culture were transferred to a 1.5 mL sterile tube. After the cells had settled, the culture medium was removed and substituted by 500 µL (0.2 mg/mL) of the *M. perniciosa* extracellular protein extract (10 mM PB, pH 6.0). Cell suspension was maintained at 25°C for periods varying from 1 to 48 h under 25 RPM agitation.

Microscopic analyses

Induction on *N. benthamiana* cell death was accompanied by measuring the decrease in GFP fluorescence using an automated fluorescence microscope (model DM RA2, Leica, Germany), with filter cube GFP (excitation range blue, excitation filter BP 470/440 nm, dichromatic mirror 500 nm, suppression filter BP 525/550, Leica, Germany). The images were further analyzed using the Leica software IM50300. The experiments were conducted with 3 individual replicates, magnitude 200X.

The mycelium morphological assay was conducted with *M. perniciosa* grown in liquid mineral medium containing glucose or glycerol as carbon sources. After 7 days the apical region from the mycelium was excised, submitted to seriatim-alcoholic dehydration (70%, 80%, 90%, 95%, 100%) and included in historesin (Leica). The sectioned material was acquired in a rotative microtome. The material was stained with 1% astra blue, for 1

hour, and then 1% safranin for 1 h. Pictures were taken in an optical microscope (Olympus DX40), 100x magnitude.

Moniliophthora perniciosa cell wall resistance analyses

For analyses of cell wall stress resistance 3mm spots from 7 days old GLU and GLY-grown *M. perniciosa* was transferred to solid GLU or GLY mineral medium containing congo red (0.5%) or Sodium diethyl Sulfate (SDS, 0.1%), both added after the medium was autoclaved. Plates were grown for 24 h, at 25^{o} C in the dark. Pictures were acquired in an electric stereoscope (Leica EX40), with 8.5x magnitude. The experiment conducted with 10 replicates.

The mycelium resistance to the action of hydrolytic enzymes was tested with Glucanex G200 (Novozyme). Fourteen day old *M. perniciosa* culture was filtered, the mycelium washed with ultra-pure water and lyophilized for 48 h. The same amount of dry material was added to 10 mL of 50 mM Tris-HCl pH 7.5 containing 50 mM EDTA, 2% SDS and 40 mM β-mercaptoethanol and resuspended by sonication (1 pulse of 20 s, 75% output) on an Ultrasonic processor (Gex 130, 130 W). Samples were boiled for 5 min and centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was extensively washed with ultra-pure water and then lyophilized for 24 h. Thirty milligrams of the powder was resuspended in 500 µL of 50 mM acetate buffer, pH 5.5, containing glucanex G200 0.2 mg/mL (Novozyme). Since Glucanex G200 is not a pure enzyme mix, and some components could interfere with the glucose quantification conducted after the material digestion, we first resuspended the Glucanex G200 powder in 50 mM acetate buffer and washed it 4x on Amicon tubes (5KDa). The digestion was

conducted at 37°C for 24 h in the dark. After this period the glucose quantification was conducted in all samples using a glucose quantification kit according to the manufacturer´s recommendation (Gold Analisa, Brazil).

Enzymatic assays

Prior to the enzymatic analysis, 50 mL of *M. perniciosa* GLY and GLU-secretome were lyophilized for 48h. Cultures were 14 d old. The powder was resuspended in 5 mL of 100 mM phosphate buffer (pH 6.0), then desalted in a PD-10 Sephadex™ G-25M column (GE Healthcare) and recovered in 100 mM phosphate buffer, pH 6.0. Protein concentration was determined according to Bradford (1976). All enzymatic assays were conducted with 25 µg of secreted proteins.

Protease activity was determined by the use of previously described methods (Alfenas et al., 1998; Pirovani et al., 2008). Briefly, same amounts of GLY and GLU-secretome proteins were resolved on a native acrylamide gel (12.5% Bis-acrylamide), at 150v for 2 h at 4° C. After electrophoresis, proteins were transferred by superposing with a gelatin gel (0.1% gelatin, 100mM phosphate buffer pH 6.0, 2mM EDTA, 10mM β-mercapto-ethanol, 7.5% Bis-acrylamide). Gels were kept under agitation, immersed on protease activity buffer (100mM phosphate buffer pH 6.0, 2mM EDTA, 10mM β-mercapto-ethanol), for 12 hours, at 37° C. Protease activity bands were visualized after staining with 0.1% w/v Colloidal Comassie G 250 solution (Neuhoff et al., 1988). Protease colorimetric assay was conducted by mixing 25 µg of GLY and GLU secretome proteins with protease activity buffer containing 1mM N-l N-alpha-benzoyl-DL-arginine- p-nitroanilide (BAPNA) (Sigma Aldrich). Samples were kept at 37° C in a microplate reader for 10 h (Molecular Devices, USA). Reads were determined at wavelength of 210 nm.

Secreted catalase and cellulase activities were determined using the methods described by Alfenas et al. (1998). Catalase activity was revealed in a native gel containing Bio-lyte 3/10 ampholytes (GE Healthcare Bio-Science) (10% Bis-acrylamide, 0.45% Bio-lyte 3/10, 1% starch). Before sample application the gel ran for 15 min. at 200V followed by additional 15 min at 300V. After sample application, electrophoresis was conducted at 150V for 2 h at 4° C. The gel was immersed for 30 s on an activity solution (18mM sodium thiosulfate, 2.1% oxygen peroxide) and then for 1 min. in a revealing solution (90 mM potassium iodide, 0.5% acetic acid). Catalase activity bands were detected as white bands on a red gel.

Lipase activity was quantified using a laboratory kit (Bioclin, São Paulo, Brazil), as recommended by the manufacturer and chitinase activity was measured after the sample's lyophilization, as described by Lopes et al. (2008).

Protein sample preparation

Prior to protein extraction, the powder obtained by lyophilization was resuspended in 5 mL sterile ultra-pure water and filtrated through a 0.45 µm Millipore filter. Proteins from the crude sample were precipitated with an equal volume of trichloroacetic acid (20% TCA in water) at 4°C for 4 h. The samples were centrifuged for 5 min (14,000 rpm) at 4°C, and pellets were re-suspended and washed twice with ice-cold acetone (80%) and then twice in ice-cold ethanol (70%). Between each wash step, samples were centrifuged at 14,000 rpm for 5 min at 4°C. The pellet was air-dried for 1 h and resuspended in 2-DE sample buffer [7M urea, 2M Thiourea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.4% (w/v) Bio-lyte 4/7 ampholytes (GE Healthcare Bio-Science), 0.1% (w/v) Bio-lyte 3/10

ampholytes (GE Healthcare Bio-Science), and 0.002% bromophenol blue. The pellet was resuspended by sonication (1 pulse of 5 s, 40% output) on an Ultrasonic processor (Gex 130, 130 W), and then centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant was collected and protein concentration was determined using 2D quantification kit according to the manufacturer's recommendations (GE Healthcare). The protein concentration was further adjusted to 1 $mg.mL^{-1}$ and 250 µg was used for one or two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis

Thirteen centimeter immobilized pH gradient (IPG) strips, pH 4-7 (GE Healthcare) were re-hydrated with 250 µL of protein samples in 2-DE buffer for 12 h at 20°C on an Ettan IPGphor system (GE Healthcare). Iso-electric focusing (IEF) was performed with the same apparatus under the following conditions: step and hold at 500 V for 1 h, gradient 1000 V for 1 h, gradient 8000 V for 2.5 h, and step and hold 8000 V for 40 min, maintaining 50 µA/IPG strip. After IEF, the strips were stored, immersed in an equilibration buffer (6 M urea, 2% SDS, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 0.002% Bromophenolblue), at –80°C until the second dimensional analyses.

Before the second-dimensional run, the strips were incubated for 15 min in an equilibration buffer containing 65 mM DTT and then for 15 min in the same buffer containing 70 mM iodoacetamide. Equilibrated strips were transferred onto 12% vertical SDS-PAGE (20 x 20 cm), without stacking gels, and the second-dimensional separation was carried out on a Ruby SE600 system (GE Healthcare): 80 V for 45 min and 50 mA *per* gel for 3.5 hours for every strip at a constant temperature of 5°C. After electrophoresis, the

proteins were fixed in 40% ethanol and 10% acetic acid solution for 1 h. The gels were stained for 7 days, under agitation, in a 0.1% w/v colloidal Coomassie G 250 solution (Neuhoff et al., 1988). The 2-DE images were processed using the Kodak EDAS 290 imaging system. The High-Range Rainbow Molecular Weight Marker was used (GE Healthcare). All gel separations were conducted with 3 replicas.

Protein Digestion and sample handling

Spots of interest were excised from 2-DE gels and submitted to a series of dehydration in 2:1 (acetonitrile: 25mM ammonium bicarbonate)/re-hydration (25 mM ammonium bicarbonate) steps that proceeded, usually three times, until the spots became totally transparent. The gel spots were then dried in a concentrator (Model 5301, Eppendorf, Hamburg) and submitted to an in-gel-digestion procedure carried out using a proteomics sequencing grade-modified trypsin (Promega), according to the manufacturer's recommendation. Peptides were extracted using Millipore ZipTip® pipette tips (Millipore, Bedford-MA, U.S.A.) and were further recovered in a 75% acetonitrile: 0.1% Trifluoroacetic acid (TFA) solution. The volume was reduced in the concentrator and samples were then used for MALDI-QTOF MS/MS analysis.

High performance liquid chromatography separations

The secretome of *M. perniciosa* was lyophilized and the powder resuspended in 10 mM PB, pH 6.0. The sample was desalted in a PD-10 Sephadex™ G-25M column (GE Healthcare) and recovered in 20 mM Tris-HCl, pH 9.0. Protein quantification was carried out according to Bradford (1976). One mg of protein, from the glucose and glycerol treatment, was fractionated in a High Performance Liquid Chromatography equipment

(HPLC – ÄKTA GE Healthcare) using an ion exchange MonoQ^{TM} 5/50 GL column (GE Healthcare). Fifty fractions of 1 mL were collected and desalted in PD-10 Sephadex^{TM} G-25M columns (GE Healthcare). The proteins were recovered in 10 mM PB, pH 6.0 and each fraction was infiltrated in *N. benthamiana*- GFP cell suspension, in order to proceed the biological activity assay. The fraction 4, from both treatments, were selected for further analyses. The fractions were concentrated in Amicon ultra centrifugal tubes, cut off 5 KDa (Millipore, Billerica-MA, USA.), as recommended by the manufacturer, and digested with proteomics sequencing grade-modified trypsin (Promega). Sample volume was reduced in a concentrator (Model 5301, Eppendorf, Hamburg) to be further analyzed in a *nanoESI-MS/MS* .

Determination of peptide sequences by bi-dimensional UPLC-nano-electrospray ionization mass spectrometry (nanoESI-MS/MS)

The nanoESI-MS/MS analysis was performed in a quadrupole time of flight hybrid mass spectrometer (Q-Tof Waters – Micromass Manchester, UK) equipped with a nano Z-spray source operating in a positive ion mode coupled to a Ultra-performance liquid chromatographic system UPLC-nanoAcquity. (Waters, Manchester, UK). The samples (1 mg/mL, w/v) GusF1ose, GusF1erol, GusF4ose, GusF4erol were desalted on-line using a Waters Opti-Pak C18 trap column. Trapped peptides were eluted at a flow rate of 200 ηL/min using a water/acetonitrile 0.1% formic acid gradient and separated by a 75 μm ID capillary Acquity NanoEase column packed with C18 silica (Waters, Manchester, UK). The ionization conditions of usage included a capillary voltage of 3.0 kV, a cone voltage of 35 V, extractor voltage of Z-spray source set to 4 V and LM / HM resolution of 5 V and 15 V,

respectively, with different collision energies, depending on the mass and charge state of the precursor ions. The source temperature was 80°C and the cone gas was nitrogen at a flow rate of 80 L/h; nebulizing gas was not used to obtain the sprays. Argon was used for the fragmentation of ions in the collision cell – (collision induced dissociation - CID). External calibration with phosphoric acid 0.1% with acetonitrile: water 1:1 (V/V) was over a mass range from 50 to 2500 m/z and the resolution obtained was 7000 and in a frequency of 20 s. All spectra were acquired with the TOF analyser in "V-mode" (TOF= 7.2 kV) and the MCP voltage set at 2250 V with a scan of 1 spectra/s and MS profile in a range of 300 to 2700 m/z. Acquisition was carried out in data-dependent mode (DDA), and multiple charged ions (such as double and triple charged) were subjected to the MS/MS experiments. The MS/MS spectra were processed using ProteinLynxGlobalServer 2.3 (Waters, Manchester, U.K.) and output files with pkl extension were generated. The combined pkl file was submitted to PLGS/MASCOT search and the resulting spectra were predicted and the product-ion MS/MS spectra were deconvoluted using MaxEnt3 and manually sequenced using the PepSeq application included in the MassLynx (Waters, Manchester, UK).

Quantitative PCR assays

Total RNA was isolated from 14 d old culture of *M. perniciosa*, isolates 487 and 553, grown in mineral medium containing glucose or glycerol as carbon source. Extraction was performed using an RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. RNA was separated in a 1% agarose gel and stained with ethidium bromide in order to confirm its integrity. The cDNA was obtained using a first cDNA strand synthesis kit with M-MuL V reverse transcriptase (Fermentas), as recommended by the manufacturer.

RT-qPCR was performed with four genes identified in the *M. perniciosa* libraries; two of them presumably involved in oxidative stress protection in this fungus: *CTT1* and *CTA1,* catalases (Pungartinik et al., 2009)*;* one gene involved on morphological changes and virulence on others fungi: *RHO1* (Exchange factor for rho1 GTPase) (Martínez et al., 2008) and non-inducible *ACT1* actin as the control (Pungartinik et al., 2009). Expression values were estimated for these 4 genes based on Q-PCR using $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) at 4 sampling periods (0, 0.5, 1 and 2 h). Quantitative real-time amplifications from reversed-transcribed samples (Q-PCR) was performed in 20 µL reactions containing cDNA (10 ng/reaction), 200 pM of each primer (Table 3), taq platinum (Invitrogen), SYBR-green ER® (Applied Biosystems) and ROX (Invitrogen). Amplification was performed in triplicates in the ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with initial incubation at 50° C for 5 min, 95 ºC for 10 min, followed by 40 cycles at 95°C for 15s and 60ºC for 1min, with fluorescence detection at the end of extension cycles. After cycling, a melting curve between 60 and 80 ºC for each amplicon was determined (data not shown). Experiments included a negative control (no template DNA), and amplification efficiency of each primer pair was determined by a standard curve derived from 3 serial ten-fold dilutions of cDNA (data not shown). Real-time data provided the values of cycle threshold (C_t) and PCR efficiency (E). The average C_t from the triplicates was applied to determine differences in expression using the internal standard of gene expression of non-inducible Mp*ACT1* as reference based on $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001). Results are the mean of at least 3 independent experiments, and error bars represent the standard deviation; data were analyzed using two-way ANOVA and Student's t test analysis of variance as calculated by the Instat® program.

Table 3 Primers and melting points (MP) for real time polymerase chain reaction

Gene	Gene code	5' primer	3' primer	MP
Guanine exchange factor for Rho1	RHO	TCGCACTGCATTCTCCGTATA	TCGAGGTGTCGGATTTCGA	82,5
Peroxissomal catalase	CTA	CTCCACTCAAGCCTCCTGTC	CGCTCAGTAATCTCGCTCAA	85,2
Secreted catalase	CTT	GTTGTTCCTGGCATCGATTT	TTGAGTGTGTTCGGGGTGTAT	87,9
Actin	ACT*	CCATCTACACCACAATGGAGGA	CCCGACATAGGAGTCCTTCTG	88,2

(*) transcripts used for normalization; MP of the amplicon is expressed in Celsius degrees.

Statistical analyses

All results are the mean of at least 3 independent experiments and error bars represent standard deviation; when necessary, data were analyzed using two-way ANOVA and Student test analysis of variance as calculated by the Instat program®. $p<0.01$.

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CONSIDERAÇÕES FINAIS

O corrente trabalho demonstrou que em *M. perniciosa*, assim como observado em outros fungos, as fontes de carbono podem exercer a função de sinalizadores moleculares. Os resultados demonstraram que características morfológicas do micélio são relacionadas com o metabolismo geral de carbono (fonte fermentável ou não fermentável). Contudo, a atividade secretora do fungo, o perfil protéico do secretome e a sua capacidade em induzir morte celular em tecidos vegetais são variáveis a depender da fonte de carbono utilizada para o crescimento micelial. Esta observação sugeriu que as fontes de carbono podem também atuar como sinalizadoras durante a VB, como em outros patossistemas, modulando modificações morfológicas e fisiológicas do patógeno. Dentre todas as fontes de carbono testadas o glicerol foi a que induziu em *M. perniciosa* a produção do secretoma com maior atividade em induzir morte celular em plantas modelo, como o fumo, e no hospedeiro natural do patógeno, o cacau. Esta atividade necrótica só é aumentada em *M. perniciosa* dicariótico, que é o tipo celular padrão da fase necrotrófica deste fungo hemibiotrófico (Evans & Bastos, 1980, Griffith et al., 2003). Contudo, o glicerol é utilizado *in vitro* visando manter as culturas do *M. perniciosa* na fase monocariótica (Meinhardt et al., 2006) e portanto deve servir de sinalizador para outros eventos nesta fase micelial.

Análises proteômicas conduzidas com os secretomas de *M. perniciosa* revelaram que de fato a composição protéica (qualitativa e quantitativa) difere a depender da fonte de carbono utilizada para o crescimento do patógeno. A espectrometria de massas identificou algumas das proteínas mais abundantes em secretoma induzido por glicerol. Os cDNAs que codificam para estas proteínas foram isolados das bibliotecas funcionais de *M. perniciosa*. Análises funcionais serão conduzidas visando caracterizar se estas proteínas apresentam a

capacidade de induzir morte celular em tecidos vegetais. Dentre as proteínas mais abundantes presente no secretoma induzido em resposta a glicerol encontra-se a MGG 01215, uma proteína hipotética de M. grisea. O cDNA que codifica para esta proteína foi o único identificado apenas em uma biblioteca representativa da interação compatível cacau: *M. perniciosa*. O fato de apenas 2% dos transcritos desta biblioteca de cDNA pertencer ao patógeno sugere que este gene deva ser altamente expresso durante a VB. Este clone já foi isolado e será submetido a análises funcionais.

O micélio dicariótico crescido em glicerol apresentou também uma maior resistência a tratamentos com indutores de estresse em parede celular, como o vermelho congo e SDS. Além disso, as quantidades de transcritos de catalase intramicelial bem como a atividade de catalases secretadas aumentam mediante o crescimento do fungo em glicerol, em comparação com glicose. Em conjunto, esses resultados sugeriram que o glicerol induz simultaneamente a um aumento na atividade necrótica do secretoma de *M. perniciosa*, modificações relacionadas com a maior proteção do fungo. O modo como o glicerol induz as modificações morfológicas e fisiológicas em *M. perniciosa* não foi elucidado. Os nossos resultados identificaram ao menos uma das rotas de sinalização genética ativada por glicerol: a CWI. Contudo, a comprovada complexidade e interligação molecular entre elementos das rotas de sinalização em outros patógenos sugerem que mais eventos devam ser desencadeados por glicerol. A maneira como o glicerol induz a ativação da CWI em *M. perniciosa* também não foi estabelecida. Em estudos realizados com leveduras mutantes comprovou-se que a quantidade interna de glicerol está diretamente relacionada com a ativação coordenada das rotas HOG e MKC (Wojda et al., 2003). Esta informação, aliada ao conhecimento que o glicerol é um poliol que apresenta livre passagem através da membrana plasmática, sugere que um aumento no nível intramicelial de glicerol possa ser o

sinal que desencadeia as rotas bioquímicas em *M. perniciosa*. Novos estudos deverão ser realizados visando elucidar estes mecanismos.

Os nossos resultados revelaram também que as respostas induzidas por glicerol são conservadas nos diferentes genótipos do *M. perniciosa* testados. Além disso, a sensibilidade ao glicerol, medida em função do aumento tanto na quantidade de transcritos da rho-gef e catalases quanto na atividade de enzimas hidrolíticas, é maior nos genótipos mais patogênicos de *M. perniciosa*. Recentemente, Caribé e colaboradores (2009) desenvolveram um protocolo para silenciar genes em *M. perniciosa* via RNA interferente. Esta técnica será aplicada visando silenciar genes da CWI com o propósito de caracterizar e comprovar a relação desta rota com a patogenicidade de *M. perniciosa*. Apesar dos experimentos terem sido todos conduzidos *in vitro*, o nível de glicerol aumenta em ramos de cacau infectados por *M. perniciosa*, o que sugere que o mesmo pode atuar como sinalizador para o patógeno também durante a VB (resultados da dissertação, Scarpari et al., 2005). Em uma interação VB compatível (genótipo catongo: *M. perniciosa*) o nível de glicerol no fluido apoplástico de cacau começa a aumentar no início da interação, ainda na fase de colonização da planta por *M. perniciosa*. Durante o mesmo período, o nível de glicerol não aumentou em fluido apoplástico de plantas resistentes ao patógeno (genótipo TSH 1188: *M. perniciosa*). Em conjunto, essas observações sugerem que o nível de glicerol ou o seu metabolismo possam estar relacionados a um mecanismo de defesa do hospedeiro, como já observado em outros patossistemas, (Chandra-Shekara et al., 2006; Chanda et al., 2008). Novos experimentos serão conduzidos visando dosar a quantidade de glicerol e glicerol-3-fosfato em fluido apoplástico de cacau, genótipos resistentes (TSH 1188 e outros) e susceptíveis (catongo e outros) à *M. perniciosa*, durante todo o ciclo da VB

(aproximadamente 90 dias). Estima-se, com esta estratégia, identificar se o nível de glicerol em fluido apoplástico de cacau tem relação com a resistência da planta ao patógeno.

Todos os experimentos desta tese foram conduzidos com culturas do *M. perniciosa* estabelecidas *in vitro*. Cabe agora desvendar a importância do glicerol no decorrer da interação VB visando elucidar se existe relação entre o nível deste composto e a patogenicidade do fungo e/ou a resistência do hospedeiro.

APÊNDICE

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ANEXO

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada "Identificação de proteínas secretadas por *Moniliophthora perniciosa* relacionadas com a patogenicidade em *Theobroma cacao*", da aluna Fátima Cerqueira Alvim:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

(x) está inserido no **Projeto CIBio/UESC** (Protocolo n°13/2005), intitulado Rede Nacional de Proteômica: Projeto Proteoma do *Crinipellis perniciosa*" OBS: Este projeto é desenvolvido no laboratório de Genética e Biologia Molecular e Proteômica da UESC, e o projeto da tese da aluna é parte integrante do mesmo, desenvolvido nos mesmos laboratórios;

( ) tem autorização da **Comissão de Ética em Experimentação Animal/IB/UNICAMP** (Protocolo n°);

( ) tem autorização do **Comitê de Ética para Pesquisa com Seres Humanos/FCM/UNICAMP** (Protocolo n°);

( ) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP.
Especificar:

Aluno:

Orientador:

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Nome:
Função:

Universidade Estadual de Santa Cruz
Comissão Interna de Biossegurança

DECLARAÇÃO

Declaro, para os devidos fins, que o número do Certificado em Biossegurança (CQB) dos laboratórios que compõem o Centro de Biotecnologia e Genética (CBG) da Universidade Estadual de Santa Cruz é 0134/01. Dentre os laboratórios do CBG encontram-se os de proteômica e Cultura de Tecidos, locais onde foram desenvolvidos os trabalhos de pesquisa do projeto de tese "Identificação de proteínas secretadas por *Moniliophthora perniciosa* relacionadas com a patogenicidade em *Theobroma cacao*" executado pela estudante Fátima Alvim. Este número de CQB consta na página eletrônica da Comissão Técnica Nacional de Biossegurança (CTNBio, www.ctnbio.gov.br).

Atenciosamente,

Dr. Martin Brendel
Presidente CIBio/UESC

Dr. Martin Brendel
Comissão Interna de Biossegurança
Presidente - CIBIO / UESC