ANTONIO CARLOS MOTA PORTO

EXPRESSÃO DE GENES CANDIDATOS DO FEIJÃO RELACIONADOS À RESISTÊNCIA AO MOFO BRANCO E COMPARAÇÃO DE MÉTODOS DE FENOTIPAGEM DA REAÇÃO AO PATÓGENO

LAVRAS-MG

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento de Plantas, para a obtenção do título de Mestre.

Prof. Dr. João Bosco dos Santos
Orientador

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ANTONIO CARLOS MOTA PORTO

EXPRESSÃO DE GENES CANDIDATOS DO FEIJÃO RELACIONADOS À RESISTÊNCIA AO MOFO BRANCO E ESCOLHA DO MÉTODO DE FENOTIPAGEM DA REAÇÃO AO PATÓGENO

EXPRESSION OF CANDIDATE GENES RELATED TO WHITE MOLD RESISTANCE IN BEAN AND CHOICE OF THE PHENOTYPING METHOD FOR REACTION TO THE PATHOGEN

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento de Plantas, para a obtenção do título de Mestre.

APROVADA em 08 de fevereiro de 2018.

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2018

Aos meus pais Antonio Lima Porto e Ana Maria Porto e aos meus sobrinhos Miguel Oliveira, Pedro Porto, Julia Oliveira e Antonio Porto.

Dedico

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‘‘Não há vergonha em não saber. O problema surge quando o pensamento e a conduta irracional se encarregam de preencher o vazio deixado pela ignorância.’’

(Neil deGrasse Tyson)

RESUMO

Prospectar genótipos de feijão resistentes ao mofo branco (*Sclerotinia sclerotiorum* (Lib.) de Bary) têm sido prioridade para que seja possível o controle barato e eficiente desse importante patógeno. Nesta missão, diversas ferramentas podem ser integradas para melhorar a eficiência de seleção, e dessa forma, aumentar o ganho genético nos programas de melhoramento que têm buscado genótipos resistentes a esse patógeno. O mapeamento e validação de genes de resistência é de interesse direto na seleção assistida nos programas de melhoramento, enquanto que os métodos precisos e eficientes de fenotipagem são importantes em todo o programa de melhoramento, desde o *screening* de germoplasma até as gerações avançadas de seleção. Assim, esta dissertação teve como objetivos: (*i*) analisar a expressão de genes localizados dentro de Meta-QTLs em linhagens contrastantes quanto ao nível de resistência a *S. sclerotiorum* e (*ii*) comparar os métodos de fenotipagem *straw* e *seedling test*, utilizando aspectos do progresso da doença por meio de modelos não lineares, bem como avaliar a interação do método de fenotipagem com o nível de resistência dos genótipos e a época de avaliação. No primeiro trabalho, verificou-se na avaliação fenotípica, alta suscetibilidade da linhagem Beryl, e que grande parte da interação linhagem vs época de avaliação ocorreu pelo rápido crescimento dos sintomas nessa linhagem a partir do terceiro dia após inoculação. Dos genes avaliados quanto à transcrição, os genes *Pv*PKF e *Pv*POD se mostraram os mais promissores quanto à sua relação com a resistência da linhagem Cornell 605. As diferenças no *background* genético das populações e o fato da avaliação ter sido feita em casa de vegetação podem explicar o fato de não necessariamente haver maior expressão dos genes candidatos na linhagem Cornell 605. Os resultados do segundo trabalho demostraram que os dois diferentes métodos de fenotipagem são bem correlacionados dentro dos grupos de resistência do feijão, no entanto, fazem inferências distintas sobre a resistência dos genótipos, sendo que o progresso da doença está intimamente ligado ao nível de resistência do genótipo avaliado. Os dois métodos de fenotipagem são eficientes, porém em situações distintas, bem como os modelos de progresso dos sintomas testados têm ajuste parecido aos níveis de sintomas observados nos dois métodos.

Palavras-chave: Avaliação fenotipica. *Phaseolus vulgaris* L. *Sclerotinia sclerotiorum* (Lib.) de Bary. Modelos não lineares. Meta-QTLs de resistência. RT-qPCR.

ABSTRACT

Prospecting bean genotypes resistant to white mold (*Sclerotinia sclerotiorum* (Lib.) de Bary) have been a priority for cheap and efficient control of this important pathogen. In this mission, several tools can be integrated to improve selection efficiency, and thus increase the genetic gain in breeding programs that have sought genotypes resistant to this pathogen. The mapping and validation of resistance genes are of direct interest in assisted selection in breeding programs, whereas accurate and efficient phenotyping methods are important throughout the breeding program, from germplasm screening to the advanced breeding generations. The objectives of this dissertation were: (*i*) to analyze the expression of genes located within Meta-QTLs in contrasting lines in terms of resistance to *S. sclerotiorum*; and (*ii*) to compare the straw and seedling test phenotyping methods, using aspects of the disease progression through nonlinear models, as well as to evaluate the interaction of the phenotyping method with the level of resistance of the genotypes and the evaluation period. In the first work, high susceptibility was observed for the Beryl line in the phenotypic evaluation, and that a large part of the interaction between lines and evaluation period occurred due to the rapid growth of symptoms in this line from the third day after inoculation. Of the genes evaluated for transcription expression, the *Pv*PKF and *Pv*POD genes were the most promising in relation to the resistance of the Cornell 605 line. Differences in the genetic background of the populations and the fact that the evaluation was done in a greenhouse could explain the fact that there was not a necessarily greater expression of the candidate genes in the Cornell 605 line. The results of the second work demonstrated that the two different phenotyping methods are well correlated within the bean resistance groups, however, they make distinct inferences about the resistance of the beans genotypes, and the progress of the disease is closely related to the resistance level of the evaluated genotype. The two methods of phenotyping are efficient, but in different situations, as well as the progress models of the symptoms tested have adjustment similar to the levels of symptoms observed in both methods.

Keywords: Phenotyping. *Phaseolus vulgaris* L. *Sclerotinia sclerotiorum* (Lib.) de Bary. Nonlinear models. Resistance Meta-QTLs. RT-qPCR.

SUMÁRIO

1 INTRODUÇÃO GERAL

O feijão (*Phaseolus vulgaris* L.) é um dos principais alimentos dos brasileiros, sendo o grande responsável na composição proteica vegetal. A produção do feijão não vem crescendo nos últimos anos e uma das causas dessa estagnação é o ataque de várias doenças (CONAB, 2017). Dentre essas doenças, umas das principais é o mofo branco, que tem como agente etiológico o fungo *Sclerotinia sclerotiorum* (Lib.) de Bary.

Em regiões de clima temperado, onde este fungo tem as condições ideiais para seu desenvolvimento, o mofo branco pode causar perdas de até 90% (SCHWARTZ et al., 2006). No Brasil, esse patógeno causa maiores problemas durante as safras de inverno nas regiões Sul e Sudeste do Brasil, onde o feijão é produzido em áreas irrigadas com pivô central, levando frequentemente a perdas significativas de produção (PAULA JÚNIOR et al., 2009; VIEIRA et al., 2010). O uso de medidas integradas, incluindo fungicidas, aração profunda e antecipada, rotação de culturas com plantas não hospedeiras, maior espaçamento entre linhas, baixa população, uso de fertilizantes, irrigação reduzida e uso de cultivares de porte ereto podem reduzir a incidência da doença (ANDO et al., 2007; KOLKMAN; KELLY, 2003a; REIS; CASA; BIANCHIN, 2011). Contudo, o uso de tais medidas integradas pode reduzir a produtividade e o retorno econômico (SCHWARTZ; SINGH, 2013).

Embora tais mecanismos de controle sejam utilizados, o emprego de cultivares resistentes é a medida de menor custo, que exige um manejo menos intensivo do agricultor e causa menor impacto ambiental. No entanto, o melhoramento para resistência ao mofo branco tem sido muito lento devido à baixa herdabilidade, às técnicas complexas de avaliação, à grande influência ambiental e mecanismos de escape da planta que confundem a expressão e detecção de resistência fisiológica em campo (BARDIN; HUANG, 2001; TERÁN; SINGH, 2009b).

Assim, o entendimento das relações patógeno-hospedeiro, identificando na planta fenótipos estáveis, bem como a utilização de ferramentas biotecnológicas como marcadores de DNA, podem auxiliar de forma indireta na identificação de genótipos superiores (RESENDE et al., 2008). Com isso, pode-se lançar mão de técnicas que permitam identificar genes ligados à resistência, como o mapeamento de regiões genômicas ligados à variação fenotípica para caracteres quantitativos, como é a resistência do feijão a *S. sclerotiotum*. Assim, Geldermann (1975) propôs o termo QTL (*quantitative trait loci*) para designar locos controladores desse tipo de caráter. A identificação de QTLs tem sido largamente utilizada em diversos patossistemas de interação horizontal. No feijão, por exemplo, já foram

identificados mais de 36 QTLs relacionados a características que influenciam a resistência a este patógeno (MIKLAS et al., 2013).

A identificação de QTLs pode apresentar problemas de reprodutibilidade pela interação de QTLs por ambientes e por populações. Assim, para contornar essa dificuldade, um novo termo foi cunhado, que é a identificação de Meta-QTLs, representando um grande avanço no estudo de QTLs, por buscar regiões genômicas que tenham efeitos em diferentes locais, em populações distintas ou em diferentes condições ambientas (GOFFINET; GERBER, 2000; VASCONCELLOS et al., 2017). Para validação de um QTL ou Meta-QTL em uma população, pode-se utilizar estudos de expressão dos mesmos. Para isso, a validação desses QTLs pode ser feita por RT-qPCR, técnica amplamente utilizada na validação de alelos de resistência do hospedeiro por meio da detecção de níveis de transcrição totais ou relativos (BORRAS-HIDALGO et al., 2012; BÜTTOW; BONOW, 2013; DE KEYSER et al., 2013; LU et al., 2014; MACIEL et al., 2009; MARKAKIS et al., 2009).

Os falsos positivos para resistência fisiológica podem resultar principalmente da redução da severidade da doença em cultivares de porte arbustivo com ramificação aberta e crescimento determinado verticalmente (Tipo I) e indeterminado (Tipo II) (ANDO et al., 2007; KOLKMAN; KELLY, 2003b; MIKLAS; GRAFTON, 1992). Por essas razões, os métodos de avaliação fisiológica em casa de vegetação vêm sendo utilizados. Além disso, o crescimento da utilização desses métodos se deve à fácil condução, eficiência, reprodutibilidade, o que permite testar um grande número de genótipos sem contaminar o campo com o patógeno (TERÁN; SINGH, 2008). Dessa forma, o desenvolvimento de métodos confiáveis de avaliação de resistência de feijoeiro ao mofo branco permanece como uma das prioridades no melhoramento dessa espécie.

As técnicas de fenotipagem para triagem da resistência devem ser sempre otimizadas e avaliadas para que não ocorram inferências incorretas sobre o exato nível de resistência do hospedeiro sob determinada condição ambiental (MAHLEIN et al., 2012; MUTKA; BART, 2015). Tanto para o melhoramento visando resistência quanto para outros caracteres, é importante que haja integração entre as ferramentas disponíveis para maximização dos ganhos (FINCKH, 2008). Desse modo, informações moleculares e fenotípicas precisas devem ser integradas com eficiência para geração de dados consistentes (CATTIVELLI et al., 2008).

Diante do exposto, essa dissertação teve por objetivos: (*i*) analisar a expressão de genes localizados dentro de Meta-QTLs em linhagens contrastantes quanto ao nível de resistência à *S. sclerotiorum* e (*ii*) comparar os métodos de fenotipagem *straw* e *seedling test*, utilizando aspectos do progresso da doença por meio de modelos não lineares, bem

como avaliar a interação do método de fenotipagem com o nível de resistência dos genótipos e a época de avaliação.

2 REVISÃO DE LITERATURA

2.1 Cultura do Feijão

O feijão (*Phaseolus vulgaris L.*) é uma espécie da família Fabaceae, diplóide, que tem ciclo anual e que se reproduz majoritariamente por autofecundação (DE RON et al., 2015). O feijão tem centro de origem na América, com dois distintos centros de diversidade, sendo um *pool* gênico Mesoamericano e outro Andino, caracterizados pelo isolamento reprodutivo parcial, que incluem populações selvagens e cultivares (DE RON et al., 2015; GEPTS, 1988; SCHOONHOVEN; VOYSEST, 1991).

O feijão é cultivado praticamente em todo o mundo, com variedade de sistemas de cultivo em ambientes bastante diversos como as Américas, África, Oriente Médio, China e Europa (BLAIR et al., 2010). Essa cultura tem grande importância na segurança alimentar de diversos países da África, Ásia e América latina por ser uma fonte barata de proteína (BURUCHARA, 2003). Em alguns países da África, estima-se que cerca de 20 a 30% da produção seja de subsistência, com consumo correspondente a 65% total de proteína e 32% do suprimento de carboidratos (BLAIR et al., 2010).

Além de fonte de proteína e carboidratos, a inclusão do feijão na dieta humana provê quantidades significativas de ácidos graxos insaturados (ácido linoleico), fibra alimentar, especialmente fibra solúvel, e é uma excelente fonte de alguns minerais e vitaminas (BERRIOS; SWANSON; ADELINE CHEONG, 1999; KUTOŠ et al., 2003). De acordo com Bennink (2002); Geil e Anderson (1994); Suárez-Martínez et al. (2016) a inclusão do feijão na dieta reduz o risco de obesidade, diabetes, doenças cardiovasculares e alguns cânceres.

O Brasil é protagonista em relação à produção e consumo de feijão, com 19,7% do consumo mundial de acordo com a FAOSTAT (2013), seguido de Índia (19,5%), México (7,7%), EUA (6,6%), Tanzânia (2,7%), e Uganda (2,7%) e 3º maior produtor, respondendo por 12% da produção, atrás de Myanmar com 16,4% e da Índia com 15,7% (CONAB, 2015).

Sempre presente no prato dos brasileiros, o feijão é a principal fonte de proteína, seguido, em importância pela carne bovina e pelo arroz, além de ter uma grande expressão socioeconômica do Brasil, tendo consumidores nas diferentes classes (DELFINO; CANNIATTI-BRAZACA, 2010; MESQUITA et al., 2007).

Quase a totalidade do que é produzido no Brasil é destinado ao consumo interno. O estado de Minas gerais é o segundo maior produtor do País, com produção estimada na safra 2016/2017 em 558,4 mil toneladas, área de 351,4 mil ha^{-1} e produtividade de 1.589 kg ha^{-1}, atrás apenas do estado do Paraná (CONAB, 2017).

2.2 Mofo branco em Feijão

O mofo branco é uma doença causada pelo fungo *Sclerotinia sclerotiorum* (Lib.) de Bary, que é um dos mais cosmopolitas e devastadores patógenos de plantas do mundo, sendo capaz de colonizar mais de 400 espécies (BOLTON; THOMMA; NELSON, 2006). Embora colonize monocotiledôneas e dicotiledôneas, a maioria das espécies que são colonizadas por esse agente etiológico pertence à classe das dicotiledôneas e, dentre estas há, várias espécies de importância agronômica, como a soja, feijão, girassol, lentilha, batata, alface, chicória, repolho, couve-flor, cenoura e outras (BOLAND; HALL, 1994; KIMATI, 1997; LEITE, 2005; MARCUZZO; SCHULLER, 2014). Em monocotiledôneas uma exceção importante é a família Poaceae (JULIATTI et al., 2015).

S. sclerotiorum pertence ao reino Fungi, filo Ascomycota, classe Discomyces, ordem Helotiales e familia Sclerotiniaceae. As hifas são hialinas, septadas, ramificadas e multinucleadas (BOLTON; THOMMA; NELSON, 2006); não são produzidos conídios assexuados e sua estrutura de sobrevivência a longo prazo é chamada de escleródio, que é uma massa de hifas pigmentada que pode permanecer viável durante longos períodos em condições ambientais inapropriadas para sua germinação (BOLTON; THOMMA; NELSON, 2006; CLARKSON; WHIPPS, 2002; KIMATI, 1997; NATTI, 1971).

Os escleródios podem sobreviver até 11 anos no solo em condições ambientais adversas, onde a germinação somente ocorre em condições ambientais favoráveis como temperaturas de 4 a 20ºC, alta umidade e quando sob profundidade do solo de até 5 cm (CLARKSON; WHIPPS, 2002; KIMATI, 1997; MERRIMAN, 1976). Essa germinação pode ocorrer de duas formas: pela germinação carpogênica, onde há produção de apotécios (órgãos de reprodução sexuada) dando origem a ascósporos que, quando maduros, serão ejetados para o meio ambiente, infectando a planta acima da linha do solo; e pela germinação miceliogênica que promove a produção de hifas a partir de micrósporos, presentes nos escleródios, que atacam diretamente os tecidos da planta (BARDIN; HUANG, 2001; NATTI, 1971).

A infecção primária, como é chamada por muitos autores, ocorre através da germinação carpogênica, sendo responsável pelas epidemias em condições de campo

(ABAWI; GROGAN, 1979; BOLTON; THOMMA; NELSON, 2006). Essa germinação produz um ou vários apotécios, que são um tipo de corpo de frutificação em forma de taça, sendo responsável pela liberação de milhares de ascósporos que iniciam a doença na parte aérea da planta. O apotécio tem coloração branca, amarelo ou marrom, chegando a medir até 1,3 cm de comprimento a partir do topo do solo e 2 cm abaixo do solo (ABAWI; GROGAN, 1979; BARDIN; HUANG, 2001; BOLTON; THOMMA; NELSON, 2006).

O processo de infecção geralmente tem início na junção do pecíolo com a haste, aproximadamente 10 a 15 cm acima da linha do solo, onde flores, pétalas e folhas desprendidas geralmente ficam retidas (BOLAND; HALL, 1994). Rapidamente, as lesões são cobertas por micélio branco de aspecto cotonoso, bem característico da doença. O micélio branco, em alguns pontos, vai escurecendo até atingir coloração preta, formando os escleródios que podem ser produzidos interna ou externamente aos tecidos do hospedeiro. Quando a lesão circunda a haste, a parte aérea da planta sofre murcha e morte de folhas. Em folhas e pecíolos, os sintomas ocorrem sob alta umidade relativa e são caracterizados por apodrecimento total das folhas, com presença de micélio branco e escleródios. Em frutos, observa-se podridão mole aquosa com produção de micélio branco e escleródios (BOLTON; THOMMA; NELSON, 2006; HEFFER LINK; JOHNSON, 2007)

O mofo branco é uma doença monocíclica, ou seja, tem apenas um ciclo primário de infecção, e uma vez que o tecido hospedeiro está doente, não há produção de esporos dentro de um mesmo ciclo da cultura (HEFFER LINK; JOHNSON, 2007).

Em feijão, *S. sclerotiorum* se apresenta como um grave patógeno durante o inverno em lavouras irrigadas, fazendo com que existam condições ambientais de temperatura e umidade ideais para a epidemia. O processo epidemiológico pode variar de acordo com o genótipo empregado, a densidade de plantio, o tipo de solo e a quantidade de água de irrigação utilizada (ABAWI; GROGAN, 1979; JULIATTI et al., 2013).

No estado de Minas Gerais, no Brasil, onde aproximadamente 35% do feijão colhido é produzido em áreas irrigadas com pivô central durante a safra de outono-inverno, o mofo branco ganha grande importância, já que muitas dessas áreas são infestadas com escleródios de *S. sclerotiorum*, que sob condições favoráveis como estas, levam frequentemente a perdas significativas de produção (PAULA JÚNIOR et al., 2009). Essa perda de produção é consequência direta da diminuição do estande de plantas e má qualidade do grão produzido pelas plantas infectadas, como a redução do peso e número de sementes por vagem (HOFFMAN et al., 1998; KOLKMAN; KELLY, 2003a).

Embora haja um grande esforço no sentido de controlar as epidemias de *S. sclerotiorum*, não existe nenhum método de controle totalmente eficaz para este patógeno.

Heffer Link e Johnson (2007) elencam vários métodos que podem ser implementados para controle, devendo haver um manejo integrado. Dentro do manejo integrado, se destacam o manejo cultural e o controle químico com o uso de fungicidas, porém, o ideal seria incluir a utilização de cultivares com maior resistência, principalmente para reduzir o uso de fungicidas, e até dispensá-lo, nas áreas com menor risco de epidemia (ANTONIO, 2011).

O controle químico com uso de fungicidas é o método mais utilizado pelos produtores, contudo, devido ao seu custo elevado, baixa eficiência dos princípios ativos e impossibilidade de aplicação em todas as safras e regiões, vem tornando seu uso cada vez mais dispendioso e com alto custo-benefício (SCHWARTZ et al., 2004). Outro fato importante é que o uso de determinados princípios ativos existentes no mercado de fungicidas proporcionam forte pressão de seleção sobre isolados insensíveis (JULIATTI et al., 2015). Quanto ao manejo cultural, pode-se lançar mão do uso de sementes livres do patógeno, controle da população de plantas, rotação de culturas, uso de cultivares de porte ereto, controle biológico e regulação da frequência de irrigação (REIS; CASA; BIANCHIN, 2011; STEADMAN, 1979; TU, 1997).

No feijão a herança da resistência ao mofo branco é poligênica ou quantitativa, apresentando de baixa a moderada herdabilidade (CARNEIRO et al., 2011; MEINERS, 1981; MIKLAS, 2006; MIKLAS et al., 2001; PÉREZ-VEGA et al., 2012). Essa herança complexa é fator limitante no sucesso da seleção em favor da resistência (LEITE et al., 2016). Atualmente, nos programas de melhoramento, têm-se buscado cultivares com resistência fisiológica e mecanismos de escape. Trabalhos como os de Miklas et al. (2013), Soule et al. (2011) e Hoyos-Villegas et al. (2015) são exemplos da busca por QTLs (*Quantitative Trait Loci*) para caracteres de escape, como porte ereto e hábito de crescimento determinado. A busca pela resistência fisiológica pode ser vista em trabalhos como os de Lara et al. (2014); Pamplona et al. (2015); Souza et al. (2016) e Vasconcellos et al. (2017), nos quais buscaram identificar marcadores ligados a QTLs que explicassem a variação genética para o caráter.

2.3 Resistência de plantas a patógenos

2.3.1 Mecanismos pré e pós-formados

As plantas vivem em associação com uma grande diversidade de microrganismos, incluindo vírus, bactérias e fungos, o que favoreceu o desenvolvimento de complexo sistema de comunicação entre eles (DALIO et al., 2014; KRATTINGER; KELLER, 2016).

Contudo, para as plantas essas interações podem ser benéficas ou maléficas, como no caso dos agentes fitopatogênicos (KRATTINGER; KELLER, 2016). As plantas, por sua vez, apresentam uma série de mecanismos de resistência a esses patógenos, que podem ser (*i*) passivos ou pré-formados e (*ii)* ativos ou pós-formados.

Tanto os mecanismos de resistência pré quanto os pós-formados podem ser subdivididos em estruturais e bioquímicos (PASCHOLATI; LEITE, 1994). Os mecanismos estruturais são barreiras físicas ou morfo-anatômicas à penetração e/ou colonização do patógeno, enquanto que os mecanismos bioquímicos englobam substâncias capazes de inibir o desenvolvimento do patógeno ou gerar condições adversas para a sobrevivência nos tecidos do hospedeiro, devendo estar presentes em concentração adequada nas partes invadidas e em forma acessível ao patógeno (PASCHOLATI; LEITE, 1994; SCHWAN-ESTRADA; STANGARLIN; PASCHOLATI, 2008).

Pascholati e Leite (1994) então classificaram os mecanismos de resistência utilizados pelas plantas da seguinte forma (adaptado de Stangarlin, Kuhn e Toledo (2011):

I) Pré-formados:

I.II) estruturais: cutícula, tricomas, estômatos e vasos condutores;

I.II) bioquímicos: fenóis, alcaloides glicosídicos, lactonas insaturadas, glicosídeos fenólicos e cianogênicos, inibidores proteicos, quitinases e β-1,3 glucanases.

II) Pós-formados:

II.I) estruturais: papilas, halos, lignificação, glicoproteínas ricas nos aminoácidos hidroxiprolina (HRGP) e glicina (GRP), camadas de cortiça, camadas de abscisão e tiloses;

II.II) bioquímicos: fitoalexinas, proteínas relacionadas à patogênese e espécies ativas de oxigênio.

Para cada patossistema, os fatores estruturais e bioquímicos nas respostas de resistência são particulares, o que também varia de acordo com a idade da planta hospedeira (STANGARLIN; KUHN; TOLEDO, 2011). Com isso, as plantas podem desenvolver estratégias especificas contra patógenos biotróficos e necrotróficos. Contra patógenos biotróficos podem ser elicitados genes de defesa que asseguram o desencadeamento, em um dado momento, de mecanismos que mantêm a resistência ao patógeno, ao passo que, contra os necrotróficos, os genes de resistência buscam anular os mecanismos de patogenicidade do invasor (JOHAL et al., 1995; MALENČIĆ et al., 2010).

Dentre as defesas utilizadas pelas plantas estão a resposta de hipersensibilidade (HR), resistência sistêmica adquirida (SAR) e indução de compostos sinalizadores, como por exemplo, ácido salicílico e peróxido de hidrogênio e indução das PRPs (proteínas relacionadas à patogênese) (ANTONIO, 2011). A HR ou reação de hipersensibilidade em

plantas é considerada um dos principais eventos da resposta de defesa da planta contra o ataque de patógenos, se caracterizando por ser uma resposta rápida e localizada, ou seja, que ocorre no sítio de infecção do patógeno (LINTHORST et al., 2000).

Comum a todas as plantas e a diferentes patógenos, a HR constitui na primeira etapa da resposta de defesa da planta, sendo seguida de outras alterações, quer seja no sítio de infecção ou em toda a planta. Dentre as principais alterações decorrentes da HR, a mais evidente é a indução à síntese de um grande número de PRPs, destacando-se as peroxidases, quitinases e β-1,3- glucanases (ANTONIO, 2011; BARROS et al., 2010). Como conceito geral, pode-se dizer que as PRPs são induzíveis no hospedeiro em resposta à infecção por um patógeno ou por estímulos abióticos, e podem estar correlacionadas com a resistência não específica do hospedeiro ao patógeno (STANGARLIN; KUHN; TOLEDO, 2011). A indução destas proteínas é mediada pela ação de substâncias sinalizadoras que são classificadas em dois tipos, conforme sua origem: elicitores endógenos, da própria planta; e elicitores exógenos, do patógeno (CHRISTENSEN et al., 2002). As PRPs podem atuar na resistência de plantas contra patógenos de forma direta ou indireta. Na forma direta, pode-se citar por exemplo, a inibição do crescimento do patógeno ou da germinação de esporos, o qual representa uma concepção simplificada da defesa de plantas contra a entrada de patógenos (GORJANOVIĆ, 2009; STANGARLIN; KUHN; TOLEDO, 2011).

Gorjanović (2009) classificou as PRPs em 17 categorias de acordo com suas estruturas e funções, onde dentro de cada família de PRPs existem várias classes que compreendem diferentes isoformas, quer básicas ou ácidas (Tabela 1).

Tabela 1. Famílias de proteínas relacionadas à patogênese.

Categorias (Famílias)	Propriedade
PR-1	Antifúngica
PR-2	β- 1,3 - glucanase
PR-3	Quitinase I-II, IV, VII
PR-4	Quitinase I-II
PR-5	Osmotina
PR-6	Inibidores de Protease
PR-7	Endoproteinases
PR-8	Quitinase III
PR-9	Peroxidases
PR-10	Análogas a ribonuclease
PR-11	Quitinase V
PR-12	Defensinas
PR-13	Tioninas
PR-14	Proteínas relacionadas ao transporte de lipídeos
PR-15	Oxalato oxidases
PR-16	Análogas a oxalato oxidase
PR-17	Desconhecida

2.3.2 Interação molecular planta-patógeno

Uma abordagem alternativa do sistema de resistência ou sistema imune da planta é a sua organização em duas linhas de defesa principais. A primeira se dá por meio do reconhecimento de padrões moleculares associados ao patógeno (PAMPs, *Pathogen-Associated Molecular Patterns*) por proteínas receptoras presentes na membrana plasmática do hospedeiro (PRRs- *Pattern Recognition Receptors*), sendo chamada de imunidade desencadeada por PAMPs (PTI, PAMP *Triggered Immunity*) (DODDS; RATHJEN, 2010; JONES; DANGL, 2006; KRATTINGER; KELLER, 2016). Esse reconhecimento se caracteriza por ser não específico ou de amplo espectro (BOUTROT; ZIPFEL, 2017).

A primeira linha de defesa pode ser suplantada pelo patógeno por meio do transporte de proteínas efetoras para o citoplasma do hospedeiro. Em resposta, as plantas desenvolveram uma segunda linha de defesa que é chamada de imunidade desencadeada por efetores (ETI, *Effector Triggered Immunity*). Nesta, o reconhecimento de proteínas efetoras produzidas pelo patógeno é específico, direto ou indireto e ocorre por meio de proteínas codificadas pelos genes R. Os alelos que codificam esses efetores são normalmente chamados de alelos de avirulência ou Avr (DODDS; RATHJEN, 2010; JONES; DANGL, 2006) e são essenciais para o reconhecimento do patógeno pela planta.

Sabe-se da existência de vários genes R que reconhecem um grande número de patógenos diferentes. Apesar dessa diversidade, esses genes codificam proteínas que

compartilham domínios estruturais e que apresentam considerável semelhança entre si, levando ao entendimento de que as interações proteína-proteína, os componentes de sistemas receptores e a sinalização nas respostas de defesa das plantas sejam semelhantes (MILLER et al., 2008). A partir desta homologia, os produtos proteicos dos genes R puderam ser classificados em famílias (DANGL; JONES, 2001), oito classes já são reconhecidas (BOYD et al., 2013; GURURANI et al., 2012), algumas delas são NBS- LRR, RLP, RLK, Ser/ Thr quinase e CC ancorado na membrana.

Dentre as família conhecidas, a NBS-LRR (sítios de ligação a nucleotídeos - NBS, proteínas ricas em leucina - LRR), representa o maior grupo (DANGL; JONES, 2001; GUIMARÃES et al., 2005; ROMMENS; KISHORE, 2000), sendo a resistência a doenças sua única função relatada, (DANGL; JONES, 2001; MEYERS; KAUSHIK; NANDETY, 2005), o que indica que a maioria dos genes de resistência em planta são membros desta família (YAISH; SÁENZ DE MIERA; PÉREZ DE LA VEGA, 2004).

As proteínas receptoras de membrana e NBS-LRR explicam interações verticais em diversos patossistemas, contudo, esses modos de interação não conseguem esclarecer como ocorre a resistência quantitativa ou horizontal (KRATTINGER; KELLER, 2016), como é o caso do feijão – *S sclerotiorum.* É possível ainda que os RLKs (*quinases receptor-like)* e os RLPs (*proteínas receptor-like*) localizados na membrana possam ser identificados em estudos de QTL, e existem até mesmo exemplos de genes NBS-LRR raça específica que foram identificados nesse tipo de estudo (MAGO et al., 2015; PAILLARD et al., 2012).

Pesquisas recentes utilizando QTLs levaram à identificação de genes de resistência quantitativa que codificam para proteínas de resistência incomuns (KRATTINGER; KELLER, 2016). Dentre estas proteínas estão fatores de transcrição (TROGNITZ et al., 2002); elementos transponíveis (HOLESKI; JANDER; AGRAWAL, 2012; WANG et al., 2017) e transportadores de membrana (KRATTINGER et al., 2009; MOORE et al., 2015). Esse fato leva melhoristas e fitopatologistas a se debruçarem cada vez mais em pesquisas visando elucidar interações complexas de genes responsáveis por resistência horizontal.

2.4 Análise de expressão gênica

As tecnologias utilizadas na análise de expressão gênica podem ser empregadas no estudo dos mecanismos moleculares envolvidos em processos biológicos importantes como a identificação e caracterização de genes ligados aos mais diversos caracteres (WANG;

GERSTEIN; SNYDER, 2009). A análise da expressão gênica auxiliou a melhor compreensão das respostas das plantas a diferentes tipos de estresses bióticos a abióticos e, através destes estudos, numerosos genes de resposta a estresses foram descobertos (BÜTTOW; BONOW, 2013).

As aplicações para a quantificação dos níveis de expressão de mRNAs evoluíram mais lentamente, em parte devido às dificuldades no desenvolvimento de protocolos experimentais apropriados, mas também porque alguns estudos de expressão tem o objetivo de identificar diferenças quantitativas (talvez sutis) entre amostras, enquanto que outros têm objetivo de detectar a ausência ou a presença de um evento, tal como uma ligação de um fator de transcrição (MARIONI et al., 2008).

Outro fator que influencia diretamente as análises de expressão gênica é o tempo (BÜTTOW; BONOW, 2013; STEGLE et al., 2010); alguns genes têm sua expressão aumentada em determinado período, permanecendo nesse nível, enquanto que outros são "ligados e desligados"(BÜTTOW; BONOW, 2013). A sobreposição de genes induzidos sob várias condições também é outro fator importante, e sugere que existam intersecções entre as vias de sinalização (NICOT et al., 2005).

A PCR em tempo real ou qPCR é uma das metodologias que podem ser usadas para quantificação de mRNA em baixos níveis, permitindo a detecção direta dos produtos da PCR durante a fase exponencial da reação, combinando amplificação e detecção em um só passo (BELTRÁN et al., 2009; HEID et al., 1996), através da incorporação de moléculas fluorescentes covalentemente ligadas ou não a nucleotídeos, as quais podem ser quantificadas durante a cinética da reação. Esta leitura é capaz de determinar a quantidade absoluta ou relativa do número de cópias de um fragmento de DNA num dado momento (HEID et al., 1996).

Diversas plataformas de instrumentação foram criadas e comercializadas, no entanto, a maioria é composta por um termociclador, com sistema óptico para emissão e detecção da fluorescência e um computador com *software* próprio para a aquisição de dados e análise final da reação (MACKAY et al., 2007; VALASEK; REPA, 2005).

A técnica qPCR é considerada mais precisa e confiável do que outros métodos de análise de expressão gênica, sendo que muitas vezes serve para validar dados obtidos por outros métodos (KOZERA; RAPACZ, 2013). Assim, a qPCR vem sendo amplamente utilizada no estudo da interação para diversos patossitemas, principalmente para a validação de alelos de resistência do hospedeiro, estudos de expressão em diferentes tempos após inoculação com o patógeno e perfil transcricional de genes em resposta à mudanças do ambiente da patogênese (BORRAS-HIDALGO et al., 2012; BÜTTOW; BONOW, 2013;

DE KEYSER et al., 2013; LU et al., 2014; MACIEL et al., 2009; MARKAKIS et al., 2009). Outra importante funcionalidade dessa técnica é a possibilidade de uso na validação de QTLs de resistência a patógenos (FREI et al., 2010; GUNNAIAH et al., 2012; OLIVEIRA et al., 2002; VASCONCELLOS et al., 2016), tendo perspectiva de aumento de uso, já que a medida que QTLs são identificados, pode haver necessidade de serem validados.

2.5 Fitopatometria

Fitopatometria é a quantificação da intensidade de doença presente em um determinado tempo em uma população de plantas (LARGE, 1966). A fitopatometria é requisitada em diversos estudos fitopatológicos, tais como o levantamento de doenças de um local, comparação de resistência genética de cultivares a doenças, comparação de eficiência de defensivos agrícolas e práticas de manejo no controle de doenças (GOMES; MICHEREFF; MARIANO, 2004; LIBERATO, 2003).

Os métodos de quantificação precisam ser os mais exatos e precisos de avaliação de doenças para que seja possível uma quantificação confiável de sintomas (MADDEN; HUGHES; BOSCH, 2007). As doenças podem ser quantificadas por métodos diretos de avaliação da incidência e da severidade, e métodos indiretos, tais como a determinação da população do patógeno, sua distribuição espacial e danos e perdas por meio da desfolha causada (HORSFALL; COWLING, 1978). A quantificação de uma doença pode ser realizada uma única vez ou várias vezes durante o ciclo de vida de uma cultura, a depender dos objetivos. A estimativa da severidade de uma doença ao longo do tempo é feita plotando-se os valores de intensidade da doença versus tempo e obtendo-se a curva de progresso da doença, resumindo os efeitos da interação entre hospedeiro, patógeno e ambiente (MADDEN, 1986).

A simples observação da ocorrência ou a avaliação da doença utilizando-se critérios subjetivos, na maioria das vezes induz erro, seja na avaliação da resistência de cultivares, seja na tomada de decisão de controle, sendo assim necessária a adoção de critérios precisos na quantificação de doenças (LENZ et al., 2009). Dessa forma, ao longo do período de pesquisa em fitopatometria, diversos métodos têm sido propostos e empregados para aumentar a qualidade fitopatométrica de vários patossistemas.

2.6 Fenotipagem do patossistema feijão – *Sclerotinia sclerotiorum*

Em condições de campo, é difícil discriminar entre resistência fisiológica, como por exemplo a reação bioquímica de resistência da planta, ou como evasão ao mofo branco, como porte arbustivo (MIKLAS; GRAFTON, 1992). Os falsos positivos para resistência fisiológica podem resultar principalmente da redução da severidade da doença em cultivares de porte arbustivo com ramificação aberta e crescimento determinado verticalmente (Tipo I) e indeterminado (Tipo II) (ANDO et al., 2007; KOLKMAN; KELLY, 2003b; MIKLAS; GRAFTON, 1992). Por essas razões, os métodos de avaliação fisiológica em casa de vegetação vêm sendo utilizados. Além disso, o crescimento da utilização desses métodos se deve também por serem de fácil condução, eficientes, reprodutíveis e permitem testar um grande número de genótipos sem contaminar o campo com o patógeno (TERÁN; SINGH, 2008).

Várias metodologias de avaliação por meio da inoculação artificial do patógeno utilizam-se do micélio do fungo ou método direto (SCHWARTZ; SINGH, 2013). Entre eles destaca-se o *straw test* ou teste do canudo, que é eficiente para auxiliar na identificação, caracterização e seleção de genótipos resistentes ao mofo branco, sendo o mais utilizado em programas de melhoramento e que pode ser usado em casa de vegetação e campo (SINGH; SCHWARTZ; STEADMAN, 2014; TERÁN; SINGH, 2008). O *straw test* possui como vantagem o fato de ser um método não destrutivo, o que facilita o avanço de programas de melhoramento para a obtenção de progênies resistentes. Este método é considerado mais eficiente, pois, é inoculado o próprio micélio no ápice do caule com um canudo ou ponteira plástica de micropipeta e se avalia o progresso da doença na planta (PETZOLDT; DICKSON, 1996; SCHWARTZ; SINGH, 2013; TERÁN; SINGH, 2009a).

Outro método de avaliação em casa de vegetação foi recentemente proposto por Arkwazee e Myers (2017). Chamado de *seedling test*, esse método propõe a avaliação em estágio de plântula (estágio vegetativo V2). De forma similar ao *straw test*, a inoculação com micélio é feita em haste cortada logo após o primeiro nó com uso de ponteira e a avaliação do progresso dos sintomas é feita por meio de escala de notas.

A seleção de métodos eficientes de inoculação é imprescindível para a identificação de potenciais fontes de resistência e configura como uma grande preocupação para os melhoristas. Na avaliação dos genótipos de feijão ao mofo branco, como em qualquer outro patossistema, deve-se escolher o método que identifica de forma correta a resistência requerida dos genótipos. Um dos entraves para a solução desse problema é que alguns genes de resistência têm uma interação muito grande com o órgão avaliado e estágio da planta. Assim, a escolha do estágio de avaliação incorre diretamente também sobre a época de quantificação dos sintomas após inoculação (VITERI et al., 2015), ou seja, se a avaliação

ocorrer de forma precoce, talvez não estejam selecionados os alelos de interesse para uma resistência de planta adulta, que a maioria das vezes é de maior interesse (TROCH et al., 2013) .

2.7 Modelagem do progresso de doenças

2.7.1 Modelos não lineares

Um modelo é uma simplificação da realidade e tenta resumir o principal processo, apresentar hipóteses e verificar suas coerências e consequências (VAN MAANEN; XU, 2003). Ele também deve apresentar um teste que determine as hipóteses mínimas que permitiriam representação matemática mais próxima possível de processos reais (VAN MAANEN; XU, 2003). Na epidemiologia, a modelagem do progresso de doenças visa compreender as principais determinantes do desenvolvimento epidêmico, a fim de desenvolver métodos sustentáveis para estratégias e táticas de gestão de doenças, níveis futuros previstos de doença e simuladores verificados (BERGAMIN FILHO, 2011; VAN MAANEN; XU, 2003).

Van der Plank, entre 1960 e 1963, foi o primeiro a modelar o desenvolvimento temporal do progresso de doenças, que desde então constituiu a base para a modelagem de doenças de plantas (BERGAMIN FILHO, 2011; CAMPBELL; MADDEN, 1990; VAN MAANEN; XU, 2003). Posteriormente, vários modelos foram desenvolvidos (JEGER; TAMSETT, 1983). A comparação de curvas de progresso de doenças em cultivares com diferentes níveis de resistência tem sido um dos objetos de estudo da epidemiologia comparativa por meio do ajuste de modelos e a comparação das áreas abaixo da curva do progresso da doença (CAMPBELL; MADDEN, 1990; SILVA; VALE; ZAMBOLIM, 1998; SPÓSITO; BASSANEZI; AMORIM, 2004). Assim, pode-se comparar a resistência de cultivares por meio da taxa de progresso da doença, desde que as plantas sejam submetidas a condições similares de clima e sob a mesma população patogênica (CUNNIFFE et al., 2015; SPÓSITO; BASSANEZI; AMORIM, 2004).

Independentemente do patossitema considerado, os parâmetros importantes da curva de progresso da doença podem ser caracterizados, como: época de início da epidemia, a quantidade de inóculo inicial (x_0), a taxa de aumento da doença (r), a forma e a área sob a curva de progresso da doença, as quantidades máxima ($xmax$) e final (xf) de doença e a duração da epidemia (BERGAMIN FILHO, 2011).

Alguns modelos não lineares são comumente utilizados para descrever o progresso temporal das doenças. Os principais modelos são o exponencial, o monomolecular, o logístico, o de Gompertz e de Richards (BERGAMIN FILHO, 2011; CAMPBELL; MADDEN, 1990; MADDEN; HUGHES; BOSCH, 2007). Abaixo são apresentados modelos, onde x_0 é a severidade inicial da doença, r_L é a taxa de progresso da doença e t corresponde à época de avaliação, seguidos de uma breve descrição com base em (BERGAMIN FILHO, 2011; CAMPBELL; MADDEN, 1990; NESI, 2013):

Logistico

Este modelo incorpora aspectos dos modelos monomolecular e exponencial, sendo o mais empregado para descrever o progresso de doenças policíclicas. A plotagem de *x* contra o tempo origina uma curva em forma de S, conhecida como curva logística, simétrica em torno de *x* = 0,5.

$$x_L = 1/(1 + ((1/\, x_0) - 1)\, \exp(-r_L t)$$

Gompertz

Apesar do modelo de Gompertz ter sido introduzido na epidemiologia vegetal algum tempo depois do modelo logístico, sua origem é mais antiga. Apropriado para doenças policíclicas como alternativa ao modelo logístico, com ponto de inflexão em torno de y = 0,37, fazendo com que a curva alcance um máximo mais rapidamente que o modelo logístico.

$$x_G = \exp\,(-(-\ln(x_0))\, \exp(-r_G t))$$

Monomolecular

No modelo monomolecular, diferente dos modelos logístico e de Gompetz, a velocidade de aumento da doença é proporcional ao inóculo inicial e a uma taxa, não sendo proporcional à própria quantidade da doença. Com isso, este modelo é apropriado para epidemias em que não há dispersão de inóculo secundário na mesma estação de crescimento, o que significa que o aumento da doença se deve ao inóculo produzido em epidemias anteriores ou epidemias em outros locais.

$$x_M = 1 - (1 - x_0)\, \exp\,(-r_M t)$$

Richards

O modelo de Richards difere dos demais citados anteriormente por ser o único flexível, ou seja, o único que pode tomar várias formas. Esse modelo pode acomodar uma ampla variedade de formas e incluir modelos mais simples como casos especiais.

$$x_R = (1 + ((x_0{}^{(1-m)}) - 1)\exp(-r_B t))^{1/(1-m)}$$

2.7.2 Estimação dos parâmetros do modelo

O método dos mínimos quadrados consiste em encontrar os melhores preditores, ajustando-os para um conjunto de dados tentando minimizar a soma de quadrados dos resíduos entre a curva ajustada e os valores observados. Esse método pode ser utilizado na estimação dos parâmetros em modelos não lineares, da mesma maneira que em modelos lineares (GALLANT, 1987).

O método dos mínimos quadrados pode ser classificado e caracterizado em função do vetor dos erros em modelos ordinários, ponderados e generalizados. Os modelos ordinários são os mais utilizados na maioria das situações, onde a estrutura dos erros não viola nenhuma das pressuposições *i.e*, ε~N (0, Iδ^2) (SAVIAN, 2005).

Seja uma equação de regressão não linear:

$$Y_t = f(x_t, \theta) + \varepsilon_t$$

em que: t = 1,2,3,...,n.

Na obtenção das estimativas de mínimos quadrados dos parâmetros do modelo são propostos e utilizados vários métodos iterativos. Os métodos iterativos mais utilizados são o método de Gauss-Newton, também conhecido como método da linearização, o método de Steepest-Descent ou método gradiente e o método de Marquardt (BATES; WATTS, 1988; BATY et al., 2015).

Abaixo, de acordo com Sousa (2012) são listados os critérios de alguns métodos iterativos:

Gradiente: $\theta - \theta^\circ = X'\varepsilon$

Gauss-Newton: $\theta - \theta^\circ = (X'X)X'\varepsilon$

Newton: $\theta - \theta^\circ = G^- X'\varepsilon$

Maquardt: $\theta - \theta^\circ = [X'X + \sigma diag(X'X)^- X'\varepsilon$

em que $(X'X)^{-}X'$ é uma inversa generalizada.

Vale ainda a observação de que os métodos de iteração de Gauss-Newton e Marquardt são os mais utilizados por conta da sua implementação no *software* computacional R, que é um software de código aberto (BATY et al., 2015; SOUSA, 2012).

2.7.3 Avaliadores da qualidade do ajuste

Segundo Jeger (1986) o coeficiente de determinação (R^2) obtido da regressão linear entre os valores transformados da proporção de doença em função do tempo, apesar de largamente empregado na literatura, não pode servir como critério de escolha do modelo. Contudo, o R^2 obtido da regressão linear entre os valores previstos e observados, ambos não transformados, é um indicador de qualidade aconselhável (BERGAMIN FILHO, 2011). Os desvios padrões dos parâmetros do modelo, bem como a avaliação do resíduo do modelo também são procedimentos indicados (CAMPBELL; MADDEN, 1990). O fato desses avaliadores de qualidade de ajuste serem amplamente utilizados pode ser explicado pelo fato deles serem apresentados diretamente nos arquivos de saídas de *softwares* estatísticos computacionais como SAS® e R (SILVEIRA, 2010).

A exploração de vários avaliadores com o intuito de selecionar os melhores modelos de regressão não linear vem perpetuando no decorrer dos últimos anos (MATTOS, 2016). Com isso, além do coeficiente de determinação (R^2), Quadrado médio do resíduo (QMR) e desvios padrões dos parâmetros estimados, outros avaliadores como porcentagem de convergência (CG%) (SILVEIRA et al., 2011), Critério de Informação de Akaike (AIC) (AKAIKE, 1974), Critério de informação Bayesiano (BIC) (SCHWARZ, 1978), Desvio médio absoluto (DMA) (PUIATTI et al., 2013) podem ser encontrados na literatura.

Quanto maior o número de avaliadores de qualidade, mais adequada será a indicação do modelo (SILVEIRA, 2010). Contudo, quando há um grande número de avaliadores, o processo de escolha do modelo pode se tornar complexo. Exemplifica-se, nesse caso, quando um modelo tem alto desempenho em um avaliador, como o coeficiente de determinação, por exemplo, e baixo desempenho onde os modelos parametrizados são bastante penalizados, como os critérios AIC e BIC (MATTOS, 2016).

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4 ARTIGO 1 - Expression of candidate genes related to white mold resistance in bean

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Abstract: White mold (*Sclerotinia sclerotiorum*), under favorable environmental conditions, can cause crop loss. The bean resistance to *S. sclerotiorum* is quantitative, which implies a significant environmental effect and difficulty in selection. In this case, the QTLs identification, of higher effect and stable in several populations and environments (Meta-QTLs), is a methodology that can be used in breeding programs. The aim of this study was to verify the phenotypic reaction of bean lines, contrasting in the resistance level, inoculated with *S. sclerotiorum*, at different evaluation periods, and to analyze the expression of Meta-QTL genes in these lines. Two experiments were carried out: (*i*) phenotypic analysis, in which the lines were evaluated at 3, 7 and 11 days after inoculation; (*i*) expression of the resistance-associated alleles in Meta-QTLs intervals: *Pv*PKF, *Pv*F-Box, *Pv*ERF1, *Pv*PGIP4, *Pv*PR1, *Pv*POD and *Pv*MYB in the Cornell 605 (resistant) and

Beryl (susceptible) lines, at 0, 24, 48 and 72 hours after inoculation. The phenotypic evaluation showed high susceptibility of the Beryl, and that a large part of the interaction between lines and evaluation period occurred due to the rapid development of symptoms in this line. Regarding transcription, the genes *Pv*PKF and *Pv*POD were the most promising in the resistance line Cornell 605. The differences in the genetic background of the lines used in this study, and the fact that the evaluation has been performed in a greenhouse can explain the fact that there was not necessarily a higher expression of the candidate genes in the Cornell 605 line.

Keywords: Meta-QTLs*, Phaseolus vulgaris* L., Resistance genes, RT-qPCR, White mold

Introduction

In Brazil, white mold is an important disease in bean crops irrigated during winter since the temperature and humidity conditions are ideal for the development of its pathogen, the fungus *Sclerotinia sclerotiorum*. The epidemiological process may vary according to the genotype employed, crop density, soil type, and the amount of water used in irrigation (Abawi & Grogan 1979; Juliatti et al. 2013).

Although there is a significant effort to control epidemics of *S. sclerotiorum*, there is no fully effective control method. Heffer Link and Johnson (2007) listed some methods that can be implemented for this control; however, an integrated management is necessary. Among them, the cultural management, chemical control with fungicides and, especially the use of cultivars with high resistance, which is the most effective strategy for bean crop, are indicated (Antonio, 2011).

In bean plant, the inheritance of white mold resistance is polygenic or quantitative, presenting low to moderate heritability (Meiners 1981; Miklas et al. 2001; Miklas 2006; Carneiro et al. 2011; Pérez-Vega et al. 2012). There is no evidence of quantitative or non-

specific interaction between genes of small effect and others of more significant effect, effective for several pathogens. These non-specific interactions between pathogen and host are not yet well known as specific interactions. Krattinger; Keller (2016) report that plasma membrane receptors and LRRs (Leucine-rich repeat receptors proteins) are not sufficient to explain resistance to disease in cereal plants.

The complex inheritance of bean resistance to *S. sclerotiorum* is a limiting factor in the success of selection. Currently, cultivars with physiological resistance and escape mechanisms have been sought in breeding programs. Studies such as those of Miklas et al. (2013) and Soule et al. (2011) are examples of the search for QTLs (Quantitative Trait Loci) for escape characters such as determined growth habit and upright plant cultivars. The search for physiological resistance can be seen in studies such as those carried out by Lara et al. (2014), Pamplona et al. (2015), Souza et al. (2016), and Vasconcellos et al. (2017), which aimed to identify markers linked to QTLs that explain the genetic variation for the character.

The identification of Meta-QTLs represents a significant advance in the study of QTLs because it searches for genomic regions that have effects in different locations, in different populations or in different environmental conditions (Goffinet & Gerber 2000; Vasconcellos et al. 2017). Thus, it is possible to perform selection assisted by markers with a less interference of environmental factors.

The RT-qPCR is a technique that has been widely used in the study of the interaction for several pathosystems, mainly for the validation of host resistance alleles and studies of temporal differential expression, and in response to environmental gradients (Maciel et al. 2009; Markakis et al. 2009; Borras-Hidalgo et al. 2012; Büttow & Bonow 2013; De Keyser et al. 2013; Lu et al. 2014). Another important functionality of this technique is the possibility of using it to validate QTLs of resistance to pathogens (Oliveira et al. 2002; Frei et al. 2010; Gunnaiah et al. 2012; Vasconcellos et al. 2016), with

a perspective of increasing the use, since as QTLs are identified, there may be a need to validate them.

In view of the above, the aim of this study was to evaluate the levels of early expression of genes selected by meta-QTLs of resistance to white mold. Seven putative resistance genes were selected for this pathosystem, and they were evaluated by RT-qPCR, at different periods after inoculation, in two different lines based on the infection response to white mold.

Method

Two experiments were carried out. The first one for inoculation and phenotypic evaluation, and the second for inoculation, RNA extraction, and quantification of transcription of the genes studied by RT-qPCR in the lines.

Genetic materials

Two lines were used in this study, the Cornell 605 that is resistant to white mold, and derived from the cross between the Redkote line (Cornell University, NY, USA) and Cornell 6603 (Griffiths et al. 2012). The Cornell 605 has light red color grain, erect architecture and determined growth. The Beryl line is highly susceptible to white mold, and it is used as a negative control in several tests involving this pathosystem (Griffiths; Sandsted; Halseth, 2012; Lehner et al., 2015).

Inoculation and phenotypic evaluation.

The experiment was conducted in a greenhouse, located in the Department of Biology of the Federal University of Lavras (Lavras, state of Minas Gerais, Brazil). The temperature (25 ± 2

°C) and humidity (60 ± 5%) were controlled, covering the period from September to October 2016.

The sclerotia of the aggressive isolate UFLA 27 (Silva et al. 2014) were obtained from the mycological collection of the Laboratory of Plant Resistance and Molecular Genetics of the Department of Biology at UFLA. Initially, the sclerotia were surface-disinfested by consecutive immersion in 70% ethanol for 1 minute, 20% bleach solution for 3 minutes, and immersed three times in demineralized and sterilized water. They were then placed in Petri dishes containing PDA (potato-dextrose-agar) medium and then incubated in BOD at 23 °C for three days and a photoperiod of 12 hours. Subsequently, the colony was chopped up and placed in plates with PDA medium, for inoculum multiplication, and incubated at 23 °C and photoperiod for 12 hours. After the third day of incubation, the plates with homogeneous mycelial growth were used for inoculation.

The Straw test described by Petzoldt & Dickson (1996) and modified by Terán et al. (2006) was used to evaluate the reaction of the genotypes to *S. sclerotiorum*. This method consists of cutting the stem apex at about 2.5 cm from the node, where a micropipette tip is inserted, with a PDA disc containing *S. sclerotiorum* mycelium.

The evaluation of the induced symptoms was performed at three times (3, 7 and 11 DAI = days after inoculation) by the method proposed by Singh, Schwartz, and Steadman (2014), in which the severity of the symptoms in the inoculated stem was quantified using grades from 1 to 9, in which: 1 = plants without symptoms, 2 = fungus invasion beyond the inoculation site, 3 = when the fungus reaches the first node; 4 = fungus invasion beyond the first node; 5 = fungus invasion near the second node, 6 = fungus invasion reaches the second node, 7 = fungus invasion beyond the second node, 8 = fungus invasion near the third node, and 9 = invasion of the third node by the fungus and/or death of the plant.

A completely randomized experimental design was used, with five replicates and a plot consisting of a pot with three plants, which had their main stem inoculated. After verifying the presuppositions for parametric analysis, the variance analysis was carried out, using the model Y_{ij}

$= \mu + l_i + a_j + (la)_{ij} + e_{ij}$, in which Y_{ij}, is an observation relating to line i in the DAI j, μ is the general mean, l_i is the fixed effect of the line i, a_j is the fixed effect of the DAI j, $(la)_{ij}$ is the fixed effect of the interaction between the line I and the DAI, and e_{ij} is the random effect of the experimental error, $e_{ij} \cap N(0, \sigma_e^2)$.

Molecular evaluation

The experiment was set up in a greenhouse under controlled temperature conditions (25 ± 2 °C) and humidity (60 ± 5%) in September and October 2016. Total RNA extraction and synthesis of cDNA of the samples were carried out in the Laboratory of Molecular Genetics of Plants, and the procedures of RT-qPCR were conducted in the Laboratory of Molecular Physiology of Plants, both located in the Department of Biology of the Federal University of Lavras – UFLA.

The two lines were evaluated in T0, T1, T2, and T3, and these treatments consisted of stems obtained from non-inoculated plants and stems collected from inoculated plants at 24, 48 and 72 hours after inoculation (HAI), respectively. Three biological replicates per line were used at each time. Each biological replicate consisted of a pool of three stems, from three plants of the pot. The stems collected for RNA extraction were cut just below the necrotic tissue at 5 cm below the first cut, thus removing a 5 cm piece of the stem and they were immediately frozen in liquid nitrogen until the extraction of RNA.

RNA extraction and cDNA synthesis

The Concert^{TMP} (Invitrogen) reagent was used to extract total RNA following the manufacturer's recommendations, with modifications. The samples were washed twice with NaCl and chloroform to increase RNA quality. Then the samples were treated with DNase using the Turbo DNA-free™ kit (Ambion) to remove the DNA residues. Afterward, the samples were quantified using NanoVue v2.0.4 4282, observing the nucleic acid concentration and purity ratios

A260\280 and A260\230, prioritizing values between 1.8 and 2.2. Additionally, the RNA integrity was verified on 1% agarose gel with the TAE buffer (Tris, EDTA and boric acid).

Only samples with high integrity and purity were used for cDNA synthesis, using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and stored at -20 °C.

Resistance candidate genes within the Meta-QTL

The candidate alleles tested were mapped by Vasconcellos et al. (2017), aiming at identifying resistance-associated genes in stable QTL intervals in 14 segregating populations of *Phaseolus vulgaris* (Table 1).

The primers were drawn from the expressed sequences of each allele deposited in the GenBank Home – NCBI. The Primer3Plus – Bioinformatics software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to generate fragment amplification primers, from 80 to 120 bp, and their quality was analyzed from the parameters generated by the OligoAnalyzer 3.1 (https://www.idtdna.com/calc/analyzer) (supplementary data).

Efficiency of primers and RT-qPCR

Serial cDNA dilutions (1: 5, 1: 25, 1: 125, 1: 625, 1: 3125) were performed to evaluate the amplification reaction efficiency of each gene. The reactions were prepared with standardized reagents for real-time PCR, using the Rotor-Gene Q Real-Time PCR (Qiagen) thermal cycler, by the SYBR® Green detection system. The final reaction volume for each sample was 15 µL, constituted of 7.5 µL of SYBR Green (QuantiFast SYBR Green PCR Kit – Qiagen), 3 µL of specific primers to each target gene (1.5 µL primer forward + 1.5 µL primer reverse), 1.5 µL of 10 ng/µL cDNA, and 3 µL of H_2O RNAse Free. The reaction conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, and

40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The PCR amplifications were performed in triplicate for each biological sample, and the quantification readings of the amplified fragments were performed by the Qiagen Rotor-Gene apparatus at each amplification cycle, and then the melting curve was analyzed by the Rotor-Gene Series Software (v.2.1.0).

The graph generated by the cDNA concentration and the respective CT values (Threshold Cycle) were used to calculate the regression equation and the correlation between the variables by the coefficient of determination (R^2), and to observe the coefficient of efficiency $R^2 \geq 0.98$ and efficiency between 0.90 and 1.10 were standardized for each gene (Taylor et al., 2010) (supplementary data).

For the reactions of qPCR, the samples were processed in triplicates under the same reaction conditions used in the efficiency of primers, and the results were normalized using CTs obtained by expression of the endogenous genes, α-actin and insulin-degrading enzyme (IDE) (Borges; Tsai; Caldas, 2012). The CT was determined by the number of cycles, in which the fluorescence generated within a reaction crosses the threshold. The comparative CT method was used, and the relative quantification was obtained by the formula $2^{-\Delta\Delta CT}$ (Livak; Schmittgen, 2001; PfaffL, 2001).

Results

Phenotypic evaluation

According to the analysis of variance of the phenotypic data, it was possible to observe significant effects of lines, times after inoculation, and interaction between these two factors (Table 2). The lines showed different reactions to the white mold. There was a progress of the symptoms specially after the first evaluation, mainly because of the advance of the symptoms in the Beryl line. As was observed in the analysis of variance, in

the unfolding of the line factor within the DAI, there were no differences regarding symptoms for the Cornell 605 line, and significant differences for the Beryl, by the F test at 5% probability level. Likewise, the effect of each DAI within the lines was decomposed, and both differed at all analyzed times.

Expression of mapped candidate genes

The strong interaction between lines and DAI can be explained by the increased severity of symptoms in the Beryl, especially at 7 and 11 DAI. Of the seven genes studied as the relative expression (Fig. 1), only the *Pv*MYB did not have its expression detected in any line at any time of evaluation, indicating this gene is not related to the studied pathosystem.

The *Pv*PGIP4 gene, at 0 HAI and in the Beryl, had a relative expression higher than that presented by the Cornell 605. However, after 24 HAI, an opposite behavior was observed. Initially, the expression of this gene significantly decreased in the Beryl, whereas in the Cornell 605 its expression was similar to the control and higher than that observed in the Beryl (Fig. 1a).

Regarding the expression of the *Pv*PR1 gene, there was a significant increase in the relative expression levels, after inoculation. At all times, the Beryl had higher levels of expression in absolute values. There was a difference between the lines at 24 HAI; however, between 48 and 72 HAI, the relative expression of this gene was statistically equal for both lines. The Cornell 605 also had an increase in gene expression levels over time but at levels lower than those observed in the Beryl (Fig. 1b).

When analyzing the *Pv*ERF1 gene, it was observed that, at all times for the two lines, there was no high level of relative expression, with all evaluations below 2. They differed only at 72 HAI, with a higher level for the Beryl (Fig. 1c).

At 0 HAI, the level of expression of the *Pv*PKF gene was higher in the Beryl than in the Cornell 605. However, in the first evaluation after inoculation, the Cornell 605 showed a significant increase in the transcription of this gene in comparison to the Beryl. The level of expression in the Beryl remained practically stable during the evaluation times (Fig. 1d). At 48 and 72 HAI, the Cornell 605 had levels similar to those observed in the Beryl (Fig. 1d).

The *Pv*POD gene had the highest levels of relative expression for both lines at 0 HAI, although it has had an expressive gradient in the Cornell 605 (50.84) compared to the Beryl (5.83). At 24 HAI, the Beryl had an expression similar to that found at 0 HAI, although it was 6.63 times higher than the one observed in Cornell 605, in which there was practically no expression. At 48 and 72 HAI, the two lines were similar for the expression of this gene (Fig. 1e).

Regarding the *Pv*FBox gene, the Beryl had higher expression levels than the Cornell 605 at 24 and 72 HAI. For the other genes, these levels remained similar. This gene had a higher expression level than the control treatment only at 72 HAI (Fig. 1f).

Discussion

The results of the analysis of variance showed that early evaluations of the genotypes can lead to the identification of those presumably resistant. Viteri et al. (2015) evaluated lines and progenies of bean at different times after inoculation and verified the underestimation of the susceptibility of some genotypes in earlier evaluations (7 and 14 DAI). This result also corroborates the hypothesis that the resistance factors in the Cornell 605 are related to the delayed progression of symptoms, which is a phenomenon characteristic of horizontal resistance.

The protein family of MYB transcription factors is extensive and has diversified functions, which associates these transcription factors with several mechanisms of the stress response by biotic and abiotic factors in plants (Cominelli et al., 2005; Dubos et al., 2010; Jin; Martin, 1999). Among these functions, this transcription factor is one of the critical pieces in the pathway for the recognition of pathogenic infections and subsequent programming of cell death in the infected area to stop the disease progress to other regions of the plant (Raffaele et al. 2008). In addition, a higher expression of this gene has already been found in soybean and *Brassica napus* genotypes infected with *S. sclerotiorum* (Dubos et al., 2010; Zhao et al., 2007, 2009). However, in our study, none of the genotypes responded to inoculation with *S. sclerotiorum*, suggesting that the allele present in the bean lines does not recognize the pathogen or it is even absent.

The PGIPs family consists of extracellular proteins with LRR-leucine-rich repeats (Jones; Jones, 1997), with the function of recognizing the polygalacturonase (PGs) and preventing their enzymatic action during the invasion and release of nutrients necessary for the pathogen growth (D ’Ovidio et al., 2004).

In *P. vulgaris* there are four genes coding for the domain of PGIP genes; however, D ’Ovidio et al. (2004) showed by transcript analysis that these four genes have different responses to elicitors, salicylic acid or even mechanical damage. Among these genes, PvPGIP4 has already been observed as a moderately induced gene in the initial phases of mechanical damage in bean plant (Oliveira et al. 2010). This fact may explain the higher level of expression at 0 HAI, observed in our study, when the stem was cut, especially in the Beryl line; however, at 24 HAI, the expression was higher in the Cornell 605, and this fact should be related to the higher resistance of this line. The expression of this gene reduced over time in both lines.

Vasconcellos et al. (2016) quantified the expression of the four genes of the family *Pv*PGIP (*Pv*PGIP1, *Pv*PGIP2, *Pv*PGIP3, and *Pv*PGIP4). However, using lines of different

resistant levels, they found that when uninoculated, the susceptible line also had a higher level of expression of the PvPGIP4 gene. In our study, at 24 HAI, the Cornell 605 line had higher expression levels. However, after 48 HAI, this line had a decrease in gene expression, differing from the results found by Vasconcellos et al. (2016).

Among the Meta-QTL genes where the PvPGIP family is located, the PvPGIP4 gene was selected to study the expression since it is the best candidate based on the research carried out by Vasconcellos et al. (2016), in which this gene showed a better relationship between the level of transcription in a resistant line when it is inoculated with an aggressive isolate, such as the isolate 27 used in the present study.

The PR1 proteins are encoded by plants under induction of biotic and abiotic stresses; however, they are more studied in pathosystems (Dansana et al. 2014; Kothari et al. 2016; Gamir et al. 2017). Although they are locally induced by pathogen infection, these proteins do not only accumulate locally in the infected tissue, but they are also induced systematically, associated with the development of acquired systemic resistance (ASR) against new infection by fungi, bacteria, and virus (Van loon & Van strien 1999).

There was an increase in *Pv*PR1 gene expression after inoculation of *S. sclerotiorum* isolate. It is also possible to observe that there was a low expression at 0 HAI, evidencing the elicitation of gene transcription in response to infection with the pathogen. These results agree with those obtained by Oliveira et al. (2015a, 2015b), although the similar increase of expression in both lines is probably not related to the explanation of the difference in their reaction to the pathogen. The high expression of the PR1 gene is associated with the concentration of H_2O_2 in the cell. In Arabidopsis, this gene is inhibited by the ATAF2 gene, which is highly expressed as a response to mechanical injury (Delessert et al., 2005; Guo; Stotz, 2007). Thus, this may explain why at 0 HAI there was a lower expression of the gene, increasing with the disease progress.

The plants recognize the presence of pathogens from *pattern recognition receptors* (PRR). Kinase family Proteins, usually containing leucine-rich repeats (LRR) or lysine (Lysm) in the extracellular domain, work as one of these receptors, transmitting the signal through the membranes, which is involved with signal recognition (Beck et al., 2012; Dalio et al., 2014). For some pathosystems, the activities of some kinases in the recognition and response of plants to pathogens are already elucidated. In rice, the expression and activity of the MAPK5 kinase protein were activated by pathogen attack (Xiong & Yang 2003). In the same culture, the Xa21 gene (receptor kinase-like) by transgene conferred resistance to *Xanthomonas oryzae pv. oryzae* (Song et al., 1995). In beans, there are no studies reporting the relationship of some kinase protein associated with the pathogenesis of *S. sclerotiorum*. In other pathosystems involving beans, some responses have already been found, especially in response to *Fusarium* spp. infection, with mapping, isolation, expression analysis (Lange et al. 1999), and identification of analogs resistance genes (López et al. 2003).

In the present study, the significant increase of the *Pv*PKF gene, at 24 HAI in the Cornell 605 resistant line, may be related to the better performance of this genotype in the rapid triggering of signals in the infection of the pathogen from the effectors. Thus, the effectors can trigger the transcription of Kinase genes.

The ERF1 (*Ethylene response factor* 1) gene encodes a transcription factor that acts by increasing the production of ethylene by the cell, causing senescence and, consequently, cell death, preventing the progression of the disease to other tissues. The importance of ethylene in the resistance to white mold has already been identified in several species such as lettuce, *Brassica napus*, and Arabidopsis (Fatouros et al., 2017; Perchepied et al., 2010; Zhao et al., 2009); In this study, there was no significant difference in the expression of this gene up to 48 HAI. However, at 72 HAI, the line Cornell 605 had a reduction in the expression of this gene, and this may have occurred due

to the rapid response of this genotype to control the progress of the white mold, no longer being necessary the cell death strategy of the plant to control the pathogen.

Peroxidases, such as lignin and suberin, which that reinforce the cell wall, are enzymes involved in several metabolic processes, metabolism of auxins and formation of compounds that act as barriers against pathogens (Almagro et al. 2009). Although Oliveira et al. (2015b) have observed a higher expression of the *Pv*POD gene at 12 HAI in beans, Leite et al. (2014) verified that this enzyme had a higher production only at 120 HAI. In our study, the fact that no higher expression of the *Pv*POD gene was detected in the evaluated treatments can be explained by the maximum time of evaluation performed at 72 HAI. Another important point is that there is no information in the literature on the expression of this gene in non-inoculated plants, as was done in the present study. A significant gene expression was observed in the resistant line Cornell 605 at 0 HAI, indicating that this gene should partially explain the resistance in a constitutive way, or even expresses itself very early in the infection process.

The F-box family proteins play a crucial role in the plant development and growth as well as in its response to biotic and abiotic stresses (Jia et al., 2013). Regarding biotic stresses, this protein induces the production of jasmonic acid, representing an important factor in the recognition and combat of the white mold invasion (Guo & Stotz 2007). The ubiquitination of proteins is another important process related to the F-box proteins; it is little elucidated in pathogenesis processes, but it may reveal the involvement of these proteins in the interaction and degradation of proteins produced by the pathogen (Niu et al., 2016; Wang; Deng, 2011). Oliveira et al. (2015a) also detected F-Box expression in beans inoculated with *S. sclerotiorum*; however, a higher relative expression was detected at 12 HAI, with a significant reduction at 72 HAI, differently from the results found in the present study. However, the use of different isolates and line may be related to different responses to the expression of this gene. Another critical factor to be observed is that the

susceptible line had higher levels of expression, which may be related to the non-specificity of its expression with the resistance response to the pathogen under the evaluated conditions. It is also possible that even though with less expression, the line has a more efficient signaling pathway in the process of signaling of other resistance factors, with the production of jasmonic acid or ubiquitination of other proteins. This latter approach is found in a study carried out by Oliveira et al. (2015a), in which the evaluated bean line is susceptible to *S. sclerotiorum*.

The PR1 proteins are associated with the acquired resistance system (ARS), and they are elicited by hormones such as salicylic acid, ethylene, and jasmonic acid (Ferreira et al. 2007; Leon-Reyes et al. 2009). Oliveira et al. (2015a) found that the treatment of bean plants inoculated with jasmonic acid promoted a reduction in the F-Box gene expression since this gene is an essential elicitor in the production chain of jasmonic acid. The exogenous treatment led to feedback in the regulation process. In Arabidopsis, in the absence of ethylene, the NPR1 monomers, activated by salicylic acid, can bind a positive regulator of sensitive genes expression to the jasmonic acid in the cytosol, stopping this acid from entering the nucleus, resulting in the suppression of the expression of genes responsive to the jasmonic acid (Leon-Reyes et al., 2009). That is, alternatively, the NPR1 can activate a negative regulator in the jasmonic acid pathway. Therefore, in the present study, in which the plants were not treated with jasmonate, it may have occurred no expression of the F-Box gene, and thus, a positive correlation was verified, although it is not significant between the PvF-Box and PvERF1 genes (Fig. 2).

The lack of high levels of genetic resistance to white mold in beans has caused a limited progress in breeding for this characteristic (Schwartz & Singh 2013). Numerous studies have identified only a partial resistance in bean germplasm, and higher levels of resistance are found only in related species, such as *Phaseolus coccineus* L. (Abawi et al., 1978), which makes difficult the introgression into adapted genotypes of plant bean (Singh

et al., 2014). Inheritability studies have demonstrated that partial resistance is quantitative, making more difficult the reproduction of resistance (Miklas et al., 2004). In addition, most studies fail to show a strong correlation between greenhouse and field works, suggesting that additional factors found in field conditions are absent in greenhouse trials (Terán; Singh, 2009a). As an example, in sources of resistance, as it was reported for the line "Ex Rico 23", also known as ICA Bunsi, Tu; Beversdorf (1982) have shown moderate levels of disease resistance in the field over the years (Kolkman; Kelly, 2003b), but they cannot show the same levels of resistance in greenhouse trials (Singh et al., 2014). In addition to the seasonal variation in rainfall, humidity, and temperature in the field, the role played by plant size is also an important factor in the field, which cannot be evaluated in greenhouse studies (Coyne et al. 1974; Ando et al. 2007; Miklas et al. 2013).

Due to differences in the genetic background of the mapping populations and the lines used in this study, and because of the fact that the evaluation was performed only in the greenhouse, it is plausible to admit that the QTLs identified by Vasconcellos et al. (2017) cannot be identified in the studied populations and, consequently, they may not necessarily present a higher candidate gene expression in the resistant line Cornell 605, since the mapping of QTLs is generally very dependent on the environment and the population in which the study was carried out. However, considering the complexity of bean resistance to white mold, the identified promising alleles should be included in the selection process. Thus they will probably also contribute to the resistance in the field.

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Table 1. Genes selected for expression analysis.

Meta-QTL	Chromosome	Identification	Gene
WM1.1	Pv 01	Phvul.001G236600	*Pv*PKF - protein kinase family protein
WM1.1	Pv 01	Phvul.001G240400	*Pv*F-Box - RNI-like superfamily protein
WM2.2	Pv 02	Phvul.002G055800	*Pv*ERF1 - ethylene responsive element binding factor 1
WM2.2	Pv 02	Phavul.002G201700	*Pv*PGIP4 - polygalacturonase-inhibiting proteins 4
*	Pv 03	Phavul.003G109100	*Pv*PR1 - pathogenesis-related protein 1
WM3.1	Pv 03	Phvul.003G164600	*Pv*POD - Peroxidase
WM5.5	Pv 05	Phvul.005G115500	*Pv*MYB - MYB transcription factor

*Gene not located within Meta-QTLs.

Table 2. Analysis of variance for the evaluation of the white mold severity in bean lines at different times.

Sources of Variation	**DF**	**MS**	**Fc**
Lines	1	80.29	149.95 **
DAI	2	17.29	32.30 **
Lines vs DAI	2	7.44	13.89 **
Lines			
DAI in the line Cornell 605	1	0.02	0.035
DAI in the line Beryl	1	3.61	6.74 *
DAI			
Lines in 3 DAI	1	4.34	8.11 **
Lines in 7 DAI	1	38.34	71.50 **
Lines in 11 DAI	1	52.49	98.02 **
Error	24	0.54	
CV (%)	14.16		

DAI: Days after inoculation, DF: Degrees of freedom, MS: Mean square.

*: Significant at 5% probability, **: Significant at 1% probability.

Fig. 1. Relative expression of selected genes in the Beryl and Cornell 605 lines at 0, 2, 48 and 72 hours after inoculation (HAI) with the isolate 27 of *Sclerotinia sclerotiorum*.

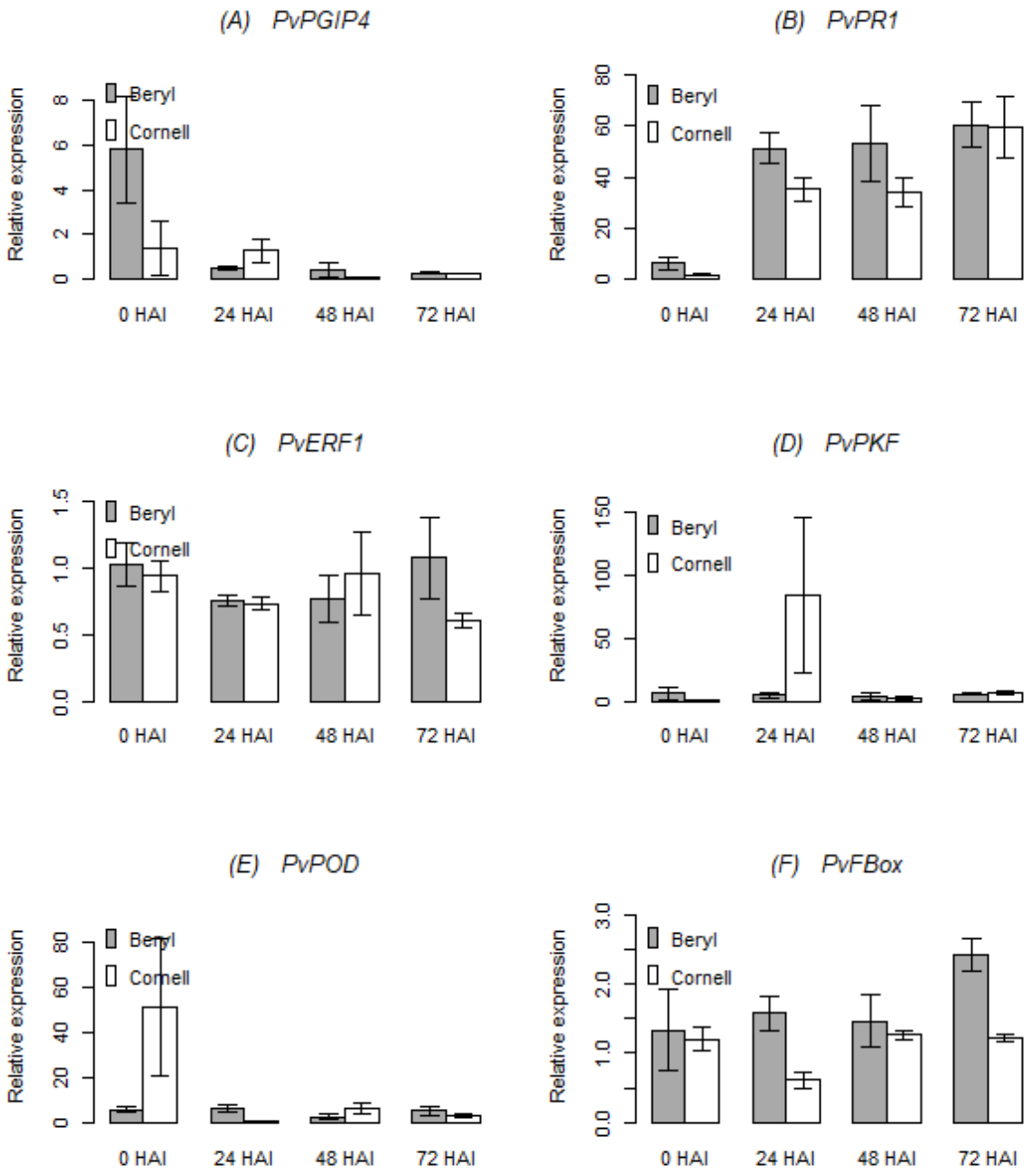
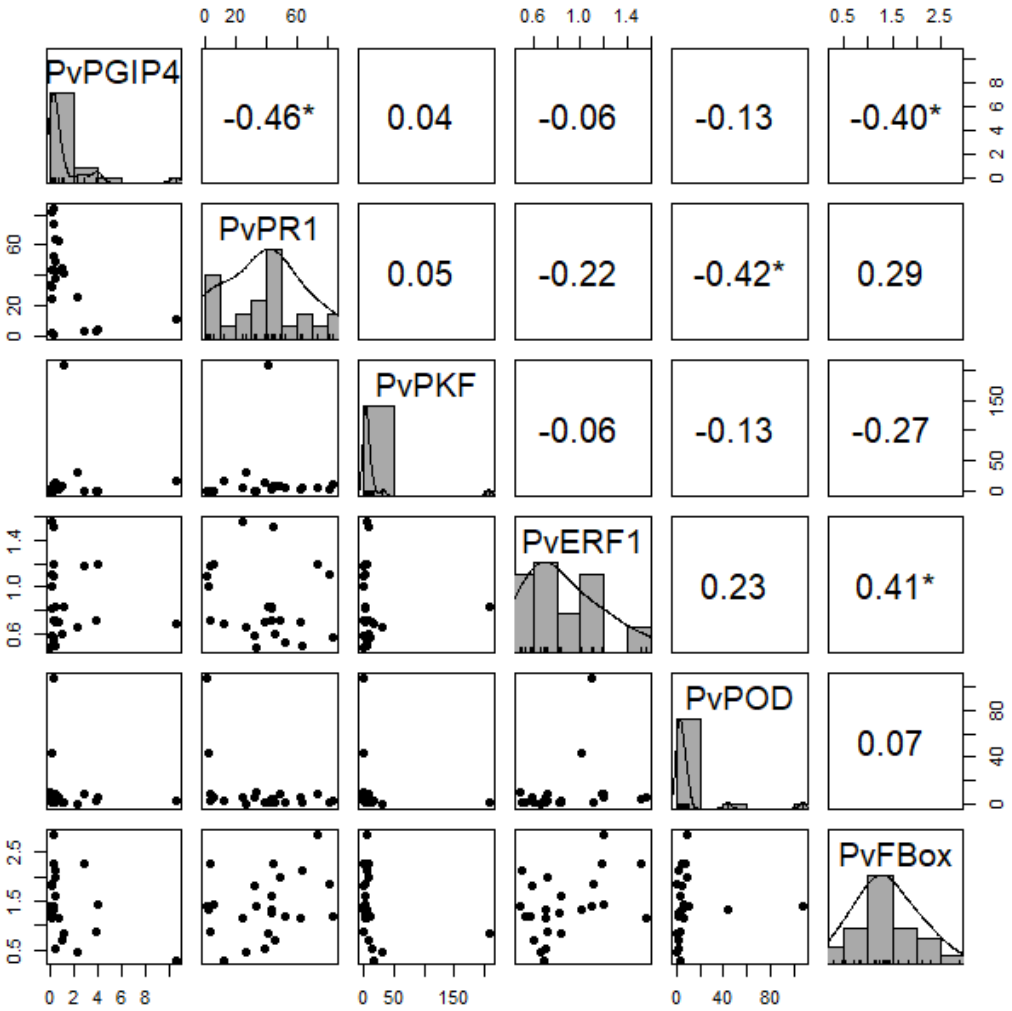


Fig. 2. Correlations between global data of the genes relative expressions. * significant Pearson correlation at 5% probability.



Supplementary data: Genes selected for expression analysis and reference

Meta-QTL	Cromossome	Identififcation	Gene	Sequence 5' - 3' (Forward/Reverse)	Amplification efficiency
WM1.1	Pv 01	Phvul.001G236600	*Pv*PKF - protein kinase family protein	CCAAGGGGTGTGCTCTAAAA/ AGCCCCACATACTGAACCTG	1.03
WM1.1	Pv 01	Phvul.001G240400	*Pv*F-Box - RNI-like superfamily protein	ACGACGGAGAATGGCTACAC/ GCCAGAAGTTCAAGGTCCTG	1.04
WM2.2	Pv 02	Phvul.002G055800	*Pv*ERF1 - ethylene responsive element binding factor 1	CCCTCTGTTCCCAATCTTCA/ AGAGGGGGAACTGGTTTTGT	1.04
WM2.2	Pv 02	Phavul.002G201700	*Pv*PGIP4 - polygalacturonase-inhibiting proteins 4	TCCTTCCCGAAGCATTTCAC/ GCCAGCGTCGTCGGAATAT	1.02
*	Pv 03	Phavul.003G109100	*Pv*PR1 - pathogenesis-related protein 1	GGTGTTGCCTTGCCAGAAAC/ TCATCTTCATTGGGTGGAGCA	0.98
WM3.1	Pv 03	Phvul.003G164600	*Pv*POD - Peroxidase	TCCTTTTCAGCACTTTCACT/ AGAAAGCAGTGTTCTTGTGG	1.09
WM5.5	Pv 05	Phvul.005G115500	*Pv*MYB - MYB transcription fator	GGACTTGAAAACCAGACCA/ GTTCCAGCAAGTTCCGAGAG	1.04
___	Pv 08	Phvul.008G011000	*Pv*Act11 - Actin-11	TGCATACGTTGGTGATGAGG/ AGCCTTGGGGTTAAGAGGAG	0.97
___	Pv 01	Phvul.001G1332000	*Pv*IDE - insulin-degrading enzyme	GCAACCAACCTTTCATCAGC/ AGAAATGCCTCAACCCTTTG	1.03

*Gene not located within Meta-QTLs.

5 ARTIGO 2 - The importance of choosing an adequate phenotyping method for subsequent evaluations of bean plant reactions to white mold by non-linear model adjustments of symptom progression

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Abstract White mold, caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is currently one of the major diseases affecting bean cultivars, especially in irrigated crops, being extremely difficult to control. Genetic improvement in the form of more resistant lines has been sought ought for inclusion in the integrated management of this disease. When selecting resistant genotypes, an efficient, fast and simple evaluation method regarding plant reactions to the pathogen is required. In this context, the aim of the present study was to compare the straw and seedling phenotyping methods in the evaluation of disease progression by nonlinear models and evaluate the interaction of the phenotyping method with genotype resistance and evaluation timeframes. The results indicate that both phenotyping methods are well correlated regarding bean resistance groups. The methods carry out distinct inferences on genotype resistance, and disease progress is closely linked to the resistance level of the evaluated genotype. Both phenotyping methods are efficient, but in different situations. The symptom progression models display similar adjustments to the symptom levels of both methods.

Key words: *Phaseolus vulgaris* L. *Sclerotinia sclerotiotum.* Growth models. Quantification. Resistance.

Introduction

White mold is caused by the *Sclerotinia sclerotiorum* (Lib.) de Bary fungus, one of the most cosmopolitan and devastating plant pathogens in the world, capable of colonizing over 400 plant species (Bolton et al. 2006). One host species for this fungus is beans (*Phaseolus vulgaris* L.), where production becomes limited or impossible in environmental conditions adequate for the growth of this pathogen. In Brazil, white mold is noteworthy in bean crops irrigated during the winter, since temperature and humidity conditions in winter are ideal for *S. Sclerotiorum* development.

There is currently no effective control method to prevent *S. sclerotiorum* epidemics. Thus, the integrated use of several strategies to contain white mold progress in bean crops should rely on a combination of different techniques, such as fungicide use, low plant population density, decreased use of irrigation and fertilizers and the production of cultivars with a more upright architecture and more open canopies (Heffer Link and Johnson 2007). However, these measures, while useful in controlling the disease, can cause decreased productivity. In this scenario, the use of fungus-resistant lines could contribute to decreased production costs (Schwartz and Singh 2013), and is the most effective method for bean cultivation (Antonio 2011).

Considering the adoption of resistant lines as the most efficient control method, it is important to chose an efficient, fast and simple evaluation method regarding plant reactions to the pathogen when carrying out genotype selection, i.e. the symptom quantification method should be the most accurate and precise, so that reliable symptom quantification is obtained (Madden et al. 2007).

Subsequently, symptom severity at different timeframes may be evaluated, thus obtaining a disease progression curve. The comparison of disease progress curves in cultivars with different resistance levels has been studied in comparative epidemiology through model adjustments and comparison of areas below the curve (Campbell and Madden 1990; Silva et al. 1998; Spósito et al. 2004). To model plant disease progression curves, certain nonlinear models are commonly used, such as the Logistic, Exponential, Monomolecular, the Gompertz and the Richards models (Campbell and Madden 1990; Madden et al. 2007; Bergamin Filho 2011). Cultivar resistance can, thus, be compared through different models by evaluating disease progression rates, provided that plants are submitted to similar climate conditions and affected by the same pathogenic population (Spósito et al. 2004; Cunniffe et al. 2015).

In this context, this study aims to (i) assess the best phenotyping method for evaluating bean reactions to *S. sclerotiorum* as a function of the adjustment of symptom growth to nonlinear models; (ii) model the temporal progress of white mold after artificial inoculation in greenhouse conditions; (iii) verify nonlinear model fits based on the resistance level of each genotype group and (iv) infer the best evaluation timeframe after *S. sclerotiorum* inoculation for each method.

Methodology

Preparation of the *S. sclerotiorum* inoculum

Sclerotia of the aggressive isolate 27 (Silva et al. 2014) were obtained from the Plant Resistance Laboratory mycological collection at the Department of Biology of the Federal University of Lavras - UFLA. Sclerotia were superficially disinfested by consecutive immersion in a 70% ethanol solution for 1 minute, followed by immersion in a 20% bleach solution for 3 minutes and three immersions in demineralized and sterilized water. The sclerotia were then placed in Petri dishes containing BDA (potato-dextrose-agar) medium and incubated in a BOD incubator at 23 °C for three days under a 12-hour photoperiod. The colony was then harvested once in BDA medium for inoculum multiplication and incubated again at 23 °C under a 12-hour photoperiod. After the third day of incubation, plates with homogeneous mycelial growth were used for inoculation.

Genotypes

Twelve bean genotypes were used for inoculation (Table 1), chosen based on their heterogeneity regarding white mold reaction.

Evaluation methods

Two methods were applied to evaluate bean genotype reactions to *S. sclerotiorum*,

the straw test and seedling test. In the straw test, two experiments were carried out in a complete randomized block design with three replicates, each plot consisting of a 7 L pot containing four plants. Inoculation was carried out by a cut made about 2 cm above the last node of the main stem, and insertion of a micropipette tip with a BDA disc containing *S. sclerotiorum* mycelium 30 days after plant emergence. Symptom quantifications were performed at 4, 7, 11, 14, 21 and 28 days after inoculation (DAI), using the diagrammatic scale proposed by Singh; Schwartz and Steadman (2014) (Table 2).

In the seedling test, two experiments were carried out in a randomized complete block design with three replicates, each plot consisting of a 1 L pot containing four seedlings. At 12 days after emergence (stage V2-V3), a cut at the apical stem portion was made, about 1 to 2 cm above the primary leaves, and a micropipette tip with a BDA disc was inserted. Symptom quantification was performed at 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 days after inoculation (DAIs) using the descriptive key proposed by Arkwazee and Myers (2017) (Table 2).

All experiments were conducted in a greenhouse in the Department of Biology at the Federal University of Lavras - UFLA, located in the municipality of Lavras, in the state of Minas Gerais, Brazil. Control conditions for temperature (25 ± 2 ° C) and humidity (60 ± 5%) were applied in September and October 2017.

Data analyses

For each evaluation method, the data were submitted to analysis of variance and later grouping test of each genotypes by their means, applying the Scott-Knott test (Scott and Knott 1974), using the following fixed model:

$$y_{yjgk} = \mu + b(r)_{jk} + g_i + t_g + gt_{ig} + e_{ijgk}$$

Where Y_{ijgk} is the genotype observation i, at timeframe g, in block j of experiment k; $b(r)_{jk}$ is the effect of the observation of block j within experiment k; g_i is the effect of genotype i; t_g is the effect of time point g; gt_{ig} is the effect of the interaction between genotype i and time g and e_{ijgk} is the random effect of the experimental error associated to observation $Y_{ijgk.}$

After grouping the genotype means regarding disease severity, the means of each group, for each evaluation timeframe, were calculated for each phenotyping method, and the suitability of each group to the following non-linear models was assessed:

Logistic model: $y_L = b_1/(1 + b_2) \exp(-r_L t)$

Gompertz model: $y_G = b_1(\exp((-b_2) \exp(-r_G t)))$

Monomolecular model: $y_M = b_1(1 - b_2) \exp(-r_M t)$

Von Bertalanffy model: $y_V = b_1((1 - b_2) \exp(-r_V t))^3$

The models were adjusted by the ordinary least squares method using the iterative Gauss-Newton algorithm. For all models, b_1 is the estimated maximum asymptotic component of the model, b_2 is the initial disease severity estimated by the model, r is the disease progress rate estimated by the model and t corresponds to the evaluation timeframe. An assumption that the errors are independent and homocedastic was accepted ($\varepsilon \sim N(0, I\delta^2)$).

Five adjustment evaluators were calculated for the defined models, namely the coefficient of determination (R^2), mean square of the residue (MSR), mean absolute deviation of the residuals (MAD), and the Akaike (AIC) and Bayesian (BIC) information criterions. The higher the coefficient of determination, the better the model fit, while lower values represent better adjustments for the other evaluators. Their equations are:

$$R^2 = 1 - \frac{MSR}{TMS}$$

Where MSR is the square sum of the residue and TMS is the total sum of squares.

$$MSR = \sum_{i=1}^{n} \frac{(y_i - \hat{y}_i)}{n - p}$$

where n represents the number of observations; p the number of parameters; y_i the observed value of the severity of observation i and $\hat{y}_i$ the estimated value of the total severity of the observation i for the given model.

$$MAD = \sum_{i=1}^{n} \frac{|y_i - \hat{y}_i|}{n}$$

where n represents the number of observations; y_i the observed value of the severity of observation i and $\hat{y}_i$ the estimated value of the total severity of the observation i for the given model.

The Akaike (AIC) and Bayesian (BIC) information criteria were applied to compare the quality of the model fit based on the maximum of the likelihood function, which is dependent on the number of model observations and parameters. Thus,

$$AIC = 2k - 2logL(\hat{\theta})$$

$$BIC = klog(n) - 2logL(\hat{\theta})$$

where n represents the number of observations, k the number of model parameters and $logL(\hat{\theta})$ the maximum of the likelihood function.

Results

The analysis of variance indicated a significant difference mainly between genotypes and the evaluation timeframe after inoculation, while, to a lesser extent, interactions were also observed between genotypes and significant evaluation timeframes. A means clustering test was applied after the detection of significant genotype effects, and the significance of the interaction between genotypes and

evaluation timeframes was evaluated, in order to verify nonlinear model adjustments to the different genotype groups, since this interaction demonstrates that genotypes do not exhibit similar behavior at each evaluation timeframe, besides being generally non-linear (Table 3).

The analysis of variance also indicated no significant experiment effects, therefore demonstrating the robustness of the results and revealing that both phenotyping methods present similar results at the timeframes of the experimental assembly. The general means, as well as the coefficients of variation, were similar between both methods (Table 3).

Genotype clustering led to six groups (a-f) in both phenotyping methods (Table 4). This partly indicates the ability of both evaluation methods to distinguish genotypes due to their resistance to white mold. The methods showed partially agreement, with correlation close to 0.7, mainly due to the species that comprise the intermediate genotype group, which are more difficult to discriminate. However, the methods showed good agreement concerning the most contrasting genotypes, confirming their efficiency (Table 4; Fig. 1).

All evaluated nonlinear models converged for all groups comprising both phenotyping methods. The seedling test generally presented the best model parameters and fit quality evaluator values (Tables 5-14). The straw test, on the other hand, exhibited decreased asymptotic parameter ($\hat{b}_1$), initial inoculum ($\hat{b}_2$) and disease progression rate ($\hat{r}$) values in the genotype groups with higher resistance. The seedling test modeling also demonstrated decreases in the asymptotic parameter ($\hat{b}_1$), and initial inoculum ($\hat{b}_2$) values. However, an unreliable oscillation was noted for disease progression rate ($\hat{r}$) (Tables 5-14).

The Logistic model was better suited to the data generated by the seedling test. Although parameter $\hat{b}_1$ was well estimated in both evaluation methods, $\hat{b}_2$ and $\hat{r}$ were not, especially in the resistant genotype group (Table 5). Although non-significant, a greater trend towards a decrease in disease progression rate was observed in the resistant genotype groups by the straw test method. This was not observed in the seedling test method, although this parameter was estimated. The best estimation of the seedling test parameters led to better fit quality evaluator values, except for group 'a', the most susceptible genotype group (Tables 5 and 6).

The Gompertz model followed the same quality parameter trend as the Logistic model. A slight gain regarding the initial inoculum estimation for the straw test was observed, improving the quality of fit indicators for these groups (Tables 7 and 8).

Regarding the Monomolecular model, a better estimation for the seedling test was also observed (Table 14). However, and inversion as to the fit quality of the model was detected: while the model displayed a better fit for the 'a', 'b' and 'c' groups in the straw test, the best fit in the seedling test method were groups 'd', 'e' and 'f' (Tables 9 and 10).

Inferences for the Von Bertalanffy model were very similar to those for the Monomolecular model. The Von Bertalanffy model presented the lowest initial severity estimates among all models, as well as decreasing disease progression rates with increasing resistance levels in the straw test. This was not observed for the seedling test, corroborating the other evaluated models (Tables 11 and 12).

When adjusting the models for the means of all genotypes, better parameter estimations were observed for the seedling test phenotyping method. In general, the best estimation quality of seedling test parameters is observable by the smaller confidence interval of the models (Figs 2 and 3). The maximum asymptote estimation ($\hat{b}_1$) was

robustly estimated in both phenotyping methods, with lower estimates for the Logistic and the Gompertz models and higher estimates for the Monomolecular and Von Bertalanffy models, although the mean estimates of this parameter in both methods were quite similar, at 7.17 and 7.29 for the straw and seedling test, respectively (Table 13). The symptom progression rates ($\hat{r}$) estimated by the straw test, were, on average, 48% lower than those estimated by the seedling test, although with a lower estimation accuracy (Table 13).

The Logistic and the Gompertz models better modeled the seedling test data, as observed by the adjustment quality indicators, mainly by the AIC and BIC criteria and the MSR. On the other hand, the Monomolecular and Von Bertalanffy models were more suitable for the straw test method (Table 14).

Discussion

Even with very similar means and coefficients of variation, the coefficient of determination ($R^2 = 0.67$) between both methods, although significant, did not represent the expected linearity between the two methods (Fig. 1). (Arkwazee et al. 2017) reported an $R^2 = 0.55$ between both methods with evaluations at 7 and 4 DAI, respectively, and stated that this was due to the greater classification change for intermediate resistance genotypes between both methods. In the present study, a change of genotype position among all resistance levels was observed, and consequently, no similarity between genotype group components and sizes was detected (Table 4). However, if the classification listed in Table 1 is to be considered, obtained through previous studies, genotypes are exactly ranked according to their classification as resistant, moderately resistant and susceptible. In addition, the Spearman correlation coefficient of the present study (Fig. 1) corroborates the conclusions reported by Arkwazee et al. (2017), where,

although changes are observed in genotype ranking, they are subtle between both evaluated methods.

The model parameters did not exhibit the same estimation quality for two methods. The lower estimation quality of initial inoculum parameters and symptom progression rate (Tables 5-14) in the straw test method was probably due to thelower number of timeframe evaluations carried out (6) to feed the model. The t test applied to these parameters corresponds to the confidence interval (in this case, the distribution percentiles). Thus, the probability of adequately estimating these parameters decreases with a lower degree of freedom, due to an increased confidence interval (Baty et al. 2015). This can be proven by the greater confidence interval of the straw test models (Figs 2 and 3). This degree of unreliability in parameter estimation in the straw test had a direct effect on the MSR and MAD evaluators and on the AIC and BIC criteria in all models (Tables 5-14), since they all displayed the same parameterization level, with no penalty for greater parameterization in a given model (Aho et al. 2014).

The resistance of beans to white mold throughout its entire cycle is of paramount importance for the success of a strain, especially under field conditions, where the same plant can be infected several times (Viteri et al. 2015). Thus, the symptom growth rates of a given genotype are an important estimator of ‘field resistance’ (Spósito et al. 2004). In the present study, the evaluated phenotype methods provided different information on disease progression rates: the genotype groups in the straw test displayed decreasing rates as a function of increased resistance levels, as expected in horizontal resistance, while no pattern was detected in the seedling test.

The different disease progression rate behaviors observed in both methods may be related to the different defense mechanisms of adult plants and seedlings. The symptom progression rate in the 605 Cornell strain (resistant) is much lower than in the

susceptible Beryl strain evaluated by the straw test (Fig. 4), whereas, these same lines display curves with greater parallelism when applying the seedling test. Thus, the same pattern of symptom progression reduction was not detected as in the straw test.

However, when inoculation/early natural infection of resistant genotypes occurs, they may display similar behavior as the susceptible genotypes, due to the fact that pathogen resistance-related genes in the seedling stage are usually not the same genes that confer resistance in adult plants, as reported for certain patosystems, such as *Puccinia striiformis* - wheat (Zegeye et al. 2014), *Pyrenophora teres f. sp. teres* and *Cochliobolus sativus* – Barley (Steffenson et al. 1996) and *Fusarium pseudograminearum* – wheat (Martin et al. 2015). This has also been observed in a study evaluating the phenotypic resistance of soybean cultivars to *S. sclerotiorum*, where white mold severity decreased in direct proportion to plant age (Garcia et al. 2012).

Although estimated, parameter ($\hat{b}_2$) cannot be applied in this case for initial inoculum estimation, since the inoculation was performed artificially with a uniform amount of inoculum. However, this parameter may be related to greater or lesser plant susceptibility at the beginning of the pathogen infection. In the study carried out by Spósito et al. (2004), the 'Hamlin' *Citrus* spp. variety displayed the highest initial inoculum values, probably due to the fact that this variety initiates fruit maturation before other varieties. Thus, this variety displays certain defense mechanisms, such as acid and carotene compounds, due to ripening. In other words, more efficient preformed mechanisms in certain genotypes may lead to lower parameter estimates, and logically, to higher estimates in susceptible genotypes. This was observed more clearly in the straw test method (Tables 5-14), where estimates decreased as group resistance levels increased.

The parameter ($\hat{b}_1$), maximum asymptote of the model curve, was adequately estimated in both methods for all models. The observed decreasing pattern as measured by increases in resistance level clearly indicates the ability of resistant genotypes in this dystrophic system to maintain low symptom levels until later timeframe evaluations, also due to decreasing symptom progression rates ($\hat{r}$).

Taking into account aspects of both evaluated phenotyping methods and breeding program characteristics, where initial populations display greater variability, which decreases with later generations, making selection difficult and leading to small genetic gains, considerations regarding the choice of phenotyping method are required: the seedling test, as it displays disease progression parallelism between resistant and susceptible lines, can be applied in initial selection generations, with evaluations carried out between 8 and 12 days after inoculation, when discrimination between resistant and susceptible genotypes becomes more evident. This is reinforced by the fact that initial populations are numerous, so this method allows for evaluations of the same number of genotypes using less logistical resources and in less time. The straw test, as it reveals different symptom progression rates according to the resistance level of the evaluated genotypes, allows for a greater variation range between susceptible and resistant genotypes when phenotyping both early and more advanced generations. However, this method requires more logistic resources and is slower that the seedling test. For both methods, evaluations should be conducted as late as possible, when the progress curve reaches its maximum asymptote. However, assessments prior to the last evaluation already reach steady symptom progression levels (Figs 2,3 and 4).

Most studies on the temporal progression of white mold have been carried out with artificial inoculation, where, in most cases, both the pathogen and the disease progress depend on the existence of environmental variables to express their infective

potential and maximum intensity, respectively (Juliatti et al. 2015). According to Melzer and Boland (1994), the epidemiology of white mold in canola (*Lactuca canadensis*) conforms to the Monomolecular model. In soybean, adjustments of different models regarding epidemic progress have been carried out applying the Monomolecular, Exponential and Logistic models (Mila et al. 2004; Harikrishnan and Río 2008; Beruski 2013).

In the present study the Monomolecular model showed generally better adjustments for the straw test method, while the Logistic model showed better adjustments for the seedling test. However, the progress curves of monocyclic pathogen symptoms, that lead to the production of resistance structures, are attributed to Monomolecular model adjustment (Vanderplank 1968; Bergamin Filho 2011). However, to the best of our knowledge, no modeling studies on the progression of white mold symptoms in beans by artificial inoculation are available. Thus, the present study sheds new light on the relationship between the inoculation and quantification methods and the diagnosis that nonlinear symptom progression models can carry out regarding inoculation methods and the interaction between the applied method and genotype resistance.

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Table 1. Genotypes used for inoculation and evaluation in the present study.

Genotype	**Reaction**	**Reference**
56/x	Resistant	Lopes (2017)
53/3	Resistant	Lopes (2017)
11/185	Resistant	Lopes (2017)
Cornell 605	Resistant	Griffiths et al. (2012)
59/6	M. Resistant	Lopes (2017)
64/9	M. Resistant	Lopes (2017)
61/12	M. Resistant	Lopes (2017)
64/8	M. Resistant	Lopes (2017)
Beryl	Susceptible	Lehner et al. (2015)
CNFC 9506	Susceptible	Lehner et al. (2015)
Corujinha	Susceptible	Leite et al. (2016)
M20	Susceptible	Antonio (2011)

M. Resistant: Moderately resistant

Table 2. Descriptive key applied to evaluated symptom severity by the straw and seedling test phenotyping methods.

Score	Description	
	Seedling test Arkwazee and Myers (2017)	Straw test Singh, Schwartz and Steadman (2014)
1	No symptoms beyond the inoculation site.	No symptoms beyond the inoculation site.
2	Injury to an intermediate point between the cut stem and the primary leaf node.	Infected internode, but the fungus invasion does not reach the first inoculated node.
3	The lesion reaches the primary leaf node.	Infected internode and fungus invasion in the first inoculated node.
4	The lesion passes through the primary leaf node (in the first quarter of the distance between the primary leaf node and the cotyledon node).	The fungus invasion moves after the first node, but symptoms stop at ≤ 50% of the length of the second internode.
5	The lesion reaches the intermediate point between the primary leaf node and the cotyledonary node.	The fungus invasion moves at ≥ 50% of the length of the second internode, but does not reach the second node.
6	The lesion passes through the intermediate point between the primary leaf node and the cotyledonary node	The fungus invasion reaches the second node.
7	The lesion reaches the cotyledonary node.	The fungus invasion moves after the second node, but the symptoms do not reach ≤ 50% of the length of the third internode.
8	The lesion passes through the cotyledonic nodule, the first half between the cotyledonic nodule and the soil surface.	The fungus invasion moves to ≥ 50% of the length, but the symptoms do not reach the third node.
9	The seedling completely collapses and dies.	Fungus invasion is observed in the third internode and the symptoms reach or pass the third node, leading to eventual plant death.

Table 3. Summary of the analysis of variance for the two methods applied in the evaluation of bean genotype reactions to inoculation with *S. sclerotiorum* in greenhouse conditions.

Variance source	Straw test		Seedling test	
	GL	QM	GL	QM
Block (Experiment)	4	1.29	4	5.92**
Experiment	1	0.1	1	0.71
Days after inoculation (DAI)	5	159.96**	9	152.82**
Genotype (G)	11	63.07**	11	71.94**
G x DAI	55	2.82**	99	1.72**
Residue	355	0.93	595	1.21
General means	5.63		5.41	
CV (%)	17.11		20.33	

** significant at 1% probability by the F test.

Table 4. Aggregation of bean genotypes by the Scott-Knott test for the two methods applied in the evaluation of bean genotype reactions to inoculation with *S. sclerotiorum* in greenhouse conditions.

Straw test			Seedling test		
Group	Genotype	Means	Group	Genotype	Means
a	Beryl	7.6	a	Beryl	7.03
b	CNFC	6.97	a	Corujinha	6.92
b	M20	6.76	b	M20	6.42
b	Corujinha	6.63	c	CNFC	5.98
b	61/12	6.53	c	64/8	5.86
c	53/3	6.18	d	61/12	5.43
d	64/8	5.56	d	53/3	5.25
e	56/x	4.67	e	64/9	4.93
e	11/185	4.56	e	56/x	4.79
e	64/9	4.44	e	59/6	4.51
e	59/6	4.13	e	Cornell 605	4.4
f	Cornell 605	3.51	f	11/185	3.34

Means followed by the same letter do not differ at a 5% probability level by the Scott-Knott test.

Table 5. Maximum asymptote ($\hat{b}_1$), initial inoculum ($\hat{b}_2$) and disease progress rate ($\hat{r}$) estimated by the Logistic model for white mold severity data for each genotype group in each evaluation method.

Group	Straw test			Seedling test		
	$\hat{b}_1$	$\hat{b}_2$	r_L	$\hat{b}_1$	$\hat{b}_2$	r_L
a	9.000**	8.231**	0.442**	9.269**	12.974*	0.572**
b	8.538**	3.177*	0.233*	8.632**	5.742**	0.418**
c	7.627**	4.209*	0.294*	7.669**	6.966**	0.484**
d	9.61**	3.808*	0.126	7.272**	4.866**	0.382**
e	5.483**	1.514	0.169	6.066**	5.120**	0.432**
f	4.653**	1.030*	0.093	4.012**	3.047*	0.424**

* significant at 5% by the t test; ** significant at 1% by t-test

Table 6. Estimates of fit quality assessors of the Logistic model for each genotype group in each evaluation method.

Group	Straw test					Seedling test				
	R^2	MRS	MAD	AIC	BIC	R^2	MRS	MAD	AIC	BIC
a	0.999	0.002	0.025	-15.841	-16.682	0.9664	0.221	0.305	17.71	18.91
b	0.979	0.123	0.222	8.324	7.491	0.985	0.06	0.148	4.694	5.904
c	0.981	0.093	0.18	6.649	5.816	0.99	0.032	0.133	-1.373	-0.162
d	0.952	0.41	0.335	15.529	14.696	0.985	0.037	0.146	-0.016	1.193
e	0.918	0.121	0.234	8.197	7.364	0.988	0.02	0.099	-6.212	-5.002
f	0.938	0.04	0.127	1.581	0.747	0.962	0.018	0.076	-6.884	-5.674

R^2 = Coefficient of determination; MRS = Mean square of the residue; MAD = mean absolute deviation; AIC = Akaike Information Criterion; BIC = Bayesian information criterion.

Table 7. Maximum asymptote ($\hat{b}_1$), initial inoculum ($\hat{b}_2$) and disease progress rate ($\hat{r}$) estimated by the Gompertz model for white mold severity data for each genotype group in each evaluation method.

Grupo	Straw test			Seedling test		
	$\hat{b}_1$	$\hat{b}_2$	r_L	$\hat{b}_1$	$\hat{b}_2$	r_L
a	9.046**	3.626**	0.351**	9.561**	4.163*	0.400**
b	8.618**	0.272**	0.187**	8.951**	2.552**	0.302**
c	7.692**	2.201*	0.235**	7.888**	2.923**	0.354**
d	10.328*	1.817**	0.083	7.580**	2.263**	0.274**
e	5.524**	1.049*	0.143	6.222**	2.437**	0.325**
f	4.763**	0.755**	0.073	4.053**	1.844**	0.353**

* significant at 5% by the t test; ** significant at 1% by t-test

Table 8. Estimates of fit quality assessors of the Gompertz model for each genotype group in each evaluation method.

Grupo	Straw test					Seedling test				
	R^2	MRS	MAD	AIC	BIC	R^2	MRS	MAD	AIC	BIC
a	0.999	0.003	0.037	-12.755	-13.588	0.957	0.285	0.37	20.268	21.479
b	0.986	0.082	0.183	5.884	5.051	0.985	0.058	0.152	4.414	5.624
c	0.988	0.06	0.148	4.063	3.23	0.985	0.047	0.158	2.411	3.622
d	0.958	0.363	0.324	14.802	13.969	0.986	0.035	0.146	-0.586	0.623
e	0.927	0.107	0.22	7.501	6.668	0.987	0.022	0.105	-5.022	-3.812
f	0.939	0.039	0.128	1.55	0.717	0.965	0.017	0.074	-7.775	-6.565

R^2 = Coefficient of determination; MRS = Mean square of the residue; MAD = mean absolute deviation; AIC = Akaike Information Criterion; BIC = Bayesian information criterion.

Table 9. Maximum asymptote ($\hat{b}_1$), initial inoculum ($\hat{b}_2$) and disease progress rate ($\hat{r}$) estimated by the Monomolecular model for white mold severity data for each genotype group in each evaluation method.

Group	Straw test			Seedling test		
	$\hat{b}_1$	$\hat{b}_2$	r_L	$\hat{b}_1$	$\hat{b}_2$	r_L
a	9.096**	1.780**	0.274**	10.218**	1.503**	0.238*
b	8.753**	1.036**	0.143**	9.634**	1.182**	0.187**
c	7.789**	1.208**	0.179**	8.319**	1.310**	0.229**
d	12.21	0.921**	0.041	8.213**	1.100**	0.168**
e	5.586**	0.735*	0.117	6.514**	1.211**	0.220**
f	4.929*	0.561**	0.054	4.113**	1.135**	0.282**

* significant at 5% by the t test; ** significant at 1% by t-test

Table 10. Estimates of fit quality assessors of the Monomolecular model for each genotype group in each evaluation method.

Group	Straw test					Seedling test				
	R^2	MRS	MAD	AIC	BIC	R^2	MRS	MAD	AIC	BIC
a	0.997	0.019	0.089	-2.906	-3.739	0.944	0.372	0.442	22.935	24.146
b	0.992	0.045	0.136	2.281	1.448	0.984	0.063	0.167	5.303	6.513
c	0.993	0.034	0.111	0.75	-0.082	0.979	0.069	0.196	6.108	7.319
d	0.964	0.307	0.302	13.799	12.966	0.986	0.035	0.145	-0.643	0.566
e	0.937	0.094	0.203	6.681	5.848	0.985	0.026	0.114	-3.322	-2.112
f	0.939	0.039	0.128	1.504	0.671	0.968	0.015	0.073	-8.785	-7.575

R^2 = Coefficient of determination; MRS = Mean square of the residue; MAD = mean absolute deviation; AIC = Akaike Information Criterion; BIC = Bayesian information criterion.

Table 11. Maximum asymptote ($\hat{b}_1$), initial inoculum ($\hat{b}_2$) and disease progress rate ($\hat{r}$) estimated by the Von Bertalanffy model for white mold severity data for each genotype group in each evaluation method.

Group	Straw test			Seedling test		
	$\hat{b}_1$	$\hat{b}_2$	r_L	$\hat{b}_1$	$\hat{b}_2$	r_L
a	9.062**	0.941**	0.324**	9.719**	0.973*	0.344**
b	8.655**	0.494**	0.172**	9.118**	0.654**	0.264**
c	7.720**	0.597**	0.216**	7.998**	0.739**	0.312**
d	10.736*	0.479**	0.069	7.737**	0.590**	0.238**
e	5.542**	0.310*	0.135	6.298**	0.640**	0.290**
f	4.810**	0.227**	0.067	4.070**	0.522**	0.329**

* significant at 5% by the t test; ** significant at 1% by t-test

Table 10. Estimates of fit quality assessors of the Von Bertalanffy model for each genotype group in each evaluation method.

Group	Straw test					Seedling test				
	R^2	MRS	MAD	AIC	BIC	R^2	MRS	MAD	AIC	BIC
a	0.999	0.007	0.054	-8.771	-9.604	0.953	0.311	0.394	21.159	22.37
b	0.988	0.069	0.168	4.841	4.008	0.985	0.059	0.155	4.569	5.779
c	0.989	0.051	0.136	3.03	2.197	0.983	0.054	0.17	3.668	4.879
d	0.960	0.346	0.318	14.504	13.671	0.986	0.035	0.146	-0.674	0.535
e	0.930	0.103	0.214	7.242	6.409	0.986	0.023	0.108	-4.51	-3.299
f	0.939	0.039	0.128	1.537	0.704	0.966	0.016	0.074	-8.1	-6.889

R^2 = Coefficient of determination; MRS = Mean square of the residue; MAD = mean absolute deviation; AIC = Akaike Information Criterion; BIC = Bayesian information criterion.

Table 13. Maximum asymptote ($\hat{b}_1$), initial inoculum ($\hat{b}_2$) and disease progress rate ($\hat{r}$) estimated by the Logistic, Gompertz, Monomolecular and Von Bertalanffy models for white mold severity data for each genotype group in each evaluation method.

Group	Straw test			Seedling test		
	$\hat{b}_1$	$\hat{b}_2$	r_L	$\hat{b}_1$	$\hat{b}_2$	r_L
Logistic	7.093**	2.648*	0.218*	7.033**	6.631**	0.474**
Gompertz	7.156**	1.575*	0.178*	7.229**	2.852**	0.349**
Monomolecular	7.261**	0.963**	0.138*	7.611**	1.301**	0.228**
Von Bertalanffy	7.186**	0.444*	0.164*	7.327**	0.726**	0.308**
Means	7.174	1.407	0.174	7.291	2.877	0.339

* significant at 5% by the t test; ** significant at 1% by t-test

Table 14. Estimates of fit quality assessors of the Logistic, Gompertz, Monomolecular and Von Bertalanffy model for each genotype group in each evaluation method.

Group	Straw test					Seedling test				
	R^2	MRS	MAD	AIC	BIC	R^2	MRS	MAD	AIC	BIC
Logistic	0.967	0.119	0.216	8.133	7.3	0.991	0.024	0.111	-4.476	-3.265
Gompertz	0.975	0.09	0.19	6.423	5.59	0.988	0.033	0.133	-1.282	-0.071
Monomolecular	0.983	0.061	0.157	4.11	3.277	0.983	0.046	0.153	2.165	3.375
Von Bertalanffy	0.978	0.08	0.179	5.728	4.895	0.986	0.037	0.14	-0.141	1.068
Means	0.976	0.088	0.186	6.099	5.266	0.987	0.035	0.134	-0.933	0.276

R^2 = Coefficient of determination; MRS = Mean square of the residue; MAD = mean absolute deviation; AIC = Akaike Information Criterion; BIC = Bayesian information criterion.

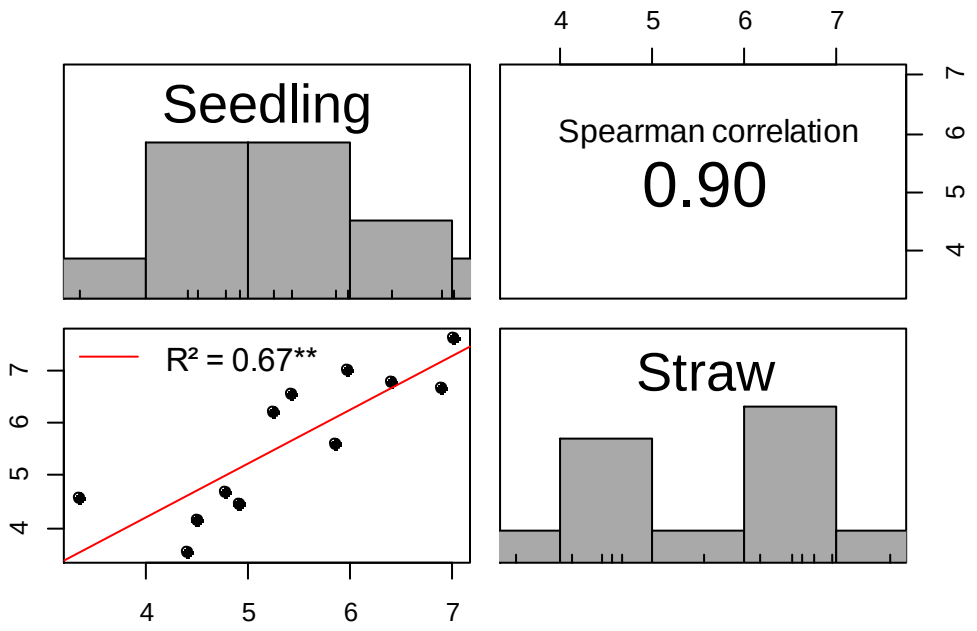

Fig. 1. Correlation between the genotype means of both phenotyping methods. (**p = 1%).

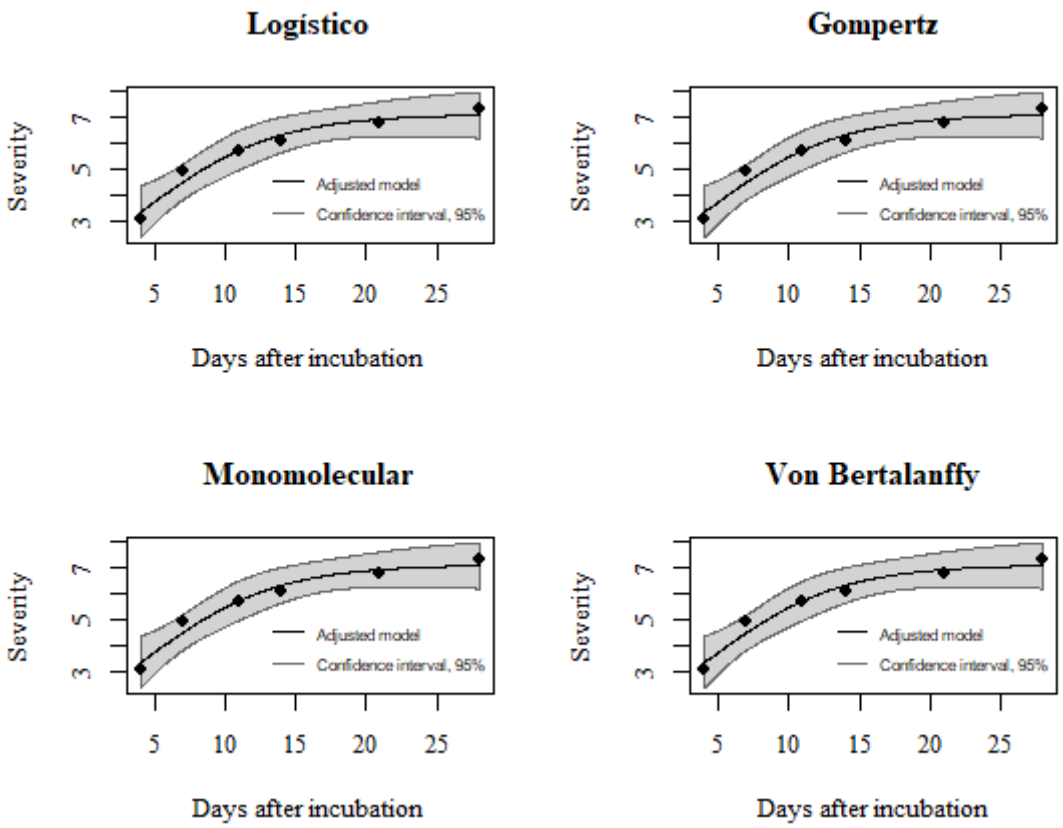

Fig. 2. Adjustment of the Logistic, Gompertz, Monomolecular and Von Bertalanffy models for white mold severity data in the straw test method.

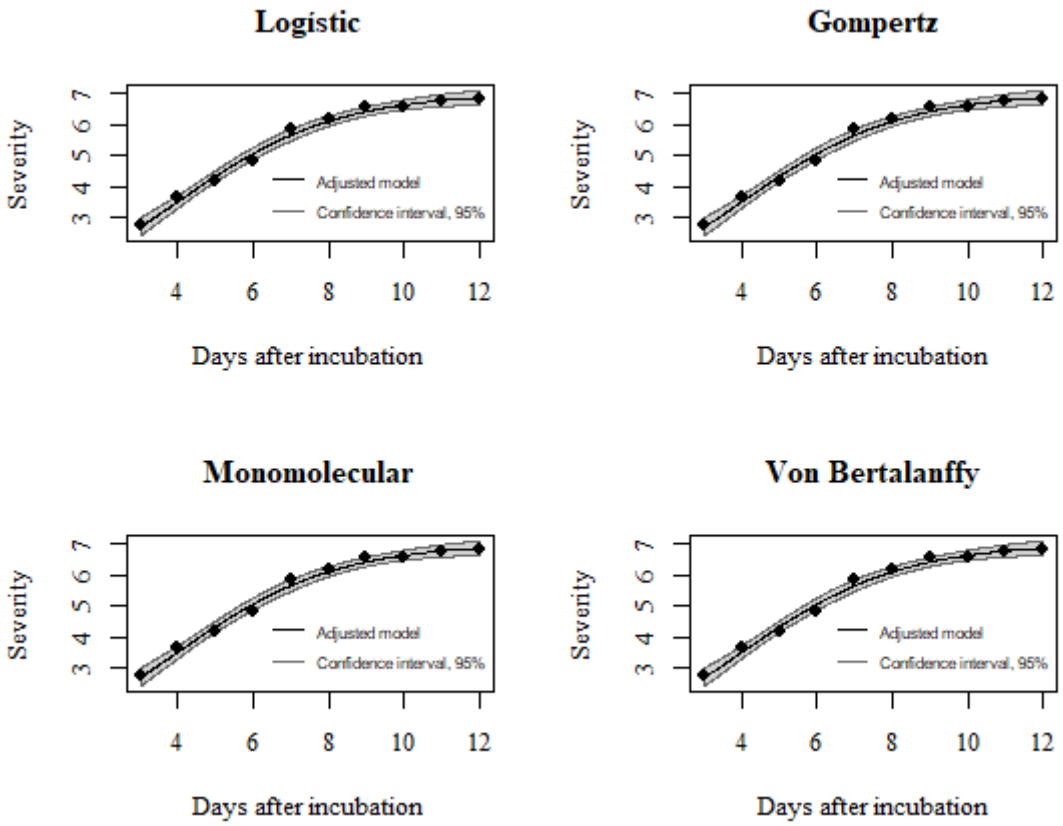

Fig. 3. Adjustment of the Logistic, Gompertz, Monomolecular and Von Bertalanffy models for white mold severity data in the seedling test method.

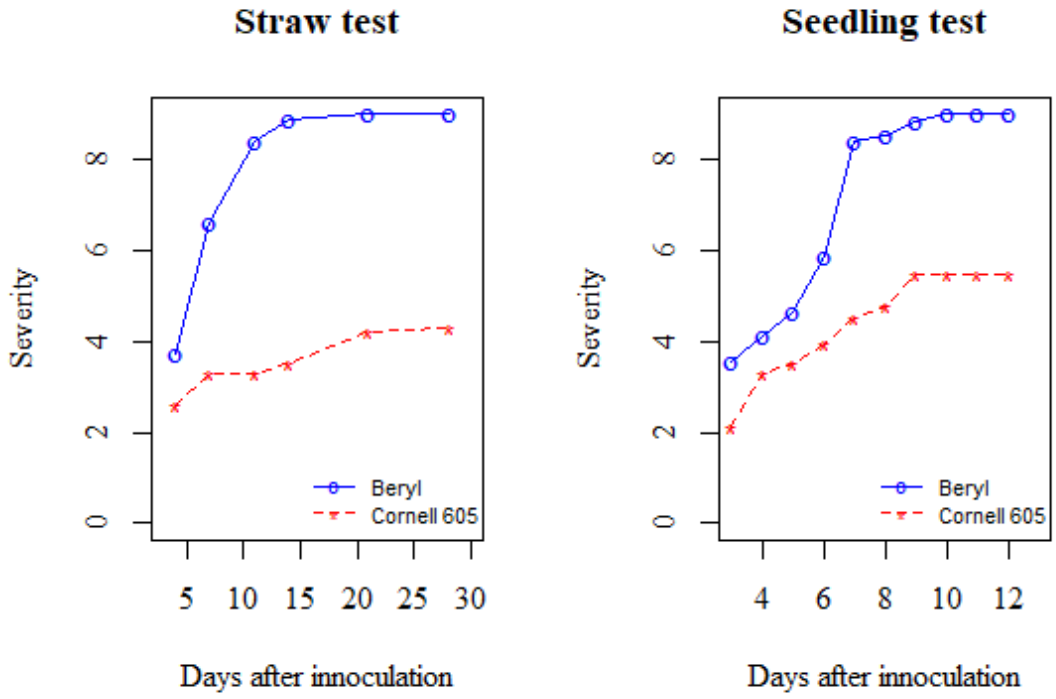

Fig. 4. Disease progression curves for contrasting genotypes regarding *S. sclerotiorum* severity by both phenotyping methods.