UNIVERSIDADE FEDERAL DE GOIÁS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA

MARÍLIA BARROS OLIVEIRA

Caracterização da expressão de inibidores de poligalacturonases (PGIPs) em resposta aos estresses biótico e abiótico em plantas de feijão comum (*Phaseolus vulgaris* L.*)*

Goiânia

2011

MARÍLIA BARROS OLIVEIRA

Caracterização da expressão de inibidores de poligalacturonases (PGIPs) em resposta aos estresses biótico e abiótico em plantas de feijão comum (*Phaseolus vulgaris* L.*)*

Dissertação apresentada ao Programa de Pós-Graduação em Biologia da Universidade Federal de Goiás, como requisito parcial para a obtenção do grau de Mestre em Biologia – Área de concentração Biologia Celular e Molecular

Orientadora: Profª Drª Silvana Petrofeza da Silva

Goiânia

2011

Dissertação desenvolvida no Laboratório de Biologia Molecular de Fungos Filamentosos, do Departamento de Bioquímica e Biologia Molecular, do Instituto de Ciências Biológicas da Universidade Federal de Goiás.

Apoio financeiro disponibilizado pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); e à Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG).

AGRADECIMENTOS

A **Deus**, presença constante em minha vida, pelas bênçãos concedidas.

Aos meus pais **Dioripes e Isvaldeni**, e meus irmãos **Cláudia e Ricardo** que sempre me deram força acreditaram em mim. Ao meu namorado **Júnior**, que esteve comigo durante toda esta trajetória, acompanhando, ouvindo meus desabafos e transmitindo sempre uma palavra de tranquilidade. Amo muito todos vocês.

À minha orientadora Profª. Drª. **Silvana Petrofeza da Silva**, pelo voto de confiança e pela dedicação. Estes anos de convivência foram constituídos de muita aprendizagem e crescimento profissional. Sou eternamente grata a você por tudo.

Aos meus queridos amigos do laboratório: **Ana Paula, Deyze, Camilla, Elvira, Andressa, Elda, Amarildo e Silvio** pelos anos de convivência, pela amizade e pelo carinho que sei que tiveram por mim. Vou guardar vocês sempre em meu coração.

À **UCB** na pessoa da Profª Drª **Rosângela Vieira de Andrade** pela colaboração na execução de experimentos através do PROCAD UFG-UCB.

Ao Dr. **Murillo Lobo Júnior** da Embrapa Arroz e Feijão pela colaboração nos experimentos realizados em casa de vegetação.

Aos meus **colegas de mestrado** da UFG, em especial Rachel, pelos momentos de convivência e que compartilharam comigo não só momentos de tensão, mas também momentos de alegria.

Agradeço à **UFG** pela oportunidade de formação e pela bolsa concedida.

SUMÁRIO

RESUMO

Sclerotinia sclerotiorum (Lib.) de Bary é um fungo necrotrófico patogênico causador da doença conhecida como mofo branco em muitas plantas. Durante a infecção, secreta várias endopoligalacturonases (endo-PGs) que degradam a pectina da parede celular da planta hospedeira. Para contrapor à ação das PGs, as plantas produzem proteínas inibidoras de poligalacturonases (PGIPs) que reduzem a atividade hidrolítica das endo-PGs e favorecem o acúmulo de oligogalacturonídeos (OGs) que são indutores de uma variedade de respostas de defesa. As PGIPs pertencem à superfamília de proteínas ricas em repetições de leucina (LRR), e desempenham um papel importante na resistência à infecção de patógenos. Neste estudo, RT-PCR em tempo real foi usada para avaliar a expressão de genes *Pvpgip* em plantas de feijão comum (*Phaseolus vulgaris* L.) submetidas a diferentes condições de estresse. A análise transcricional mostrou que estes genes são ativados por fatores bióticos (infecção por *S. sclerotiorum*) e abióticos (injúria ou tratamento com metil jasmonato) e que existe uma variação em termos de expressão. *Pvpgip1* foi induzido nos estágios iniciais da infecção, principalmente para as plantas tratadas com metil jasmonato (MeJA) em que o acúmulo deste transcritos foi maior. Altos níveis de expressão dos genes *Pvpgip2* e *Pvpgip3* foram observados nas plantas tratadas ou não com MeJA. Todos os tratamentos mostraram indução do gene *Pvpgip4.* Estes resultados mostraram que os quatro genes *Pvpgip* respondem diferentemente ao tratamento com indutor de resistência, infecção por fungo e injúria.

Palavras-chave: Endopoligalacturonases; metil jasmonato; feijão comum; PGIP; *Sclerotinia sclerotiorum.*

ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen that causes the disease known as white mold in many plants. During infection, it secretes several endopolygalacturonases (PGs) to degrade cell wall pectin. To counteract the action of PGs, plants express polygalacturonases-inhibiting proteins (PGIPs) that reduce the hydrolytic activity of endo-PGs and favor the accumulation of oligogalacturonides (OGs) with are elicitors of a variety of defense responses. PGIPs belong to the superfamily of leucine rich repeat (LRR) proteins and play important roles in resistance to infection of pathogens. In this study, real time RT-PCR was used to evaluate the expression of *Pvpgip* genes in dry bean plants (*Phaseolus vulgaris* L) submitted to different stress conditions. Transcriptional analysis showed that these genes are differentially expressed and activated by biotic (*S. sclerotiorum* infection) and abiotic (wound or methyl jasmonate treatment) stresses. *Pvpgip1* was induced at early stages of the infection especially for plants treated with methyl jasmonate (MeJA) in which the transcript accumulation was higher. High levels of *Pvpgip2* and *Pvpgip3* expression were observed in infect plants treated or not with MeJA. All treatments showed induction of gene *Pvpgip4.* These results show that the/ four genes *Pvpgip* respond differently to treatment with the resistance inducer, fungal infection and wound.

Key words: Endopolygalacturonases; methyl jasmonate; Dry bean; PGIP; *Sclerotinia sclerotiorum.*

LISTA DE FIGURAS

1-INTRODUÇÃO

O Brasil é atualmente o maior produtor de feijão comum (*Phaseolus vugaris* L.), que se constitui o alimento protéico básico da dieta do brasileiro. Para atender essa demanda, o feijão comum é plantado durante todo o ano, nos mais variados sistemas de cultivo. Na safra 2008, foram produzidos 2,8 milhões de toneladas de feijão comum em uma área de 2,3 milhões de hectares, com uma produtividade média nacional de 1.223 Kg/ha (Feijão, 2010).

O Estado de Goiás e do Distrito Federal respondem por cerca de 10% da produção nacional de feijão, o que representou 266.806 toneladas, em 2008 (Feijão, 2010). Esta produção está concentrada nas épocas de semeadura das águas correspondendo aos meses de outubro a dezembro (40%) e de inverno de abril a julho (49%). A produtividade média de 2.296 kg/ha obtida nesses Estados, é bastante superior à média nacional de 1.233 kg/ha.

O cultivo do feijão no inverno sob irrigação, em especial com o uso de pivô central, gera condições favoráveis para o desenvolvimento de doenças como, por exemplo, o mofo branco. Esta doença é uma das mais destrutivas do feijão comum, capaz de causar 100% de perdas na sua produção (Lobo Júnior et al., 2009). A doença é caudada pelo fungo habitante de solo *Sclerotinia sclerotiorum* Lib (De Bary), registrado pela primeira vez no Brasil em 1920 (Lobo Júnior et al., 2009). Mundialmente este parasita está distribuído por todos os continentes, sendo relatado na África do Sul, Austrália, Bulgária, Canadá, China, Coréia, México, Nova Zelândia, Nigéria, Portugal, Romênia e Taiwan (Farr e Rosman, 2010).

O primeiro indício da presença da doença é um aspecto de murcha da planta. Nos órgãos infectados são encontradas lesões encharcadas, de coloração parda e consistência mole, com micélio branco de aspecto cotonoso, cobrindo os tecidos da planta. Com o progresso da doença, as folhas e caules infectados tornam-se marrons e permanecem eretos

mesmo com a morte da planta. O fungo é capaz de infectar qualquer parte da planta, porém, as infecções iniciam-se com mais freqüência a partir das inflorescências e das axilas das folhas e ramos laterais (Kimati et al., 2005).

1.1-O fungo *Sclerotinia Sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary pertence ao filo Ascomycota, ordem Helotilales, família Sclerotineacea, gênero *Scleorotinia.* É um fungo de solo que ataca cerca de 75 famílias, 278 gêneros e 408 espécies ou variedades de plantas, sendo a maioria plantas herbáceas da subclasse dicotiledônea (família Angiosperma) e de grande importância econômica (Boland e Hall, 1994).

Este fungo sobrevive principalmente na forma de escleródios, que são estruturas compactas, melanizadas, podendo sobreviver por longos períodos de tempo sob condições adversas. Essas propriedades são essenciais para a sobrevivência, propagação e difusão do fungo, já que encontrando condições favoráveis como alta umidade e temperatura baixa os escleródios poderão dar início à germinação. O enorme potencial reprodutivo, juntamente com a capacidade de sobrevivência em longo prazo faz dos escleródios componentes centrais na epidemiologia de *S. sclerotiorum.* Escleródios podem germinar carpogenicamente ou miceliogenicamente, dependendo das condições ambientais, resultando em duas distintas categorias de doenças. No ciclo de vida de *S. sclerotiorum* (Figura 1) pode ser observada a importância destes mecanismos no processo inicial de patogenicidade deste fungo. A forma miceliogênica produz hifas que podem atacar diretamente os tecidos das plantas. A forma carpogênica produz apotécios e, posteriormente, ascósporos aéreos que infectam partes de plantas hospedeiras (Bardin e Huang, 2001).

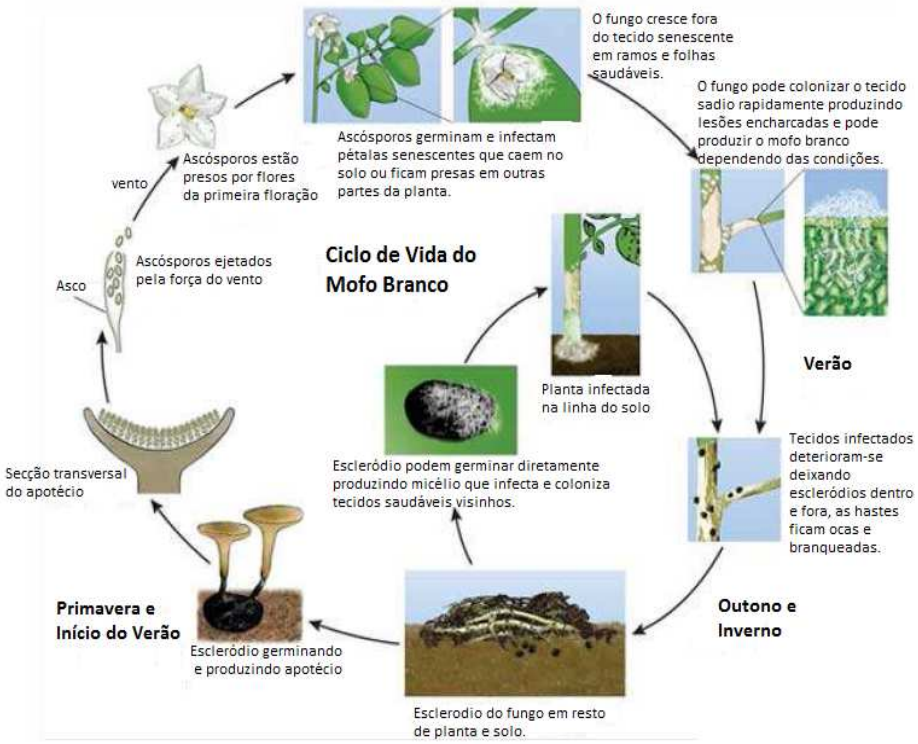

Figura 1: Esquema do ciclo de vida de *Sclerotinia sclerotiorum*. São mostradas as fases carpogênicas e miceliogênicas de propagação. Fonte: www.potatodiseases.org.

1.2 Fatores de patogenicidade

A parede celular é primeira barreira imposta pela planta para contrapor ao ataque de patógenos. A parede celular das células vegetais é formada por uma complexa rede de microfibrilas de celulose, moléculas de hemicelulose e pectina (Figura 2). Esta última é um carboidrato, formado por polímeros de ácido poligalacturônico, que funciona como um agente cimemtante fazendo a união das células (de Vries e Visser, 2001).

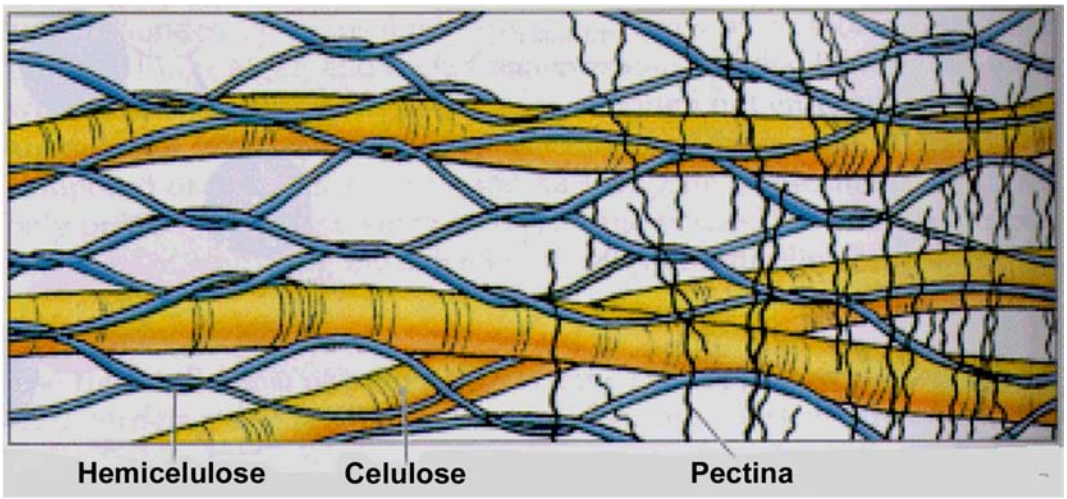

Figura 2: Esquematização da parede celular vegetal. Fonte: www.ippa.info

Para *S. sclerotiorum* vencer a barreira imposta pela parede celular das plantas e ganhar acesso à célula hospedeira, dois principais fatores de patogenicidade, secreção de ácido oxálico e enzimas hidrolíticas, agem em concerto na maceração de tecidos vegetais e geração de necrose. A degradação de componentes da parede celular da planta está ligada à produção de uma ampla e complexa variedade de enzimas líticas, como celulases, hemicelulases, cutinases, pectinases e proteases. A secreção de enzimas líticas pelo fungo facilita a penetração, colonização e maceração dos tecidos vegetais e, também, gera uma importante fonte de nutrientes (Bolton et al. 2006).

1.2.1 Ácido Oxálico

O ácido oxálico é produzido pela maioria das classes de fungos. Sua síntese é catalisada pela enzima oxalacetato acetilhidrolase e constitui um forte regulador de pH do meio. A atividade desta enzima aumenta em pH alcalino e à medida que o ácido oxálico acumula, o pH diminui limitando assim sua produção (Kubicek et al., 1988).

A síntese e a secreção de ácido oxálico têm como determinante o pH do meio via um fator de transcrição conhecido como *pacC (*Rollins e Dickman, 2001). Homólogos de *pacC* foram isolados e caracterizados em fungos, incluindo *Aspergillus nidulans, Saccharomyces cerevisiae, Candida albicans* e *Yarrowia lipolytica.* Genes ativados por este fator de transcrição em resposta ao pH ambiente estão envolvidos na virulência destes patógenos (Diez et al., 2002 Peñalva et al., 2002; El Barkani et al., 2000; Lambert et al., 1997).

Em *S. sclerotiorum* o gene *pac1,* homólogo de *pacC,* é necessário para o seu crescimento em pH neutro, virulência total, produção de ácido oxálico e desenvolvimento normal de escleródios, demonstrando claramente que a expressão de genes em resposta ao pH é um aspecto importante no desenvolvimento e patogênese deste fungo (Rollins et al.,2003).

A secreção de ácido oxálico é determinante para a patogênese em *S. sclerotiorum* (Marciano et al., 1983; Godoy et al 1990; Rollins e Dickman 2001). Esta evidência foi reforçada pela observação na qual mutantes de *S. sclerotiorum* que não produziam ácido oxálico foram incapazes de colonizar tecidos infectados (Godoy et al., 1990) e plantas de soja transgênicas capazes de eliminar oxalato, através da atividade da enzima oxalato oxidase, foram resistentes a este patógeno (Donaldson et al., 2001).

Vários são os mecanismos pelos quais a secreção de ácido oxálico reforça a virulência em *S. sclerotiorum:*

1. Várias enzimas secretadas por fungos durante a invasão de tecidos vegetais têm atividade máxima em pH baixo, assim, a redução do pH provocada pela secreção do ácido oxálico favorece a degradação enzimática da parede celular das plantas (Marciano et al., 1983).
2. Por ser tóxico para as células hospedeiras, devido à sua acidez, o ácido oxálico enfraquece a planta facilitando a invasão de patógenos (Noyes e Hancock 1981).

3. O ácido oxálico é um quelante de íons Ca^{2+} do apoplasto. Assim, desestabiliza a parede celular, além de comprometer respostas de defesa da planta dependentes de Ca^{2+} (Bateman e Beer, 1965).
4. Suprime o estresse oxidativo que constitui uma importante resposta de defesa para muitas espécies de planta (Cessna et al., 2000).
5. Diminui a afinidade da ligação entre poligalacturonases (PGs) do fungo com inibidores de poligalacturonases do hospedeiro (Favaron et al.2004).

1.2.2 Enzimas hidrolíticas

Várias enzimas que degradam a parede celular da planta são produzidas por *S. sclerotiorum* durante a infecção (Marciano et al., 1983 e Rioy et al., 1991). Dentre estas enzimas estão incluídas: pectinases, β-1,3-glicanases, glicosidases, celulases, xilanases, cutinases e proteases (Lebeda et al., 2001). A acidificação do meio provocada pela liberação de ácido oxálico induz a atividade da maioria destas enzimas (Marciano et al., 1983).

1.2.2.1 Poligalacturonases

A pectina é o maior constituinte da parede celular da planta e as pectinases produzidas por *S. scleortiorum* atuam na degradação deste polímero durante o processo de infecção. A hidrólise da pectina enfraquece a parede celular e facilita a penetração e colonização do hospedeiro. Entre estas pectinases, as poligalacturonases (PGs) têm recebido particular atenção. A atividade total dessas enzimas é aumentada com a liberação de ácido oxálico durante a infecção da planta. Tal efeito é particularmente evidenciado quando o pH está baixo, ideal para a atividade das PGs (Favaron et al., 2004).

As poligalacturonases (EC 3.2.1.15) são as primeiras enzimas a serem secretadas por patógenos quando estes encontram a parede celular da planta. Sua contribuição para a

patogenicidade foi demonstrada, por exemplo, a deleção do gene *Bcpg2* que codifica uma isoforma de PG em *Botrytis cinerea* resultou em uma forte redução da virulência deste fungo em tomate e feijão (Kars et al., 2005). PGs são importantes fatores de patogenicidade para *S. sclerotiorum* assim como para outros fungos, como: *Aspergillus flavus, Alternaria citri, Claviceps purpurea* e para bactéria como *Agrobacterium tumefaciens* e *Ralstonia solanacearum* (Di Matteo et al., 2006; Federici et al., 2006).

As endopoligalacturonases (endo-PGs) atuam clivando as ligações α(1-4) entre resíduos de ácido D-galacturônico em homogalacturona, causando a separação e maceração do tecido do hospedeiro. Já as exopoligalacturonases (exo-PGs) clivam grupos glicosil monoméricos ou diméricos dos polissacarídeos pécticos da parede celular. Um maior interesse é dado às endo-PG as quais são capazes de macerar os tecidos da planta promovendo a liberação de oligossacarídeos que são potentes sinalizadores celulares e podem ativar a resposta de defesa da planta hospedeira (Benito, et al., 1998).

Em fungos, os genes que codificam para PGs são organizados em famílias cujos membros apresentam alto grau de polimorfismo (Markovic e Janecek, 2001). A maioria dos fungos produz múltiplas PGs que diferem em propriedade enzimática, peso molecular e regulação. Várias isoformas são expressas em diferentes tecidos em diferentes tempos em resposta a diferentes estímulos (González-Cerrana et al., 2007). Esta multiplicidade pode conceder flexibilidade de adaptação aos patógenos quando infectam hospedeiros ou órgãos de plantas diferentes (De Lorenzo et al., 2001). O ascomiceto *Aspergillus Niger* apresenta onze genes que codificam para PG (de Vries e Visser, 2001; Pel *et al.*, 2007), *B. cinera* apresenta cinco genes PG (Kars et al., 2005) e o basidiomiceto *Chondrosterum purpureum* tem cinco genes que codificam para PGs (Williams et al., 2002).

Em *S. sclerotiorum*, a análise de EST (*Expressed Sequence Tags*) identificou distintos cDNAs codificando para quatro endo-PGs (SSPG1, SSPG3, SSPG5 SSPG6) e duas exo-PGs

(SSXPG1 e SSXPG2) (Li et al., 2004a). Entretanto, outros trabalhos já haviam identificado outros genes que codificam para PGs: *pg1*, *pg2* e *pg3* (Reymond *et al.*, 1994) e *pg5*, *pg6* e *pg7* (Kasza et al., 2004). As modificações pós-traducionais, como glicosilação, e pós-secrecionais explicam a multiplicidade de enzimas secretadas por fungos filamentosos.

Análises das endo-PGs de *S. scleorotiorum* mostraram que elas apresentam expressão diferencial durante a patogênese (Cotton et al., 2002; Kasza et al., 2004; Li et al., 2004b). Trabalho realizado em raiz de cenoura revelou um padrão sequencial de expressão de *pg*, em que *pg1- pg3* são expressos na fase inicial de colonização do tecido sadio da planta, *pg5* foi transcrito durante a fase final de maceração, e *pg6* e *pg7* exibiram padrão de expressão constante (Kasz et al., 2004) .

Análises de sequências promotoras indicaram que a expressão de vários genes que codificam para endo-PGs estão sujeitos a regulação transcricional em resposta à disponibilidade de fonte de carbono e/ou fatores ambientais, como pH. Dois fatores de transcrição estão envolvidos na regulação da expressão dos genes endo-PGs: *CreA* regulando a repressão catabólica de carbono e, *PacC* regulando a expressão de genes dependentes de pH (Wuben 2000). Sítios de ligação para *PacC* foram encontrados em regiões promotoras de *pg5* e *pg6* e sítios alvos para *CreA* também estão presentes em regiões promotoras desses genes em *S. sclerotiorum* (Kasza et al., 2004). O repressor de glicose CRE1 foi isolado e caracterizado em *S. sclerotiorum* e o envolvimento deste fator de transcrição na regulação de genes endo-PG foi considerado (Vautard et al., 1999). A região regulatória do gene *sspg1* possui diversos sítios de ligação do DNA para o repressor CRE1 podendo restringir a expressão deste gene sob condições saprofíticas (Reymond-Cotton et al., 1996).

No decorrer da infecção, a acidificação do meio e a degradação dos polímeros de pectina presentes na parede celular da planta por ação direta das PGs podem criar uma sequência de condições ambientais. Um rápido esgotamento de indutores tais como

poligalacturonatos ou ácido galacturônico, pode levar à repressão catabólita pelo acúmulo de produtos finais, e um pH ácido pode, através da existência de regiões em vários promotores, ativar ou reprimir a transcrição de genes endoPG, estabelecendo um padrão de cinética de expressão já caracterizado (Kasza et al., 2004; Martel et al., 1998).

Contudo, vale ressaltar que as poligalacturonases não estão envolvidas apenas no processo de degradação do tecido vegetal, as plantas também sintetizam PGs. Estas enzimas desempenham importante papel durante todo o ciclo de vida da planta, incluindo germinação, expansão celular, maturação dos grãos de pólen, deiscência da antera, abscisão, amadurecimento de frutos e quebra de vagens. (Roberts et al., 2002; Kim et al., 2006). Também foi proposto por Rose e colaboradores (2003) que essas enzimas hidrolíticas atuam na desagregação e adesão entre células vizinhas. Essas PGs são codificadas por uma família de genes que apresentam diferentes membros, cujo padrão de expressão varia de acordo com os sítios onde a separação celular ocorre (Gonzalles-Carranza et al., 2007).

1.3 Respostas de defesa da planta

As plantas estão expostas a diversos fatores de estresse, como fitopatógenos, herbívoros e condições extremas de luz, temperatura, água e nutrientes. Em resposta a estes fatores a planta possui defesas constitutivas e/ou induzidas. As respostas de defesa podem estar ausentes ou com pouca expressividade em plantas sadias, mas se tornam evidentes após a invasão do patógeno, quando a planta sofre injúria ou algum outro tipo de estresse (Ryals et al., 1996, Mur et al., 1997, 2000).

Os componentes deste sistema de defesa geralmente incluem polímeros específicos da parede celular e uma mistura de metabólitos secundários, peptídeos e proteínas antimicrobianos (Thomma et al., 1998). A indução de resposta de defesa é ativada pelo reconhecimento de moléculas elicitoras, derivadas de microrganismos invasores, por

receptores da parede celular da planta. Estes eventos de reconhecimento disparam uma cascata de transdução de sinal que resulta, dentre outras coisas, na produção de componentes endógenos de sinalização (Ryals et al., 1996).

Durante o processo de invasão, as enzimas produzidas pelo patógeno para degradar a parede celular são também capazes de induzir resposta de defesa em planta. Dentre elas estão as PGs, descritas anteriormente no tópico 1.2.2.1. A atividade elicitora das PGs tem sido demonstrada em muitos sistemas patogênicos, como: PG de *Colletotrichum lindemuthianum* em plantas de tabaco (*Nicotiana tabacum*; Boudart et al.,2003), PG de *B. cinera* em plantas de uva (*Vitis vinifera;* Poinssot et al., 2003). Hahn e colaboradores (1981) demonstraram pela primeira vez, que PGs não são responsáveis diretamente pela indução de resposta de defesa de plantas, mas provocam a liberação de elicitores da parede celular da planta chamados oligogalacturonideos (OGs), com grau de polimerização entre 10 e 15. OGs são capazes de ativar uma variedade de respostas de defesa incluindo acúmulo de fitoalexinas, síntese de lignina, inibidores de proteinases, produção de β1-3 glicanase e produção de espécies reativas de oxigênio (De Lorenzo 1997). Em *Arabidopsis thaliana*, OGs ativaram resposta de defesa contra *B. cinera* independente de ácido salicílico, etileno e jasmonato, mostrando que a sinalização mediada por OGs é diferente daquela mediada por moléculas sinalizadoras (Ferrari 2007).

1.3.1 Proteínas inibidoras de poligalacturonases – PGIP

Proteínas inibidoras de Poligalacturonases (PGIPs) são glicoproteínas associadas à parede celular das plantas e são capazes de inibir as endo-PG de fungos (De Lorenzo et al., 2001). Estas proteínas pertencem à superfamília de proteínas ricas em repetições de leucina (LRR) (Kobe e Kajava, 2001). Elas são caracterizadas pela presença de 9-10 repetições de 24 resíduos cada, que são organizados para formar duas folhas beta, sendo que uma delas ocupa a

borda côncava interna da molécula e contêm resíduos cruciais para o reconhecimento das PGs (Di Matteo et al., 2003).

Proteínas que contêm LRRs não são restritas ao reino *plantae*, mas são generalizadas entre microrganismos e animais, onde elas estão envolvidas em um importante número de funções, incluindo transdução de sinal, adesão celular, desenvolvimento, reparo do DNA, recombinação, transcrição e processamento de RNA (D'Ovideo et al., 2004b). Uma propriedade comum de proteínas LRR é a capacidade de executar interações proteína-proteína. As PGIPs mostram uma estreita relação com uma série de proteínas vegetais LRR envolvidas na resistência a patógenos e transdução de sinal (Ellis et al., 2000; Gomez-Gomez e Boller, 2000).

As PGIPs interagem especificamente com PGs pela formação de um complexo biomolecular. Foi proposto que a inibição da atividade de PGs por PGIPs pode, não somente, prolongar o acúmulo de oligogalacturonídeos e acentuar a resposta de defesa, mas também diretamente impedir a invasão do patógeno por inativação das PGs (De Lorenzo e Cervone, 1997; Federici et al., 2006).

A inibição de PG por PGIP é altamente específica. Assim, PGIP de uma determinada planta que é capaz de inibir uma PG de certo patógeno pode não ser capaz de inibir PGs de outro patógeno. Além disso, para casos onde uma PGIP é capaz de inibir PG de diferentes patógenos, a percentagem de inibição é diferente. Assim, existem em plantas pequenas famílias de genes codificam isoformas de PGIP que diferem em afinidade e especificidade para PGs secretadas por diferentes patógenos (Federice et al., 2006). Esta especificidade de reconhecimento é resultado de poucas variações na sequência de aminoácidos das PGIPs (Leckie et al., 1999).

Muitos estudos têm sido realizados mostrando que diferentes isoformas de PGIP aumentam a resistência em uma variedade de plantas. Foi visto que a supressão de dois genes

PGIP, *AtPgip1* e *AtPgip*2 de Arabidopsis limita a colonização por *B. cinera* e também reduz os sintomas da doença (Ferrari et al., 2003). Plantas de tomate (*Lycopersicon esculentum*) super expressando PGIP de pêra mostraram uma significante redução da susceptibilidade a *B. cinera* (Powell et al., 2000), e plantas de tabaco (*N. tabacum* L) transformadas com PGIP de feijão apresentaram maior resistência a *B. cinera* (Manfredini et al., 2005). O acúmulo de informações sobre as propriedades exibidas por PGIPs têm gerado um interesse em explorá-las como ferramentas para aumentar a resistência da planta. A este respeito, PGIPs de feijoeiro tem recebido atenção especial.

Em *P. vulgaris* quatro genes parálogos *Pvpgip1-4* formam dois pares *Pvpgip1-Pvpgip2* e *Pvpgip3-Pvpgip4* que codificam para classes funcionalmente distintas de PGIPs. O arranjo e similaridade entre eles sugerem que foram derivados de um antecessor comum como resultado de uma sequência de eventos divergentes de duplicação (D'Ovideo et al., 2004a). O primeiro evento pode ter gerado o antecessor dos pares, *Pvpgip1- Pvpgip2* e *Pvpgip3-Pvpgip4*, cada um dos quais sofreu diversificação de forma independente com arranjo genético limitado dentro das regiões codificantes, representadas por mutações pontuais e pequenas inserções ou deleções. Subsequentemente, a duplicação de segmentos genômicos de aproximadamente 2,000 bp e 4,000 bp pode ter gerado *Pvpgip1* e *Pvpgip2,* e *Pvpgip3* e *Pvpgip4*, respectivamente. A amplitude da identidade de nucleotídeos dentro dos dois pares gênicos incluindo as regiões regulatórias (95% a 97.6%) sugere que as duas recentes duplicações ocorreram aproximadamente ao mesmo tempo (D'Ovideo et al., 2004a). Análise filogenética indica que os grupos *Pvpgip1- Pvpgip2* com *Gmpgip3* de soja (*Glicine max*), uma *Phaseoleae* próxima de feijão, sugere que estes dois genes são provavelmente mais próximos ao gene ancestral que *Pvpgip3 e Pvpgip4* (De Lorenzo et al., 2001).

Comparação de sequências correspondentes a genes *pgip* entre feijão, soja, trigo (*Triticum aestivum)*, arroz (*Oryza sativa*) e Arabidopsis produziram dois diferentes

subgrupos, um contendo as monocotiledôneas, arroz e trigo, e o outro as dicotiledôneas feijão, soja e Arabidopsis. Dentro de cada subgrupo, os membros de cada família estão separados um do outro, exceto *Gmpgip3* que é mais próximo de *Pvpgip1/Pvpgip2* que dos outros membros de soja. Além disso, os membros *pgip* de feijão, soja e arroz formam dois distintos grupos, sugerindo que são estruturalmente classes distintas de genes *pgip* (D'Ovideo et al., 2004b).

A duplicação e diversificação dos genes *Pvpgip* resulta não somente na diversificação de sua função bioquímica, mas também na diversificação de sua regulação. Assim, a expressão dos genes *pgip* tem regulação diferente durante o desenvolvimento do processo patogênico e em resposta a diferentes estímulos de estresse. *Pvpgip3* é expresso em resposta a OGs mas não a glucanos, ácido salicílico ou injúria, a expressão de *Pvpgip4* não é alterada por nenhum destes tratamentos. *Pvpgip1* responde somente a injúria (Devoto et al., 1998) enquanto *Pvpgip2,* que codifica o mais eficiente inibidor de PGs de fungos, é induzido por todos estes estímulos de estresse (D'Ovideo et al., 2004a). Similarmente, em soja (*Glicine Max), Gmpgip1* e *Gmpgip3* tem sua expressão aumentada por injúria e infecção com *S. sclerotiorum,* enquanto que *Gmpgip2* não é induzido por injúria e é somente tardiamente *up*-regulado seguido de ataque do mesmo patógeno (D'Ovideo 2002). Em *Arabidopsis,* ambos *Atpgip1* e *Atpgip2* são *up*-regulados em resposta a *B. cinera,* mas por diferentes caminhos de transdução. *Atpgip1* é *up*-regulado por OGs mas não é alterado por outros sinais relacionados a defesa como etileno, acido salicílico e MeJA. Em contraste a expressão de *Atpgip2* não é induzida por OGs mas é mediada por MeJA (Ferrari et al., 2003). Esta capacidade de ligar diferentes genes, neste caso, *pgip,* em resposta a diferentes sinais de estresse, parece ser uma característica comum das plantas, e pode ter significado adaptativo já que garante a ativação de pelo menos um gene em diferentes situações de estresse, evitando a ativação de outras vias de defesa.

As diferenças entre os vários *pgips* estão não somente presentes na regulação de seus transcritos, mas também na especificidade bioquímica de seu substrato. Ambas as atividades inibitória e habilidade de reconhecimento específico para as quatro PGIPs em *P. vulgaris* foram significativamente diferentes em resposta a várias fontes. Por exemplo, todos os membros da família PGIP de feijão inibem, com diferente especificidade, PGs de *B. cinera* e *Colletotrichum acutatum*, mas somente PvPGIP2 inibe PG de *Fusarium phyllophilum* e somente PvPGIP3 e PvPGIP4 inibem PGs de insetos (D'Ovideo et al., 2004a; Leckie et al., 1999).

1.3.2 Ácido Jasmônico

Para se adaptar e responder às mudanças ambientais uma gama de compostos químicos é produzido pelas plantas. Entre estes compostos, os fitormônios desempenham um importante papel na fisiologia da planta (Wasternack e Hause, 2002; Chen et al., 2006). A classe dos jasmonatos, fitormônios derivados de ácidos graxos têm sido largamente estudados. Estes compostos estão envolvidos em processos cruciais do desenvolvimento e sobrevivência de plantas, incluindo respostas de defesa, metabolismo secundário, processo reprodutivo, senescência, desenvolvimento de frutos entre outros (Seo et al., 2001; Wasternack et al., 2007).

O ácido jasmônico (JA), o mais caracterizado membro da família dos jasmonatos, foi identificado e isolado da cultura do fungo *Lasiodiplodia theobromae* (Avanci et al., 2010). Recentes estudos mostraram que JA pode ser modificado em várias formas formando compostos de extrema importância para a ativação de genes relacionados a respostas de defesa (Liechti e Farmer 2006; Thines et al., 2007).

Outro jasmonato de importância chave é o metil jasmonato (MeJA), forma ester metil do ácido jasmônico, que foi isolado do óleo essencial extraído de pétalas de *Jasminum grandiflorum* (Avanci et al., 2010). MeJA é considerado um importante candidato a molécula de sinalização modulando respostas de defesa (Seo et al., 2001; Wasternack 2007).

O ácido jasmônico e seu precursor ácido 12-oxofitodiênico (OPDA) são compostos derivados de lipídios, que ao ataque de patógenos ou insetos são rapidamente sintetizados através da via de biossíntese de oxilipinas (Wasternack 2007). O ácido jasmônico pode ser rapidamente metabolizado para o composto volátil metil jasmonato, através da atividade da enzima ácido jasmônico carboxil metiltransferase (Seo et al., 2001).

O potencial dos jasmonatos na proteção de plantas contra infecção por patógenos foi mostrado após interação entre batata e *Phytophthora infestans* (Cohen et al., 1993), entre algodão e *Verticillium dahiae* (Li et al., 1996) e entre Arabidopsis e *Pythium mastophorum* (Vijayan *et al.,* 1998) e *Alternaria brassiclola* (Pennickx et al., 1996). O ácido jasmônico mostrou ser também um potente elicitor da expressão de inibidores de proteinases em tomate (Farmer e Ryan, 1992).

Trabalho realizado por Seo e colaboradores (2001) mostrou que super-expressão da enzima ácido jasmônico carboxil metiltransferase levou ao acúmulo de MeJA e aumentou a resistência de *Arabidopsis* ao ataque de patógenos. MeJA também mostrou promover a expressão de genes de resistência em plantas de uva (Belhadj et al., 2006). Tratamentos de folhas com MeJA exógeno elevou o nível de acúmulo de transcritos que codificam para proteínas relacionadas à patogenicidade, como quitinases, inibidores de serinas proteases, PGIPs e β-1,3-glicanases, além de estimular genes envolvidos na biossíntese de fitoalexinas. A capacidade de promover resistência contra o ataque de patógenos foi confirmado pelo aumento da tolerância para o patógeno *Erysiphe necator* (oídeo) após o tratamento com MeJA (Belhadj et al., 2006).

As respostas de defesa das plantas podem ser reguladas, além do ácido jasmônico, por outras moléculas sinalizadoras como o ácido salicílico (SA) e etileno (ET). O ácido salicílico tem seus níveis aumentados na resposta sistêmica adquirida (SAR) que é caracterizada pela ativação de proteínas relacionadas à patogênese (PR), algumas das quais mostraram ter atividade antimicrobiana (Smith et al., 1996). Já o ácido jasmônico e o etileno estão relacionados com a resistência sistêmica induzida (IRS) que é independente de ácido salicílico. Alguns trabalhos demonstraram que as vias de sinalização por ácido salicílico e ácido jasmônico são mutualmente antagonistas, assim, os genes que são ativados pela via do ácido jasmônico são suprimidos com a aplicação exógena de ácido salicílico e *vice versa* (Thomma et al., 1998; Pieterse et al., 2009; Leon-Reyes et al, 2010). Entretanto, foi demonstrado que o ácido jasmônico e o etileno são requeridos conjuntamente para a indução de genes de defesa (Penninckx et al., 1998).

A ativação de uma via em detrimento de outra é determinada pelo tipo de patógeno. Assim, foi demonstrado que patógenos biotróficos são geralmente sensíveis às respostas de defesa mediada pelo ácido salicílico, enquanto patógenos com estilo de vida necrotrófico são comumente sensíveis às respostas de defesa controladas por ácido jasmônico e etileno (Becker and Spoel, 2006).

Estudos recentes afirmam que as plantas, após o reconhecimento do patógeno invasor, são capazes de discriminar com precisão entre diferentes vias de sinalização por meio de interações sinérgicas e antogônicas (Pieterse et al., 2001). Este tipo de *cross-talk* entre diferentes sinais promove uma regulação eficiente, estabelecendo um estado de alerta em plantas e canalizando as respostas de defesa para patógenos específicos (Becker and Spoel, 2006).

Estes mecanismos baseado no *cross-talk* entre diferentes vias de sinalização hormonal podem melhorar ainda mais a resposta de defesa da planta, influenciando também processos relacionados a crescimento, desenvolvimento e adaptação a estresse biótico e abiótico (Kazan e Manners, 2008).

2-OBJETIVOS

2.1- Objetivo Geral:

Este trabalho teve como objetivo avaliar a expressão de genes inibidores de poligalacturonases (*Pvpgip*) em plantas de feijão comum (*Phaseolus vulgaris* L.) em resposta aos estresses biótico e abiótico.

2.2- Objetivos Específicos / Metas

2.2.1- Estabelecer o processo de infecção em:

a. Estresse biótico – infecção por *S. sclerotiorum*

- Inóculo de palito colonizados com *S. sclerotiorum* no caule da planta.
- Inóculo com "*plugs"* de BDA colonizados com *S. sclerotiorum* no caule da planta.

b. Estresse abiótico

- Injúria – inóculo de palito sem fungo no caule da planta.
- Indutor químico - Inóculo com "*plugs"* de BDA colonizados com *S. sclerotiorum* no caule da plantas submetidas ao tratamento com metil jasmonato, indutor de resistência, em diferentes concentrações.

2.2.2- Analisar a expressão de genes inibidores de poligalacturonases (*Pvpgip1, Pvpgip2, Pvpgip3* e *Pvpgip4)* em plantas de feijoeiro submetidas a diferentes situações descritas no item 2.2.1.

2.2.3- Determinar os níveis de atividade enzimática das poligalacturonases secretadas por *S. sclerotiorum* durante a interação patogênica.

2.2.4- Determinar os níveis de inibição de poligalacturonases (PG) de *S. sclerotiorum* por proteínas inibidoras de poligalacturonases (PGIP) de plantas de feijoeiro.

3-RESULTADOS

Os resultados deste trabalho serão apresentados na forma dos seguintes artigos:

1. Characterization of the dry bean polygalacturonase-inhbiting protein (PGIP) gene family during *Sclerotinia sclerotiorum* (Sclerotiniaceae) infection. Genet. Mol. Research, 9: 994-1004 (2010).

2. Polygalacturonases-inhibiting proteins genes are differentially expressed in response to biotic and abiotic stress in common bean (*Phasoelus vulgaris* L.) - a ser submetido.

3.1- Artigo 1: Characterization of the dry bean polygalacturonase-inhbiting protein (PGIP) gene family during *Sclerotinia sclerotiorum* (Sclerotiniaceae) infection.

Publicado no periódico GMR (Genetics and Molecular Research) em Junho de 2010.

Neste trabalho, foi feita a análise comparativa durante a interação parasitária do fungo *Sclerotinia sclerotiorum* em plantas de feijão (*Phaseolus vulgaris* L.). Foi analisada a expressão temporal dos genes *sspg*, que codificam para endopoligalacturonases (endoPG) do fungo e *Pvpgip* que codificam para proteínas inibidoras de polygalacturonase (PGIP) em plantas de feijão.

A análise da expressão dos genes ss*pg* (*sspg1, sspg3, sspg5* e *sspg6)* e dos genes *Pvpgip* (*Pvpgip1, Pvpgip2, Pvpgip3* e *Pvpgip4)* foi realizada por meio de transcrição reversa e reação em cadeia de polimerasse (PCR) semiquantitativa e PCR em tempo real. Os RNAs utilizados foram extraídos de hastes de plantas de feijoeiro infectadas por *S. sclerotiorum* em diferentes tempos após o inóculo.

Os resultados mostraram que os genes que codificam para PGs são diferencialmente expressos sob condições saprofíticas. O gene *sspg1* foi altamente expresso durante a infecção, enquanto que gene *sspg3* foi expresso durante a fase final da infecção. O gene *sspg5* foi o mais uniformemente expresso e *sspg6* foi fracamente expresso.

Durante o curso da infecção, transcritos de *Pvpgip1* não foram detectados nos estágios iniciais da doença, mas apareceram 72 horas após o inóculo. Altos níveis de expressão do gene *Pvpgip2* foram observados durante a fase inicial de infecção apresentando um pico com 48 horas e declinando em 72 horas após o inóculo. A expressão de *Pvpgip3* aumentou fortemente com 96 horas após o inóculo. *Pvpgip4* esteve constantemente presente desde as primeiras 24 horas até o final do experimento. Para plantas não infectadas, que receberam injúria mecânica, alto nível de acúmulo de transcritos foi observado para o gene *Pvpgip4* durante todo o período analisado (24 – 96 hpi).

Tais resultados mostraram uma expressão temporal dos genes ss*pg* e *Pvpgip* no decorrer da infecção de hastes de feijoeiro. Também foi demonstrado que as endoPGs provavelmente contribuem para o processo de infecção durante a colonização do tecido hospedeiro por promover a liberação de oligogalacturonideos da parede celular da planta, que são potentes sinalizadores podendo atuar na resposta de defesa de plantas, como PGIPs.

Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Characterization of the dry bean polygalacturonase-inhibiting protein (PGIP) gene family during *Sclerotinia sclerotiorum* (Sclerotiniaceae) infection

M.B. Oliveira[1], L.B. Nascimento[1], M.L. Junior[2] and S. Petrofeza[1]

[1]Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74.001-940 Goiânia, GO, Brasil; [2]Embrapa Arroz e Feijão, GO-462 km 12, C.P. 179, 75375-000, Santo Antônio de Goiás, GO, Brasil.

Running Title: *Pvpgip* gene expression in dry beans

Silvana Petrofeza (Corresponding author)

Departamento de Bioquímica e Biologia molecular, Instituto de Ciências Biológicas, C.P. 131, Campus Samambaia, Goiânia-GO, 74001-970, Brasil

Fax: (+55) 62 3521-1190

E-mail address: petrofez@uol.com.br

Key words: Endopolygalacturonase; *Phaseolus vulgaris*; Polygalacturonase-inhibiting protein; *Sclerotinia sclerotiorum*; White mold.

ABSTRACT

Polygalacturonase-inhibiting proteins (PGIPs) are leucine-rich repeat (LRR) proteins that inhibit fungal endopolygalacturonases (PGs). The interaction of PGPIP with PGs limits the destructive potential of PGs and may trigger plant defense responses induced by oligogalacturonides. We characterized the expression of fungal *pg* and plant *Pvpgip* genes in bean (*Phaseolus vulgaris*) stems infected with *Sclerotinia sclerotiorum* to determine whether any of them could be associated with the infection process. Transcriptional analysis was carried out by means of semi-quantitative reverse transcription polymerase chain reaction (PCR) or real time PCR. The *sspg1* gene was highly expressed during infection; *sspg3* was subjected regulation during the later phases of infection; *sspg5* was more uniform during infection, whereas *sspg6* was only weakly expressed. In the course of infection, *Pvpgip1* transcripts were not detected in early stages, but their presence was revealed 72 h post-inoculation (hpi). High levels of *Pvpgip2* expression were observed during the initial phase of infection; the transcript peaked by 48 hpi and declined by 72 hpi. *Pvpgip3* expression increased strongly at 96 hpi. *Pvpgip4* was constantly present from 24 hpi until the end of the experiment. However, we detected higher levels of the *Pvpgip4* transcript in the necrotic lesion area than in plants mechanically wounded. Remarkably, only *Pvpgip4* appeared to be moderately induced by mechanical wounding. These results provide evidence that PGs contribute to the infection process during host colonization by promoting the release of plant cell oligogalacturonides, which are powerful signaling molecules and may also activate plant defenses such as polygalacturonase-inhibiting proteins.

INTRODUCTION

White mold, caused by the ascomycete *Sclerotinia sclerotiorum* (Lib.) de Bary, is a significant cause of yield loss in Brazilian dry bean (*Phaseolus vulgaris*) crops. This pathogen survives in the soil for several years as sclerotia and has a very broad host range of more than 400 species, including many yearly crops and weeds, thus enabling fungal populations to persist and spread easily (Boland and Hall, 1994). Effective and economically feasible disease control is laborious and dry bean cultivars are at present highly susceptible to the disease.

To infect and colonize the plant tissues, most fungal pathogens need to overcome the structural barrier represented by plant cell wall. Cell wall degrading enzymes (CWDEs) are deputed to this function, and those able to depolymerize the pectic component of the middle lamellae and primary cell walls are the most intensely investigated (Alghisi and Favaron, 1995; Annis and Goodwin, 1997). The contribution of CWDEs to the pathogenicity or virulence of fungi has been studied in several pathosystems. Endopolygalacturonases (PGs) are among the first enzymes secreted during the infection process and cleave the α(1-4) linkages between D-galacturonic acid residues in homogalacturonan, causing cell separation and maceration of host tissue. The genome of *S. sclerotiorum* strain 1980 encodes five endo-PGs, four of which are expressed in culture or during infection (Li et al., 2004). The importance of polygalacturonases in plant diseases has been demonstrated for the pathogenic fungi *Botrytis cinerea* (ten Have et al., 1998), *Alternaria citri* (Isshiki et al., 2001), *Claviceps purpurea* (Oeser et al., 2002), and *S. sclerotiorum* (Kasza et al., 2004; Li et al., 2004).

Many plants possess a cell wall glycoprotein, polygalacturonase-inhibiting protein (PGIP), that is able to inhibit fungal PGs. The interaction between fungal PGs and plant PGIPs favors the accumulation of oligogalacturonides, which elicit a wide range of defense mechanisms (De Lorenzo et al., 2001; Ridley et al. 2001; De Lorenzo and Ferrari, 2002). A

direct role for PGIPs in plant defense has been demonstrated recently where transgenic tomato and tobacco plants over-expressing PGIP showed a reduction in symptoms when infected by *B. cinerea* (Powell et al. 2000; Ferrari et al. 2003; Manfredini et al., 2005).

Recently, the development of expressed sequence tag (EST) resources, together with the availability of high-quality genomic libraries and the progress in sequence analysis tools, have allowed complete or extensive characterization of the *PGIP* gene families in *Arabidopsis thaliana* (L.) Heynh. (Ferrari et al., 2003) and *Brassica napus* L. (Li et al., 2003; Hegedus et al. 2008). In *P. vulgaris*, four genes code for proteins that show sequence variation from 3% to 24%. These genes are grouped into two couples, *Pvpgip1-Pvpgip2* and *Pvpgip3-Pvpgip4*, probably originating from independent events of gene duplication (D'Ovidio et al., 2004).

In order to compare the temporal expression of *sspg* and *Pvpgip* genes, we performed semi-quantitative reverse transcription polymerase chain reaction (PCR) and real time (PCR) experiments on total RNA extracted from dry bean stems at different times following inoculation with *S. sclerotiorum.* Simultaneous analysis of *sspg* and *Pvpgip* expression during plant infection could better elucidate and substantiate the role of these different components in plant-pathogen interaction.

MATERIALS AND METHODS

Infection and bean tissue sampling

S. sclerotiorum isolate SPS was collected from a naturally infected bean plant and grown in Petri dishes containing PDA (potato-dextrose agar) culture medium for 5 days at 20°C. After this growth period, 1-cm long toothpick ends poked into culture medium were wrapped by the pathogen mycelia, which were used to inoculate dry bean plants afterwards. Plants of the dry bean cultivar Pérola were grown in a greenhouse at Embrapa Arroz e Feijão

(Santo Antônio de Goiás, GO, Brazil), in 2-kg plastic pots with red yellow latosol soil that was treated with 5 g of 4-30-16 NPK fertilizer. The inoculation was performed 10 days after emergence by introducing the toothpick ends wrapped with the pathogen mycelia into the base of the stems 1.0 cm above the soil surface. Plants that were mechanically wounded by introducing a toothpick without fungi into the base of their stems were used as an experimental control. All the plants were kept at 20ºC and 90% relative humidity for 48 h to provide adequate conditions for infection. Tissue samples were collected from both the necrotic part and the chlorotic area of each lesion within 2 cm of the lesion edges at 24, 48, 72, and 96 h post-inoculation (hpi) and immediately frozen in liquid nitrogen prior to RNA and protein extraction.

Semi-quantitative reverse transcription PCR

RNA was extracted from plant tissues and fungal material using the standard Trizol protocol (Gibco-BRL). To remove any genomic DNA contamination, RNA was treated with RNAse-free DNAse I (Promega) followed by enzyme inactivation (2.5 mM EDTA, 65°C/10 min) and ethanol precipitation.

The transcriptional analyses of fungal *PG* (*sspg*) and plant *PGIP* (*Pvpgip*) genes were carried out employing semi-quantitative reverse transcription PCR and the following oligonucleotide primers specific for *pg* sequences: ss*pg1*(AF501307) [pg1R 5’-TCT TGC AGC AGT CGA GAA GA-3’; pg1F 5’-GTG TTG TGT CCG AGG GAG TT-3’]; ss*pg3* (AY312510) [pg3R 5’-ACC CAC CAC TTT GGC TAC TG-3’; pg3F 5’-TGA GAC GGT AAG ACC CTT GG-3’]; ss*pg5* (AY496277) [pg5R 5’-TGT CCA AGT TTT CAG TAT T-3’; pg5F 5’-CTA CCA GCA TTT CCA TTA T-3’]; and ss*pg6* (AF501308) [pg6R 5’-CAA GCT TAT TGG AAT GGG TAT-3’; pg6F 5’-CTG GAG TTG ACG ATT TGA CTA-3’]. The

sizes of all amplified fragments were 495 bp, 448 bp, 452 bp, and 462 bp, respectively. The 28S rDNA gene-specific primers [rDNA-F (5'-GGT GGA GTG ATT TGT CTG-3') and rDNA-R (5'-CTT ACT AGG GAT TCC TCG-3')] were designed according to 28S rDNA gene of *S. sclerotiorum*. These pairs of primers were used to amplify a 600-bp cDNA product that acted as a control in the reverse transcription PCR experiments.

Transcripts of PGIP were amplified using specific primers for *Pvpgip*1, *Pvpgip*2 (both *Pvpgip2.1* and *Pvpgip2.2*), *Pvpgip3*, and *Pvpgip4* as described by D'Ovidio et al. (2004).

First strand cDNA was synthesized using 2 µg of DNAse-treated total RNA using superscript II Reverse Transcriptase (Gibco-BRL), following the supplier's recommendations. We amplified 5 µL of first strand cDNA in a final reaction volume of 25 µL containing 1X Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM specific primers, and 5U Taq DNA polymerase (Gibco-BRL). PCR was initially performed at 94°C for 4 min, and then 27 cycles as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR products were resolved by electrophoresis in a 1.2% w/v agarose gel. Two replicates of reverse transcription PCR experiments were performed for all analyzed genes. To quantify the gene expression levels, we employed densitometry analysis (Scion Image software) available online (http://www.scioncorp.com). Amplified product intensity was expressed as relative absorbance units (AU). The ratio between the relative AU determined for the amplified gene of interest and internal control was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency.

Real-time PCR

The sequences of primers used were: *Pvpgip1* (AJ864506.1) [pgip1 F – CGC CTC ACC GGG AAG AT; pgip1 R – TGT TCC GAG ACA AGT CAA CGA A]; *Pvpgip2*

(AJ864507.1) [pgip2 F – TTC GAC GGC AAC CGA ATC; pgip2 R – TGG TCA TCG ACG TAA ACA GCT T]; *Pvpgip3* (AJ864508.1) [pgip3 F – TCC TTC CCG AAG CAT TTC AC; pgip3 R – GCG TCG CCG GTA TAT TGC]; *Pvpgip4* (AJ864509.1) [pgip4 F – TCC TTC CCG AAG CAT TTC AC; pgip4 R – GCC AGC GTC GTC GGA ATA T]; and actin gene (*act*) of *P. vulgaris* (Q11197) [act-F 5' CCCCAGCGTTCTACGTCT 3', act-R 5' CAT GTC AAC ACG AGC AAT G 3']. The sizes of all amplified fragments were 70 bp, 76 bp, 84 bp, 106 bp, and 92 bp for *Pvpgip1-4* and actin genes, respectively.

Equal amounts of RNA (0.5 µg) were reverse-transcribed using oligo(dT)12-18 primer and submitted to real time PCR . Amplification assays were carried out with an 7900HT Sequence Detection System ABI PRISM instrument (Applied Biosystems, USA) in 12-µL reaction mixtures containing 0.4 µM of each primer described above, 6 µL SYBR Green PCR Master mix (2x) (Applied Biosystems, USA), and 0.2 µL template cDNA. After an initial denaturation step at 95°C for 10 min, amplifications were carried out for 40 cycles at 95°C for 15 s and 60°C for 1 min. To check the specificity of the PCR product, melting curves were analyzed for each data point. Relative gene expression was obtained with the formula: fold induction = $2^{-[\Delta\Delta Ct]}$, where ΔΔCt = [Ct GI (unknown sample) - Ct ACT (unknown sample)] - [Ct GI (reference sample) - Ct ATC (reference sample)]. GI is the gene of interest and ACT is the *P. vulgaris* actin gene used as internal control. The reference sample was chosen to represent 1x expression of the gene of interest.

Enzyme assay

Polygalacturonase activity was quantified by recording the increase in absorbance at 575 nm caused by the release of reducing sugars from citrus pectin. The reaction mixture contained 25 µL of macerated tissue extract and 225 µL 2 mg/ml citrus pectin dissolved in

0.1M sodium acetate buffer, pH 5. After 30 min incubation at 45ºC, the reaction was stopped by the addition of 750 μL dinitrosalicylic acid reagent, boiled for 5 min, and cooled on ice (Miller, 1959). Enzyme and substrate controls were included in all the assays. Enzyme activities are expressed as micrograms of reducing sugar released during 30 min incubation. Protein contents were determined using the Quant-iT Protein Assay kit (Invitrogen).

RESULTS AND DISCUSSION

Aiming at comparing the temporal expression of *sspg* and *Pvpgip* genes, semi-quantitative real time PCR experiments were performed on total RNA extracted from bean stem lesions at different times after inoculation with *S. sclerotiorum*. These experiments were carried out employing specific primers directed to the internal control (rDNA 28S) and to the experimental *sspg* (*sspg1*, *sspg3*, *sspg5*, *sspg6*) and *PvPpgip* (*Pvpgip1*, *Pvpgip2*, *Pvpgip3*, *Pvpgip4*) genes. Afterward, we performed reverse transcription PCR product quantification using the appropriate analyses, which revealed that both *sspg* and *Pvpgip* genes showed high expression levels as a consequence of *S. sclerotiorum* infection.

Expression of *S. sclerotiorum PG* genes during pathogenesis

Dry bean plants were effectively infected by *S. sclerotiorum* (Figure 1). During the first 24 hpi, a mycelial mass developed and invaded the tissues, but no macroscopic symptoms were detectable. At 48 hpi, a necrotic zone was observed around the inoculation site and at 72 hpi, abundant aerial mycelia emerged from plant decayed tissues, which showed maceration on the surface. From 96 hpi on, the mycelium became progressively less abundant while the plant tissues were totally macerated.

Figure. 1. *Sclerotinia sclerotiorum*-infected bean stem at different times after inoculation. Inoculation performed 10 days after emergence, by introducing mycelium-colonized toothpick ends into the base of the stems 1.0 cm above the soil surface, which were removed to take the photograph in order to show the lesions at the following times: (**A**) 24 hpi, (**B**) 48 hpi, (**C**) 72 hpi, and (**D**) 96 hpi; tissue samples collected within 2 cm of the lesion margin.

The expression of *sspg* genes was assessed in infected tissues to determine whether any of them could be associated with the infection process. Transcripts of the *sspg1, sspg3, sspg5*, and *sspg6* genes were detected by semi-quantitative reverse transcription PCR at 24 hpi (Figure 2A-B). At this point, a mycelium developed and invaded the tissues, but no macroscopic symptoms were detectable.

The most significantly induced gene during the early phases of infection was *sspg1*, and the level of its transcript increased strongly at 96 hpi, when the stems were completely invaded by mycelia. Recent evidence suggests that *sspg1* plays a role both in the initiation of the infection and in the subsequent lesion expansion during interaction of *S. sclerotiorum* with *B. napus* (Cessna et al., 2000; Li et al., 2004). In *B. cinerea*, the systematic appearance of individual PGs attests to the role that each plays in the infection process: *Bcpg1* and *Bcpg2*

are expressed in the earliest stages of infection (ten Have et al., 2001), and mutants unable to produce BCPG1 exhibit reduced rates of lesion expansion (ten Have et al., 1998).

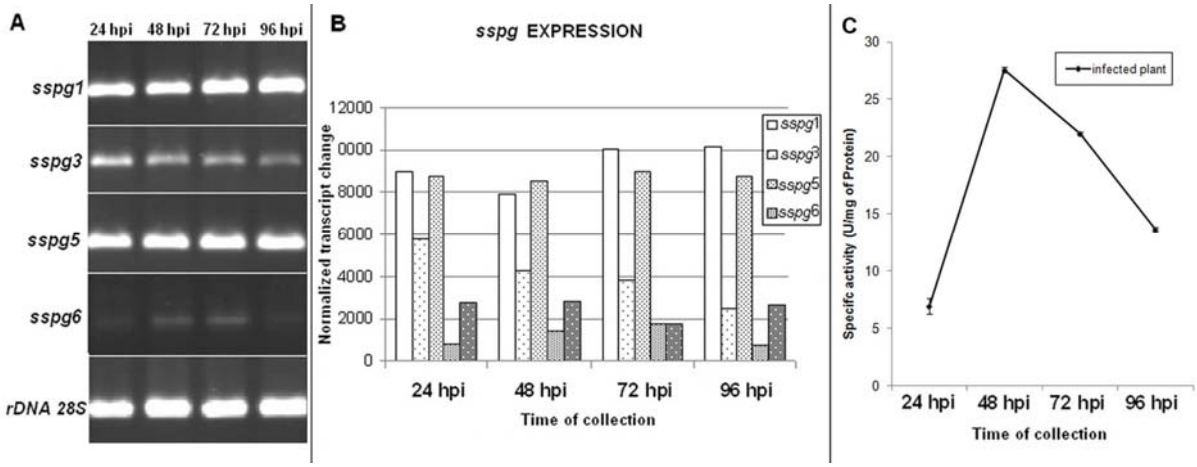

Figure. 2. Expression pattern of polygalacturonase genes (*sspg1, sspg3, sspg5, sspg6*) of *Sclerotinia sclerotiorum* during pathogenesis of bean stems. (**A**) Ethidium bromide-stained reverse transcription PCR products: Tissue samples were collected from the necrotic part of each lesion 24, 48, 72, and 96 hpi. Total RNA was extracted and used in reverse transcription PCR experiments as described in the Material and methods section. A fragment of rDNA was used to evaluate the presence of this fungus in the affected tissues. (**B**) The semi quantitative analysis of *sspg* genes expression was performed by densitometry employing Scion Image software (http://www.scioncorp.com). The *sspg* transcript levels were calculated in relation to the internal control. (**C**) PG activity during pathogenesis of bean stems (see Material and methods section).

In our study, the *sspg3* gene was expressed during the initial phase of colonization of healthy plant tissues and peaked at 24 hpi, when the fungal biomass was very low. Its expression decreased at later stages of host colonization. This pattern of expression suggests that *sspg3* may be more implicated in the early stages of host colonization than during the late stages of maceration of host tissues; *sspg5* was expressed at a high level 24 hpi and the transcripts accumulated between 72 hpi and 96 hpi, the phase of invasive growth of the pathogen and progression of the necrotic zone; *sspg6* was regulated during the final phase of maceration and its transcript reached a maximum at 72 hpi, and then slightly decreased when the host tissues were macerated (96 hpi). A fragment of fungal 28S rRNA was amplified as a control for the amount of fungal RNA present in each sample.

The expression timeframe of *sspg* genes was similar to those reported for *pgs* of other *S. sclerotiorum* isolates (Cotton et al., 2002; Kasza et al., 2004; Li et al., 2004), although the respective patterns were different. For example, the expression of *pg1* precedes that of *pg3*, *pg5*, and *pg6* during infection of carrot roots with *S. sclerotiorum* (Kasza et al., 2004). In that pathosystem, *pg1-3* genes were expressed during the phase of colonization of healthy plant tissues, whereas *pg5* was transcribed during the final phase of maceration, and *pg6* and *pg7* exhibited a more constant expression pattern (Kasza et al., 2004). Studying *B. napus* leaves infected with *S. sclerotiorum*, Li et al. (2004) reported that *sspg1* gene was highly expressed during infection; *sspg3* was also expressed during the early phase of infection, although lower less than *sspg1*, whereas *sspg5* and *sspg6* were only weakly expressed. Curiously, our work indicates that *sspg5* gene was highly expressed during the infection process.

Examination of the expression patterns of individual *sspg* genes has revealed that the interplay of PGs among themselves and with the host during the various stages of infection is finely coordinated. Early in pathogenesis, *S. sclerotiorum* secretes oxalic acid in infected tissues, which gradually lowers the neutral pH of the plant apoplasm. In the course of infection, acidification of the ambient medium and decay of the plant tissue polymers may create an environment conducive for early or constitutive PGs to attack the host cell wall, in turn releasing metabolites that serve to induce expression of other *sspg* genes (Fraissinet-Tachet and Févre, 1996; ten Have et al., 2001).

We also investigated whether the presence of multiple genes could respond to PG activity modulation. During the pathogenesis process in infected tissue, PG activity increased and peaked at approximately 48 h, and then gradually decreased at the times evaluated (Figure 2C).

In addition to previous reports (Kasza et al., 2004; Li et al., 2004), our data indicate that *sspg* genes are differentially regulated under parasitic conditions, the expression of each of them being governed by a unique set of environmental and developmental factors. However, the specific role individual PG plays in the infection process remains to be elucidated.

Members of the *Pvpgip* family are differentially regulated

In order to verify whether the four *Pvpgip* genes display different regulation patterns following wounding and pathogen inoculation, we performed a semi-quantitative reverse transcription PCR analysis using specific primers for each gene. Transcript level analyses were performed on total RNA extracted from 1- to 4-day-old bean seedlings mechanically wounded or infected with *S. sclerotiorum*.

Even with a high number of amplification cycles and at an RNA template concentration higher than that used for the analysis of *Pvpgip* genes of infected samples, amplification of *Pvpgip1-3* never occurred in RNA samples extracted from mechanically wounded plant tissues. Remarkably, only *Pvpgip4* transcripts appeared to be induced by mechanical wounding (Figure 3A).

Densitometric analyses revealed that *Pvpgip* transcripts were differentially induced in dry bean stem tissue in response to *S. sclerotiorum* infection (Figure 3B). During the early phases of infection, *Pvpgip1* transcripts were not detected, whereas their presence was revealed at 72-96 hpi, when the stems were completely invaded by mycelia. *Pvpgip2* levels were high in the initial phase of colonization and peaked at 48 hpi. The levels of transcript declined between 72 hpi and 96 hpi as the plant tissue began to undergo necrosis. *Pvpgip3* expression increased strongly at 96 hpi. A reverse transcription PCR product derived from

transcripts of *Pvpgip4* was present from 24 hpi until the end of the experiment, but its expression was detected at higher levels in the necrotic lesion area than in the mechanically wounded plant.

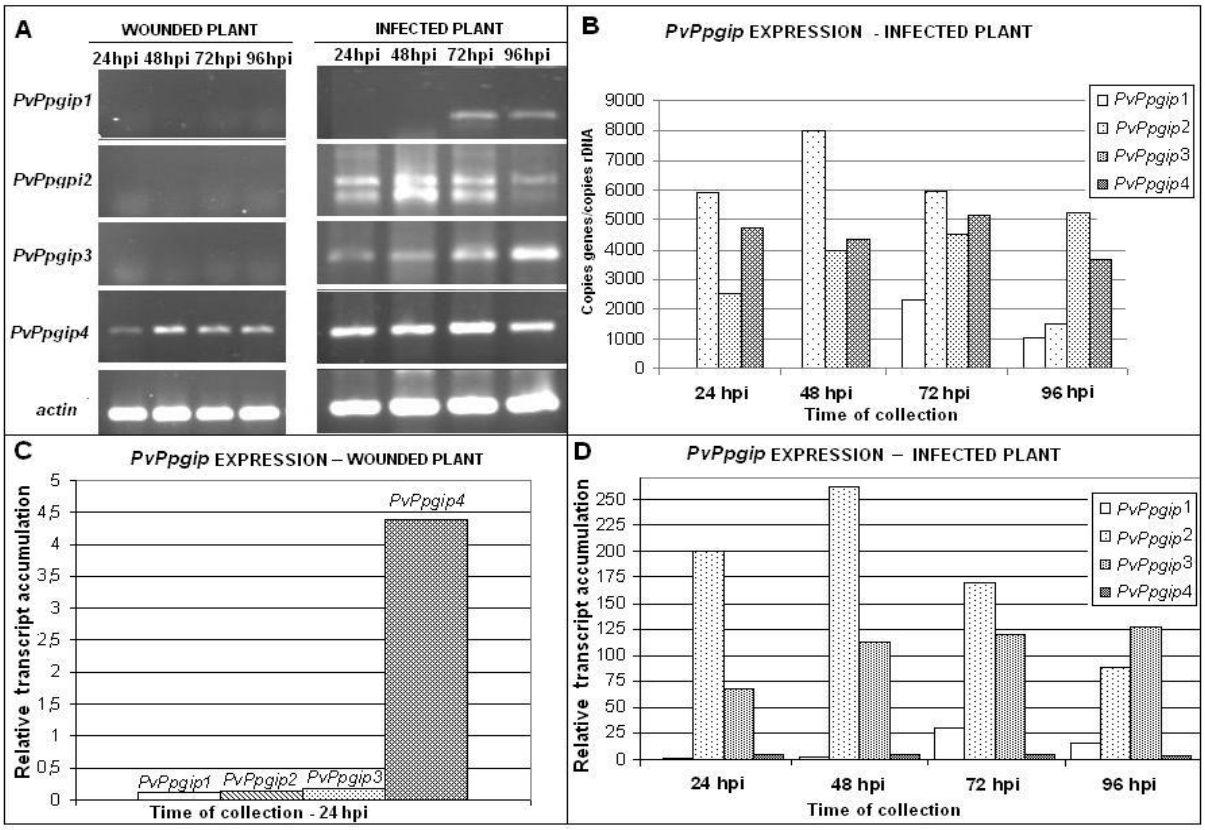

Figure. 3. Differential expression of dry bean *Pvpgip* genes in response to infection with *Sclerotinia sclerotiorum*. (**A**) Total RNA was extracted from bean stem at different times (24, 48, 72, and 96 h) after wounding (wounded plant) or infection with *S. sclerotiorum* (infected plant). Reverse transcription PCR experiments were carried out as described in the Material and methods section. The sizes of the amplified DNA fragments are as follows: *Pvpgip1* – 493 bp; *Pvpgip2* – 443 bp; *Pvpgip3* – 451 bp; *Pvpgip4* – 445 bp. *rDNA* 28S gene was used to control the amount of mRNA present in each sample. (**B**) Semi-quantitative analyses of *PvPpgip* expression in infected tissue were performed exactly as described in Figure 2. **(C and D)** Transcript accumulation of *PvPpgip* genes in wounded or infected plants. Real time PCR analyses were performed and transcript levels were calculated from triplicate data using the standard curve method and: (**C**) *P. vulgaris* actin gene as internal control and health plant (at time zero) as reference sample; (**D**) *P. vulgaris* actin gene as internal control and wounded plant (at respective time) as reference sample. The results represent the mean fold increase of mRNA level over wounded stem, plotted against the 1x □expression level. Results are expressed as mean ± standard deviation of three experiments.

These expression data clearly characterize a sequential pattern of gene transcription and suggest that *Pvpgip2* may be more involved in the early stages of host defense and

Pvpgip1 and *Pvpgip3*, during the late stages. This profile expression was confirmed by real time PCR analyses.

Pvpgip genes are regulated during development and upon wounding and pathogen infection or treatments with eliciting agents such as salicylic acid and cold (De Lorenzo et al., 2001; Ferrari et al. 2003). Analyses performed by D'Ovidio et al. (2004) resulted in the identification of four *PvPpgip*s in *P. vulgaris*. Furthermore, individual *Pvpgip*s are differentially expressed in response to pathogen infection or treatments with elicitors: the expression of *Pvpgip3* is induced by oligogalacturonides but not by fungal glucan, salicylic acid, or wounding; on the other hand, expression of *Pvpgip4* is not altered by any of these treatments. Expression of *Pvpgip1* is induced by wounding only, whereas *Pvpgip2* is upregulated by oligogalacturonides, salicylic acid, and wounding. However, except for the amplification of *Pvpgip1*, which never occurred in RNA samples extracted at time 0 from either untreated tissues or suspension cultured cells, basal levels of transcripts of *Pvpgip1*, *Pvpgip2*, *Pvpgip3*, and *Pvpgip4* were detected by reverse transcription PCR in all RNA samples extracted from bean hypocotyls at different times after wounding (D'Ovidio et al., 2004).

In contrast, we report here that transcripts of *Pvpgip1*, *Pvpgip2*, and *Pvpgip3* were not detected by semi-quantitative reverse transcription PCR in bean stem upon wounding. In order to confirm their regulation, real time PCR approach was used. Primers specific for *Pvpgip1*, *Pvpgip2*, *Pvpgip3*, and *Pvpgip4* were synthesized (see Materials and methods section).

Real time PCR analyses revealed very low basal levels of *Pvpgip1*, *Pvpgip2*, and *Pvpgip3* genes in *P. vulgaris* stems 24 h after wounding (Figure 3 C). The values represent a higher number of transcripts in relation to the healthy plants used as reference in these

analyses. Such discrepancies between data attained on bean hypocotyls (D'Ovidio et al., 2004) and those obtained on stem, however, are not surprising because additional factors occurring in plant tissue may condition PG activity or PG-PGIP interaction. Although other factors such as cultivar, phase of plant development, and cultivation conditions may have influenced the expression of *Pvpgip*, further studies are necessary to prove this.

In infected bean stem, the expression of *Pvpgip1*, *Pvpgip2*, *Pvpgip3*, and *Pvpgip4* genes was rapidly and transiently upregulated (Figure 3 D). Compared to the expression in injured plants, induction of *Pvpgip* genes during stem infection by *S. sclerotiorum* peaked at: 48 hpi for *Pvgip2*, showing a 262-fold increase in the amount of transcripts; 96 hpi for *Pvgip3*, with a 112-fold increase; 72 hpi for *Pvpgip1*, with a 27.2-fold increase; and *Pvgip4* showing an average of 4.9-fold increase at the times evaluated. Transcript accumulation of these genes slowly decreased until 96 hpi. Similar expression profiles were previously observed by semi-quantitative reverse transcription PCR.

Remarkably, *Pvpgip2* gene, which encodes the most efficient inhibitor of fungal PGs characterized so far (D'Ovidio et al., 2004; Manfredini et al., 2005), was the family member most upregulated by infection stimuli. *Pvpgip2* has been previously found to have different types of inhibition, which suggests that the inhibitor adapts the recognition capabilities of its wide concave surface in many ways and against different epitopes of different PG ligands (Sicilia et al., 2005).

Plant defenses against fungal pathogens consist of both a localized response that is often associated with an oxidative burst and a more generalized systemic response mediated by signaling molecules. PGIPs have been shown to halt fungal invasion in plants. Following inoculation with *B. cinerea*, transgenic tomato and grapevine plants overexpressing a *pgip* gene from pear (Powell et al., 2000; Agüero et al., 2005), *Arabidopsis* plants overexpressing

two endogenous *pgip* genes (Ferrari et al., 2003), and tobacco plants overexpressing bean *Pvpgip2* (Manfredini et al., 2005) developed smaller lesions than those in wild type plants, suggesting that PGIPs play a role in the innate immunity of plants and contribute to their basal resistance against fungi.

The PG-PGIP interaction limits the aggressive potential of PGs and favors the accumulation of elicitor-active oligogalacturonides in the apoplast (De Lorenzo and Ferrari, 2002). Oligogalacturonides are general elicitors of a wide number of plant defenses, including accumulation of phytoalexins, synthesis of lignin, expression of β-1,3-glucanase and proteinase inhibitors, and production of reactive oxygen species (Ridley et al., 2001; De Lorenzo and Ferrari, 2002). PGs are also capable of initiating programmed cell death in the host leading to tissue necrosis, a phenomenon that is circumvented by PGIPs (Zuppini et al., 2005). The capacity of PGs to elicit defense responses unrelated to their enzymatic activity has been recently proposed for *B. cinerea*–grapevine interaction (Poinssot et al., 2003). However, the enzymatic activity of PG from *Colletrotrichum lindemuthianum* has been shown to be essential for activating protective responses in tobacco (Boudart et al., 2003).

We showed different temporal expression of *pg* and *Pvpgis* genes in the course of bean stem infection. Our results seem to be consistent with a maximization of fungal PG activity in the host tissue. By promoting the release of plant cell wall polysaccharide fragments and releasing oligogalacturonides, which are powerful signaling molecules, this enzyme may also activate plant defenses such as the increased expression of polygalacturonase inhibitor proteins. Future studies will aim at identifying cell surface proteins specifically induced during the first steps of pathogenesis by a proteomic approach. This should help unravel the complex developmental program underlying the infection process. In order to improve plant resistance through a PGIP-based strategy, knowledge of specific relationships between PGs and PGIPs is crucial.

ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

Agüero CB, Uratsu SL, Greve C, Powell ALT, Labavitch, JM, Meredith CP, Dandekar AM. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. *Mol Plant Pathol* 6:43-51.

Alghisi P, Favaron F. 1995. Pectin-degrading enzymes and plant–parasite interactions. *Eur J Plant Pathol* **101:**365–375.

Annis SL, Goodwin PH. 1997. Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *Eur J Plant Pathol* 103:1–14.

Boland GJ, Hall R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. *Can J Plant Pathol* 16:93–108.

Boudart G, Charpentier M, Lafitte C, Martinez Y, Jauneau A, Gaulin E, Esquerre-Tugayé MT, Dumas B. 2003. Elicitor activity of a fungal endopolygalacturonase in tobacco required a functional catalytic site and cell wall localization. *Plant Physiol* 131:93-101.

Cessna SG, Sears VE, Dickman MB, Low PS. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *Plant Cell* 12:2191–2199.

Cotton P, Rascale C, Févre M. 2002. Characterization of PG2, an early endoPG produced by *Sclerotinia sclerotiorum*, expressed in yeast. *FEMS Microbiol Lett* 213:239–244.

D'Ovidio R, Raiola A, Capodicasa C, Devoto A, Pontiggia D, Roberti S, Galletti R, Conti E, O'Sullivan D, De Lorenzo G. 2004. Characterization of the complex locus of bean encoding polygalacturonase-inhibiting proteins reveals subfunctionalization for defense against fungi and insects. *Plant Physiol* 135:2424–2435.

De Lorenzo G, Ferrari S. 2002. Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. *Curr Opin Plant Biol* 5:295–299.

De Lorenzo G, D'Ovidio R, Cervone F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu Rev Phytopathol* 39:313–335.

Ferrari S, Vairo D, Ausubel FM, Cervone F, De Lorenzo G. 2003. Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *Plant Cell* 15:93–106.

Fraissinet-Tachet L, Févre M. 1996. Regulation by galacturonic acid of pectinolytic enzyme production by *Sclerotinia sclerotiorum*. *Curr Microbiol* 33:49–53.

Hegedus DD, Li R, Buchwaldt L, Parkin I, Whitwill S, Coutu C, Bekkaoui D, Rimmer SR. 2008. *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment. *Planta* 228:241–253.

Isshiki A, Akimitsu K, Yamamoto M, Yamamoto H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Mol Plant-Microbe Interact* 14:749–757.

Kasza Z, Vagvölgyi C, Févre M, Cotton P. 2004. Molecular characterization and in planta detection of *Sclerotinia sclerotiorum* endopolygalacturonase genes. *Curr Microbiol* 48:208–213.

Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G, Hegedus DD. 2004. Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of endo-and exo-polygalacturonases expressed during saprophytic and parasitic modes. *Fungal Genet Biol* 41:754-765.

Li R, Rimmer R, Yu M, Sharpe AG, Séguin-Swartz G, Lydiate D, Hegedus DD. 2003. Two *Brassica napus* polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses. *Planta* 217:299–308.

Manfredini C, Sicilia F, Ferrari S, Pontiggia D, Salvi G, Caprari C, Lorito M, De Lorenzo G. 2005. Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. *Physiol Mol Plant Pathol* 67:108–115.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*. 31, 426–428.

Oeser B, Heidrich PM, Müller U, Tudzynski P, Tenberge KB. 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea* rye interaction. *Fungal Genet Biol* 36:176–186.

Poinssot B, Vandelle E, Bentejac M, Adrian M, Levis C, Brygoo Y, Garin J, Sicilia F, Coutos-Thevenot P, Pugin A. 2003. The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defense reactions unrelated to its enzymatic activity. *Mol Plant-Microbe Interact* 16:553–564.

Powell ALT, van Kan J, ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. *Mol Plant–Microbe Interact* 13:942–950.

Ridley BL, O'Neill MA, Mohnen D. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57:929–967.

Sicilia F, Fernandez-Recio J, Caprari C, De Lorenzo G, Tsernoglou D, Cervone F, Federici L. 2005. The polygalacturonase-inhibiting protein PGIP2 of *Phaseolus vulgaris* has evolved a mixed mode of inhibition of endopolygalacturonase PG1 of *Botrytis cinerea*. *Plant Physiol* 139:1380–1388.

ten Have A, Mulder W, Visser J, van Kan JAL. 1998. The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Mol Plant–Microbe Interact* 11:1009–1016.

ten Have A, Breuil WO, Wubben JP, Visser J, van Kan JAL. 2001. *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genet Biol* 33:97–105.

Zuppini A Navazio L, Sella L, Castiglioni C, Favaron F, Mariani P. 2005. An endopolygalacturonase from *Sclerotinia sclerotiorum* induces calcium-mediated signaling and programmed cell death in soybean cells. *Mol Plant Microbe Interact* 18:849–855.

3.2- Artigo 2: Polygalacturonases-inhibiting proteins genes are differentially expressed in response to biotic and abiotic stress in common bean (*Phasoelus vulgaris* L.)

Manuscrito submetido para o periódico Physiological and Molecular Plant Pathology.

A partir dos resultados obtidos no trabalho anterior, e visando ampliar nosso estudo, este trabalho teve como objetivo avaliar a expressão de genes inibidores de poligalacturonases (*Pvpgip)* em plantas de feijão comum (*Phasoelus vulgaris*) em resposta aos estresses biótico e abiótico.

A análise comparativa da expressão dos genes *Pvpgip* de *P. vulgaris* foi realizada por meio de RT-PCR em tempo real. Foram utilizados RNAs extraídos de haste de plantas de feijão que receberam diferentes tratamentos: estresse biótico (infecção por *S. sclerotiorum)* e estresse abiótico (injúria e indução de resistência por MeJA seguida de infecção por *S. sclerotiorum*). Os ensaios de atividade de PGs, bem como os ensaios de inibição, foram feitos a partir de extrato protéico obtido de hastes de plantas de feijão.

Os resultados demonstraram que nas plantas que receberam estresse biótico, o inóculo do fungo pelo palito levou à expressão dos quatro genes *Pvpgip*. Entretanto, nas plantas inoculadas com o *plug* de BDA, a expressão dos genes *Pvpgip* foi detectada apenas nas primeiras 6 horas para *Pvpgip2* e com 24 horas para o gene *Pvpgip3*, enquanto que *Pvpgip4* foi induzido nos estágios finais da infecção. Já as plantas que receberam estresse abiótico, a injúria levou a altos níveis de expressão no período de 12 horas para os genes *Pvpgip1-3*, enquanto que o gene *Pvpgip4* apresentou níveis de expressão elevados em todo o período analisado. Em plantas infectadas por *S. sclerotiorum* que foram previamente tratadas com MeJA, a indução aconteceu inicialmente (6 e 12 hpi) para o gene *Pvpgip1*. Para os genes *Pvpgip2* e *Pvpgip3* a indução foi tardia (48 e 72 hpi). A indução do gene *Pvpgip4* foi verificada em todo o período analisado, mas foi maior nas primeiras 12 horas.

Os resultados obtidos nos ensaios de atividade enzimática demonstraram que a atividade das PGs foi maior nos estágios finais do processo infectivo, momento que o tecido vegetal estava necrosado. Os ensaios de inibição das PGIPs contra as PGs apresentaram atividade inibitória positiva em relação ao controle.

Nossos resultados demonstraram que os genes *Pvpgip* são expressos diferencialmente em resposta aos fatores bióticos como infecção por *S. sclerotiorum* e abióticos como injúria mecânica e tratamento por metil jasmonato. Isso sugere que as plantas têm diferentes mecanismos de respostas de defesa, e que um único fator de estresse pode ativar diferentes vias, resultando em vários tipos de respostas.

Polygalacturonases-Inhibiting Proteins genes are differentially expressed in response to biotic and abiotic stress in common bean (*Phasoelus vulgaris* L.)

Marília Barros Oliveira[1]; Rosângela Vieira Andrade[2]; Maria Fátima Grossi-de-Sá[2,3]; Silvana Petrofeza[1*].

[1]Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74.001-940 Goiânia, GO, Brazil; [2]Programa de Pós-graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, 70790-160, Brasília, DF, Brazil; [3]Embrapa Recursos Genéticos e Biotecnologia, Laboratório de Interação Molecular Planta-Praga, W5 Norte, 70770-900 Brasília, DF, Brazil.

*Corresponding author: S. Petrofeza

Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Caixa Postal 131, Campus Samambaia, Goiânia, GO, 74001-970, Brazil

Fax: (+55) 62 3521-1190; E-mail: petrofez@uol.com.br

ABSTRACT

The *Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic fungal pathogen that causes the disease known as white mold in many plants. During the infection, the *S. sclerotiorum* discharges several endopolygalacturonases (PGs). To counteract the action of PGs, plants express polygalacturonases-inhibiting proteins (PGIPs) that belong to the super-family of leucine reach repeat (LRR) proteins and play important role in resistance to infection of pathogens. In this study, qRT-PCR was used to evaluate the expression of *Pvpgip* genes in

dry bean plants (*Phaseolus vulgaris* L.), submitted to different stress conditions. The transcriptional analysis showed that these genes are differentially expressed by biotic (*S.sclerotiorum* infection) and abiotic (wound or methyl jasmonate treatment) stress. The P*vpgip 4* gene was strongly induced by *S. sclerotiorum* infection and *Pvpgip3* to methyl jasmonate treatment.

Keywords: Endopolygalacturonases; methyl jasmonate; dry bean; PGIPs; *Sclerotinia sclerotiorum.*

1. INTRODUCTION

The necrotrophic fungal *Sclerotinia sclerotiorum* (Lib.) De Bary is capable of infecting at least 408 plant species economically important [1]. In Brazil, the dry bean culture suffers from severe losses caused by this pathogen.

During the invasion, the pathogen faces the first obstacle to overcome the barrier imposed by the plant cell wall and most fungal pathogens produce a variety of hydrolytic enzymes that degrade the cell wall polymers. The *Endo*-polygalacturonases (PGs) secreted at the early stages of the infection, depolymerizes the homogalacturonan, the main component of pectin, by cleaving α-1,4 glycosidic bonds between the galacturonic acid units causing cell separation and maceration of host tissue [2]. It is known that the *S. sclerotiorum* produces multiple PG forms, biochemical and molecular studies have also been undertaken to determine which of these are important pathogenicity factors or what specific role each plays during the infection process [3-8]. Previously, we showed that *Sspg1* and *Sspg5* are highly expressed during the S. *sclerotiorum* infection in *P. vulgaris* plants and also that the *Sspg3*

gene is expressed during the initial phase of colonization and *Sspg6* is weakly expressed during this process [9].

To counteract the PGs many plants produce polygalacturonase-inhibiting protein (PGIP) that are glycoproteins located in plant cell, preventing cell wall degradation and therefore hampering the invasion process and the release of nutrients that are necessary for pathogen growth [10]. The interaction between fungal PGs and plant PGIP favors the accumulation of oligogalacturonides (OGs), which elicit a wide range of defense responses [11, 12].

The PGIPs belong to the super-family of leucine-rich repeat (LRR) proteins, a class of proteins specialized for protein-protein interactions [13]. They typically contain 10 imperfect LRRs with 24 residues each that are organized to form two β-sheets, one of which – sheet B1- occupies the concave inner side of the molecule owing residues crucial for PG recognition [14]. The PGIPs specifically interact with PGs by forming a biomolecular complex. The PG-PGIP interaction varies in terms of inhibition kinetics and strength, and reflects the counter-adaptation occurring in both enzymes and inhibitors [15].

In *Phaseolus vulgari,s* four paralogues *Pvpip1-4* form two pairs, *Pvpgip1-Pvpgip2* and *PvPgip3-PvPgip4,* which code for functionally distinct classes of PGIPs. The arrangement and similarity among them suggest that they derive from a common ancestor as a result of a sequence of duplication-divergence duplication events [16]. The *pgip* genes are different not in terms of recognition specificity of their products and also because of they are differentially regulated [17]. Studies have showed that *pgip* genes are differentially regulated in response to various biotic and abiotic stresses [18,19].

In this study, we compared the *P. vulgaris Pvpgip1-4* genes expression in response to the *S. sclerotiorum* infection, wounding and jasmonic acid treatment, additionally we determinate the differences in PG during infection stages of *S.sclerotiorum* in this specie.

2. MATERIAL AND METHODS

2.1 Plant material and biotic and abiotic treatment.

The plants of the dry bean cultivar Pérola (PRS) were grown in 2 kg plastic pots with soil fertilized with NPK (4-39-16) (1g/Kg of soil) in greenhouse at 24 ± 2°C temperature, 60 ± 5% relative humidity and 16 h/8 h light/ dark period.

Approximately 10–12 days after seeding, when the primary leaves were completely expanded, plants were sprayed with 5 mL of the aqueous solution of two concentrations of methyl jasmonate (MeJA) – 10 and 50 µM, respectively (100 plants/treatment). The plants received fourth subsequent applications of methyl jasmonate in the respective concentrations, or carrier solution, with an interval of six days each.

The *S. sclerotiorum* inoculation was performed 12 hours after the fourth application of the chemical inducer. The control plants were also inoculated with the pathogen.

2.2 Pathogen inoculation

The *S. sclerotiorum* isolate SPS was collected from a naturally infected dry bean plant and grown in Petri dishes containing PDA (potato-dextrose agar) culture medium for 5 days at 20°C. Two inoculation methods were used: **a) Toothpick** – 1 cm long toothpick ends poked into Petri dishes containing PDA culture medium, inoculated with *S. sclerotiorum* and grown for 5 days at 20°C. After this period, the toothpick ends were wrapped by the pathogen mycelia, which were used to inoculate dry bean plants. **b) PDA plugs** - the *S. sclerotiorum*

was grown on PDA culture medium for 5 days at 20°C, and plugs (3mm) of these cultures were used as inoculum in the axilary region of the stem of plants. The pathogen inoculation was performed 30 days after emergence by introducing the toothpick ends wrapped with the pathogen mycelia into the base of the stems 1.0 cm above the soil surface or adding PDA culture plugs in the axilary region. The control plants that were mechanically wounded by introducing a fungi free toothpick into the base of their stems. All the plants were kept at 20ºC and 90% relative humidity to provide adequate conditions for infection. The tissue samples were collected from both the necrotic part and the chlorotic area of each lesion within 2 cm of the lesion edges at 06, 12, 24, 48, and 72 hours post-inoculation (hpi) and immediately frozen in liquid nitrogen prior to RNA and protein extraction. Each treatment consisted of a pool of 20 plants from different pots.

2.3 Total RNA isolation and real time quantitative RT-PCR

The RNA was extracted from plant tissues and fungal material using the standard Trizol protocol (Invitrogen Corp. Carlsbad, CA, USA). The qRT-PCR system using SYBR Green detection (Applied Biosystems) was used to analyze gene expression in RNA samples. After treatment with DNase I (Invitrogen Corp., Carlsbad, CA, USA) in the presence of RNase inhibitor (Invitrogen Corp., Carlsbad, CA, USA), equal amounts of RNA (1 μg) were reverse transcribed using oligo(dT)12-18 primer and submitted to real time PCR. The amplification assays were carried out with a StepOnePlus (Applied Biosystems, Carlsbad, CA, USA) in 10 μL reactions containing 0.4 μM of each primer, 6 μL of SYBR Green PCR Master mix (2 x), and 0.2 μL of template cDNA. After initial denaturation at 95ºC for 10 min, the amplifications were performed for 40 cycles at 95ºC for 15 s and at 60ºC for 1 min. To check the specificity of the PCR product, the melting curves were analyzed for each data point.

The sequences of primers used were: *Pvpgip1* (AJ864506.1) [pgip1 F – CGC CTC ACC GGG AAG AT; pgip1 R – TGT TCC GAG ACA AGT CAA CGA A]; *Pvpgip2* (AJ864507.1) [pgip2 F – TTC GAC GGC AAC CGA ATC; pgip2 R – TGG TCA TCG ACG TAA ACA GCT T]; *Pvpgip3* (AJ864508.1) [pgip3 F – TCC TTC CCG AAG CAT TTC AC; pgip3 R – GCG TCG CCG GTA TAT TGC]; *Pvpgip4* (AJ864509.1) [pgip4 F – TCC TTC CCG AAG CAT TTC AC; pgip4 R – GCC AGC GTC GTC GGA ATA T]; and actin gene (*act*) of *P. vulgaris* (Q11197) [act-F - CCCCAGCGTTCTACGTCT, act-R - CAT GTC AAC ACG AGC AAT G].

The relative gene expression was obtained with the formula: fold induction = $2^{-[\Delta\Delta Ct]}$, where ΔΔCt = [Ct GI (unknown sample) - Ct ACT (unknown sample)] - [Ct GI (reference sample) - Ct ATC (reference sample)]. The GI is the gene of interest and ACT is the *P. vulgaris* actin gene used as internal control. The reference sample was chosen to represent 1x expression of the gene of interest. Three samples were analyzed for each treatment; values are given in mean ± SD.

2.4 Polygalacturonase activity and inhibition assay

The samples collected from the *S. sclerotiorum* infected plants were macerated in liquid nitrogen and homogenized in 1M NaCl and 0,1M sodium acetate buffer, pH 5.2 (3mL/g tissue). The homogenate was centrifuged at 20.000 g for 25 min at 4 °C. The supernatant obtained was considered the tissue extract. The total proteins were determined using the Quant-iT Protein Assay kit (Invitrogen Corp., Carlsbad, CA, USA).

The polygalacturonase activity was quantified by recording the increase in absorbance at 575 nm caused by the release of reducing sugars from polygalacturonic acid. The reaction mixture contained 25 μL of macerated tissue extract and 225 μL of 0,5% orange polygalacturonic acid (PGA, Sigma, USA) dissolved in 0.1M sodium acetate buffer, pH 5.

After 30 min incubation at 50ºC, the reaction was stopped by the addition of 750 µL dinitrosalicylic acid reagent, boiled for 5 min, and cooled on ice [19]. The enzyme and substrate controls were included in all the assays. The enzyme activity was expressed as micrograms of reducing sugar released during 30 min incubation.

The inhibition assay also done by the release of reducing sugars. For this we blend the culture supernatant of the fungus grown on minimal medium (2g/LNH_4NO_3, 1g/LKH_2PO_4, 0,1g/L$MgSO_4 \cdot 7H_2O$, 0,5g/L yeast extract, 3g/L DL- malic acid, 1g/L NAOH) supplemented with 1% citrus pectin under agitation for 48 hours, and tissue extract in proportion of 1:10. The enzyme activity was performed using 0,1M sodium acetate, pH 5, 0,5% PGA as substrate. The reaction conditions were the same used for the activity of PGs. Three samples were analyzed for each treatment; the values are given in meam ± SD.

2.5 Statistical analysis

The analysis of variance (ANOVA) comparison of treatment means Tukey test at 1% level of probability were performed through the statistical program ASSISTAT (2010) version 7.5 beta.

3. RESULTS

3.1 Expression of *Pvpgip* genes under biotic and abiotic stress condition

The expression of four *Pvpgip* genes was detected in dry bean plants infected with *S. sclerotiorum* by the toothpick-method inoculation: *Pvpgip*1 was induced 6 hours after infection (hpi) and persisted until 72 hpi. *Pvpgip*2 transcripts were detected 6 hpi and increased progressively peaking at 72 hpi. *Pvpgip*3 gene expression was also up-regulated

within 6 hpi, peaking at 48 hpi. *Pvpgip*4 showed high levels of transcripts in the early hours (6-12 hpi) after inoculation (Figure 1).

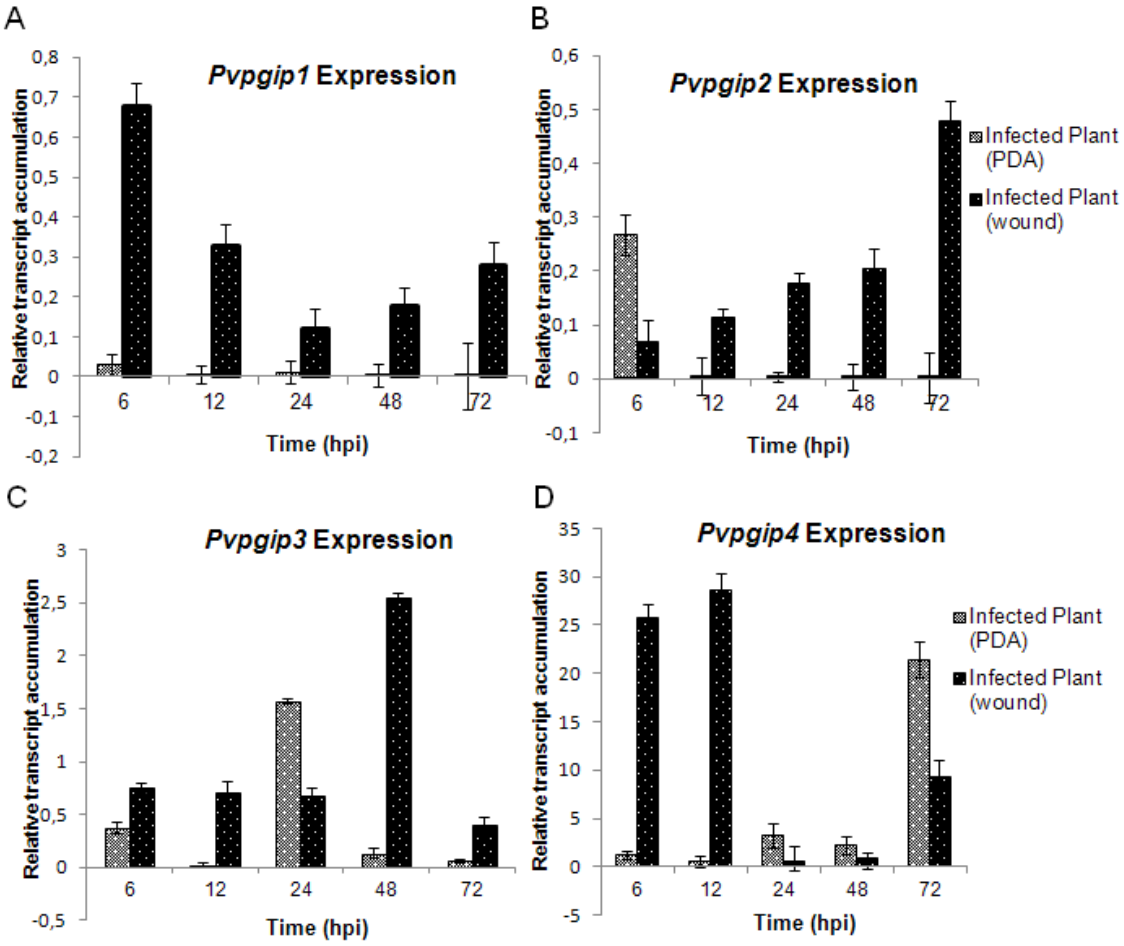

Figure 1: Expression analysis of *Pvpgip* genes of *P. vulgaris* in response to *S. sclerotiorum* infection. (**A**)- *Pvpgip1;* (**B**)- *Pvpgip2*; (**C**)- *Pvpgip3;* (**D**)- *Pvpgip4*. Expression analyses by qqrT-PCR were performed and transcript levels were calculated from triplicate data using comparative method. *P. vulgaris* actin gene as internal control and health plant as reference sample. Tissue samples were collected at 06, 12, 24, 48, and 72 h post-inoculation (hpi). Results are reported as means ± standart deviation (SD) of three experiments

However, in plants inoculated with a plug of PDA of *S. sclerotiorum* culture, different expression pattern of the *Pvpgip* genes was observed: *Pvpgip1* had no expression; the *Pvpgip*2 gene expression was detected only in the first 6 hpi; *Pvpgip*3 at 24 hours and *Pvpgip*4 gene was induced in late stages of the infection (48-72 hpi).

The *Pvpgip* gene expressions in plant stem were also examined using plants that received a foliar application of MeJA 12 hours prior to the *S. sclerotiorum* inoculation. We

observed that this abiotic stress induced the *Pvpgip* genes expression in *P. vulgaris* (Figure 2). The *Pvpgip*1 gene induction occurred in initial 6 to 48 hpi, at all MeJA concentrations tested (Figure 2A). For the *Pvpgip*2 and *Pvpgip*3 genes the induction were delayed (48 - 72 hpi), but only in plants that received the 10µM of the MeJA. For these two genes, in plants that received 50 µM of the MeJA, no significant values of accumulated transcripts were observed (Figure 2B-C). The induction of *Pvpgip*4 gene was observed in the whole period but was higher in the first 12 hours in plants treated with 10µM of MeJA. However, in plants treated with 50 µM of MeJA lower induction was observed (Figure 2D).

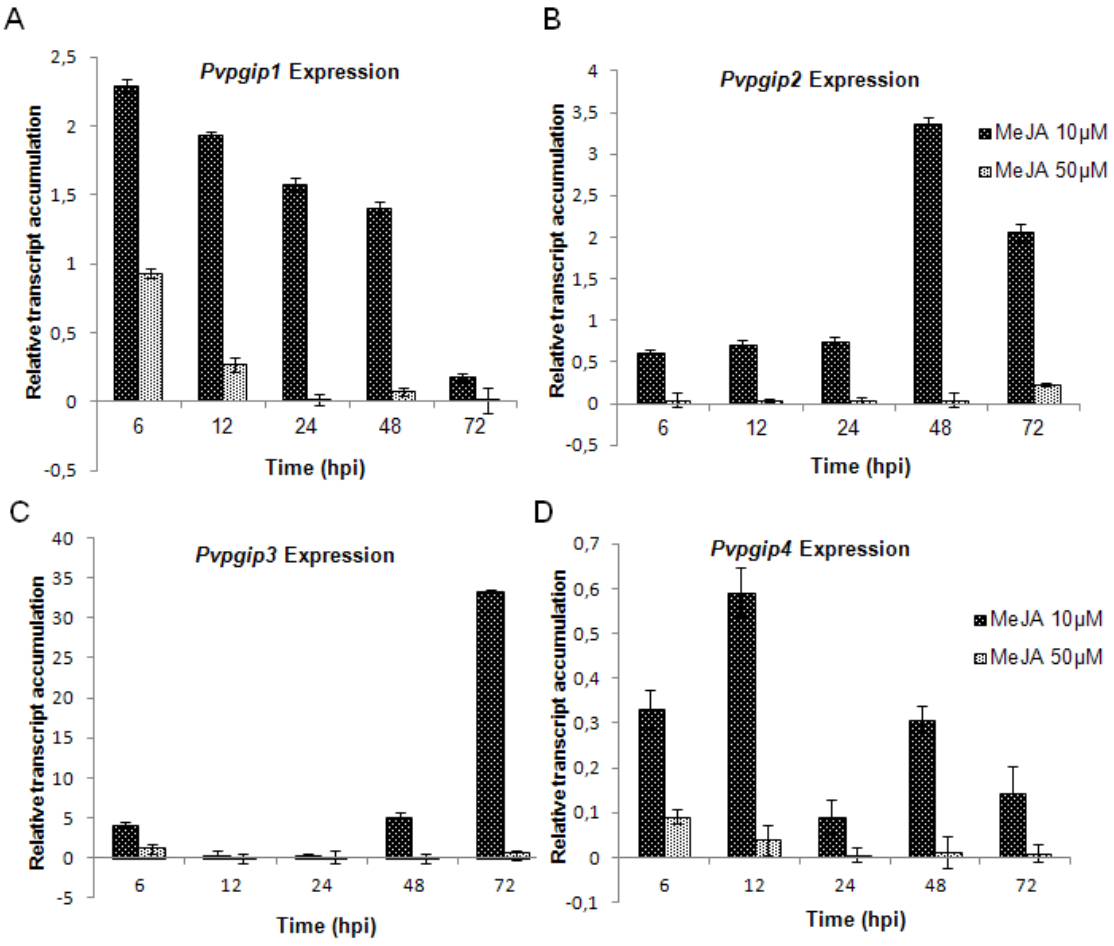

Figure 2: Expression analysis of *Pvpgip* genes of *P. vulgaris* in response to MeJA foliar treatment. (**A**)- *Pvpgip1;* (**B**)- *Pvpgip2*; (**C**)- *Pvpgip3;* (**D**)- *Pvpgip4*. Expression analyses by qqrT-PCR were performed and transcript levels were calculated from triplicate data using comparative method. The *P. vulgaris* actin gene as internal control and health plant as reference sample. The tissue samples were collected at 06, 12, 24, 48, and 72 h post-inoculation (hpi). The results are reported as means ± standard deviation (SD) of three experiments.

The results clearly show that in plant infected, PDA or wound, the *Pvpgip4* gene presented a 40-fold (25 to 0,6) increase in expression when compared with the expression in treated plant with MeJA. In contrast, the levels of *Pvpgip3* transcripts strongly increased in plants treated with MeJA (as can be seen in Figure 1D and 2C, respectively).

In conclusion the results reveal distinct patterns expression in the *Pvpgip* genes: The *Pvpgip1* gene was expressed in the early stage (6-24 hpi) of infection in all treatments. The *Pvpgip2* and *Pvpgip3* were expressed in the early stage in infected plants (PDA), and in the late stage (48-72 hpi) for infected plants (wound) and plants treated with MeJA (10 μM). The *Pvpgip4* gene was induced both in the initial stage in infected plants (wound) and plants treated with MeJA, and in the late stage in infected plants (PDA) (Figure 3D).

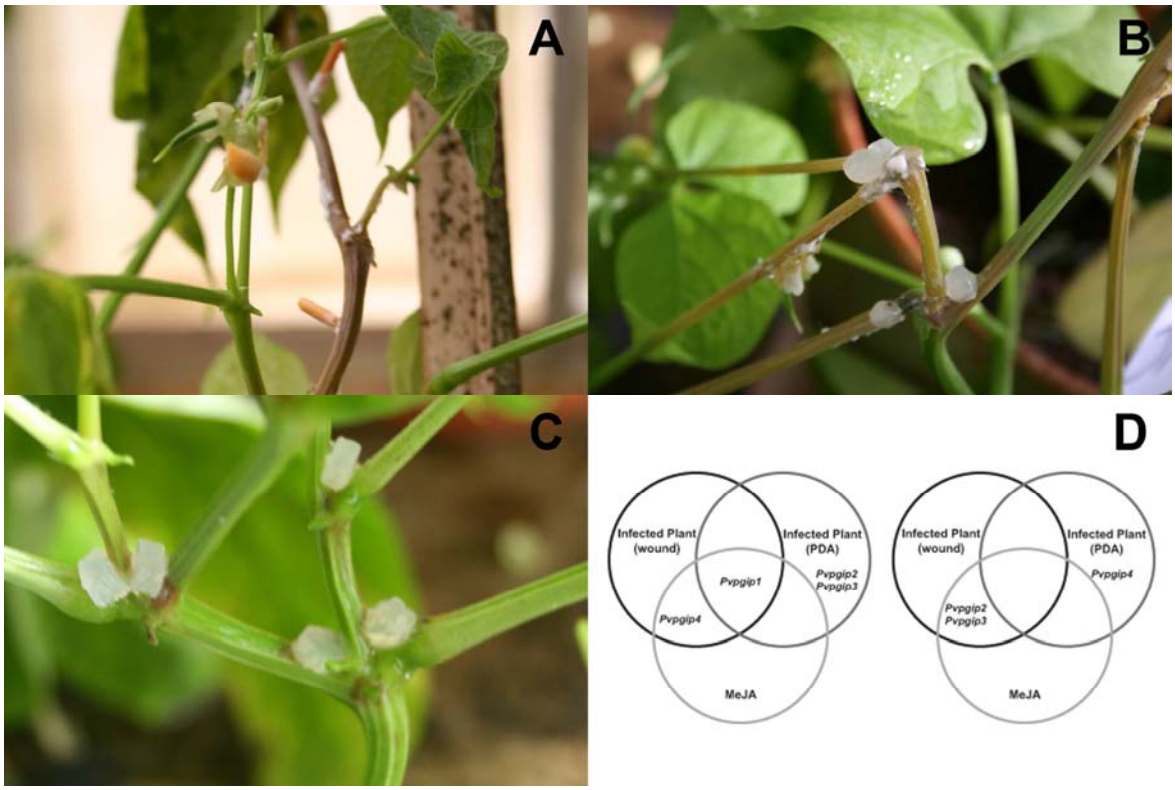

Figure 3: Model of expression differential of *Pvpgip* genes. The dry bean plants 72 hours post-infection with *S. sclerotiorum* as described in the Materials and Methods. (**A**) infected plants - Toothpick Method, (**B**) infected plants - PDA plugs, (**C**) plants infected - treated with 10mM MeJA, (**D**)- Patterns expression of *Pvpgip1-4* genes in early infection stages (until 24 hpi) and late infection stage (from 24 hpi).

A significant delay in symptom development was evident when plants treated with MeJA at 10µM or higher concentrations were inoculated with fungus (Figure 3C), suggesting that the MeJA is involved in the induction of partial resistance *S. sclerotiorum* interaction with host plants.

3.2 PGs activity and inhibition assay

The PGs enzyme assays were performed to determinate the differences in enzyme activity during infection stages of *S. sclerotiorum* in plant with/without MeJA foliar treatments as well between inoculation methods.

Was observed difference in the enzyme activity between treatments, however the peak of PGs activity started 48-72 hpi in all of them (Figure 4). There was a statistically differences between both methods of inoculation from 6 to 72 hpi (Figure 4).

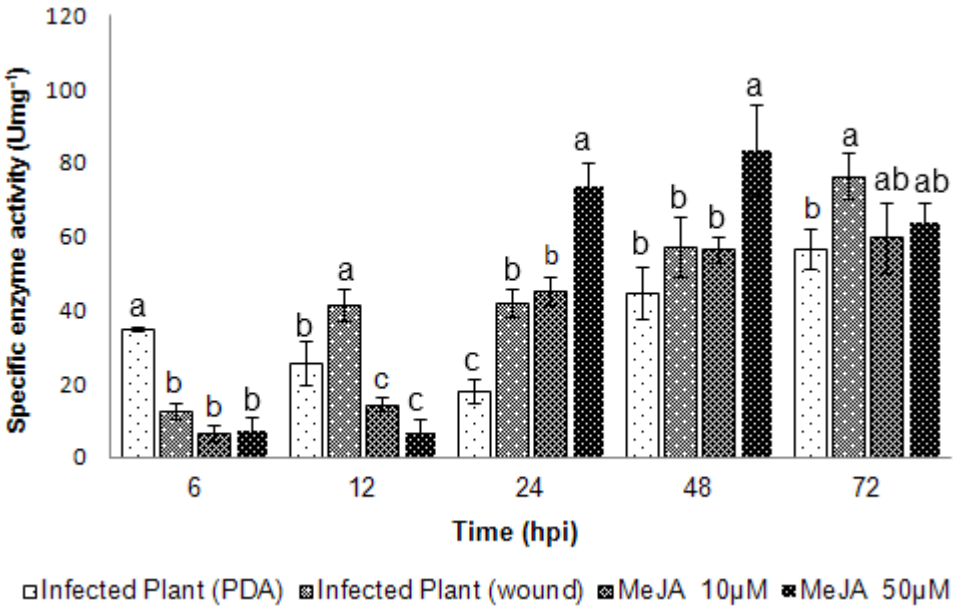

Figure 4: Polygalacturonase activity of bean plants infected by *S. sclerotiorum*. The tests were performed in triplicate and the results expressed in terms of activity unit per microgram of protein (U/mg). Since one unit of enzyme activity (U) is defined as the amount of enzyme capable of releasing one µmol of reducing sugar/min/mg protein under the experimental conditions. Three samples were

analyzed for each treatment, the values are mean ± SD. The different letters on bars indicate statistically significant at 1% probability ($p<0,01$).

The inhibition test was performed to confirm the inhibitory effect of *P. vulgaris* PGIPs on *S. sclerotiorum* PGs activities during infective process. The test was done in cell free supernatant of the *S. sclerotiorum* culture grown on minimal medium All treatments tested were positive for inhibitory activity compared to control and the highest percentage of inhibition was observed in the initial stage of the infection process (Figure 5). The highest peak, about 50% inhibition of PGs activity, was found in plants extracts treated with 50µM of MeJA – 6-12 hours post-inoculation (Figure 5).

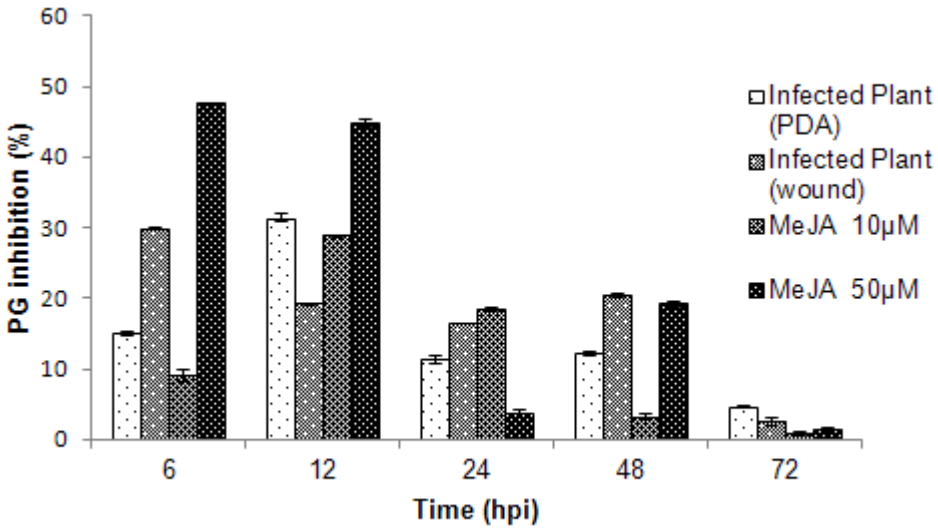

Figure 5. Polygalacturonase activity inhibition assay. The percentage of inhibition was compared to control. The test was done from the culture supernatant of the *S. sclerotiorum* grown on minimal medium and plant extract obtained from the different treatments as described in the Materials and Methods. For control, we measured only the activity of the culture supernatant. The enzyme activity was performed using polygalacturonic acid as substrate based on the release of reducing sugar. The results are reported as means ± SD of three experiments.

4. DISCUSSION

We have previously shown that high levels of *Pvpgip2* expression were observedin dry bean plant during the initial phase (48 hpi) of the *S. sclerotiorum* infective process and declined by 72 hpi. In addition, the *Pvpgip3* expression increased strongly at 96 hpi (late stage) and the *Pvpgip4* was constantly present from 24 hpi up to 96 hpi, and a highest level was detected in the necrotic lesion area than in plants that had been mechanically wounded [9]. In the present work we investigate other forms of stress, comparing inoculum methods and infective process starting from 6 hours post-inoculation. We showed that occurred differences in the patterns expression of *Pvpgip 1-4* genes and the exogenous MeJA plants treatment was able to induce the *Pvpgip* genes expression in *P. vulgaris* plants. Whereas that the P*vpgip 4* gene was strongly induced by *S. sclerotiorum* infection and *Pvpgip3* by MeJA treatment. This result strongly suggests the importance of the *Pvpgip* genes in the response to MeJA.

The difference in the *Pvpgip* gene expression in response to MeJA was also observed in other studies. In *A. thaliana* was demonstrated that the *Atpgip*1 expression was not altered by MeJA, while that of *Atpgip*2 was mediated by this compound [17]. The defense responses against pathogens infection, in plants treated with MeJA, may be activated by different routes of the polygalacturonase inhibiting proteins. Several studies have shown the role of MeJA as inducer of plants resistance, where on the exogenous application of this product increased the level of transcripts accumulation encoding proteins related to pathogenicity, such as chitinases, serine protease inhibitors and β-1,3-glucanase. In addition to activate genes involved in phytoalexins biosynthesis [21]. The importance of PGIPs in plant defense has been corroborated by *in vivo* studies. The over expression of the genes Atpgip1 and Atpgip2 in *A. thaliana* is a limiting factot for the colonization of *B. cinerea* learing to the reduction of disease symptoms [17].

The differences found in the *Pvpgip* gene expression pattern can be explained by the origin of these genes. According to Ovideo and coworkers [16], the duplication and diversification of *Pvpgip* genes resulted not only in the diversification of their biochemical function, but also in their regulation under stress condition. Thus, in *P. vulgaris* the expression of *Pvpgip3* is induced by oligogalacturonides but not by fungal glucan or salicylic acid or wounding; the expression of *Pvpgip4* is not altered by any of these treatments. The expression of *Pvpgip*1 is induced by wounding only [22] whereas *Pvpgip*2, which encodes the most efficient inhibitor of fungal PGs, is up-regulated by oligogalacturonides, salicylic acid and wounding [16]. However, the *Pvpgip* expression model as described by Ovideo et al. [16] could not be observed in our experiment. This might indicate that the inducing effect is dependent on plant cultivar, *S. sclerotiorum* isolate or a combination of both.

Farmer and Ryan [23] established that linolenic acid released in response to wound signals would be converted through several intermediates to jasmonate and led to the expression of genes proteinase inhibitors. Probably, the similarity observed in the temporal expression of genes *Pvpgip* for these two treatments (MeJA and wound – Figure 3) is explained by the activation of one pathway defense response.

The PGs, expressed and secreted by phytopathogenic fungal at the early stages of plant infection, degrade homogalacturonan, the main component of cell wall, causing cell wall degradation and tissue maceration [2, 24]. Overall, our results of PGs activities and gene expression analyses support a role of the PGs and *Pvpgips* during pathogenesis and provide elements to describe a possible sequence of events occurring during colonization of the *P. vulgaris* tissue by *S. sclerotiorum*. The early expression of PG and its sensitivity to PGIP inhibition account for its early role in tissue penetration. Furthermore, at this stage, the PGIP content in plant tissue may not be sufficient to impair significantly the PG activity [25]. Subsequently, the increased discharge of oxalic acid would parallel the expression of acidic

PGs and, by lowering the pH value toward the optimum, would better support the overall PG activity. During this infection stage, the pathogen also induces the expression of the *pgip* genes and, likely, the PGIP accumulates in the infected tissue [26, 27]. Further accumulation of oxalic acid decreases the pH level below the optimum for PG activity favoring the escape of PGIP inhibition by the acidic PGs.

Our analyses confirms the PGIPs inhibitory activity which is consistent with the observation that PvPGIP2 only partially inhibits the PG activity secreted by the fungus in untransformed tobacco tissues [28]. Similarly, it has been reported that the soybean PGIP inhibits only one of the two PGs secreted by *S. sclerotiorum* at conditions mimicking those during infection [29]. However, as we previously showed, *S. sclerotiorum* expresses different isoforms of PG with, show differential regulation [9].

To accommodate pathogenesis of different hosts and environmental conditions the fungi produces PG isoenzymes variable in terms of sequence, specific activity, pH optimum and substrate preference [2, 30]. Conversely, plants have evolved PGIPs with different recognition specificities encoded by differentially regulated *pgip* genes [10, 17]. For example, PGs produced by *B. cinera* and *Colletotrichum acutatum* are inhibited with different efficiencies by all members of PvPGIP [16, 31], while PG from *Aspergillus niger* (AnPGII) is inhibited by PvPGIP1 and PvPGIP2 but not by PvPGIP3 and PvPGIP4 [32]. PG from *Fusarium moniliforme* (FmPG) is inhibited only by PvPGIP2 [33]. The ability to interact with the same PGIP the expression of different PGs is facilitated by the LRR motifs that provide versatility for the structural protein-protein interaction. The LRR domain of plant proteins is often fused with other domains and acts in recognition of hormonal signals and pathogen derived elicitors [13].

In conclusion, the expression data, using the model of infection *P. vulgaris* cv pérola and *S. sclerotiorum*, suggests specialized roles or subfunctionalization for each *Pvpgip* gene

member under different stress condition. This makes it clear that plants have different mechanisms of defense responses, and that a single stress factor can activate different pathways, resulting in various types of responses.

Acknowledgements

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), CAPES and Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG).

REFERENCES

[1] Boland GJ, Hall R. Index of plant hosts of *Sclerotinia sclerotiorum*. Canadian Journal Plant Phathology 1994; 16 (2): 93-108.

[2] De Lorenzo G, D'Ovidio F, Cervone F. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annual Rewiew of Phytopathology 2001; 39: 313-335.

[3] Reymond P, Deléage G, Rascle C, Fèvre M. Cloning and sequence analysis of a polygalacturonase-encoding gene from the phytopathogenic fungus *Sclerotinia sclerotiorum.* Gene 1994; 146: 233-237.

[4] Fraissinet-Tachet L, Reymond-Cotton R, Fevre M. Characterization of a multigene family encoding an endopolygalacturonase in *Sclerotinia sclerotiorum.* Curr Genet 1995; 29:96-100.

[5] Fraissinet-Tachet L, Fevre m. Regulation by galacturonic acid of pectinolytic enzyme production by *Sclerotinia sclerotiorum.* Curr Microbiology 1996; 33: 49-53

[6] Cotton P, Rascale C, Fèvre M. Characterization of PG2, an early endoPG produced by *Sclerotinia sclerotiorum*, expressed in yeast. FEMS Microbiol Lett 2002; 213: 239-244.

[7] Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G, Hegedus DD. Interaction of *Sclerotinia sclerotiorum* with *Brassica napus:* cloning and characterization of endo and exo-polygalacturonases expressed during saprophytic and parasitic modes. Fungal Genet Boil 2004a; 41: 754-765.

[8] Li R, Rimmer R, Buchwaldt L, Sharpe AG, Séguin-Swartz G, Coutu C, Hegedus DD. Interaction of *Sclerotinia sclerptiorum* with a resistant *Brassica napus* cultivar: expressed sequence tag analysis identifies genes associated with fungal pathogenesis. Fungal Genet Boil 2004b; 41: 735-753.

[9] Oliveira MB, Nascimento LB, Lobo-Junior M, Petrofeza S. Characterization of dry bean polygalacturonase-inhibiting protein (PGIP) gene Family during *Sclerotinia sclerotiorum* (Sclerotiniaceae) infection. Genet and Mol Research 2010; 9 (2): 994-1004.

[10] De Lorenzo G, Ferrari S. Polygalacturonase-inhibiting proteins in defesnse against phytopathogenic fungi. Curr Opinion in Plant Biol 2002; 5: 295-299.

[11] Cervone F, Castoria R, Leckie F, DE Lorenzo G. Perception of fungal elicitors and signal transduction. In Aducci P (ed) Signal transduction in plants 1997; 153-177.

[12] Ridley BL, O'Neill MA, Mohnen D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 2001; 57: 929-967.

[13] Kobe B, Kajava AV. The leucine-rich repeats as a protein recognition motif. Curr Opin Struct Biol 2001; 11:725-732

[14] Di Matteo A, Federici L, Mattei B, Salvi G, Johnson C, De Lorenzo G, Tsernoglou D, Cervone F. The Crystal structure of polygalacturonase-inhibiting protein (PGIP), a leucine rich repeat protein involved in plant defense. Proc Natl Acad Sci USA 2003; 100: 10124-10128.

[15] Federici L, Caprari C, Mattei B, Savino C, Di Mateo A, De Lorenzo G, Cervone F, Tsernoglou D. Structural requeriments of endoplygalacturonases for the interation with PGIP (polygalacturonase-inhibiting proteins). Proc Natl Acad Sei USA 2001; 98:13425-13430.

[16] D'Ovidio R, Rayola A, Capodicasa C, Devoto A, Pontiggia D, Roberti S, Galetti R, Conti E, O'Sulivan D, De Lorenzo G. Caracterization of the complex locus of bean encoding Polygalacturonase-inhibiting proteins reveals subfunctionalization for defense against fungi and insects. Plant Physiol 2004a; 135:2424-2435.

[17] Ferrari S, Vairo D, Ausubel FM, Cervone F, De Lorenzo,G. Tandemly duplicated arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinaly by deifferent signal transduction pathways in response to fungal in fection. The Plant Cell 2003; 15: 93-106.

[18] Li R, Rimmer R, Yu M, Sharpe AG, Séguin-Swartz G, Lydiate D, Hegedus DD. Two *Brassica napus* polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses. Planta 2003; 217: 299-308.

[19] Hegedus DD, Li R, Buchwaldt L, Parkin I, Whitwill S, Coutu C, Bekkaoui D, Rimmer SR. *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment. Planta 228: 241-253.

[20] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem 1959; 31:426-428.

[21] Belhadj A, Saigne C, Telef N, Cluzet S, Bouscaut J, Corio-Costet M F, Mérillon JM. Methyl jasmonate induces defense response in gravine and triggers protection against *Erysiphe necator*. J Agric Food Chem 2006; 54: 9119-9125.

[22] Devoto A, Leckie F, Lupotto E, Cervone F, De Lorenzo G. The promoter of a gene encoding a polygalacturonase-inhibiting protein of *Phaseolus vulgaris* L. is activated by wounding but not by elicitors or pathogen infection. Planta 1998; 205: 165-174.

[23] Farmer EE, Ryan CA. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 1992; 4: 129-134.

[24] Federici L, Di Mateu A, Fernandez-Recio J, Tsernoglou D, Cervone F. Polygalacturonase inhibiting proteins: players in plat inate immunity. Trends Plant Sci 2006; 11:65-70.

[25] Favaron F, D'Ovideo R, Porceddu E, Alghisi P. Purification and molecular characterization of soybean polygalacturonase-inhibiting protein. Panta 1994; 195: 80-87.

[26] D'Ovidio R, Roberti S, Melaragni M, Capodicasa C, Sella L, Favaron F. Characterization of two closely linked soybean Pgip genes and transcript regulation following pathogen infection and wounding. Plant Protection Science 2002; 38: 480-482.

[27] Favaron F, Destro T, D'Ovideo R. Transcript accumulation of polygalacturonase inhibiting protein (PGIP) following pathogen infections in soybean. J Plant Pathol 2000; 82: 103-109.

[28] Powell A L, Van Kan J, ten Have A, Greve LC, Bennett AB, Labavitch JM. Transgenic expression of pear PGIP in tomato limits fungal colonization. Molecular Plant–Microbe Interactions 2000; 13: 942-950.

[29] Favaron F, Sella L, D'Ovidio R. Relationsships among endopolygalacturonase, oxalate, pH, and plant polygalacturonase-inhibiting protein (PGIP) in te interaction between *Sclerotinia sclerotiorum* and soybean. Mol Plant-Microbe Interact 2004; 17: 1402-1408.

[30] Poinssot B, Vandelle E, Bentejac M, Adrian M, Levis C, Brygoo Y, Garin J, Sicilia F, Coutos-Thevenot P, Pugin A. The endopolygalacturonase 1 from *Botrytis cinera* activates gravine defense resctions unrelated to its enzymatic activity. Mol Plant Microbe Interact 2003; 16: 553-564.

[31] Manfredini C, Sicilia F, Ferrari S, Pontiggia D, Caprari C, Lorito M, De Lorenzo G. Polygalacturonase-inhibiting protein 2 of *Phaxeolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinera* importante for pathogenicity, and protects transgenic plants from infection. Physiol Mol Pathol 2005; 67: 108-115.

[32] D'Ovidio R, Mattei B, Roberti S, Bellincampi D, Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions. Biochim Biophys Acta 2004b; 1696: 237-244.

[33] Leckie F, Mattei B, Capodicasa C, Hemmings A, Nuss L, Aracri B, De Lorenzo G, Cervone F. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/ β- turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. Embo Journal 1999; 9: 2352-2363.

4-CONCLUSÕES

Neste trabalho nós avaliamos a interação parasitária entre *S. sclerotiorum* e plantas de feijão (*P. vulgaris*) e concluímos que:

1. Os genes que codificam para as diferentes isoformas de PGs apresentaram expressão diferencial onde: os genes *sspg1* e *sspg5* foram altamente expressos durante todo o período analisado *sspg3* foi expresso nas fases finais e *sspg6 foi* fracamente expresso durante o processo infectivo (24 – 96 hpi).

2. A atividade enzimática das PGs variou durante o processo infectivo, apresentando um pico com 48 horas após o inóculo.

3. Durante o processo infectivo as PGs são inibidas por PGIPs, isso foi confirmado no teste de inibição em que foi demonstrado que a maior percentagem de inibição acontece na fase inicial de colonização do tecido hospedeiro pelo fungo.

4. Os genes *Pvpgip* apresentam expressão diferencial, podendo variar com a fase da infecção e em resposta a diferentes estímulos: fatores bióticos como infecção por *S. sclerotiorum* e abióticos como injúria mecânica e tratamento por metil jasmonato.

5. Tanto em plantas infectadas tratadas com metil jasmonato quanto em plantas não tratadas, o gene *Pvpgip1* foi ativado na fase inicial do processo infectivo, enquanto que os genes *Pvpgip2* e *Pvpgip3* tiveram maior indução nas fases finais. O gene *Pvpgip4* foi altamente induzido pela infecção por *S. sclerotiorum* e por injúria mecânica.

5- PERSPECTIVAS

- Identificação das isoformas de PGs no processo de interação patógeno-hospedeiro através de géis de atividade enzimática (Zimograma);
- Analisar outros elicitores que são capazes de induzir a expressão dos genes *Pvpgip,* e verificar seu papel na defesa de plantas contra invasão de patógenos;
- Expressão heteróloga em *Pichia pastoris* das proteínas inibidoras de poligalacturonases (PGIP) produzidas pela planta de feijoeiro;
- Transformação de plantas com genes *Pvpgip* visando a análise funcional destes genes;
- Comparação da expressão dos genes *Pvpgip* entre cultivares de feijão, visando seleção de cultivares resistentes ao mofo branco.

6- REFERÊNCIAS

AVANCI, N. C.; LUCHE, D. D.; GOLDMAN, G. H.; GOLDMAN, M. H. S. (2010) Jasmonates are phytormones with multiple functions, including plant defense and reproduction. **Genetics and Molecular Research,** 9: 484-505.

BARDIN, S. D.; HUANG, H. C. (2001) Research on biology abd control of *Sclerotinia* diseases in Canada. **Can. J. Plant Pathol.**, 23:88-89.

BATEMAN, D. F.; BEER, S. V. (1965) Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotinia rolfsii.* **Phytopathology,** 55: 2014-211.

BECKERS, G. J.; SPOEL, S. H. (2006) Fine-tuning defence signaling: salicylate versus jasmonate. **Plant Biology,** 8: 1-10.

BELHADJ, A.; SAIGNE, C.; TELEF, N.; CLUZET, S.; BOUSCAUT, J.; CORIO-COSTET, M. F.; MÉRILLON, J.M. (2006) Methyl jasmonate induces defense response in gravine and triggers protection against *Erysiphe necator.* **J. Agric. Food Chem.** 54: 9119-9125.

BENITO, E. P. (1998) Fungal and plant gene expression during sychronized of tomato leaves by *Botrytis cinera.* **Eur J Plant Pathol,** 104:207-220.

BOLAND, G. J.; HALL, R. (1994) Index of plant hosts of *Sclerotinia sclerotiorum.* **Canadian Journal Plant Phathology**, 16 (2): 93-108.

BOLTON, M. D.; THOMMA, B. P. H. J. (2006). *Sclerotinia sclerotiorum* (Lib) de bary: biology and molecular traits of a cosmopolitan pathogen. **Mol Plant Pathol,** 1:1-6.

BOURDAT, G.; CHARPENTIER, M.; LAFITTE, C.; MARTINEZ, Y.; JAUNEAU, A.; GAULIN, E.; ESQUERRE-TUGAYE, M. T.; DUMAS, B. (2003). Elicitor activity of a

fungal endopolygalacturonase in tobacco requires a functional catalytic site and cell wall localization. **Plant Physiol,** 131:93-101.

CESSNA, S. G.; SEARS, V. E.; DICKMAN, M. B.; LOW, P. S. (2000) Oxalic acid, a pathogenicity for *Scleortinia sclerotiorum*, suppresses the oxidative burst of the host plant. **Plant Cell,** 12: 2191-2199.

CHEN, H. JONES, A. D.; HOWE, G. A. (2006) Constituitive activation of the jasmonate signaling patway enhances the production of secondary metabolites in tomato. **FEBBS Lett,** 580: 2540-2546.

COHEN, Y.; GISI, U.; NIDERMAN, T. (1993) Local and systemic protection against *Phytophthora infestam* induce in potato and tomato plants by jasmonic acid and jasmonic acid methyl ester. **Phytopathology**, 83: 1054-1062.

COTTON, P.; RASCALE, C.; FÈVRE, M. (2002) Characterization of PG2, an early endoPG produced by *Sclerotinia sclerotiorum*, expressed in yeast. **FEMS Microbiol. Lett,** 213: 239-244.

DE LORENZO, G.; D'OVIDIO, F.; CERVONE, F. (2001). The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. **Annual Rewiew of Phytopathology,** 39:313-335.

DE LORENZO, G.; CERVONE, F. (1997) Polygalacturonase-inhibiting proteins (PGIPs) role in specifity and defense against pathogenic fungi. In STACEY, G.; KEEN, N. T. (eds) **Plant microbe interactions,** vol 3. Chapman & Hall, New York, pp 76-93.

DEVOTO, A.; LECKIE, F.; LUPOTTO, E.; CERVONE, F.; DE LORENZO, G. (1998) Thr promoter of a gene encoding a polygalacturonase-inhibiting protein of *Phaseolus vulgaris* L. is activated by wounding but not by elicitors or pathogen infection. **Planta,** 205: 165-174.

DE VRIES, R. P.; VISSER, J. (2001) *Aspergillus* enzymes involvedin degradation of plant cell wall polysaccharides. **Microbiology and Molecular Biology Reviews**, 497-522.

DIEZ, E.; ALVARO, J.; ESPESO, E. A.; RAINBOW, L.; SUAREZ, T.; TILBURN, J. (2002) Activation of the *Aspergillus* PacC zinc finger transcription factor requires two proteolitic steps. **EMBO J,** 21: 1350-1359.

DI MATTEO, A.; BONIVENTO, D.; TSERNOGLOU, D.; FEDERICI, L.; CERVONE, F. (2006) Polygalacturonae-inhibiting protein (PGIP) in plant defence: a structural view. **Phytochemistry,** 67: 528-533.

DI MATTEO, A.; FEDERICI, L.; MATTEI, B.; SALVI, G.; JOHNSON, C.; DE LORENZO, G.; TSERNOGLOU, D.; CERVONE, F. (2003) The Crystal structure of polygalacturonase-inhibiting protein (PGIP), a leucine rich repeat protein involved in plant defense. **Proc. Natl. Acad. Sci. U.S.A.,** 100: 10124-10128.

DONALDSON, P. A.; ANDERSON, T.; LANE, B. G.; DAVIDSON, A.; SIMMONDS, D. H. (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat *gf-2.8* (germin) gene are resistant to the ozalate-secreting pathogen *Sclerotinia sclerotiorum.* **Physiol. Mol. Plant. Pathol.,** 59:297-307.

D'OVIDIO, R.; RAYOLA, A.; CAPODICASA, C.; DEVOTO, A.; PONTIGGIA, D.; ROBERTI, S.; GALETTI, R.; CONTI, E.; O'SULIVAN, D.; DE LORENZO, G. (2004a) Caracterization of the complex locus of bean encoding Polygalacturonase-inhibiting proteins reveals subfunctionalization for defense against fungi and insects. **Plant Physiol.** 135:2424-2435.

D'OVIDIO, R.; MATTEI, B.; ROBERTI, S.; BELLINCAMPI, D. (2004b) Polygalacturonases, polygalacturonase-inhibiting proteins and pectc oligomers in plant-pathogen interactions. **Biochim. Biophys. Acta,** 1696: 237-244.

D'OVIDIO, R.; ROBERTI, S.; MELARAGNI, M.; CAPODICASA, C.; SELLA, L.; FAVARON, F. (2002) Characterization of two closely linked soybean Pgip genes and transcript regulation following pathogen infection and wounding. **Plant Protection Science,** 38: 480-482.

EL BARKANI, A.; KURZAI, O.; FONZI, W. A.; RAMON, A.; PORTA, A.; FROSCH, M.; MÜHLSCHLEGEL, F.A. (2000) Dominant active alleles of RIM101 (PRR2) bypass the pH restriction on filamentation of *Candida albicans*. **Mol. Cell. Biol.,** 20: 4635-4647

ELLIS, J.; DODDS, P.; PRYOR, T. (2000) Struture, function and evolution plant disease resistance genes. **Curr. Opinion in Plant Biol.,** 3: 278-284.

FARMER, E. E.; RYAN, C. A. (1990) Interplant communication: airbone methyl jasmonate induces synthesis of proteinases inhibitors in plant leaves. **Proc. Natl. Acad. Sci. USA**, 87:7713-7716.

FARMER, E. E.; RYAN, C. A. (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. **Plant Cell,** 4: 129-134.

FARR & ROSMAN, SBML Systematic Botany of Mycological Resources. Disponível em: http://www.ars.usda.gov. Acessado em: 08 de dezembro de 2010.

FAVARON, F.; SELLA, L.; D'OVIDIO, R. (2004) Relationsships among endopolygalacturonase, oxalate, pH, and plant polygalacturonase-inhibiting protein (PGIP) in te interaction between *Sclerotinia sclerotiorum* and soybean. **Mol. Plant- Microbe Interact**., 17: 1402-1408.

FEDERICI, L.; DI MATEU, A.; FERNANDEZ-RECIO J.; TSERNOGLOU D.; CERVONE, F. (2006) Polygalacturonase inhibiting proteins: players in plat inate immunity. **Trends Plant Sci,** 11:65-70.

FEIJÃO: dados de conjuntura da produção de feijão (*Phaseolus vulgaris*) no Brasil: 1985-2008. Disponível em: http://www.cnpaf.embrapa.br/apps/socioeconomia/index.htm. Acesso em 15 de março de 2011.

FERRARI, S.; GALLETTI, R.; DENOUX, C.; DE LORENZO, G.; AUSUBEL, F. M.; DEWDNEY, J. (2007) Resistance to *Botrytis cinera* induced in Arabidopsis by elicitors is independente of salicilic acid, ethylene or jasmonate signaling but requires Phytoalexin Deficient.**Plant Physiology,** 144: 367-379.

FERRARI, S.; VAIRO, D.; AUSUBEL, F. M.; CERVONE, F.; DE LORENZO, G. (2003) Tandemly duplicated arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinaly by deifferent signal transduction pathways in response to fungal in fection. **The Plant Cell,** 15: 93-106.

GOMEZ-GOMEZ, L.; BOLLER, T. (2000) FLS2: na LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. **Molecular Cell,** 5: 1003-1011.

GONZÁLEZ-CARRANZA, Z. H.; ELLIOTT, K. A.; ROBERTS, J. A. (2007) Expression of polygalacturonases and evidence to support their role during cell separation processes en *Arabidopsis thaliana.* **Journal of Experimental Botany,** 58: 3719-3730.

GODOY, G.; STEADMAN, J. R.; DICKMAN, M.B.; DAM, R. (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris.* **Physiol. Mol. Plant. Pathol.,** 37: 179-191.

HAHN, M. G.; DARVILL, A. G.; ALBERSHEIM, P. (1981) Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of plant cell wall polussaccharide that elicits phytoalexin accumulation in soy beans. **Plant Physiol,** 68-1161-1169.

KARS, I.; KROOSHOF, G. H.; WAGEMAKERS, L.; JOOSTEN, R.; BENEN, J. A. B.; KAN, J. A. L. V. (2005) Necrotizing activity of five *Botrytis cinera* endopolygalacturonases produced in *Pichia pastoris.* **The Plant Journal,** 43: 213-225.

KASZA, Z.; VAGVÖLGYI, C.; FÈVRE, M.; COTTON, P. (2004) Molecular characterization and *in planta* detection of *Sclerotinia sclerotiorum* endoplygalacturonase genes. **Curr. Microbiol.,** 48: 208-213.

KAZAN, K.; MANNERS, J. M. (2008) Jasmonate signaling: toward an integrated view. **Plant Physiol.,** 146: 1459-1468.

KIMATI, H.; AMORIM, L.; REZENDE, J.A.M. (2005) Doenças das plantas cultivadas. **Manual de Fitopatologia** 4ª ed. S. Paulo: Ed Agronômica Ceres Ltda, 2:919.

KIM, J.; PATTERSON, S. E. (2006) Expression divergence and functional redundancy of polygalacturonases in floral organa abscission. **Plant Signaling and Behaviour,** 1: 281-283.

KOBE, B.; KAJAVA, A. V. (2001). The leucine-rich repeats as a protein recognition motif curr. **Opin. Struct. Biol,** 11:725-732.

KUBICEK, C. P.; SCHREFERL-KUNAR, G.; WÖHRER, W.; RÖHR, M. (1998) Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. **Appl. Environ. Microbiol.,** 54: 633-637.

LAMBERT, M.; BLANCHIN-ROLAND, S.; LE LOUEDEC, F.; LEPINGLE, A.; GAILLARDIN, C. (1997) Genetic analysis of regulatory mutants affecting synthesis of extracellular proteinases in the yeast *Yarrowia lipolytica*: identification of a RIM101/pacC homolog. **Mol Cell Biol,** 17: 3966-3976.

LEBEDA, A.; LUHOVAL.; SEDLAROVA, M.; JANCOVA, D. (2001) The role of enzymes in plant-fungal pathogens interactions – Review. **Z. Pflanzenk. Pflanzens. J. Plant Dis. Prot.,** 108: 89-111.

LECKIE, F.; MATTEI, B.; CAPODICASA, C.; HEMMINGS, A.; NUSS, L.; ARACRI, B.; DE LORENZO, G.; CERVONE, F. (1999) The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/ β- turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. **EMBO Journal,** 9: 2352-2363.

LEON-REYS, A.; van DER DOES, D.; DE LANGE, E. S.; DELKER, C.; WASTERNACK, C.; van WEES, S. C. M.; RITSEMA, T.; PIETERSE, C. M. J. (2010) Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway. **Planta,** 232: 1423-1432.

LIECHTI, R.; FARMER, E. F. (2006) Jasmonate biochemical pathway. **Sci. STKE,** 322: 1-3.

LI, J.; ZINGEN-SELL,I.; BUCHENAUER, H. (1996) Induction of resistance of cotton plants to Verticillium wilt and of tomato to Fusarium wilt by 3-aminobutyric acid and methyl jasmonate. **J. Plant dis. Prot.,** 103: 288-299.

LI, R.; RIMMER, R.; BUCHWALDT, L.; SHARPE, A. G.; SÉGUIN-SWARTZ, G.; HEGEDUS, D. D. (2004a) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus:* cloning and characterization of endo and exo-polygalacturonases expressed during saprophytic and parasitic modes. **Fungal Genet. Boil.**, 41: 754-765.

LOBO-JUNIOR, M.; GERALDINE, A. M.; CARVALHO, D. D. C.; COBUCCI, T. (2009) Uso de cultivares de feijão comum com arquitetura ereta e ciclo precoce para escape do mofo branco (*Sclerotinia sclerotiorum*). **Embrapa Arroz e Feijão: Comunicado técnico** 182.

MANFREDINI, C.; SICILIA, F.; FERRARI, S.; PONTIGGIA, D.; CAPRARI, C.; LORITO, M.;DE LORENZO, G. (2005) Polygalacturonase-inhibiting protein 2 of *Phaxeolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinera* importante for pathogenicity, and protects transgenic plants from infection. **Physiol. Mol. Pathol.,** 67: 108-115.

MARCIANO, P.; DI LENNA, P.; MAGRO, P. (1983) Oxalic acid, cell wall degrating enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. **Physiol. Plant Pathol.**, 22: 339-345.

MARKOVIC, O.; JANECEK, S. (2001) Pectin degrading glycosidr hydrolases of family 28: sequence-structural features, specificities and evolution. **Protein Engineering,** 14: 615-631.

MARTEL, M. B.; LEËTOUBLON, R.; FEÈVRE, M. (1998) Purification of two endopolygalacturonases secreted during the early stages of the saprophytic growth of *Sclerotinia sclerotiorum.* **FEMS Microbiol. Lett.,** 158: 133-138.

MUR, L. A.; BROWN, I. R.; DARBY, R. M.; BESTWICK, C. S.; BI, Y. M.; MANSFIELD, J. W.; DRAPER, J. (2000) A loss of resistance to avirulent bacterial pathogens in tabaco is associated with the attenuation of a salicylic acid-potentiated oxidative burst. **Plant J,** 23: 609-621.

MUR, L. A. J.; BI, Y. M.; DARBY, R. M.; FIREK, S.; DRAPER, J. (1997) compromising early salicylic acid accumulation delays the hypersensitive response and increases viral dispersal during lesion estabeleciment in TMV-infected tobacco. **Plant J,** 12: 1113-1126.

NOYES, R. D.; HANCOCK, J.G. (1981) Role of oxalic acid in the Sclerotinia wilt of sunflower. **Physiol. Plant. Pathol.,** 18: 123-132.

PEL, H. J.; de WINDE, J. H.; ARCHER, D. B.; DYER, P. S. HOFMANN, G.; SHAAP, P.J.; TURNER, G.; de VRIES, R. P. ALBANG, K.; ANDERSEN, M. R.; BENDTSEN, J. D.;

BENEN, J. A.; van der BERG, M.; BREESTRAAT, S.; CADDICK, M. X.; CONTERAS, R.; CORNELL, M.; COUTINHO, P. M.; DANCHIN, E. G.; DEBETS, A. J.; DEKKER, P.; van DICK, P. W.; van DIJK, A.; DIJKHUIZEN, L.; DRIESSEN, A. J.; D'EFERT, C.; GEYSENS, S.; GOOSEN, C.; GROOT, G. S.; DE GROOT, P. W.; GUILLEMET, T.; HENRISSAT, B.; HERWEIJER, M.; van den HOMBERGH, J. P.; van den HONDEL, C. A.; van der HEIJDEN, R. T.; van der KAAIJ, R. M.; KLIS, F.M.; KOOLS, H. J.; KUBICEK, C. P.; van KUYK, P. A.; LAUBER, J.; LU, X.; van der MAAREL, M. J.; MEULENBERG, R.; MENKE, H.; MORTIMER, M. A.; NIELSEN, J.; OLIVER, S. G.; OLSTHOORN, M.; PAL, K.; van PEIJ, N. N.; RAM, A. F.; RINAS, U.; ROUBOS, J. A.; SAGT, C. M.; SCHMOLL, M.; SUN, J.; USSERY, D.; VARGA, J.; VERVECKEN, W,; van der VONDERVOOT, P. J.; WEDLER, H.; WOSTEN, H. A.; ZENG, A. P.; van OOYEN, A. J.; VISSER, J.; STAM, H. (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. **Nat. Biotechnol.,** 25: 221-231.

PEÑALVA, M. A.; ARST, H. N. JR. Regualation of gene expression by ambient pH in filamentous fungi and yeasts. **Molecular and Cellular Biology Reviews,** 426-446.

PENNINCKX, I. A. M. A.; THOMMA, B. P. H. J.; BUCHALA, A.; METRAUX, J. P.; BROEKAERT, W. F. (1998) Concomitant activation of jasmonat and ethylene response pathways is required for induction of a plant defense gene in Arabidopsis. **Plant cell,** 10:2103-2123.

PENNICKX, I. A. M. A.; EGGERMONT, K.; TERRAS, F.R.G.; THOMMA, B. P. H. J.; DE SAMBLANX, G. W.; BUCHALA, A.; MÉTRAUS, J. P.; MANNER, J. M.; BROEKAERT, W. F. (1996) Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-dependent pathway. **Plant Cell,** 8: 2309-2323.

PIETERSE, C. M. J.; LEON REYES, A.; van der ENT, S.; van WEES, S. C. M. (2009) Networking by small-molecule hormones in plant immunity. **Nat. Chem. Biol.,** 5: 308-316.

PIETERSE, C. M. J.; TON, J.; van LOON, L. C. (2001) Cross-talk between plant defense signaling pathways: boost or burden? **AgBiotechNet,** 3: ABN 068.

POINSSOT, B.; VANDELLE, E.; BENTEJAC, M.; ADRIAN, M.; LEVIS, C.; BRYGOO, Y.; GARIN, J.; SICILIA, F.; COUTOS-THEVENOT, P.; PUGIN, A. (2003) The endopolygalacturonase 1 from *Botrytis cinera* activates gravine defense resctions unrelated to its enzymatic activity. **Mol Plant Microbe Interact,** 16: 553-564.

POWELL, A. L.; van KAN, J.; ten HAVE, A.; GREVE, L. C.; BENNETT, A. B.; LABAVITCH, J. M. (2000) Transgenic expression of pear PGIP in tomato limits fungal colonization. **Molecular Plant – Microbe Interactions,** 13: 942-950.

REYMOND-COTTON, P.; FRAISSINET-TACHET, L.; FÈVRE, M. (1996) Expression of the *Sclerotinia sclerotiorum* polygalacturonase *pg1* gene: possible involvement of CREA in glucose catabolite repression. **Curr. Genet.,** 30: 240-245.

RIOY, C.; FREYSSINET, G.; FEVRE, M.; (1991). Production of cell wall-degrading enzimes by the phytopathogenic fungus *Sclerotinia sclerotiorum.* **Appl. Environ. Microbiol.**, 57: 1478-1484.

ROBERTS, J. A.; ELLIOTT, K.A.; GONZÁLEZ-CARRANZA, Z. H. (2002) Abscission, diescence and other cell separation processes. **Annual Review of Plant Biology,** 53: 131-158.

ROLLINS, J. A. (2003) The *Sclerotinia sclerotiorum pac1* gene is required for sclerotial development and virulence. **Appl. Enviromen. Microbiol.,** 67: 75-81.

ROLLIS, J. A.; DICKMAN, M. B. (2001) pH sinaling in *Sclerotinia sclerotiorum:* Identification of a *pac*C/RIM1 homolpg. **Appl. Environ. Microbiol.**, 67: 75-81.

ROSE, J. K. C.; CATALA, C.; GONZÁLEZ-CARRANZA, Z. H.; ROBERTS, J. A. (2003) Cell wall disassembly. **Annual Plant Reviews,** 8: 264-324.

RYALS, J.A.; NEUENSCHWANDER, U. H.; WILLITS, M.G.; MOLINA, A.; STEINER, H. Y.; HUNT, M.D. (1996) Systemic acquired resistance. **Plant cell,** 8: 1809-1819.

SEO, H. S.; SONG, J. T.; CHEONG, J. J.; LEE, Y. H.; LEE, Y. W.; HWANG, I.; LEE, J. S.; DO CHOI, Y. (2001). Jasmonic acid carboxyl methyltransferase: a Key for jasmonate-regulated plant response. **Proc. Natl. Acad. Sci. U.S.A.,** 98: 4788-4793.

SMITH, C. J. (1996) Accumutation of phytoalexins: defense mechanism and stimulus response system. **New Phytol.,** 132: 1-45.

THINES, B.; KATSIR, L.; MELOTTO, M.; NIU,Y.; MANDAOCAR, A.; LIU, G.; NOMURA,K.; YANG HE, S.; HOWE, G. A.; BROWSE, J. (2007) JAZ repressor proteins are targets of the SCF (COI1) complex during jasmonate signaling. **Nature,** 448: 661-665.

THOMMA, B. P. H. J.; EGGERMONT, K.; PENNINCKX I. A. M. A.; MAUCH-MANI, B.; VOGELSANG, R.; CAMMUE, B.P O. A.; BROEKAERT, W. F. (1998) Separate jasmonate-dependent and salicylte dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. **Proc. Natl. Acad. Sci. U.S.A.,** 95: 15107-15111.

VAUTARD, G.; COTTON, P.; FÈVRE, M. (1999) The glucose repressor CRE1 from *Sclerotinia scleortiorum* is functionally related to CREA from *Aspergillus nidulans* but not to the Mig proteins from *Saccharomyces cerevisiae*. **FEBS Lett,** 453: 54-58.

VIJAYAN, P.; SHOCKEY, J.; LEVESQUE, C. A.; COOK, R. J.; BROWSE, J. (1998) A role for jasmonate in pathogen defense of *Arabisopsis.* **Proc. Natl. Acad. Sci. U.S.A.,** 95: 7209-7214.

WASTERNACK, C. (2007) Jasmonates: an update on biosynthesis, signal traduction and action in plant stress response, growth and developmental. **Ann. Bot.,** 100: 681-697.

WASTERNACK, C.; HAUSE, B. (2002) Jasmonates and octadecanoids: signal in plant stress responses and development. **Prog Nucleic Acid Res Mol Biol,** 72:154-221.

WILLIAMS, L. H.; TANG, Y.; HINTZ, W. E. (2002) Endopolygalacturonase is encoded by multigene family in the basidiomycete *Chondrostereum purpureum*. **Fungal Genet. Biol.,** 36: 71-83.

WUBBEN, J. P.; ten HAVE, A.; van KAN, J. A. L.; VISSER, J. (2000) Regulation of endopolygalacturonase gene expression in *Botrytis cinera* by galacturonic acid, ambient pH and catabolite repression. **Curr. Genet.** 37: 152-157.