UNIVERSIDADE FEDERAL DO PARANÁ

ANA CLAUDIA KLOSOWSKI

SENSIBILIDADE DE ISOLADOS DE *Phakopsora pachyrhizi* AOS FUNGICIDAS TEBUCONAZOL (INIBIDOR DA DESMETILAÇÃO) E AZOXISTROBINA (INIBIDOR DA QUINONA EXTERNA)

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Orientadora: Prof. Dr. Louise Larissa May De Mio

Co-orientador: Dr. Gerd Stammler

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PARECER

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Agronomia - Produção Vegetal, reuniram-se para realizar a argüição da Tese de DOUTORADO, apresentada pela candidata **ANA CLAUDIA KLOSOWSKI**, sob o título **"SENSIBILIDADE DE ISOLADOS DE *Phakopsora pachyrhizi* AOS FUNGICIDAS TEBUCONAZOL (INIBIDOR DA DESMETILAÇÃO) E AZOXISTROBINA (INIBIDOR DA QUINONA EXTERNA)"**, para obtenção do grau de Doutor em Ciências do Programa de Pós-Graduação em Agronomia - Produção Vegetal do Setor de Ciências Agrárias da Universidade Federal do Paraná.

Após haver analisado o referido trabalho e argüido a candidata são de parecer pela **"APROVAÇÃO"** da Tese.

Curitiba, 09 de Dezembro de 2015.

Professor Dr. Cícero Deschamps
Coordenador do Programa

Dra. Claudia Vieira Godoy
Primeira Examinadora

Professora Dra. Natalia Peres
Segunda Examinadora

Professora Dra. Chirlei Glienke
Terceira Examinadora

Professor Dr. Henrique da Silva Silveira Duarte
Quarto Examinador

Professor Dr. Louise Larissa May De Mio
Presidente da Banca e Orientadora

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Adelmo e Beatriz, que me apoiaram de todas
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“A excelência pode ser obtida se você se importa mais do que os outros julgam ser necessário, se arrisca mais do que os outros julgam ser seguro, sonha mais do que os outros julgam ser prático e espera mais do que os outros julgam ser possível."

Vince Lombardi

RESUMO

A ferrugem-asiática, causada pelo fungo *Phakopsora pachyrhizi*, é uma doença presente nas principais regiões produtoras de soja no mundo. Sua importância está associada ao potencial de dano que ela pode causar devido à desfolha precoce que interfere diretamente na formação e enchimento dos grãos. Dentre as medidas de controle recomendadas, a aplicação de fungicidas é indispensável para manter a doença abaixo dos níveis de dano. Dentre os fungicidas registrados para o controle da ferrugem no Brasil, mais de 99% contém moléculas dos grupos dos inibidores da desmetilação (IDMs) e dos inibidores da quinona externa (IQe), de forma isolada, combinadas com moléculas de outros grupos e combinadas entre si. A perda de eficiência do controle da doença no campo foi relatada para ambos os grupos e está associada à perda de sensibilidade do patógeno aos fungicidas. O estudo da sensibilidade de populações aos fungicidas juntamente com a inferência da adaptabilidade de isolados menos sensíveis permitirá entender a dinâmica da resistência no campo e, por conseguinte, recomendar um manejo anti-resistência adequado para preservar e até retomar a eficiência dos produtos. Os objetivos deste estudo foram i) comparar a sensibilidade aos fungicidas tebuconazol (IDM) e azoxistrobina (IQe) e o monociclo de populações e isolados de *P. pachyrhizi* provenientes de campos de produção de soja orgânica e convencional; ii) monitorar mutações no gene CYP51 e no gene CYTB em isolados de *P. pachyrhizi*; iii) comparar a habilidade competitiva entre isolados sensíveis (sem mutação) e isolados com mutações nos genes CYP51 e CYTB. Os isolados oriundos do sistema convencional se mostraram menos sensíveis ao tebuconazol do que os isolados do sistema orgânico. Além disso, com base nos parâmetros monocíclicos, isolados do sistema convencional se mostraram menos adaptados e essa característica pode estar relacionada com a menor sensibilidade ao fungicida. Isolados do sistema orgânico e convencional do estado do Paraná e isolados do estado do Mato Grosso apresentaram mutações nos genes CYP51 e CYTB, relacionados à menor sensibilidade aos IDMs e aos IQes, respectivamente. Dentre as mutações no gene CYP51, prevaleceu a combinação F120L+Y131H. A frequência de isolados apresentando a mutação F129L no CYTB aumentou consideravelmente, de nove para 47%, de uma safra para outra. Além disso, 58% dos isolados testados apresentaram mutações em ambos os genes. Os isolados com mutações no gene CYP51 apresentaram desvantagens competitivas em relação ao isolado sensível quando cultivados juntos em folhas de soja sem fungicida e tratadas com um fungicida multi-sítio, e sua frequência diminuiu após quatro ciclos do fungo. A mutação F120L+Y131H foi associada à menor desvantagem competitiva em relação às outras mutações, o que explica sua prevalência dentre os isolados. O isolado com a mutação F129L no gene CYTB competiu igualmente bem com o isolado sensível, nas mesmas condições. As mutações nos dois genes foram estáveis em isolados monourediniais durante quatro ciclos do fungo. A menor adaptabilidade de isolados com mutações no gene CYP51 indica que, na ausência do fungicida, a frequência de isolados mutados pode diminuir e isso será determinante para as estratégias de manejo da doença.

Palavras-chave: Ferrugem-asiática. Tebuconazol. Azoxistrobina. Resistência. Mutações. Adaptabilidade.

ABSTRACT

Asian soybean rust, caused by *Phakopsora pachyrhizi*, is a widespread foliar disease and has potential for great damage due to premature defoliation interfering directly in the grain formation and filling. Among the recommended measures for control, the use of fungicides is indispensable to keep the disease below damaging levels. Among the fungicides registered for the rust control in Brazil, more than 99% contain compounds in the groups of demethylation inhibitors (DMIs) and quinone-outside inhibitors (QoI) alone or combined with products in the other or the same group. The loss of efficacy for disease control in the field has been reported for fungicides of both groups and is associated with lower sensitivity of the *P. pachyrhizi* to fungicides. Study of population sensitivity to fungicides and the knowledge of fitness penalties of less sensitive strains is important to understand the dynamics of resistance in the field and therefore recommend a suitable resistance management program to preserve and to retain the efficacy of the products. The objectives of this study were i) to compare the sensitivity to tebuconazole (DMI) and azoxystrobin (QoI) and the monocycle of populations and isolates of *P. pachyrhizi* from organic and conventional soybean production systems; ii) to monitor the mutations in the CYP51 and CYTB genes of isolates of *P. pachyrhizi*; iii) to compare the competitive ability between sensitive isolates (without mutations) and isolates with mutations in the CYP51 and CYTB genes. Isolates from the conventional system were less sensitive to tebuconazole than isolates from the organic system. Furthermore, based on the monocyclic parameters, isolates from the conventional system were less fit, and this feature can be related to lower sensitivity to fungicide. Isolates from organic and conventional systems from the State of Paraná and isolates from the State of Mato Grosso showed mutations in CYP51 and CYTB genes, associated to lower sensitivity to DMIs and QoIs, respectively. Among the mutations in the CYP51 gene, haplotype F120L+Y131H predominated. The frequency of isolates with the F129L mutation in CYTB increased considerably, from 9 to 47%, from the 2013-2014 to the 2014-2015 season. Moreover, 58% of tested isolates had mutations in both genes (CYP51 and CYTB). The isolates containing mutations in the CYP51 gene were at a competitive disadvantage in relation to the sensitive isolates when co-cultivated on detached soybean leaves without fungicide and leaves treated with a multi-site fungicide. Their frequency decreased after four cycles of the disease. The F120L+Y131H mutation was associated with lower competitive disadvantage in relation to other mutations. Isolates with F129L mutation in the gene CYTB competed equally well with sensitive isolates under the same conditions. The mutations in the two genes in monouredinial isolates were stable during four cycles of the disease. The fitness penalties associated with mutations in the CYP51 gene indicates that the frequency of mutated isolates tends to decrease in the absence of the fungicide and this would affect the management strategies of the disease.

Key-words: Soybean rust. Tebuconazole. Azoxystrobin. Resistance. Mutations. Fitness.

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1 INTRODUÇÃO GERAL

A soja está entre os quatro grãos mais produzidos e consumidos no mundo e foi o grão mais plantado no Brasil no ano de 2013 (FAO, 2015). Sua importância está relacionada às diversas finalidades dos seus produtos e subprodutos, que incluem a alimentação humana e animal e a produção de biodiesel (HIRAKURI; LAZZAROTTO, 2014). O Brasil é o segundo maior produtor mundial de soja com uma área de 32.093,1 mil hectares e produção de 96.243,3 mil toneladas, tendo como principais produtores nacionais os estados do Mato Grosso e do Paraná (CONAB, 2015). As condições climáticas das regiões produtoras no Brasil, muitas vezes, são propícias ao desenvolvimento de doenças, que podem limitar a produtividade da cultura, como é o caso da ferrugem-asiática que é favorecida por chuvas bem distribuídas e longos períodos de molhamento (DEL PONTE *et al*., 2006).

A ferrugem-asiática, causada pelo patógeno *Phakopsora pachyrhizi* Syd & P. Syd., é a principal doença foliar da soja no Brasil e pode causar danos de até 80% na produção quando medidas de controle não são aplicadas (BROMFIELD, 1984; HARTMAN *et al*., 1991; YANG *et al*., 1991). Em estágios avançados da doença ocorre a desfolha precoce, impedindo a plena formação de grãos e causando perda no rendimento e na qualidade do produto (REIS *et al*., 2006).

O controle da ferrugem-asiática está baseado no uso de fungicidas, no Vazio Sanitário, medida adotada pelos estados brasileiros que proíbe a presença de plantas de soja vivas no período de entressafra (60 a 90 dias) visando reduzir o inóculo inicial, fuga da época mais propícia ao desenvolvimento da doença, incluindo o plantio antecipado e o uso de cultivares precoces, e a utilização de cultivares com resistência à ferrugem, que devem ser usadas associadas ao controle químico para evitar suplantação da resistência (GODOY, 2012). O uso de fungicidas ainda se constitui na principal medida utilizada pelos produtores, que fazem aplicações calendarizadas, independentemente das condições de clima, diante do potencial de dano que a doença pode causar (GODOY *et al*., 2009). O controle químico está baseado principalmente no uso de fungicidas do grupo dos inibidores da desmetilação (IDMs), representado pelos triazóis e triazolintione, e dos inibidores da quinona externa (IQes), também chamados de estrobilurinas, de forma isolada ou em mistura. Mais de 99% dos fungicidas registrados no Ministério da Agricultura, Pecuária e Abastecimento (MAPA) para o controle da ferrugem-asiática da soja no Brasil incluem moléculas de um destes dois grupos ou de ambos (MAPA, 2015).

O uso intensivo e inadequado de fungicidas, como uso repetido de moléculas com o mesmo mecanismo de ação, utilização de doses diferentes das recomendadas na bula, sejam elas sub ou sobredoses, e o uso do produto de forma curativa, quando a doença já está estabelecida, pode contribuir com a seleção de isolados fúngicos menos sensíveis, e, por consequência, diminuir a eficiência dos fungicidas no controle da doença (BRENT; HOLLOMON, 2007a). A comparação entre populações provenientes de áreas com manejo intensivo de fungicidas e áreas onde não ocorre aplicação dos mesmos poderia ser usada para confirmar tal hipótese.

A perda de eficiência dos fungicidas do grupo dos IDMs no controle da ferrugem-asiática vem sendo observada desde a safra de 2007-2008 (GODOY, 2012) e o mesmo problema foi relatado para os fungicidas do grupo dos IQes e para os produtos compostos da mistura de fungicidas de ambos os grupos na safra 2013-2014 (GODOY *et al*., 2014; GODOY *et al*., 2015). Esta menor eficiência está associada à menor sensibilidade de *P. pachyrhizi* aos fungicidas, que já foi relatada em relação ao grupo dos IDMs em trabalho conduzido em folhas destacadas utilizando isolados provenientes de diferentes regiões do Brasil da safra 2009-2010 (SCHMITZ *et al*., 2014). As menores sensibilidades foram associadas às mutações no gene da enzima 14-α- desmetilase dependente do citocromo P450 (CYP51) que codifica a proteína alvo dos IDMs, envolvendo mutações em cinco pontos distintos, que ocorreram preferencialmente combinadas duas a duas (SCHMITZ *et al*., 2014). Assume-se que os efeitos de mutações combinadas no gene CYP51 podem ser aditivos ou possivelmente sinérgicos (SANGLARD *et al*., 1998).

No mesmo trabalho conduzido por Schmitz *et al*. (2014) não foi constatada a perda de sensibilidade dos isolados aos IQes, tampouco a presença de mutações no gene do citocromo *b* (CYTB), que codifica a proteína alvo deste grupo de fungicidas. No entanto, o monitoramento de populações de safras mais recentes deve ser feito para verificar uma possível mudança no perfil de resistência, que pode ser a causa da menor eficiência dos IQes no campo.

As mutações que causam resistência a fungicidas podem reduzir a eficiência de importantes processos fisiológicos e bioquímicos no agente patogênico, o que conduz à diminuição da sua adaptabilidade em relação aos isolados sensíveis (ZHAN; MCDONALD, 2013). Isolados resistentes com menor adaptabilidade tendem a ter desvantagens competitivas quando comparados aos isolados sensíveis, na ausência do fungicida, e assim, a redução no número de aplicações e a alternância com fungicidas de resistência não cruzada, podem favorecer a redução da frequência de isolados resistentes na população (BRENT;

HOLLOMON, 2007a). O estudo comparativo da adaptabilidade de isolados sensíveis e resistentes durante vários ciclos da doença pode determinar o manejo mais adequado para preservar ou retomar a eficiência dos fungicidas ao longo do tempo. Isso significa que se isolados resistentes apresentarem desvantagem competitiva em relação aos isolados sensíveis ou se a mutação não for estável, na ausência do fungicida, populações predominantemente resistentes podem se tornar predominantemente sensíveis a uma molécula específica se o produto não for aplicado durante um certo período de tempo.

Com base no exposto acima, este trabalho foi organizado em três capítulos que apresentam os seguintes objetivos: Capítulo I - comparar a sensibilidade aos fungicidas tebuconazol (IDM) e azoxistrobina (IQe) de populações e isolados monouredíniais de *P. pachyrhizi* provenientes de campos de produção de soja orgânica e convencional e comparar o monociclo entre isolados dos dois sistemas sob condições de temperatura e umidade ideais para o desenvolvimento da doença; Capítulo II - monitorar mutações no gene CYP51 e no gene CYTB em isolados de *P. pachyrhizi* e desenvolver um ensaio de pirosequenciamento que forneça uma detecção rápida e quantitativa da mutação F129L; Capítulo III - comparar a habilidade competitiva entre isolados sensíveis (sem mutação) e isolados com mutações nos genes CYP51 e CYTB e determinar a estabilidade de genótipos mutados de isolados de *P. pachyrhizi* durante quatro ciclos da doença.

2 REVISÃO DE LITERATURA

1.1 IMPORTÂNCIA ECONÔMICA DA SOJA

A soja (*Glycine max* (L.) Merr) foi o quarto grão mais produzido e consumido globalmente, atrás apenas do milho, trigo e arroz, segundo dados do ano de 2013 da Organização das Nações Unidas para a Alimentação e a Agricultura (FAO, 2015). A importância da cultura da soja está relacionada a vários fatores, dentre eles, o alto teor de proteína (aproximadamente 40%) utilizada para o consumo humano e animal, o teor considerável de óleo (aproximadamente 20%) usado para o consumo humano e a produção de biodiesel, por ser uma *commodity* padronizada e uniforme e poder ser produzida e negociada por produtores de diferentes países e pelo seu cultivo ser altamente mecanizado e automatizado (HIRAKURI; LAZZAROTTO, 2014).

O Brasil é o segundo maior produtor de soja no mundo, apenas atrás dos EUA, com uma área plantada de 32.093,1 mil hectares e uma produção de 96.243,3 mil toneladas, na safra de 2014-2015 (CONAB, 2015). A soja ocupa a maior área plantada com grãos no país e o principal produtor nacional é o estado do Mato Grosso (área de 8.934,5 mil hectares e produção de 28.018,6 mil toneladas) seguido pelo estado do Paraná (área de 5.224,8 mil hectares e produção de 17.210,5 mil toneladas) (CONAB, 2015) (Figura 1).

Embora a área plantada com a soja e a sua produtividade tenham expandido nos últimos anos, as adversidades climáticas aliadas a problemas causados por pragas e doenças, como a ferrugem-asiática, ainda causam danos na produção (WRATHER; KOENNING, 2009; WESTCOTT; JEWISON, 2013).

1.2 FERRUGEM-ASIÁTICA DA SOJA

A ferrugem-asiática da soja é causada pelo fungo *Phakopsora pachyrhizi* Syd. & P. Syd., que foi relatado pela primeira vez no Japão em 1902, onde o patógeno foi considerado endêmico (KITANI; INOUE, 1960). A doença causa severos danos na região de clima

tropical e subtropical na Ásia, que equivale ao sul da China, onde a soja é pouco plantada (TAN *et al*., 1996).

A ferrugem-asiática foi encontrada na América do Sul na safra de 2000-2001 no Paraguai, em seguida foi relatada no Brasil após o término da safra, ainda no ano de 2001, e na Argentina no ano de 2002, se mostrando altamente agressiva e se tornando uma das principais doenças foliares da soja (YORINORI *et al*., 2005).

A ferrugem está presente em todos os estados brasileiros produtores de soja, exceto em Roraima, de acordo com informações de monitoramento da doença do Consórcio Antiferrugem. Além disso, a doença ocorre em todos os países produtores de soja no mundo, em regiões com condições climáticas diversas, incluindo climas tropical, subtropical e temperado, em diferentes continentes (LI *et al*., 2010). Os maiores danos na produção ocasionados pela doença são observados na América do Sul, onde a soja é a cultura que ocupa a maior área plantada e a ausência de um inverno rigoroso permite que o inóculo sobreviva na entressafra, em plantas vivas cultivadas ou plantas espontâneas, favorecendo a ocorrência da doença na safra da soja (LI *et al*., 2010).

O nível de dano causado pela ferrugem depende do momento em que ela ocorre no ciclo da soja, das condições climáticas, do nível de resistência ou tolerância e ciclo da cultivar utilizada. Foram relatados danos de até 80% da produção em situações em que medidas de controle não foram aplicadas (BROMFIELD, 1984; HARTMAN *et al*., 1991; YANG *et al*., 1991). No Brasil, danos de até 75% da produção foram relatados na safra de 2003-2004, na região central do país, onde as condições climáticas são altamente favoráveis para o desenvolvimento da doença (YORINORI *et al*., 2005)

Os danos causados pela ferrugem da soja, no período de 2001-2002 à 2007-2008, foram estimadas em 31,56 milhões de toneladas e o custo total, incluindo danos na produção, custo com controle e redução da arrecadação de impostos sobre grãos perdidos, atingiu U$ 13,42 bilhões (YORINORI *et al*., 2009).

1.2.1 Sintomas

Os primeiros sintomas da ferrugem-asiática são caracterizados por minúsculas pontuações de coloração esverdeada a cinza-esverdeada, mais fácil de serem observadas contra a luz, progredindo para lesões angulares de cor marrom-avermelhada ou marrom

escura (Figura 2), com uma ou mais urédias globosas principalmente na face abaxial das folhas (HARTMAN *et al*., 1999). A urédias rompem e liberam urediniósporos de cor hialina através de um pequeno poro (Figura 3), que podem ser disseminados pelo vento e causar novas infecções (ALMEIDA *et al*., 2005). Em geral, as primeiras lesões são encontradas nas folhas baixeiras, do terço inferior da planta, próximo ou após o florescimento, progredindo para as folhas do terço médio e superior (REIS *et al*., 2006), embora os sintomas possam aparecer em qualquer estágio do desenvolvimento da soja e em diferentes partes da planta, como cotilédones, folhas e haste (ALMEIDA *et al*., 2005). Em estágio avançado da doença observa-se amarelecimento geral das folhas com intensa desfolha, comprometendo a formação e o enchimento de grãos (REIS *et al*., 2006).

1.2.2 Epidemiologia e ciclo da doença

O agente causal *P. pachyrhizi* é um patógeno biotrófico, também denominado de parasita obrigatório, que só se desenvolve em tecido vivo do hospedeiro. Mais de 90 espécies de leguminosas tem sido relatadas como hospedeiras de *P. pachyrhizi*, mas apenas aproximadamente 31 espécies são hospedeiras naturais e 60 espécies são hospedeiras artificiais, com infecções produzidas por inoculações artificiais (RYTTER *et al*., 1984; ONO *et al*., 1992; SLAMINKO *et al*., 2008).

Após sobreviver na entressafra, principalmente em plantas de soja espontâneas originadas dos grãos perdidos na colheita, a dispersão do inóculo ocorre pelo vento e por meio de pessoas que podem transportar urediniósporos nas roupas (ISARD *et al*., 2005; HARTMAN; HAUDENSHIELD, 2009).

Para que ocorra a infecção no tecido do hospedeiro, o fungo precisa de molhamento, água livre ou orvalho, mínimo de 6 horas e severas epidemias da ferrugem-asiática estão diretamente relacionadas com a alta frequência de chuvas ao longo do ciclo da cultura da soja (DEL PONTE *et al*., 2006). Além disso, a temperatura propícia para a infecção pode variar de 8 a 30 °C, com uma faixa mais favorável entre 15 e 25 °C. Temperaturas constantes maiores ou iguais a 35 °C impedem a colonização de *P. pachyrhizi*, mesmo se o processo de infecção for iniciado em temperaturas ideais (ALVES, 2007). Sob condições adequadas, os urediniósporos podem germinar e penetrar seis horas após a deposição, diretamente na

epiderme da folha, ao contrário da maioria das outras ferrugens, que penetram através de aberturas estomatais (Figura 3) (MAGNANI *et al*., 2007).

A colonização das hifas do patógeno ocorre nos espaços intercelulares do tecido infectado e a formação das urédias se dá por uma agregação de hifas, formando o primórdio uredial (MAGNANI *et al*., 2007). Em trabalho desenvolvido no Brasil, o período de incubação da doença foi de 6 dias e o período de latência, de 6 a 12 dias, quando ocorre a produção de urediniósporos (ZAMBENEDETTI *et al*., 2007).

Embora teliósporos do fungo tenham sido observados na Ásia em vários hospedeiros, incluindo a soja, sua germinação nunca foi relatada no campo (BROMFIELD, 1984). Sua formação é rara em regiões tropicais, onde não há temperatura favorável, que fica em torno de 10 ºC (BROMFIELD, 1984).

1.2.3 Controle

Medidas de controle da ferrugem-asiática estão baseadas no controle químico e no manejo de fontes de inóculo, representado pelo vazio sanitário, medida adotada pelas Secretarias de Agricultura de vários estados brasileiros, que determina a erradicação de plantas vivas de soja no período de 60 a 90 dias, para evitar a sobrevivência do fungo *P. pachyrhizi* no hospedeiro durante a entressafra (SEIXAS; GODOY, 2007). Além disso, tem-se recomendado medidas que visam à fuga da época mais favorável ao desenvolvimento da doença, como o uso de cultivares de ciclo precoce e o plantio antecipado, no início do período recomendado (YORINORI *et al*., 2004; REIS *et al*., 2006). Apesar de existirem cultivares com resistência à ferrugem, o seu uso é recomendado associado com o controle químico, devido à alta variabilidade genética do patógeno, que já foi relatada em vários estudos (SINCLAIR; HARTMAN, 1999; YAMAOKA *et al*., 2002; BONDE *et al*., 2006; FREIRE *et al*., 2008, TSCHURTSCHENTHALER *et al*., 2012) e pode suplantar essa resistência.

Embora a utilização de fungicidas não se constitua na única medida de controle para a ferrugem da soja, esta ainda tem sido a prática mais eficiente, auxiliando, desta forma, na manutenção da produtividade da cultura. Diante do risco de danos que a ferrugem representa, o produtor tem realizado aplicações de fungicidas para o controle da doença de forma calendarizada, independentemente das condições de clima, iniciando no estádio de florescimento da soja e repetindo em intervalos de 14 a 21 dias (GODOY *et al*., 2009). Os

ingredientes ativos dos fungicidas registrados para o controle da ferrugem da soja no Brasil, até a presente data, são, em sua maioria, pertencentes aos grupos dos inibidores da desmetilação (IDMs), representado pelo triazóis, e dos inibidores da quinona externa (IQe), também chamados de estrobilurinas, formulados de forma isolada ou em mistura (MAPA, 2015). A perda da eficiência dos fungicidas destes grupos no controle da ferrugem-asiática já foi relatada em experimentos de campo conduzidos pela EMBRAPA SOJA em parceria com outras instituições de pesquisa e universidades e, está sendo associada à menor sensibilidade de *P. pachyrhizi* aos fungicidas, possivelmente ocasionada pela intensa utilização de produtos do mesmo grupo químico, com o mesmo modo de ação (GODOY *et al*., 2015).

1.3 RESISTÊNCIA A FUNGICIDAS

A resistência a fungicidas é definida como uma alteração herdável e estável de um fungo em resposta a aplicação de um fungicida, resultando na redução da sensibilidade do mesmo a um produto (BRENT; HOLLOMON, 2007a). A resistência pode ser induzida de forma artificial em laboratório ou ela pode ocorrer de forma natural nas populações do patógeno no campo. Nos casos em que a resistência de campo resulta em perda de eficiência dos fungicidas para o controle da doença, denomina-se como resistência prática (BRENT; HOLLOMON, 2007a).

O surgimento de isolados com menor sensibilidade aos fungicidas é explicado pelas forças genéticas da mutação e da seleção, ou seja, mutações espontâneas podem ocorrer em alguns isolados pertencentes a uma população, levando a sua resistência a um determinado produto, que, quando aplicado, irá selecionar os isolados resistentes, resultando no aumento da frequência dos mesmos dentro da população (DEKKER, 1995; GHINI; KIMATI, 2000). A seleção dos isolados resistentes e o aumento da sua frequência de forma significativa está relacionada ao uso inadequado dos fungicidas, como o uso repetido de um mesmo produto ou o uso de diferentes fungicidas relacionados química e/ou bioquimicamente por meio de um mecanismo de ação em comum, além da utilização de doses diferentes da recomendada, sejam elas sub ou sobredoses, do uso do produto de forma curativa, dentre outros fatores que favorecem a seleção de indivíduos resistentes (GHINI; KIMATI, 2000; BRENT; HOLLOMON, 2007a; BRENT; HOLLOMON, 2007b).

Existem vários mecanismos, genéticos e bioquímicos, que podem levar ao surgimento de resistência dos fungos aos fungicidas, dentre eles a alteração do sítio alvo devido à mutação no gene que o codifica; a redução da absorção ou aumento do efluxo do produto; a detoxificação da molécula; a falta de conversão para o composto ativo; a compensação por meio do aumento da produção da enzima alvo; o desenvolvimento de vias metabólicas alternativas que não incluem o sítio alvo do fungicida, dentre outros (LEROUX *et al*., 2002; YAMAGUCHI; FUJIMURA, 2005; BRENT; HOLLOMON, 2007a). Segundo Brent e Hollomon (2007a), o mecanismo mais comum de resistência é a alteração do sítio alvo dos fungicidas, ocasionada por mutações no gene que codifica a proteína e, isso explica a razão da resistência ser um problema para os fungicidas modernos, também chamados sítio-específicos. Enquanto fungicidas antigos, de ação multi-sítio, agem inibindo enzimas em geral, atuando em diversos mecanismos bioquímicos do fungo, os fungicidas de ação sítio-específica agem primariamente em um único sítio alvo e, uma única mutação no gene pode alterar esse sítio, tornando-o menos ou não sensível ao fungicida.

A resistência de patógenos a fungicidas pode ser avaliada e monitorada por meio de diferentes métodos. De acordo com Russel (2004), testes *in vitro* podem ser utilizados para parasitas obrigatórios e não obrigatórios. Normalmente, utiliza-se meio de cultura sólido com adição de diferentes concentrações do fungicida e se avalia a porcentagem de inibição do crescimento em relação ao meio sem fungicida, sendo que, para os parasitas obrigatórios, o método se restringe à avaliação da germinação de esporos. Testes *in vivo* são desenvolvidos para patógenos e/ou moléculas que possuem propriedades que tornam o teste *in vitro* inadequado. Estes testes podem ser realizados em partes destacadas da planta, especialmente folhas, e suas avaliações normalmente estão baseadas no controle da doença expresso pelo desenvolvimento de lesões e/ou produção de esporos nas lesões.

O parâmetro de avaliação geralmente utilizado em ambos os métodos é a CE_{50} (concentração efetiva), utilizado para expressar o grau de toxicidade de uma substância, que representa a concentração de um composto químico onde 50% de seu efeito máximo é observado (OGA, 2008). Se a CE_{50} de um fungicida, ao longo do tempo, apresentar alteração para valores superiores aos inicialmente estabelecidos, visando ao controle de um determinado patógeno, poderá indicar redução na sensibilidade àquele fungicida (REIS *et al*., 2007).

1.3.1 Resistência aos IDMs

A resistência aos IDMs é denominada de resistência quantitativa ou contínua, porque ela se manifesta gradualmente, de forma parcial e variável em grau. Dessa forma, uma população com condição menos sensível pode ser revertida para mais sensível se o fungicida for usado de forma menos intensa ou se fungicidas alternativos forem utilizados para o controle da doença (BRENT; HOLLOMON, 2007a).

Relacionado a esta característica, evidências bioquímicas indicam que a resistência aos IDMs é poligênica, envolvendo pelo menos quatro mecanismos. Em estudos com *Candida albicans*, um patógeno humano que é referência nos estudos da resistência aos IDMs, foram encontradas diferentes mutações no mesmo gene alvo em um único isolado, e seus efeitos podem ser aditivos ou possivelmente sinérgicos (SANGLARD *et al*., 1998).

A perda de sensibilidade aos IDMs tem sido relatada há muitos anos para vários fitopatógenos, dentre eles *Botrytis cinerea*, *Uncinula necator*, *Venturia inaequalis*, *Erysiphe graminis*, *Cercospora beticola*, *Mycosphaerella graminicola*, *Colletotrichum cereale*, *Podosphaera aphanis* e *Monilinia fructicola* (STEHMANN; WAARD. 1995; DÉLYE *et al*., 1997; KÖLLER *et al*., 1997; DÉLYE *et al*., 1998; KARAOGLANIDIS *et al*., 2000; STAMMLER *et al*., 2007; WANG; MIDLAND, 2007; SOMBARDIER *et al*., 2010; MAY DE MIO *et al*., 2011).

O primeiro trabalho sobre sensibilidade de isolados de *P. pachyrhizi* aos IDMs foi desenvolvido no Brasil, onde foi observada oscilação nos valores de CE_{50} para os fungicidas ciproconazol, metconazol, tebuconazol e protioconazol, variando de 0,02 a 3,89 ppm, sendo considerada uma resposta normal a partir de diferentes genótipos que constituem populações de diversas regiões do Brasil (KOGA *et al*., 2011). Somente em 2014 foi relatada a perda de sensibilidade de isolados de *P. pachyrhizi* da safra de 2009-2010 aos IDMs, associada a mutações no gene da enzima 14-α- desmetilase dependente do citocromo P450 (CYP51), um gene nuclear que codifica para a proteína alvo do fungicida (SCHMITZ *et al*., 2014). Neste trabalho, foram detectadas mutações em diferentes pontos do gene CYP51, sendo elas: substituição de fenilalanina por leucina no códon 120, tirosina por histidina/fenilalanina no códon 131, lisina por arginina na posição 142, isoleucina por fenilalanina no códon 145 e isoleucina por treonina na posição 475 (SCHMITZ *et al*., 2014).

O mecanismo de ação dos IDMs envolve a inibição da desmetilação do carbono 14-α do lanosterol ou 24-metileno dihidrolanosterol (eburicol), que são substratos para a enzima 14-α desmetilase dependente do citocromo P450 na biossíntese de esteróis como ergosterol, prejudicando a síntese deste na membrana citoplasmática e levando ao acúmulo de 14-α-metilesteróis. Esses metilesteróis levam à formação da membrana com propriedades alteradas,

que não desempenha as funções básicas necessárias ao desenvolvimento do fungo (GISI *et al*., 2000).

1.3.2 Resistência aos IQes

A resistência aos IQes está normalmente associada à substituição de uma glicina por uma alanina na posição 143 do gene do citocromo *b* (CYTB), que está relacionada a altos níveis de resistência e, consequentemente, a menor eficiência no controle da doença (GISI *et al*., 2002). Com base nessa mutação, a resistência aos IQes é denominada qualitativa ou discreta, caracterizando a ocorrência de apenas duas classes de sensibilidade, sensível e resistente. Uma vez desenvolvida, ela tende a ser estável, mesmo se o fungicida for usado de forma menos intensa. Este tipo de resistência é regulada por um gene principal, e uma mutação pontual que cause mudança em um único aminoácido da proteína alvo pode resultar em alto nível de resistência, caso da mutação G143A (BRENT; HOLLOMON, 2007a).

Outras mutações podem ocorrer no gene CYTB, como F129L (substituição de fenilalanina por leucina) e G137R (substituição de glicina por arginina), ambas relacionadas a baixos níveis de resistência ou resistência quantitativa, com pequeno ou nenhum efeito na eficiência do fungicida para o controle da doença (GISI *et al*., 2002; FRAC, 2006).

A análise da sequência do gene CYTB revelou que ferrugens, inclusive *P. pachyrhizi*, contem um íntron tipo I logo após o códon 143 e a substituição do nucleotídeo nesta posição impediria a remoção do íntron, sendo letal para o fungo (GRASSO *et al*., 2006). Além das ferrugens, patógenos como *Monilinia fructicola*, *Monilinia laxa*, *Alternaria solani* e *Pyrenophora teres* também apresentam o íntron logo após o códon 143 sendo que os dois últimos desenvolveram a mutação F129L, que tem se mostrado menos efetiva com relação ao nível de resistência (MIESSNER; STAMMLER, 2010; STAMMLER, 2012).

O mecanismo de ação dos IQes envolve a inibição da respiração mitocondrial por meio da ligação ao sítio Qo do complexo do citocromo *bc1* (complexo III), impedindo a transferência de elétrons e, consequentemente, a produção de ATP, levando a deficiência energética nas células fúngicas. A proteína alvo dos IQes, citocromo bc1-ubiquinol oxidase, é codificada por um gene mitocondrial e, como os mecanismos de reparo do DNA são menos efetivos para o DNA mitocondrial em relação ao nuclear, os genes mitocondriais são mais suscetíveis a mutações (BRENT; HOLLOMON, 2007a).

1.4 ADAPTABILIDADE ASSOCIADA À RESISTÊNCIA A FUNGICIDAS

A adaptabilidade é definida como a habilidade de um isolado sobreviver, desenvolver e reproduzir quando comparado a outro isolado sob as mesmas condições. A evolução da resistência a fungicidas em uma população fúngica é altamente dependente da adaptabilidade, que afeta a dinâmica da competição entre isolados resistentes e sensíveis, e assim, tem implicações importantes no manejo da doença (PARNELL *et al*., 2005).

As mutações que resultam em resistência aos fungicidas podem reduzir a eficiência de importantes processos fisiológicos e bioquímicos do patógeno, levando a uma menor adaptabilidade (GHINI; KIMATI, 2000). Portanto, quando o mesmo ponto de mutação que confere resistência aos fungicidas está relacionado com a perda de eficiência de algum mecanismo fisiológico assume-se que a resistência acarreta custos ou penalidades adaptativas ao patógeno (GHINI; KIMATI, 2000). Como exemplo, mutações no códon 240 do gene da β-tubulina, que confere resistência aos benzimidazóis em isolados de *M. laxa*, também acarretam maior sensibilidade dos isolados ao calor (MA *et al*., 2005).

Custos adaptativos associados com a resistência a fungicidas são frequentemente previstos, mas raramente relatados (ZHAN; MCDONALD, 2013). Evidências experimentais sugerem que mutantes resistentes aos azoles se mostraram menos adaptados que isolados sensíveis, assumindo-se que os mesmos tem pequena chance de sobrevivência nas condições de campo (KÖLLER; SCHEINPFLUG, 1987). A resistência aos IDMs foi associada com penalidades adaptativas relacionadas à menor agressividade e produção de esporos e desvantagem competitiva em relação aos isolados sensíveis para *Cercospora beticola* (KARAOGLANIDIS *et al*., 2001). Para *Penicillium expansum*, isolados resistentes apresentaram menor crescimento micelial e menor agressividade em relação aos isolados sensíveis (KARAOGLANIDIS *et al*., 2011). Isolados resistentes de *Monilinia fructicola* também apresentaram menor adaptabilidade em relação aos isolados sensíveis, com base na taxa de crescimento, esporulação e tamanho da lesão (CHEN *et al*., 2012). Para isolados de *Pyrenophora teres* resistentes não foi observado nenhum custo adaptativo com relação aos componentes período de latência e esporulação (PEEVER; MILGROOM, 1994).

A associação entre a resistência aos IQes e as penalidades adaptativas ainda não está bem elucidada. Sugere-se que as substituições que ocorrem em isolados resistentes afetam a adaptabilidade de formas diferentes (FERNÁNDEZ-ORTUÑO *et al*., 2008). A mutação F129L, que pode ocorrer em ferrugens, foi associada a custos adaptativos para *Plasmopora*

viticola, *Alternaria solani* e *Botrytis cinerea*, casos em que isolados resistentes apresentaram desvantagem em ensaios de competição com isolados sensíveis (TOFFOLATTI *et al*., 2007; PASCHE; GUDMESTAD, 2008; KIM; XIAO, 2011).

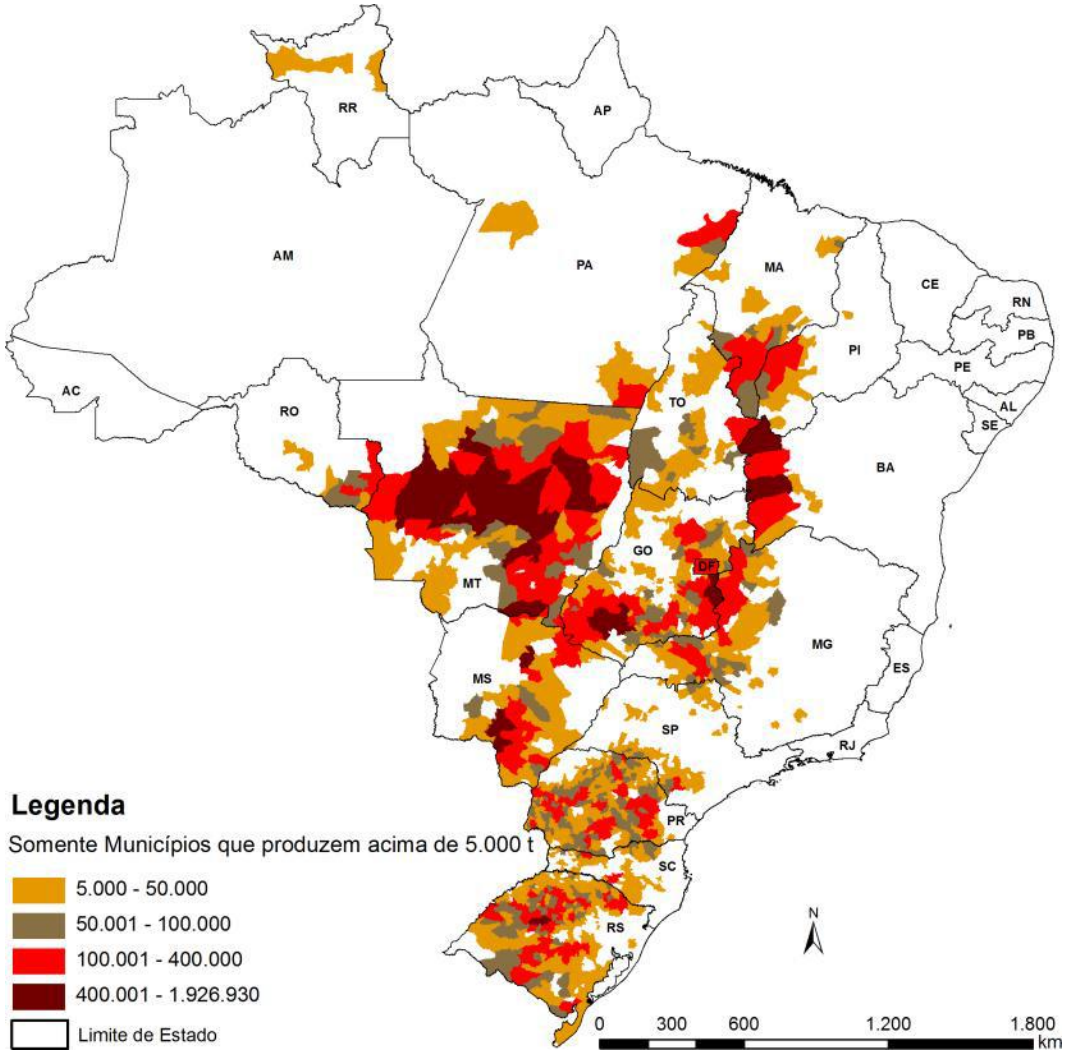

FIGURA 1. Distribuição da produção de soja no Brasil na safra de 2014-2015. Fonte: CONAB, 2015.

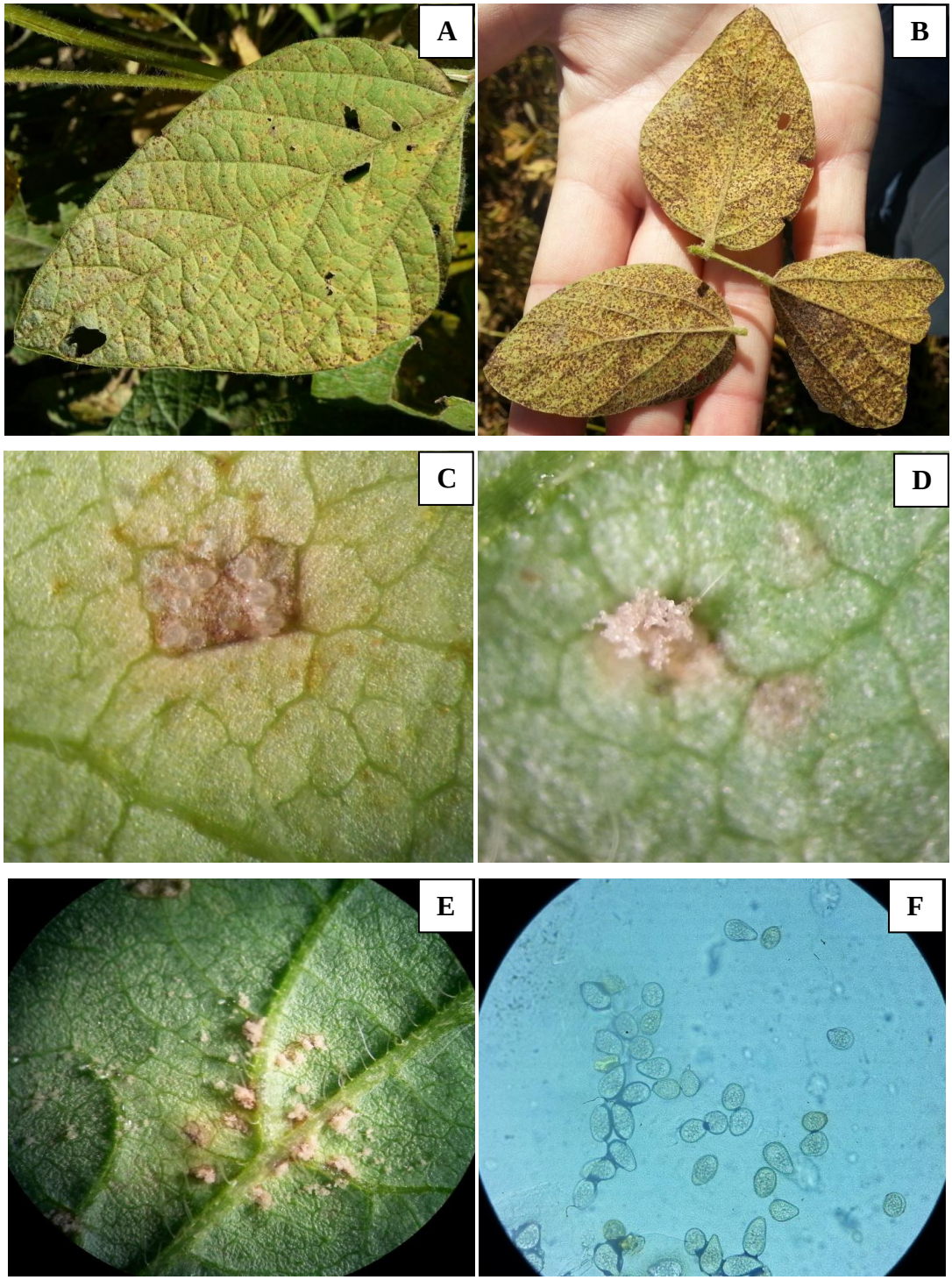

FIGURA 2. Sintomas (A) e sinais (B) da ferrugem-asiática da soja (*Glycine max*) causada por *Phakopsora pachyrhizi*; urédias iniciando a liberação de urediniósporos de *P. pachyrhizi* em folha de soja, visualizadas sob microscópio estereoscópico com aumento de 40x (C); urediniósporos hialinos de *P. pachyrhizi* em folha de soja com sintomas de ferrugem-asiática, visualizados sob microscópio estereoscópico com aumento de 40x (D) e 20x (E); urediniósporos de *P. pachyrhizi* observados em microscópio de luz com objetiva de aumento de 10x (F). Fotos: O autor (2013).

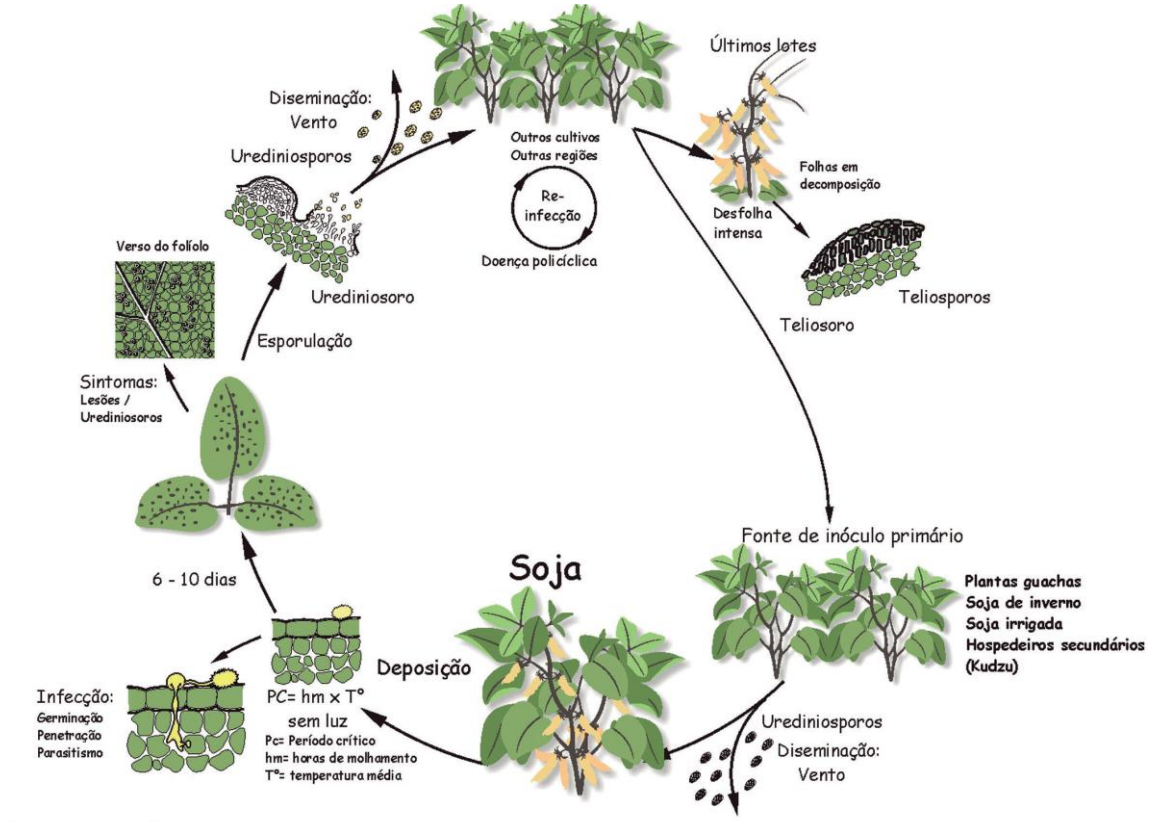

FIGURA 3. Ciclo da ferrugem-asiática da soja (*Glycine max*) causada por *Phakopsora pachyrhizi*. Fonte: Reis e Carmona, 2005 citado por Reis *et al*., 2006.

3 CAPÍTULO I - FUNGICIDE SENSITIVITY AND MONOCYCLIC COMPONENTS OF *Phakopsora pachyrhizi* ISOLATES FROM ORGANIC AND CONVENTIONAL SOYBEAN PRODUCTION SYSTEMS

Soybean rust, caused by *Phakopsora pachyrhizi*, is mainly controlled by the use of fungicides from the group of demethylation inhibitors (DMI) and quinone-outside inhibitors (QoI) and failures of control has been observed in different regions of Brazil, which may be related to the selection of less sensitive isolates. The aims were to compare the sensitivity to tebuconazole and azoxystrobin and the monocycle of populations and isolates of *P. pachyrhizi* from organic and conventional soybean fields in the 2012-2013 and 2013-2014 seasons. To assess the sensitivity to tebuconazole and azoxystrobin, the methodologies of detached leaf and *in vitro* germination, respectively, were used. To calculate the EC_{50}, the fungicide concentrations were: 0; 0.05; 0.10; 0.50; 1.0; 2.5; 5.0; 10.0 µg mL^{-1}. To evaluate the monocycle, detached leaves were inoculated with a urediniospore suspension and evaluated daily by counting the number of uredia. In both seasons, the EC_{50} to tebuconazole was lower for the organic system population (0.41 and 0.10 ug mL^{-1}) compared to the conventional system (1.60 and 4.44 ug mL^{-1}) and to azoxystrobin was similar for both populations. There were a higher proportion of sensitive isolates to tebuconazole in the organic than in the conventional system. The monomolecular model fitted to monocycle data and parameters related to the maximum asymptote and the initial inoculum, besides the AUDPC, were higher for organic than in the conventional system. The lower sensitivity to tebuconazole of the isolates of the conventional system may be related with lower fitness, based on monocycle parameters, related to the isolates from organic fields.

Key-words: Resistance. Tebuconazole. Azoxystrobin. Soybean rust. Fitness.

SENSIBILIDADE A FUNGICIDAS E COMPONENTES MONOCÍCLICOS DE ISOLADOS DE *Phakopsora pachyrhizi* ORIUNDOS DE CAMPOS DE PRODUÇÃO DE SOJA ORGÂNICA E CONVENCIONAL

A ferrugem-asiática da soja, causada pelo fungo *Phakopsora pachyrhizi*, é principalmente controlada por meio da aplicação de fungicidas dos grupos dos inibidores da desmetilação (IDM) e dos inibidores da quinona externa (IQe) e falhas no controle da doença já foram observadas em diferentes regiões do Brasil, podendo estar relacionadas com a seleção de isolados menos sensíveis a esses fungicidas. Os objetivos deste trabalho foram i) comparar a sensibilidade aos fungicidas tebuconazol (IDM) e azoxistrobina (IQe) de populações e isolados monouredinias de *P. pachyrhizi* oriundos de campos de produção de soja orgânica e convencional e, ii) comparar o monociclo de isolados dos dois campos sob condições ideais de temperatura e umidade. Para avaliar a sensibilidade ao tebuconazol e à azoxistrobina, seguiram-se as metodologias de folhas destacadas e de germinação *in vitro*, respectivamente. Para calcular a CE_{50}, as concentrações utilizadas dos fungicidas foram: 0; 0,05; 0,10; 0,50; 1,0; 2,5; 5,0; 10,0 µg mL^{-1}. Para avaliar o monociclo, folhas destacadas de soja foram inoculadas com uma suspensão de urediniósporos e avaliadas diariamente contando-se o número de urédias. Nas duas safras avaliadas, a CE_{50} do tebuconazol foi inferior para isolados do sistema orgânico (0,41 e 0,10 ug mL^{-1}) em relação aos isolados do sistema convencional (1,60 e 4,44 µg mL^{-1}) e a CE_{50} da azoxistrobina foi similar para as duas populações. Foi observada maior proporção de isolados sensíveis ao tebuconazol no sistema orgânico comparado com o sistema convencional. O modelo monomolecular se ajustou aos dados do monociclo e os parâmetros relacionados à assíntota máxima e ao inóculo inicial, além da AACPD, foram superiores para os isolados oriundos do campo de produção orgânica. A menor sensibilidade ao tebuconazol dos isolados do sistema convencional pode estar associada com a menor adaptabilidade, com base nos componentes monocíclicos, com relação aos isolados do sistema orgânico.

Palavras-chave: Resistência. Tebuconazol. Azoxistrobina. Ferrugem-asiática. Adaptabilidade.

3.1 INTRODUCTION

Soybean is one of the most important crops grown in the world due to its high socio-economic value and the several applications of its products and byproducts. Brazil is the second largest soybean producer (FAO, 2013) and, in the 2014-2015 season, the national production was the highest recorded to date corresponding to 96.243 millions tons from a harvested area of 32.093 millions hectares (CONAB, 2015).

Currently, *Phakopsora pachyrhizi* Syd. & P. Syd., the causal agent of Asian soybean rust is one of the most important economic threats to soybean growers in South America due to its potential yield loss. The pathogen can cause defoliation in soybean fields in a few days (Goellner et al., 2010). Yield loss up to 80% has been reported in the absence of control measures (Hartman et al., 1991; Yang et al, 1991).

Since obtaining resistant cultivars to all isolates of *P. pachyrhizi* is a complex process, due to the great genetic variability of the population (Sinclair & Hartman, 1999; Yamaoka *et al*., 2002; Bonde *et al*., 2006; Freire *et al*., 2008, Tschurtschenthaler *et al*., 2012), the use of fungicides is the main control measure.

Nowadays rust can be controlled through the use of several different groups of fungicides with different modes of action. The use of sterol demethylation inhibitors (DMIs) and quinine-outside inhibitors (QoIs), especially in commercial mixtures, has shown more consistent control of the disease (Miles et al., 2007; Scherm et al., 2009). Depending on the time of occurrence and the pressure of the disease, four or more fungicide applications may be needed to control soybean rust (Siqueri, 2005).

Failure to control soybean rust has been observed in recent years in different regions of Brazil and a lower efficiency was observed already in control of soybean rust using DMIs and QoIs (Godoy et al., 2014; 2015).

Fungicide-resistant isolates, in some cases, are less fit than sensitive isolates. Experimental evidence suggests that this occurs with mutant isolates that have resistance to DMIs (Chin et al., 2001) and QoIs (Fernández-Ortuño et al., 2008). Fitness is defined as the ability of isolates to survive, develop and reproduce when compared to other isolates under the same conditions. The evolution of fungicide resistance in a fungal population is highly dependent on this feature, which affects the dynamics of competition between resistant and sensitive isolates, and thus has important implications for disease management (Parnell et al., 2005). The fitness of the pathogen is a relative concept that can be measured on the basis of

epidemiological parameters related to their development and their reproduction (Zadoks & Schein, 1979). In the laboratory, monocyclic parameters such as incubation and latent period, number of lesions and disease progression can be assessed to compare sensitive and resistant isolates.

The explanation above raises the hypothesis that the intensive use of fungicides to control soybean rust may be selecting resistant, and potentially less fit, isolates in the populations and making them predominantly less sensitive. The same process could not occur in populations that do not come into contact with fungicides.

The objectives of this study were i) to compare the sensitivity to tebuconazole (DMI) and azoxystrobin (QoI) fungicides of *P. pachyrhizi* populations and monouredinial isolates from organic and conventional soybean production systems and; ii) to compare the monocycle components using isolates from both systems under ideal temperature and humidity to the disease development.

3.2 MATERIAL AND METHODS

3.2.1 *P. pachyrhizi* isolates

The experiment was conducted using two kinds of inoculum: first, *P. pachyrhizi* populations coming directly from the field; second, *P. pachyrhizi* isolates, obtained through monouredinial culture (Tab.1). Infected leaves by *P. pachyrhizi* were collected in the 2012-2013 and 2013-2014 seasons from organic and conventional soybean production systems.

In the tests with *P. pachyrhizi* populations, the pathogen inoculum was removed from soybean leaves infected by *P. pachyrhizi* from commercial fields located in the state of Paraná, in southern Brazil, using a vacuum pump (Insight, Brazil). Leaves were collected in fields of organic and conventional soybean production from Planalto-PR (southwestern Paraná, geographic coordinates 25º44’22.7”S 53º40’43.2”W) and Ponta Grossa-PR (east central Paraná, geographic coordinates 25º07’24,0”S 50º10’41.8”W), respectively. The fields are located at a distance of approximately 400 km from each other. Samples were collected in the end of the season, around soybean stage R6, after the fungicide applications in the conventional field.

The pathogen was inoculated on healthy unifoliate leaves from 17- to 20-day-old soybean plants kept in a greenhouse, and uredinospores from the lesions produced were used for the tests which follow to standardize the age of the inoculum.

In the tests with isolates, monouredinial isolates of *P. pachyrhizi* were obtained from the both systems of soybean production (i.e. organic and conventional production systems) by transferring spores from single uredia to healthy unifoliate leaves using an inoculation needle (Tab. 1).

Twenty isolates were obtained, ten from organic production system (5 from the 2012-2013 and 5 from the 2013-2014 season) and ten from conventional production system (5 from the 2012-2013 and 5 from the 2013-2014 season). The isolates were multiplied by transferring the spores to healthy leaves every two weeks by inoculation of a spore suspension in water with Tween (0.01%) to the abaxial surface of unifoliate leaves, using an airbrush (0.3 mm nozzle; alpha Arprex® 3).

In the organic production system, prevention of Asian soybean rust is performed through the application of products based on sulfur and copper. Fungicides of the DMI and QoI groups were never applied in this area. In the conventional production system, the rust control is usually achieved using two to four fungicide applications of the DMI and QoI groups. In the 2012-2013 season, the fungicide spray program included two applications of products based on tebuconazole and two of products based on azoxystrobin + cyproconazol. In the 2013-2014 season, one application of azoxystrobin + cyproconazol, one of picoxystrobin + cyproconazol and one of pyraclostrobin + epoxiconazol were used.

3.2.2 Sensitivity to tebuconazole

The assay to test the tebuconazole sensitivity of *P. pachyrhizi* was conducted following the FRAC (Fungicide Resistance Action Committee) methodology on detached leaves (Scherb & Mehl, 2006). Unifoliate soybean leaves (cultivar BMX Potência RR) were dipped for 3 sec in tebuconazole solutions at concentrations: 0; 0.05; 0.10; 0.50; 1.0; 2.5; 5.0; 10.0 µg mL^{-1}. After drying for 2 hours, the leaves were placed in transparent polystyrene Petri dishes (90-mm diameter), containing water agar (1%) including streptomycin sulfate (30 mg L^{-1}). The inoculation of the pathogen was performed on the abaxial surface using an airbrush (0.3 mm nozzle; alpha Arprex® 3). The suspension was prepared with distilled water+Tween

20 (0.01%) at $1x10^4$ urediniospores mL^{-1}, adjusted with the aid of a hemacytometer. Each fungicide concentration had eight replicates and one Petri dish containing one unifoliate leaf was considered an experimental unit.

After inoculation, leaves were kept in the dark during the first 48 hours at room temperature and then were incubated in a growth chamber at 23 °C and a 12-hour photoperiod. After 15 days, the rust severity was assessed using a standard area diagram (Godoy et al., 2006) and the tebuconazole concentration at which reduction 50% of population activity (EC_{50}) was calculated to compare populations from the both systems of soybean production. The test was conducted in duplicate.

3.2.3 Sensitivity to azoxystrobin

The assay to test the azoxystrobin sensitivity of *P. pachyrhizi* was carried out following the FRAC methodology of *in vitro* germination (Buzzerio, 2006) The fungicide was added to the 1% water agar (approximately 45 °C) at 0; 0.05; 0.10; 0.50; 1.0; 2.5; 5.0; 10.0 µg mL^{-1}. After the medium was poured into transparent polystyrene Petri dishes (40 mm diameter), the suspensions were spread on a surface with Drigalsky's loop (100 µL per dish) at $1x10^4$ uredospores mL^{-1} adjusted with the aid of a hemacytometer. Each fungicide concentration had 3 replicates (i.e. 3 Petri dishes). The dishes were incubated in the dark for 24 hours at 23 ºC when germination was stopped by adding 0.1 mL of lactophenol. The evaluation was performed by counting the germinated spores from 100 spores per dish using a light microscope, establishing the percentage of germination. Spores that produced germ tubes equal or longer than their diameter were considered to have germinated. The test was conducted in duplicate.

3.2.4 Monocycle assay

The assay to evaluate the disease monocycle was conducted in detached unifoliate soybean leaves (cultivar BMX Potência RR). The leaves were placed in transparent polystyrene Petri dishes (90 mm diameter) containing water agar (1%) including streptomycin

sulfate (30 mg L^{-1}). Pathogen inoculation was performed using an airbrush (0.3 mm nozzle; alpha Arprex® 3). The suspension was prepared with distilled water+Tween 20 (0.01%) at $3x10^4$ urediniospores mL^{-1}, adjusted with the aid of a hemacytometer. After inoculation, the leaves were kept in the dark during the first 48 hours at room temperature and then were incubated in a growth chamber at 23 °C and 12-hour photoperiod. Each Petri dish containing one unifoliate leaf was considered one repetition and the experimental design was completely randomized with 15 replicates for populations of *P. pachyrhizi* and four replicates for monouredinial isolates. The leaves were evaluated daily by determining the incubation (time between the inoculation and the onset of symptoms in the 50% of leaves) and latency period (time between the inoculation and the sporulation in 50% of leaves) and by counting the total number of sporulated uredia until it stabilized, using a magnifying glass.

3.2.5 Data analysis

The 50% effective concentration (EC_{50}) value for each population/isolate was obtained using a linear regression between percentage of inhibition of rust severity on leaves (tebuconazole) and of spore germination (azoxystrobin), calculated relative to the control (i.e. without fungicide) that represented 0% inhibition, and the corresponding log_{10} concentrations of the fungicide. The EC_{50} values were subject to analysis of variance ($F \leq 0.05$).

The monomolecular model (y = b1*(1-exp(-log(2)*x/b2))) was fitted to the monocycle data of populations and monouredinial isolates, where y = disease severity, b1 = maximum asymptote estimated by the model, b2 = parameter related to the initial inoculum and x = time (days after pathogen inoculation), using statistical software R (R Development Core Team, 2012). The area under the disease progress curve (AUDPC) (Shaner & Finney, 1977) for each isolate was calculated and the data were transformed by cube root (x) to comply with the assumptions of the statistical analysis, and then the Student's t test was applied ($p < 0.05$), using the statistical software R (R Development Core Team, 2012). Each monouredinial isolate was considered a replicate within the each soybean production system thus, comparing the two systems.

3.3 RESULTS

3.3.1 Populations of *P. pachyrhizi*

In the 2012-2013 season, the tebuconazole EC_{50} value for *P. pachyrhizi* population from the soybean conventional production system was significantly ($p<0.05$) higher (1.61 µg mL^{-1}) than the EC_{50} of population from the organic production system (0.41 µg mL^{-1}). The azoxystrobin EC_{50} was similar in both *P. pachyrhizi* populations and the value was approximately 1.40 µg mL^{-1} (Tab. 2).

In the 2013-2014 season, the tebuconazole EC_{50} value of the population from the conventional production system was also significantly ($p<0.05$) higher (4.44 µg mL^{-1}) than the tebuconazole EC_{50} of the population from the organic system (0.10 µg mL^{-1}) but the difference was greater than that found in the first season. The azoxystrobin EC_{50} was between 1.0 and 2.0 µg mL^{-1} and there was no significant difference ($p<0.05$) between the systems (Tab. 2).

There was no difference in the incubation and the latent periods that were 5 and 6 days, respectively, for both populations. The monomolecular model was the best fit for the monocycle data but there was high variability in monocycle data between replicates within the population of the same system (data not shown). Although the population from the organic system has reached greater severities in some replicates in relation to the population from the conventional system, the model parameters did not differ between the two populations due to high variability between replicates (data not shown).

3.3.2 Monouredinial isolates of *P. pachyrhizi*

There was variability among the EC_{50} values for tebuconazole between monouredinial isolates within each system. However, in the conventional system, the EC_{50} values of isolates were generally higher than those of the organic system (Fig. 1), and, in the second season, 60% of isolates from the conventional system showed EC_{50} values higher than 6.0 µg mL^{-1}, whereas in the organic system, all isolates showed values lower than this.

For azoxystrobin, it was not possible to determine the EC_{50} for monouredinial isolates in the first season because the compound showed 100% inhibition of germination from the third or fourth concentration tested (0.5 or 1.0 µg mL^{-1}), depending on the isolate. Therefore it was only possible to confirm that the EC_{50} for azoxystrobin was below 1.0 µg mL^{-1} for the monouredinial isolates of both systems. In the 2013-2014 season, isolates showed EC_{50} values for azoxystrobin ranging from 0.14 to 3.0 µg mL^{-1} and one isolate of the conventional system showed an EC_{50} value above 10 µg mL^{-1} (Fig. 2).

There was no difference in the incubation and the latent period, that were 7 and 8 days, respectively, for all isolates. The monomolecular model was fit to the monocycle data of the monouredinial isolates, as shown in Figure 3.

In 2012-2013 season, the parameters related to the maximum asymptote and initial inoculum of monomolecular model were significantly ($p<0.05$) greater for isolates from the organic system than isolates from the conventional system (Tab. 3). In 2013-2014 season, only the parameter related to the maximum asymptote was higher ($p<0.05$) for isolates from the organic field. In general, the maximum asymptote was higher for the isolates from the 2013-2014 season than isolates of previous season (Tab. 3). The AUDPC mean of isolates from the organic system was higher ($p<0.05$) than the mean for isolates from the conventional system in both seasons (Tab. 3).

3.4 DISCUSSION

The results suggest that the population of *P. pachyrhizi* from the conventional soybean production system is less sensitive to tebuconazole than the population from the organic soybean production system whereas there was no difference in the sensitivity to azoxystrobin between the populations.

Improper use of the fungicide, as repeated use of the same compound during the season, using unlabeled doses (lower or higher than recommended), late and curative application, among other factors, may be contributing to the selection of less fungicide sensitive isolates (Brent & Hollomon, 2007) in the conventional system.

In a population there is a mixture of sensitive and resistant isolates, and its analysis is more closely related to the practical resistance which is observed through the control effectiveness with the fungicide in the field. Working with monouredinial isolates, it is

possible to know the extreme cases that are occurring in the field and thus better analyze the structure of the population.

The EC_{50} values of tebuconazole for monouredinial isolates suggest that there is a higher proportion of less sensitive individuals in the population from the conventional system than the population from the organic system. This explains the results found in the tests with populations coming directly from the field which showed higher EC_{50} values for the population from the conventional system. In the organic system, two of ten isolates were less sensitive than the others because they had EC_{50} values higher than 1.0 µg mL^{-1} (2.80 and 5.94 µg mL^{-1}). This may be related to the intrinsic variability of isolates that constitute a population and to wind dispersal of inoculum coming from conventional areas, which receive application of fungicides. The primary inoculum in the organic system can come from the conventional soybean fields present in the region, however, there was no selection pressure of fungicides in this system. Therefore, the frequency of less sensitive isolates was lower, especially because the mutations associated with lower sensitivity to DMIs are related with fitness penalties (chapter 3 of this thesis), so, mutated isolates have competitive disadvantages in the absence of fungicide.

In work by Schmitz et al. (2014) with isolates of *P. pachyrhizi* from different regions of Brazil in the 2009-2010 season, the EC_{50} values for tebuconazole ranged from 0.01 to 10.0 µg mL^{-1} (mean = 2.5 µg mL^{-1}), within the range of values found in this work. Using the classification of Schmitz et al. (2014) for the sensitivity of *P. pachyrhizi* isolates based on the EC_{50} values, in the organic and in the conventional systems, in the first season, 60% and 40% of sensitive isolates, 40% and 40% of sensitive to moderately resistant isolates, 0% to 20% of moderately to highly resistant isolates were found, respectively (Fig. 1). In the 2013-2014 season, the proportions in the organic and conventional systems were, respectively, 40% and 0% sensitive isolates, 20% and 20% of sensitive to moderately resistant isolates, 40% and 20% of moderately to highly resistant isolates and 0% and 60% of highly resistant isolates (Fig. 1). In the same work in Germany, a single strain of the year 2004 was considered as a highly sensitive reference, being controlled with EC_{50} value of 0.01 µg mL^{-1}. So, two isolates (isolates 7 and 33) from the organic system and one (isolate 54) from the conventional system, with EC_{50} values of 0.02 µg mL^{-1}, were considered highly sensitive.

In addition, a tendency to increase the proportion of less sensitive isolates from the first to second season in both production systems can be seen in Figure 1, which may indicate that the sensitivity to tebuconazole is decreasing through the seasons probably due to repeated use of DMIs.

The lack of success to estimate the EC_{50} to azoxystrobin for isolates in the first season, indicated that all monouredinial isolates had EC_{50} values below that of the population (values < 1.0 µg mL^{-1}). The result may have been influenced by the limited number of isolates, indicating that sensitive isolates might have been selected and that some lower concentrations should be tested.

In the second season, some monouredinial isolates had higher EC_{50} values for azoxystrobin than populations, such as EC_{50} values of 3.0 ug mL^{-1} (organic system) and 10.0 ug mL^{-1} (conventional system) (Fig. 2).

In a work conducted in Brazil, with a population of *P. pachyrhizi* collected in the 2004-2005 season, the EC_{50} value for azoxystrobin was 0.07 µg mL^{-1} and the author asserts that this value can be considered as the baseline (Blum & Reis, 2013). Although some research groups claim no loss of sensitivity to QoIs in the populations of *P. pachyrhizi* (Miles *et al*., 2007; Scherm *et al*., 2009; Schmitz *et al*., 2014), higher values than the reference values have been found, including in this work, wherein the EC_{50} value of the population was higher than 1.0 ug mL^{-1} in both systems and both seasons (Tab. 2).

Despite the mutation in codon G143A of the mitochondrial cytochrome b gene that is the main QoIs resistance mechanism in most fungi, it is a lethal mutation in *P. pachyrhizi* (Grasso et al., 2006). The F129L mutation has been found in *P. pachyrhizi* isolates from Brazil (Klosowski et al., 2015) and may be confering lower sensitivity to QoIs. In chapter 3 of this thesis, the results indicated that the F129L mutation is not associated with fitness penalties and mutated isolates competed equally well with sensitive isolates even in the absence of fungicides. Therefore, the sensivity of population from the organic field was not different from the population of the conventional field.

Epidemiological findings through the monocycle and AUDPC data of soybean rust, assessed on detached leaves, showed a higher level of disease for isolates from the organic field in both seasons. It suggests that monouredinial isolates from the conventional system are less fit in relation to the isolates from the organic system. This result may be related to the lower sensitivity to tebuconazole. This finding is in agreement with the results described in the chapter 3 of this thesis, where isolates with mutations associated with lower sensitivity to DMIs have competitive disadvantage in relation to sensitive (wild type) isolates.

The higher level of disease (maximum asymptote) found for isolates of the 2013-2014 season, regardless the production system, can be related to higher germination rates (data not shown), that can be associated with the time of collection and the age of uredia and urediniospores.

Resistant isolates to fungicides with fitness penalties only compete well in nature under selection pressure of the fungicide. Thus, the resistance may be, at least, partially reversible when the selection pressure of fungicide is removed or minimized using resistance management measures (Parnell et al., 2005).

There are no reports in the literature correlating the resistance of *P. pachyrhizi* to DMIs with fitness costs and it is rarely reported for other rusts. However, for other pathogens, such as *Cercospora beticola* (Karaoglanidis et al., 2001) and *Fusarium graminearum* (Becher et al., 2010), there is evidence that this occurs.

In conclusion, the EC_{50} values for tebuconazole of *P. pachyrhizi* population and monouredinial isolates from the conventional soybean production system were higher than those from the organic system, which suggests that those isolates are less sensitive than these, whereas the QoI sensitivity was similar for isolates of the both systems. Isolates from the conventional system were less fit, based on monocycle components, in relation to the organic system, and this feature may be related to less sensitivity to tebuconazole and indicates that the DMI resistance is associated with fitness penalties.

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TABLE 1. Origin of isolates of *Phakopsora pachyrhizi* that were used in tebuconazole and azoxystrobin sensitivity tests and/or assay to evaluate the monocycle components.

Isolate	Origin	Management	Tebuconazole sensitivity test	Azoxystrobin sensitivity test	Monocycle assay
3	Southwestern Paraná	Organic	x	x	
7	Southwestern Paraná	Organic	x	x	
11	Southwestern Paraná	Organic	x	x	X
12	Southwestern Paraná	Organic			X
13	Southwestern Paraná	Organic	x	x	
15	Southwestern Paraná	Organic			X
16	Southwestern Paraná	Organic	x	x	X
17	Southwestern Paraná	Organic			X
21	Southwestern Paraná	Organic	x	x	X
22	Southwestern Paraná	Organic			X
28	Southwestern Paraná	Organic	x	x	X
29	Southwestern Paraná	Organic			X
33	Southwestern Paraná	Organic	x	x	
37	Southwestern Paraná	Organic	x	x	
40	Southwestern Paraná	Organic			x
41	Southwestern Paraná	Organic	x	x	
52	East Central Paraná	Conventional	x	x	
54	East Central Paraná	Conventional	x	x	x
57	East Central Paraná	Conventional	x	x	
61	East Central Paraná	Conventional	x	x	x
62	East Central Paraná	Conventional			x
65	East Central Paraná	Conventional	x	x	x
67	East Central Paraná	Conventional			x
90	East Central Paraná	Conventional	x	x	
91	East Central Paraná	Conventional			x
92	East Central Paraná	Conventional	x	x	
93	East Central Paraná	Conventional	x	x	
94	East Central Paraná	Conventional			x
95	East Central Paraná	Conventional			x
96	East Central Paraná	Conventional			x
97	East Central Paraná	Conventional	x	x	
98	East Central Paraná	Conventional			x
100	East Central Paraná	Conventional	x	x	

TABLE 2. Sensitivity of *Phakopsora pachyrhizi* populations to the demethylation inhibitor (DMI) tebuconazole and quinone-outside inhibitor (QoI) azoxystrobin.

Population	Season	Tebuconazole EC_{50}* ($\mu g\ mL^{-1}$)		Azoxystrobin EC_{50} ($\mu g\ mL^{-1}$)	
		Range	Mean ± SE[a]	Range	Mean ± SE[a]
Organic	2012-2013	0.21-0.55	0.41 ± 0.10 bA	1.12-1.74	1.37 ± 0.19 aA
Conventional	2012-2013	1.51-1.73	1.61 ± 0.06 aB	0.90-2.00	1.41 ± 0.32 aA
Organic	2013-2014	0.02-0.24	0.10 ± 0.06 bA	0.71-1.58	1.08 ± 0.26 aA
Conventional	2013-2014	2.90-6.75	4.44 ± 1.17 aA	1.46-2.31	1.77 ± 0.27 aA

*$EC_{50:}$ effective concentration which reduces population activity by 50% .
[a] Means followed by a different lowercase letter in the column between management systems in the same season and by a different uppercase letter between seasons in the same management system are significantly different according to a F-test at P=0.05.

TABLE 3. Maximum asymptote of the disease progress curve (b1) and parameter related to the initial level of inoculum (b2) estimated by the monomolecular model fit to monocycle data and means of area under the disease progress curve (AUDPC) on detached soybean (*Glycine max*) leaves of monouredinial isolates of *Phakopsora pachyrhizi* from organic and conventional soybean production systems.

Monouredinial isolates	Season	b1[a]	b2[a]	Means of AUDPC*[a]
Organic	2012-2013	552.03 ± 99.66 aB	6.22 ± 0.80 aA	12.03 ± 0.75 aB
Conventional	2012-2013	227.86 ± 89.43 bB	3.57 ± 0.47 bA	9.42 ± 1.44 bB
Organic	2013-2014	1663.30 ± 249.37 aA	5.24 ± 1.22 aA	20.07 ± 0.95 aA
Conventional	2013-2014	1056.74 ± 243.81 bA	3.94 ± 0.61 aA	17.73 ± 0.67 bA

*Cube-root-transformed data.
[a]Data are expressed as mean ± SE (n = 5). Means followed by a different lowercase letter in the column between management systems in the same season and by a different uppercase letter between seasons in the same management system are significantly different according to a F-test at p=0.05.

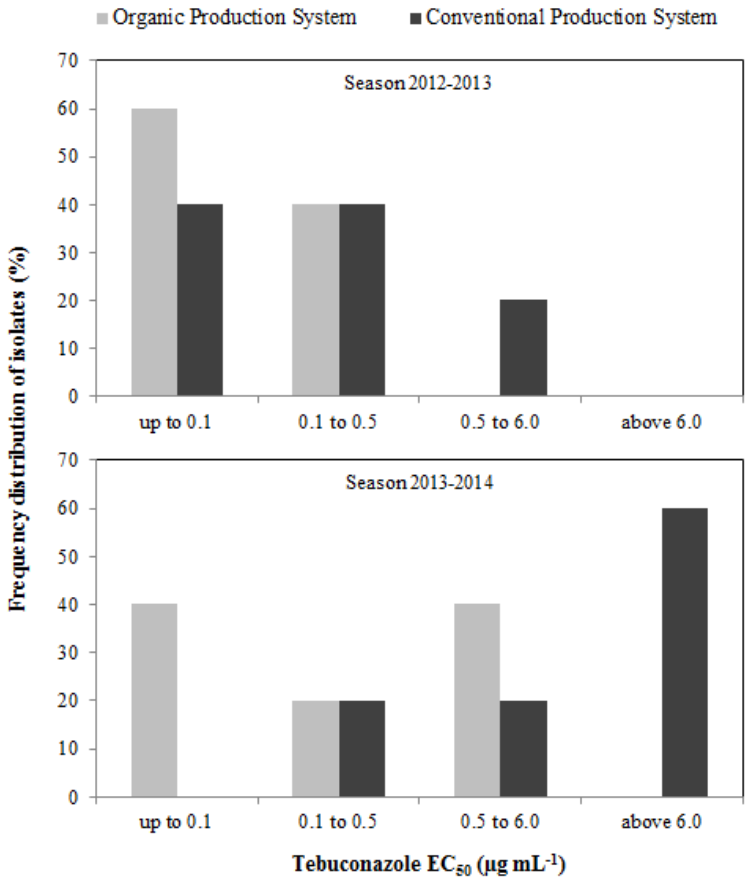

FIGURE 1. Sensitivity distribution of *Phakopsora pachyrhizi* monouredinial isolates from organic and conventional soybean (*Glycine max*) production systems according to their tebuconazole effective concentration which reduces population activity by 50% (EC_{50}). Total of 20 isolates (10 from organic system – five of the 2012-2013 season and five of the 2013-2014 season; 10 from conventional system – five of the 2012-2013 season and five of the 2013-2014 season).

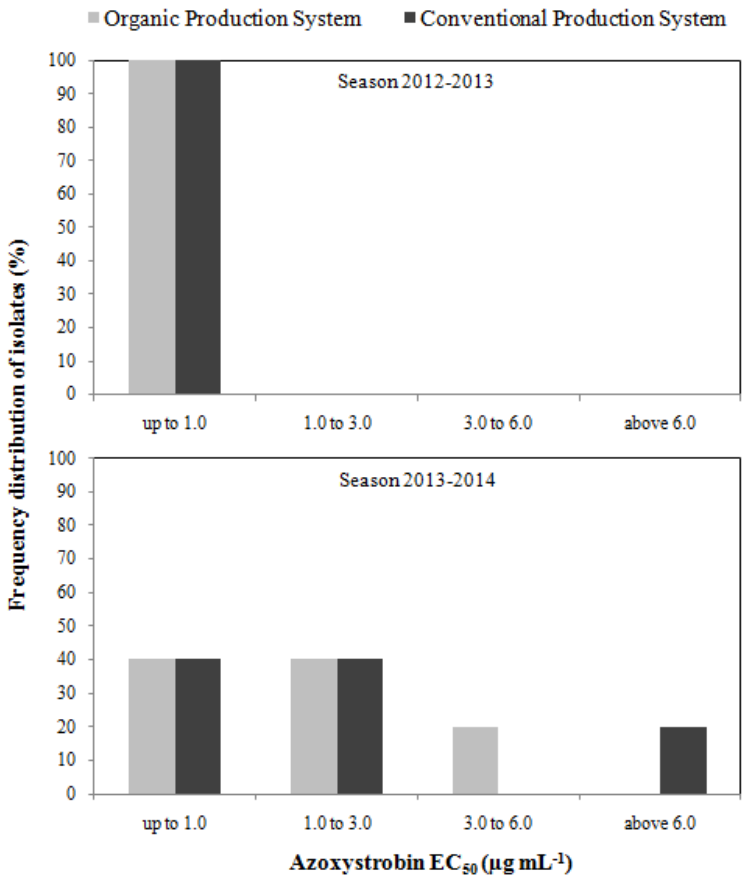

FIGURE 2. Sensitivity distribution of *Phakopsora pachyrhizi* monouredinial isolates from organic and conventional soybean (*Glycine max*) production systems according to their azoxystrobin effective concentration which reduces population activity by 50% (EC_{50}). Total of 20 isolates (10 from organic system – five of the 2012-2013 season and five of the 2013-2014 season; 10 from conventional system – five of the 2012-2013 season and five of the 2013-2014 season).

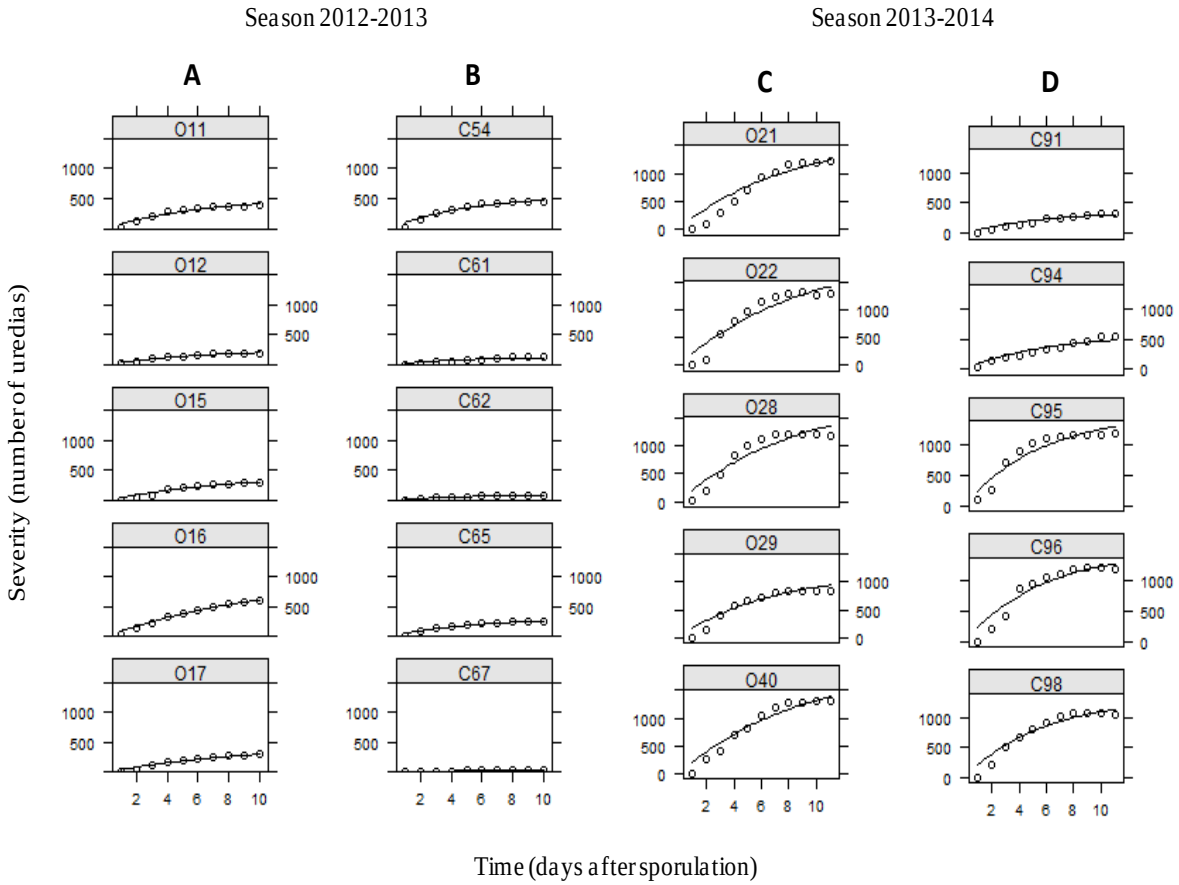

FIGURE 3. Monomolecular model fit to monocycle data of *Phakopsora pachyrhizi* monouredinial isolates from organic (A and C) and conventional (B and D) soybean (*Glycine max*) production systems in 2012-2013 (A and B) and 2013-2014 (C and D) seasons.

Numbers preceded by the letter "C" designate isolates from the conventional fields and numbers with letter "O", isolates from the organic field.

4 CAPÍTULO II - DETECTION OF MUTATIONS IN CYTOCHROME P450 14*α*-STEROL DEMETHYLASE AND THE CYTOCHROME *b* GENES IN *Phakopsora pachyrhizi*

Asian soybean rust, caused by *Phakopsora pachyrhizi*, is mostly controlled by DMI and QoI fungicides. Mutations in CYP51 and CYTB genes can lead to pathogen resistance to DMIs and QoIs. The occurrence of the mutations in both genes was investigated and a pyrosequencing assay was developed for a rapid and quantitative detection of the F129L mutation. Isolates showed three different combined mutations in the CYP51 gene including a triple combination. The analysis of the CYTB gene showed the presence of the F129L mutation, but other mutations (G143A and G137R) were not found. The pyrosequencing was an effective method for detection of the F129L mutation. This is the first report of occurrence of the F129L mutation in the CYTB gene and triple mutation of the CYP51 gene in *P. pachyrhizi*. The practical relevance of these mutations for field efficacy of DMIs and QoIs needs further investigation.

Key-words: Asian soybean rust. DMI fungicides. QoI fungicides. Fungicide resistance.

DETECÇÃO DAS MUTAÇÕES NOS GENES DO CITOCROMO P450 14 α-ESTEROL DESMETILASE E DO CITOCROMO *b* EM *Phakopsora pachyrhizi*

A ferrugem-asiática da soja, causada por *Phakopsora pachyrhizi*, é principalmente controlada por meio do uso de fungicidas dos grupos dos IDMs e IQes. Mutações nos genes CYP51 e CYTB de fungos podem levar à resistência aos fungicidas. Neste trabalho, a ocorrência de mutações em ambos os genes foi investigada e um ensaio de pirosequenciamento foi desenvolvido para uma detecção rápida e quantitativa da mutação F129L no gene CYTB. Isolados de *P. pachyrhizi* apresentaram três diferentes mutações no gene CYP51, incluindo uma combinação tripla (F120L+Y131F+I475T). A análise do gene CYTB revelou a presença da mutação F129L, enquanto as outras mutações testadas não foram encontradas (G143A e G137R). O ensaio de pirosequenciamento foi um método efetivo para detecção da mutação F129L. Este é o primeiro relato da ocorrência da mutação F129L no gene CYTB e da mutação tripla no gene CYP51 em isolados de *P. pachyrhizi*. A relevância prática destas mutações para a eficiência dos IDMs e dos IQes no campo deve ser investigada.

Palavras-chave: Ferrugem asiática. Fungicidas. Resistência a fungicidas. IDMs. IQes.

4.1 INTRODUCTION

Asian soybean rust, caused by *Phakopsora pachyrhizi* Syd. & P. Syd., is one of the most important economic threats to soybean growers in South America due to its potential to damage crop yield. Reduction of up to 80% in yield has been reported as result of poor pod filling [1,2] associated with early defoliation caused by the pathogen in the absence of control measures. [3]

Both sterol demethylation inhibitor (DMI) and quinone outside-inhibiting (QoI) fungicides represent the most important classes of fungicides currently used for Asian soybean rust control. [4,5]

DMIs inhibit demethylation at the 14-α carbon of lanosterol or 24-methylene dihydrolanosterol (eburicol), which are the substrates for the cytochrome P450-dependent 14-α demethylase in the biosynthesis of fungal sterols such as ergosterol. [6]

The QoI fungicides inhibit mitochondrial respiration by binding to the Qo site of the cytochrome *bc*1 complex (complex III), preventing the transfer of electrons and, consequently, ATP production, leading to an energy deficiency in fungal cells. [7]

Mutations in the target cytochrome P450 14α-sterol demethylase (CYP51) and cytochrome *b* (CYTB) genes may result in reduced sensitivity of pathogens to DMI and QoI fungicides, respectively, because of reduction of fungicide binding affinity due to the changes in amino acid sequence.[6,8]

In the CYP51 gene, amino acid substitutions at positions 120 (F120L – phenylalanine to leucine), 131 (Y131H/F – tyrosine to histidine/phenylalanine), 142 (K142R – lysine to arginine), 145 (I145F – isoleucine to phenylalanine) and 475 (I475T – isoleucine to threonine) have been reported for *P. pachyrhizi*, and these substitutions were related with decreased DMI sensitivity.[5] With the exception of I145F, all mutations occurred in combinations (i.e. Y131H + F120L, Y131F + K142R, Y131F + I475T). [5]

Three mutations, located at positions 129, 137 and 143, have been detected in the CYTB gene of several phytopathogenic fungi and oomycetes that are less sensitive to QoIs. The substitution of alanine for glycine at position 143 (G143A) is the most common mutation in QoI-resistant pathogens and is associated with high levels of resistance to QoI. [9-11] The F129L (change of phenylalanine to leucine) and the G137R (substitution of glycine to arginine) mutations are reported to be associated with low to moderate levels of sensitivity decrease. [11]

Sequence analyses of the CYTB gene have revealed that rusts, including *P. pachyrhizi*, have a type I intron after codon 143 and the nucleotide substitution in this codon would prevent splicing of the intron, being lethal for the fungus. [12] Meanwhile other species have been identified with such an intron sequence directly after codon 143. [13] Since QoI-resistance via G143A is not possible for such "intron pathogens", some of these pathogens (e.g. *Alternaria solani*, *Pyrenophora teres*) have made the alternative, but less effective adaption by the F129L mutation.

The aims of this work were to sequence the CYTB gene to identify mutations, to develop a pyrosequencing assay that provides a rapid and quantitative detection of the F129L mutation in CYTB gene and to monitor mutations in the CYP51 and CYTB gene in *P. pachyrhizi* isolates from Brazil using pyrosequencing assay.

4.2 MATERIAL AND METHODS

4.2.1 Isolation of monouredinial *P. pachyrhizi* strains

Leaves infected with *P. pachyrhizi* were collected from Brazilian soybean fields in the 2012-2013 and 2013-2014 seasons. A total of 41 isolates of *P. pachyrhizi* were obtained from the States of Paraná and Mato Grosso by transferring spores from single uredia to healthy unifoliate leaves with an inoculation needle to soybean plants grown in the greenhouse. Among the 28 isolates from State of Paraná, 12 were from organic soybean production fields, where the control of soybean rust is done with copper and sulfur (DMI and QoI are not applied). The other isolates from Paraná and Mato Grosso were collected in conventional soybean production fields, where fungicides from the DMI and QoI groups are used for rust control.

The inoculum of monouredinial isolates was multiplied by transferring the spores to new leaves every three weeks by inoculation of a spore suspension in water with Tween (0.01%) to the abaxial surfaces of unifoliate leaves, using an airbrush (0.3 mm nozzle).

The leaves were kept in Petri dishes with water agar (1%) including streptomycin sulfate (30 mg L^{-1}) and kinetin (0.2 mg L^{-1}). Soybean leaves were incubated in the dark during

the first 24 hours after inoculation at room temperature and afterwards at 23°C with a photoperiod of 12 hours.

4.2.3 DNA and RNA extraction

Molecular analyses were conducted for monouredinial isolates of *P. pachyrhizi* from the 2012-2013 and 2013-2014 seasons.

DNA of *P. pachyrhizi* spores was extracted using the NucleoSpin DNA Plant II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the instructions of the manufacturer for CTAB based DNA extraction.

Total RNA of *P. pachyrhizi* spores was extracted and reverse transcribed to cDNA following the procedures described by Schmitz *et al.* [5]. This procedure was supported by Biology Molecular Laboratory of BASF SE (Limburgerhof, Germany).

4.2.4 Pyrosequencing for mutations in CYP51 gene

Pyrosequencing assays to detect and quantify CYP51 mutations were applied according to methodology described by Schmitz *et al.* [5] using the same PCR and sequencing primers and the same PCR and pyrosequencing conditions described by them. [5] The mutations F120L, Y131H/F, K142R, I145F and I475T were evaluated in 31 isolates.

4.2.5 Cytochrome *b* sequencing

The whole cytochrome *b* gene was sequenced according to the method described by Schmitz *et al.*[5], using the primer pair KES 1158 fw (5' CAGTAGCCTAAAGAAGGGTGTTAA 3') and KES 1159 rv (5' CCCGTTGAATATCTTGACATCTTAC 3') for amplification of the whole gene with the following PCR program for amplification: initial heating at 98°C for 15 s, 35 cycles at 98°C

for 10 s, 64°C for 5 s. and 72°C for 45 s, followed by a final amplification step at 72°C for 1 min. This procedure was supported by Biology Molecular Laboratory of BASF SE (Limburgerhof, Germany).

4.2.6 Pyrosequencing for F129L mutation in CYTB gene

Pyrosequencing assay was developed in the Biology Molecular Laboratory of BASF SE (Limburgerhof, Germany), using the Pyrosequencing Assay Design Software (Qiagen, Hilden, Germany). In a first step, the CYTB gene fragment, which contained the target sequence was amplified in PCR reaction using 12.5 µl of 2x Maxima Mastermix (Fermentas GmbH), 7.5 µl of bidestilled water, 2.5 µl of DNA and 1.25 µl of each primer: KES 498 (5' GGACACTAGTATGGGCGATTG 3') and KES 499 (5' Biotin – CATGTGAGGCGGTCTCATT 3'), under the following conditions: an initial heating step for 4 min at 95°C followed by 40 cycles with 15 s at 94°C, 30 s at 55°C and 15 s at 72°C and a final step of 5 min at 72°C. Every template was applied in duplicate.

The subsequent pyrosequencing reaction of codon 129 was performed using the specific sequencing primer: KES 500 (5' TTGTAATAATAGCGACAGC 3'). For single strand preparation, PCR products were immobilized on Streptavidin Sepharose Beads (GE Healthcare, Buckinghamshire, GB) and cleaned up with ethanol (70%), denatured with sodium hydroxide (0.2 M), washed in tris-acetate (10 mM) and released into a mixture of annealing buffer with the sequencing primer, implementing the Vacuum Prep Worktable (Qiagen), following the instructions of the manufacturer. Then, the samples were heated at 80°C for 3 min in an incubator and cooled to room temperature. Pyrosequencing was performed for sequence determination and allele quantification using PyroMark Gold Q96 Reagents (Qiagen) on a PSQ 96MA (Qiagen) machine as described by the manufacturers.

The mutation F129L was evaluated for 41 monouredinial isolates of *P. pachyrhizi.*

4.3 RESULTS

Most of *P. pachyrhizi* isolates (80%) showed the mutation F120L+Y131H in the CYP51 gene. In the State of Paraná, one of 19 isolates tested was wild type (no mutations in CYP51 gene) and others, including isolates from organic and conventional fields, showed mutations F120L+Y131H or Y131F+I475T (Table 1). In addition, a triple combination (F120L+Y131F+I475T) was found in two isolates from conventional fields in Paraná from 2013-2014 season (Table 1). In the State of Mato Grosso, all of the 12 isolates showed the mutation F120L+Y131H. The frequency of mutated alleles in CYP51 gene was between 16 and 57% (Table 1).

The F129L mutation was found in the CYTB gene sequencing in some isolates (Table 1), whereas no other mutations were detected, including G143A and G137R. The change of phenylalanine to leucine at position 129 in all isolates and samples tested results from the substitution of the first nucleotide of codon 129 "T" to "C" (TTT to CTT). The intron after codon 143 was present in all isolates tested (Fig 1).

Monouredinial isolates showed 0% (wild type) or approximately 100% of F129L mutation (F129L mutated isolate) (Table 1). Overall, the F129L mutation was detected in 21 of 41 isolates (51%, Table 2). From the State of Mato Grosso, 92% of strains carried the mutation (all from the 2013/14 season) and 32% from the State of Paraná (50% of total isolates from the organic field and 19% of the total isolates from the conventional field). From season 2012/13 to season 2013/14, the frequency of F129L strains in Parana increased from 9% to 47%.

Multiple resistance, i.e. resistance to both DMI and QoI fungicides, resulting from mutations in both target genes, was observed for seven of 19 isolates of Paraná and 11 of 12 isolates from Mato Grosso (Table 1).

4.4 DISCUSSION AND CONCLUSIONS

The F120L+Y131H and Y131F+I475T mutations in CYP51 gene found in this work, has been reported as the most frequent mutations occurring in *P. pachyrhizi* isolates from Brazil and were related to less sensitivity to DMI. [5] The mutations F120L+Y131H and Y131F+I475T were associated with ED_{50}-range of 0.06 to 10 mg L^{-1} and 1.4 to 10 mg L^{-1}, respectively. [5] Isolates showing triple-point mutations in CYP51, F120L+Y131F+I475T, are

reported for the first time for *P. pachyrhizi* and are rarely found in other plant pathogens and its impact on DMI sensitivity should be investigated.

In studies with *Candida albicans*, Sanglard *et al.* [14] assumed that the effects of mutations in combination in the CYP51 gene may be additive or possibly synergistic, so it is possible that the triple combination is associated with a higher level of resistance. The prevalence of the F120L+Y131H mutation and the appearance of isolates showing a triple combination in the second season assessed (2013-2014) suggests that these mutations might be related to the evolutionary adaptability of isolates, which may be associated with a lower fitness cost relative to other mutation. That is in agreement with the results of chapter 3 of this thesis that showed the mutation F120L+Y131H has a lower fitness cost than others or even no fitness cost and that should be investigated for triple mutations.

As described by Schmitz *et al.*, [5] the mutant alleles frequency in the CYP51 gene never was 100% but approximately 33 or 50%. In this work, the frequency ranged between 16 and 57%, indicating that more than one copy of the CYP51 gene exists in the genome of *P. pachyrhizi* and that mutations occur in some but not all copies.[5]

Just one of 31 isolates was wild type, i.e., no mutations tested in CYP51 gene were found, whereas in Schmitz *et al.* [5], with isolates collected in 2009-2010 season, 22% of isolates (20 of 88) were wild type. This means that from 2009-2010 to 2013-2014 season, the proportion of mutated isolates increased and currently most isolates have mutations related with less sensitivity to DMIs in the two states that are the largest soybean producers in Brazil (Mato Grosso and Paraná).

The findings of this work also revealed the occurrence of F129L mutation in the CYTB gene in *P. pachyrhizi* from soybean fields in Brazil. This is the first report of mutations in CYTB gene related to less sensitivity to QoI fungicides, not only in *P. pachyrhizi*, but in any rust species. In an earlier study of QoI sensitivity of *P. pachyrhizi*, with isolates from 2009 and 2010, no F129L or any other mutation in the CYTB gene was found. [5]

The F129L mutation has been reported in different plant pathogenic fungi, such as *A. solani*, [15] *Pyricularia grisea*, [16] *P. teres* [17-19] and *P. tritici-repentis*, [18,19] as a mechanism for decreased QoI sensitivity. In species where isolates with F129L and G143A have been found (*e.g. P. tritici-repentis, P. grisea*), a direct comparison of such mutants on the effect on QoI sensitivity showed that the F129L mutation leads to a much lower sensitivity decrease than the G143A mutation. [16,19] Glasshouse and field studies with *P. teres* have shown that the effect of the F129L mutation on the field performance of pyraclostrobin is rather limited and that efficacy was good even in fields with a high frequency of F129L in the population. [17,19] A

greater decrease in efficacy of QoIs has been described for F129L mutants of *A. solani.* [20] The effect of the F129L mutation on the sensitivity of *P. pachyrhizi* and on the efficacy of QoIs needs further investigation.

Our data show that the pyrosequencing assay is an appropriate method for rapid and reliable detection of F129L mutation in *P. pachyrhizi* isolates. As it is a quantitative method, it is a valuable tool to assess the mutation in populations, since different frequencies of the F129L mutation can be detected in field samples. All monouredinial isolates had an F129L frequency of either 0 or approximately 100%. A heteroplasmy of the CYTB gene, as has been reported in single cases for *Botrytis cinerea,* [21] has so far not been found in *P. pachyrhizi*. This shows that none or all of the CYTB genes in a monouredinial isolate are mutated. Since CYTB is mitochondrially inherited and therefore present in multiple copies in a cell, this finding is of importance for the interpretation of quantitative results of F129L detection in field samples (populations). In fact, that means that in a sample with 60% F129L, 60% of the strains making up this sample population carry the F129L and 40% are wild type.

The dynamic of the F129L mutation in *P. pachyrhizi* populations in Brazil and the impact of this mutation on field efficacy of QoI fungicides should be monitored in future seasons.

The significant frequency of mutated isolates in organic fields can be related to wind dispersion from conventional fields in the same region, especially because in southwest Paraná, there are normally two subsequent seasons of soybean when growers apply up to eight fungicide applications for rust control only in the second season because the disease occurs at the early vegetative stages. [22] However, in organic fields, there is no selection pressure of fungicides on the population and, depending on the fitness of mutated isolates related to wild type isolates, the mutated isolates could maintain or decrease in frequency and, consequently, the population of organic fields could be more sensitive than population from conventional fields. According to chapter 1 of this thesis, the EC_{50} to tebuconazole is higher for isolates from the conventional field than isolates from organic field and we showed that mutations in CYP51 gene are associated with a fitness cost (chapter 3). These results suggest that, in the organic field (no selection pressure of fungicide), the frequency of isolates with mutations in CYP51 gene decreased after many disease cycles and, consequently, the population was more sensitive to tebuconazole than the population from the conventional field. Since the results of chapter 3 indicated that the mutation F129L was not associated with fitness penalties, the frequency of mutated isolates can be stable even in the absence of fungicide. Therefore, there

was no difference between the EC_{50} values with azoxystrobin in populations from organic and conventional fields.

The relatively high frequency of mutated monouredinial isolates, especially the isolates with multiple resistance, indicates that the continued use of DMIs and QoIs to control soybean rust has led to the selection of mutated individuals. It is positively influenced by selection pressure by the fungicides on the one hand and negatively by any fitness costs associated with the mutation conferring resistance on the other hand. Therefore further investigations should be conducted to determine the impact of these mutations in the field.

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TABLE 1. Origin of Brazilian monouredinial isolates of *Phakopsora pachyrhizi* and the presence of mutations in cytochrome P450 14α-sterol demethylase (CYP51) and cytochrome *b* (CYTB) genes.

Isolate	Origin	CYP51		CYTB	
		Genotype	Mutant alleles %	Genotype	Mutant alleles %
3 [a]	Southwestern Paraná	NA [d]	NA	Wild type [c]	0
7 [a]	Southwestern Paraná	NA	NA	F129L	99
11 [a]	Southwestern Paraná	NA	NA	Wild type	0
12 [a]	Southwestern Paraná	Y131F+I475T	41	Wild type	0
14 [a]	Southwestern Paraná	Y131F+I475T	35	Wild type	0
52 [a]	East Central Paraná	NA	NA	Wild type	0
54 [a]	East Central Paraná	NA	NA	Wild type	0
57 [a]	East Central Paraná	Y131F+I475T	55	Wild type	0
61 [a]	East Central Paraná	NA	NA	Wild type	0
62 [a]	East Central Paraná	NA	NA	Wild type	0
63 [a]	East Central Paraná	F120L+Y131H	57	Wild type	0
21 [b]	Southwestern Paraná	NA	NA	F129L	98
22 [b]	Southwestern Paraná	F120L+Y131H	35	F129L	99
23 [b]	Southwestern Paraná	NA	NA	Wild type	0
28 [b]	Southwestern Paraná	F120L+Y131H	20	F129L	96
29 [b]	Southwestern Paraná	F120L+Y131H	50	F129L	100
40 [b]	Southwestern Paraná	F120L+Y131H	34	F129L	100
41 [b]	Southwestern Paraná	F120L+Y131H	32	Wild type	0
91 [b]	East Central Paraná	F120L+Y131H	33	Wild type	0
92 [b]	East Central Paraná	F120L+Y131H	37	Wild type	0
93 [b]	East Central Paraná	F120L+Y131H	21	F129L	96
94 [b]	East Central Paraná	F120L+Y131H	33	F129L	96
95 [b]	East Central Paraná	F120L+Y131H	37	F129L	90

96 [b]	East Central Paraná	F120L+Y131H	36	Wild type	0
97 [b]	East Central Paraná	F120L+Y131H	34	Wild type	0
98 [b]	East Central Paraná	F120L+Y131F+I475T	36	Wild type	0
99 [b]	East Central Paraná	F120L+Y131F+I475T	31	Wild type	0
100 [b]	East Central Paraná	Wild type [c]	0	Wild type	0
71 [b]	South Central Mato Grosso	F120L+Y131H	32	F129L	95
72 [b]	South Central Mato Grosso	F120L+Y131H	16	F129L	97
73 [b]	South Central Mato Grosso	F120L+Y131H	27	F129L	98
76 [b]	South Central Mato Grosso	F120L+Y131H	35	F129L	99
77 [b]	South Central Mato Grosso	F120L+Y131H	38	F129L	97
78 [b]	South Central Mato Grosso	F120L+Y131H	35	Wild type	0
81 [b]	Central Mato Grosso	NA	NA	F129L	99
82 [b]	Central Mato Grosso	F120L+Y131H	50	F129L	100
84 [b]	Central Mato Grosso	F120L+Y131H	39	F129L	93
85 [b]	Central Mato Grosso	F120L+Y131H	52	F129L	98
86 [b]	Central Mato Grosso	F120L+Y131H	33	F129L	99
87 [b]	Central Mato Grosso	F120L+Y131H	46	F129L	100
88 [b]	Central Mato Grosso	F120L+Y131H	34	F129L	100

[a] Season 2012-2013; [b] Season 2013-2014; [c] Wild type: mutation not detected; [d] NA: not assessed.

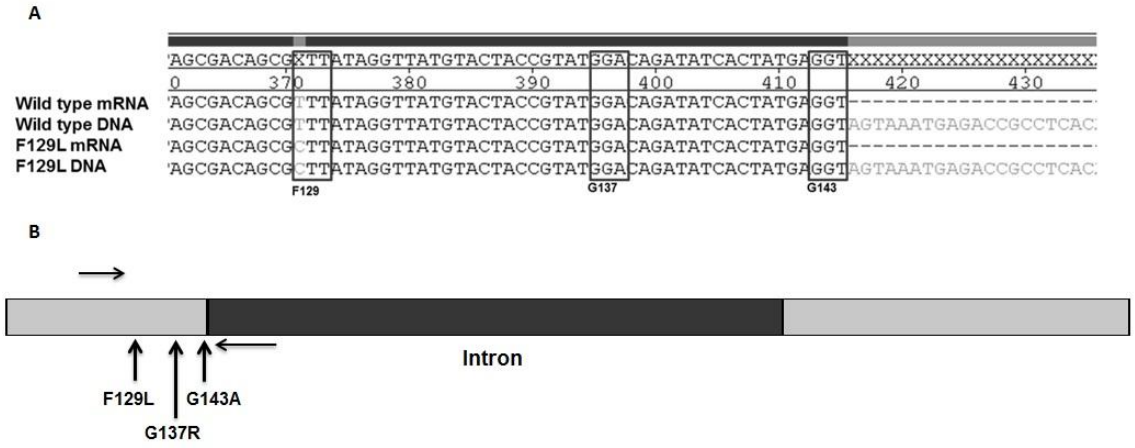

FIGURE 1. Gene sequencing of *Phakopsora pachyrhizi* cytochrome *b* and detection of the F129L mutation (A) and schematic structure showing the position of codons 129, 137 and 143 and intron (B).

(A) Nucleotide sequences are alignments of cDNA and DNA of wild type and isolates with F129L. The alignment shows fragments of the sequence containing codons at positions 129, 137 and 143. Only the mutation F129L was detected, but not any other e.g. at 137 or 143. Intron starts directly after codon 143 and was present in all sequenced isolates. (B) Schematic structure shows the cytochrome *b* gene (2524 bp) and the interruption by the intron (1336 bp), which is located directly after codon 143. Arrows indicate where primer was designed for the PCR amplification step of the pyrosequencing analysis.

5 CAPÍTULO III - COMPETITIVE ABILITY OF *Phakopsora pachyrhizi* ISOLATES ASSOCIATED WITH DMI AND QoI RESISTANCE

Soybean rust (*Phakopsora pachyrhizi*) in Brazil is mainly controlled with fungicide applications, including demethylation inhibitors (DMIs) and quinine outside inhibitors (QoIs). Less sensitive isolates to both, DMIs and QoIs have been reported, with mutations in the CYP51 and CYTB genes, respectively. There are no studies on fitness costs in isolates with mutations in CYP51 and CYTB available and the aim of this work was to compare the competitive ability between sensitive (wild type) isolates and isolates with lower DMI and QoI sensitivity. Spores of sensitive wild type isolates and isolates with different CYP51 and/or CYTB haplotypes were mixed and inoculated on detached soybean leaves. After three weeks, spores were harvested and used as inoculum for the next disease cycle. Frequency of relevant target-site mutations were followed up by the pyrosequencing method over four disease cycles. Isolates with lower DMI sensitivity and different CYP51 haplotypes had competitive disadvantages compared with sensitive and CYP51 wild type isolates. The isolate with the F129L mutation in the CYTB competed equally well with the QoI sensitive and CYTB wild type isolate under the conditions of this experiment. CYP51 and CYTB haplotypes were stable in all isolates over four disease cycles when cultivated alone.

Key-words: Asian soybean rust. Fungicide resistance. CYP51. CYTB. Fitness cost.

HABILIDADE COMPETITIVA DE ISOLADOS DE *Phakopsora pachyrhizi* ASSOCIADA COM A RESISTÊNCIA AOS IDMs E AOS IQes

A ferrugem-asiática da soja, causada por *Phakopsora pachyrhizi*, no Brasil, é controlada principalmente com o uso de fungicidas do grupo dos IDMs e dos IQes. Isolados menos sensíveis tanto aos IDMs quanto aos IQes tem sido relatados, apresentando mutações nos genes CYP51 e CYTB, respectivamente. Como não há estudos na literatura sobre custos adaptativos associados às mutações nos genes CYP51 e CYTB, o objetivo desse trabalho foi comparar a habilidade competitiva entre isolados sensíveis (sem mutações) e isolados com menor sensibilidade aos IDMs e aos IQes. Urediniósporos de isolados sensíveis (sem mutações) e isolados com diferentes haplótipos dos genes CYP51 e/ou CYTB foram misturados e inoculados em folhas destacadas de soja. Três semanas depois, os urediniósporos foram coletados e utilizados como inóculo para o próximo ciclo da doença. A frequência das mutações relevantes foi acompanhada pelo método do pirosequenciamento durante quatro ciclos da doença. Isolados com menor sensibilidade aos IDMs e diferentes haplótipos do gene CYP51 apresentaram desvantagens competitivas comparados com os isolados sensíveis. O isolado com a mutação F129L no gene CYTB competiu igualmente bem com o isolado sensível (tipo selvagem), sob as condições deste experimento. Os haplótipos dos genes CYP51 e CYTB foram estáveis em todos os isolados monouredíniais durante quatro ciclos da doença quando cultivados sozinhos.

Palavras-chave: Ferrugem-asiática. Resistência a fungicidas. CYP51. CYTB. Custo adaptativo.

5.1 INTRODUCTION

Asian soybean rust is a widely dispersed foliar disease caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd. (Ivancovich 2005; Pretorius et al. 2001; Schneider et al. 2005; Yorinori et al. 2005). The disease has potential to cause large reductions in soybean (*Glycine max*) yield and severe economic losses to soybean growers (Hartman et al. 1991; Yang et al. 1991; Yorinori et al. 2005).

The control of the disease in Brazil, where the environmental conditions are conducive to disease epidemics, is based on fungicides (Yorinori et al. 2005) and host-free period, during which farmers are restricted for planting soybean to delay the primary inoculum in the season (Seixas and Godoy 2007). Besides that, the use of resistant cultivars and early planting is recommended. Due to the genetic variability of the pathogen (Bonde et al. 2006; Freire et al. 2008) and hence its ability to overcome the resistance genes, the use of resistant cultivars has been recommended associated with the use of fungicides (Godoy 2012).

Sterol demethylation inhibitor (DMI) and quinone outside-inhibiting (QoI) fungicides are widely used for soybean rust control, especially in mixtures (Miles 2003, 2007; Scherm et al. 2009) in South America and Southern Africa. In central Brazil, where the environmental conditions can lead to early disease onset and high disease pressure, up to 12 applications of those fungicides might be needed (Godoy and Meyer 2014).

The intensive use of fungicides can select fungicide-resistant isolates and *P. pachyrhizi* isolates from Brazil less sensitive to both, DMIs and QoIs, have been reported by Schmitz et al. (2014), Klosowski et al. (2015) and the Fungicide Resistance Action Committee (FRAC 2015). Reduced efficiency of fungicides for soybean rust control in Brazil has been reported and has been associated with a lower DMI and QoI sensitivity (Godoy et al. 2014; Godoy et al. 2015).

Although there are some resistance mechanisms keeping the intracellular fungicide concentration below a critical level, such as increased drug efflux and metabolism, the most important and best-characterized resistance mechanism is the alteration of target site due to point mutations in most pathogens (Chen et al. 2012; Fernández-Ortuño et al. 2008). Additionally target-site overexpression has been identified in plant pathogens including *P. pachyrhizi*, as a resistance mechanism (Schmitz et al. 2014). QoI resistance is mainly related to mutations in the cytochrome *b* (CYTB) gene (Gisi et al. 2000) and F129L is the only mutation detected for *P. pachyrhizi* associated with QoI resistance. DMI resistance is caused

by different mechanisms, mainly mutations in the target site cytochrome P450 14α-sterol demethylase (CYP51), such as F120L, Y131F/H, K142R and I475T, overexpression of CYP51, both mechanisms reported for *P. pachyrhizi* (Schmitz et al. 2014) and enhanced efflux, reported for *Mycosphaerella graminicola* (Cools and Fraaije 2013).

Resistance mutations can disrupt or reduce the efficiency of important physiological and biochemical processes in the pathogen, leading to lower fitness. Fitness costs associated with fungicide resistance are often predicted but rarely reported (Zhan and McDonald 2013). Experimental evidence suggested that azole-resistant mutants were less fit than azole-sensitive strains assuming that they have little chance of survival in field conditions (Köller and Scheinpflug 1987) and the DMI resistance was associated with fitness penalties for *Cercospora beticola* (Karaoglanidis et al. 2001), *Penicillium expansum* (Karaoglanidis et al. 2011) and *Monilinia fructicola* (Chen et al. 2012), based on competition assays with sensitive isolates and features such as sporulation, pathogenicity and mycelium growth, whereas no fitness cost was observed for DMI-resistant isolates of *Pyrenophora teres* (Peever and Milgroom 1994). The association between QoI resistance and fitness penalties is not well elucidated. It has been suggested that the substitutions of resistant isolates affect the fitness of plant pathogens differentially (Fernández-Ortuño et al. 2008). Fitness costs of QoI resistance have been described earlier for *Botrytis cinerea* (Kim and Xiao 2011) and *Plasmopara viticola* (Toffolatti et al. 2007) and the F129L mutation, that occur in *P. pachyrhizi* isolates (Klosowski et al. 2015), was associated with fitness costs for *Alternaria solani*, based on competition assays between mutants and wild type isolates (Pasche and Gudmestad 2008).

For optimized resistance management strategies, it is of importance to characterize the fitness costs in mutated strains (Mikaberidze and McDonald 2015). Resistant isolates with lower fitness tend to have competitive disadvantages compared to sensitive isolates in the absence of the fungicide and thus reducing the total number of applications, alternating use with non-cross-resistant fungicides and use of mixtures of non-cross resistant fungicides can favor a decline of resistant strains that have a fitness deficit (Brent and Hollomon 2007, Mikaberidze and McDonald 2015). We investigated in a first approach if fitness costs are connected with mutations F120L, Y131F/H, K142R, I475T, in the CYP51, and/or F129L, in CYTB gene, in competition trials, where non-treated and wild type strains were mixed, inoculated, harvested and inoculated again over four disease cycles. This was done under optimal growth conditions and under stress conditions by using sublethal doses of the multi-site fungicide mancozeb. The stability of the mutations in the various isolates was examined for stability by cultivation of single strains on soybean leaves over four cycles.

5.2 MATERIALS AND METHODS

5.2.1 *P. pachyrhizi* isolates

Monouredinial isolates of *P. pachyrhizi* were obtained from the BASF SE (Limburgerhof, Germany) collection. The isolates with mutations in the CYP51 gene were collected in different regions of Brazil, in 2009-2010 season, and the mutations were previously characterized by Schmitz et al. (2014). The isolate with a mutation in the CYTB gene was from Brazil obtained in 2013-2014 season and the mutation F129L was characterized by the BASF SE group. The isolates are described in Table 1.

To multiply the inoculum, spore suspensions prepared in water with Tween (0.01%) were inoculated on the abaxial surface of unifoliate soybean leaves using an airbrush (0.3 mm nozzle). The leaves were kept in Petri dishes containing water agar (1%), with kinetin (0.2 mg liter^{-1}) and streptomycin sulfate (30 mg liter^{-1}). After inoculation, the leaves were kept in the dark for 24 hours at room temperature followed by 12 hours light at 23°C. Every 3 weeks the spores were transferred to new leaves.

5.2.2 DNA extraction

The DNA was extracted from urediniospores of seven *P. pachyrhizi* isolates (Table 1), that were stored in a freezer at -80 ºC, using the NucleoSpin DNA Plant II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the instructions of the manufacturer for CTAB-based DNA extraction.

5.2.3 Pyrosequencing assay

To confirm the presence and quantify the mutations in the isolates, the pyrosequencing assay was performed. The point mutations F120L, Y131F, Y131H, K142R

and I475T in CYP51 gene and F129L in CYTB gene were analyzed. Therefore, PCR amplifications and pyrosequencing assay were carried out using the primers described by Schmitz et al. (2014) and Klosowski et al. (2015) and following procedures and conditions previously published by them. Isolates and their mutations are described in Table 1. “S” means wild type for CYP51 and CYTB. “M” means mutation in CYP51 and/or CYTB. M1, M2, M3 and M4 describe different CYP51 and/or CYTB haplotypes. A detailed description of isolates and their mutations follows in the Table 1.

5.2.4 Stress caused by multi-site fungicide.

Mancozeb pretreated leaves were used in the fitness assays to verify the behavior of *P. pachyrhizi* isolates under stress conditions caused by the multi-site fungicide. Therefore, mancozeb was sprayed on soybean plants one day before inoculation at 50 mg l^{-1}, which was determined in a previous sensitivity test as a concentration causing 39.8% inhibition (data not shown).

5.2.5 Competition assay

5.2.5.1 Mutations in CYP51 gene

The suspensions of spores were prepared in water with Tween (0.01%) for each isolate and were adjusted to 2.5 x 10^4 urediniospores mL^{-1}. Mixtures of the sensitive isolate (“8”) and mutated isolates (“72”, “62”, “63”, “27” and “28”) were made as follows: 20%S + 80%M1, 20%S + 80%M2, 20%S + 80%M3, according to the designations in Table 1 and Figure 1. For the mutations M2 and M3, a mixture of two isolates (“62” and “63”; “27” and “28”, respectively) were used (Table 1). Since the frequencies of the relevant CYP51 mutations in *P. pachyrhizi* were about 30% and 50% (Schmitz et al. 2014), mixtures with the proportion 50% of sensitive isolate + 50% of mutant isolates were not used because the detection limit of pyrosequencing assay is about 5%. Therefore, about 80% of mutated

isolates in the mixture was targeted for a reliable monitoring of the frequency of mutations over the disease cycles.

The spore suspension was inoculated using the same procedure as for inoculum multiplication described earlier. For each mixture, six non-treated leaves and six mancozeb pretreated leaves were inoculated.

After 21 days of incubation, the sporulating lesions were placed in 5 mL of water with Tween (0.01%) and shaken to release the spores. The resulting suspension was used to inoculate new six non-treated leaves and six mancozeb pretreated leaves (6 mL), starting a new disease cycle, and to extract DNA (2 mL) for subsequent pyrosequencing assay. The procedure was made after every disease cycle and the experiment was terminated after four cycles.

5.2.5.2 Mutation in CYTB gene.

This experiment involved three isolates, “8”, “GWH-B” and “72”. Mixtures were made as follows: 50%S + 50%M4, 20%S + 80%M4, 50%M1 + 50%M4, 20%M1 + 80%M4, as designated in Table 1 and Figure 1.

The procedures of this experiment were the same as described for competition assay for mutations in CYP51 gene. In this case, the pyrosequencing always showed 100% of F129L frequency in CYTB gene of *P. pachyrhizi* DNA (Klosowski et al. 2015) and therefore it was possible to use the proportions 50% of sensitive isolate + 50% of mutated isolate and 20% of sensitive isolate + 80% of mutant isolate, without compromising the quantification of the mutation in the mixture.

5.2.5.3 Quantification of the resistance allele using pyrosequencing.

To quantify the frequency of mutations in the isolates and mixtures, the pyrosequencing assay was done at the outset of the experiment and after every disease cycle, following the procedures described by Schmitz et al. (2014) and Klosowski et al. (2015).

5.2.6 Stability of the mutations

To determine the stability of mutations in isolates during the cycles, suspensions of spores were made in water with Tween (0.01%) for each isolate and they were adjusted to 2.5 x 10^4 urediniospores mL^{-1}. Single isolates were inoculated on four non-treated leaves and four mancozeb pretreated leaves. The same procedures for spore transfer and pyrosequencing analysis described above were made after every cycle and the experiment was completed after four cycles. The assay was performed twice.

5.2.7 Data analysis

In the competition assays, the frequency of resistant isolates in the last cycle was compared with the initial frequency by the pairwise Student's *t* test. The same test was used to determine the stability of the mutations, comparing the frequency of mutations for single isolates in the last cycle with initial frequency. The data analysis was performed using the statistical software R (R Development Core Team, Vienna, Austria).

5.3 RESULTS

5.3.1 Competition assay

5.3.1.1 Mutations in CYP51 gene

The frequency of isolates with CYP51 mutations decreased in the mixtures with wild type isolate after four disease cycles ($p \leq 0.01$) both on non-treated leaves and mancozeb pretreated leaves (Fig. 2).

The decrease was higher for the Y131F+K142R and Y131F+I475T mutations than for the F120L+Y131H mutation (Fig. 2). For the Y131F+K142R mutation, the frequency of resistant isolates declined from 27% to 8% both on non-treated leaves and mancozeb-treated leaves. For the Y131F+I475T mutation, the proportion declined from 26% to 12% on non-treated leaves and to 8% on mancozeb-treated leaves. For the F120L+Y131H mutation, the frequency decreased from 17% to 12% and 11%, on non-treated and mancozeb-pretreated leaves, respectively.

5.3.1.2 Mutation in CYTB gene.

The frequency of isolate with F129L mutation,"GWH-B", in the mixture with sensitive isolate "8" after four disease cycles was not different from the initial frequency ($p \leq 0.01$) both on non-treated and mancozeb-pretreated leaves. In the mixture of isolates "GWH-B" and "72", the frequency of the first increased during the four disease cycles both in the 50%M1 + 50%M4 and in the 20%M1 + 80%M4 proportions and the results were similar for non-treated and treated leaves (Fig. 3).

5.3.2 Stability of the mutations.

The proportion of mutations in CYP51 and CYTB genes was stable during four disease cycles for all isolates of *P. pachyrhizi*, both on non-treated leaves and mancozeb-pretreated leaves (Fig. 4).

5.4 DISCUSSION

Isolates with lower DMI sensitivity and three different CYP51 haplotypes had competitive disadvantages compared with the sensitive CYP51 wild type isolate. The isolate

with the F129L mutation in CYTB competed equally well with the sensitive, CYTB wild type isolate under the conditions of this experiment.

The use of multi-site fungicide mancozeb did not have effect on the dynamics of competition among wild type isolate and isolates with mutation in CYP51 and CYTB genes. Although lower disease severity has been observed on treated leaves compared to non-treated leaves (data not shown), the sensitivity of wild type and mutated isolates to fungicide mancozeb seems to be similar because the frequency of isolates was the same in the absence of fungicide.

A more significant competitive disadvantage was observed for CYP51 haplotypes Y131F + I475T and Y131F + K142R than for F120L+Y131H. According to Schmitz et al. (2014), the occurrence of Y131F + I475T and Y131F + K142R combinations is related to higher ED_{50} values, whereas a larger range of ED_{50} values was observed in the presence of the F120L+Y131H combination, ranging from 0.06 mg L^{-1} to 10.0 mg L^{-1}. Similar results were found for *C. beticola*, which showed the frequency of the resistant isolate decreased significantly after four disease cycles in pairs with DMI-resistant and sensitive isolates in the greenhouse. Moreover, in the field, results showed that at the end of the growing period, the frequency of the resistant isolates had decreased slightly ($p \leq 0.05$) (Karaoglanidis et al. 2001). The same tendency was found for *M. fructicola* after nine transfer cycles on acidified potato dextrose agar (Lichtemberg 2015). The frequency of resistant isolates decreased in the absence of fungicide in all of these experiments but, even after many pathogen cycles, they have not been eliminated from the population.

Other studies involving other pathogens, such as *M. fructicola*, *Alternaria alternata* and *P. teres* that evaluated parameters related to fitness, such as mycelial growth rate, latent period, spore production, spore germination and pathogenicity, showed conflicting results about the relationship between resistance to DMIs and fitness costs (Chen et al. 2012; Karaoglanidis et al. 2011; Peever and Milgroom, 1994).

The competitive disadvantage of isolates with CYP51 mutations to sensitive isolates with CYP51 wild type in *P. pachyrhizi* might be used for resistance management strategies. Selection of resistant isolates is determined by selection pressure of the fungicide on the one hand and by fitness costs connected with resistance on the other hand. Tools that reduce the selection pressure such as limitation of number of applications, alternation and mixing with different modes of action should be implemented in disease control strategies. Monitoring of DMI sensitivity is essential to follow up the usefulness of these strategies. Since FRAC reports the DMI sensitivity situation as more or less stable (FRAC 2015), the current

resistance management strategies seem to be effective in preventing a further shift of the population to lower DMI sensitivities.

There were no fitness costs detectable for the F129L mutation in CYTB versus the CYTB wild type. The newest internal monitoring data indicate moderate to high levels of F129L in the Brazilian soybean rust population (data not shown), which indicates a relatively rapid increase of this mutation. However, our studies on F129L fitness costs were performed with only one mutated isolate. Different other properties, independent of the F129L mutation, might have influenced this outcome. Therefore, more extensive competition studies, involving a larger number of resistant and sensitive isolates might be addressed. Properties such as stability of spores, temperature tolerance, incubation period, virulence on other hosts could also be valuable objectives of future fitness studies.

The mutations both in CYP51 and CYTB gene were stable in isolates over four disease cycles. In addition to the biological meaning that the mutation is transmitted to the next generations, this finding is important to confirm that the pyrosequencing method is a reliable method to quantify mutations.

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TABLE 1. Isolates of *Phakopsora pachyrhizi* of Brazil, season 2009-2010, and their mutations in cytochrome P450 14α-sterol demethylase (CYP51) and cytochrome *b* (CYTB) genes.

Isolate	Location	Season	Mutation		Designation
			CYP51 gene	CYTB gene	
8[a]	Goiás	2009-2010	Wild type	Wild type	S[c]
72[a]	Paraná	2009-2010	F120L+Y131H	Wild type	M1[d]
62[a]	Goiás	2009-2010	Y131F+K142R	Wild type	M2 [d]
63[a]	Goiás	2009-2010	Y131F+K142R	Wild type	M2 [d]
27[a]	Goiás	2009-2010	Y131F+I475T	Wild type	M3 [d]
28[a]	Goiás	2009-2010	Y131F+I475T	Wild type	M3 [d]
GWH-B[b]	São Paulo	2013-2014	F120L+Y131H	F129L	M4 [d]

All isolates belong to the BASF SE collection. [a] Isolates with mutations described by Schmitz et al. (2014); [b] Isolate with mutations characterized by BASF SE group; [c] S = sensitive isolate; [d] M1, M2, M3 and M4 = different haplotypes with mutations in CYP51 and/or CYTB genes.

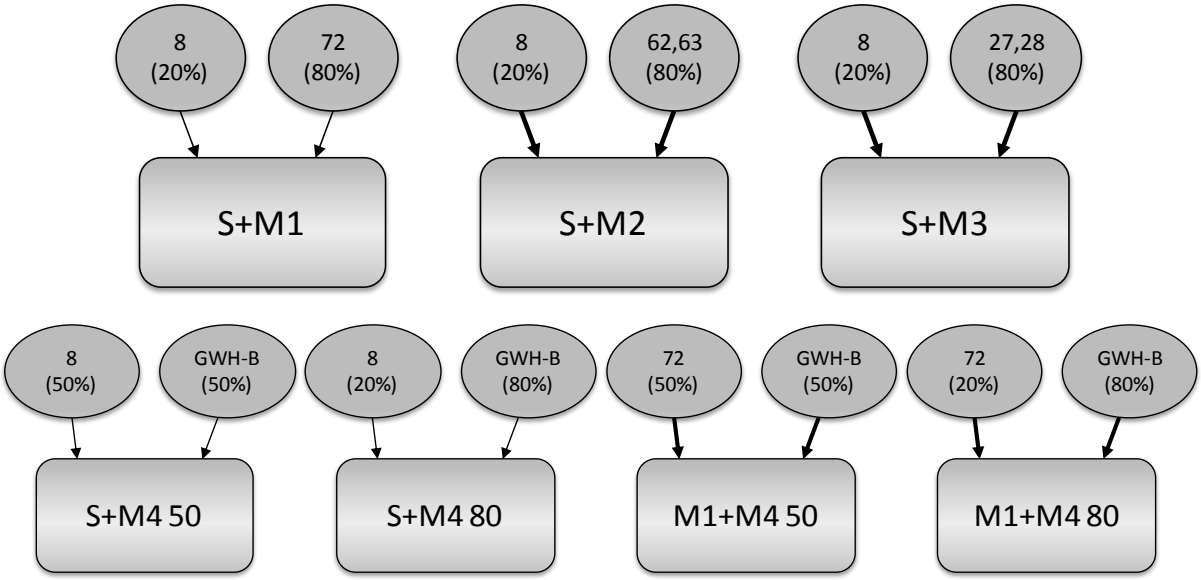

FIGURE 1. Mixtures between the sensitive isolate (S = wild type) and mutated isolates (M1, M2, M3 and M4) in the cytochrome P450 14α-sterol demethylase (CYP51) and cytochrome *b* (CYTB) genes of *Phakopsora pachyrhizi* prepared for the competition assays on detached leaves of soybean (*Glycine max*). The proportions used in the suspensions of urediniospores were 50% of sensitive isolate + 50% of mutated isolates and/or 20% of sensitive isolate + 80% of mutated isolates

S = sensitive isolate (wild type) = 8; M1 = mutated isolate in CYP51 gene (F120L+Y131H) = 72; M2 = mutated isolate in CYP51 gene (Y131F+K142R) = 62 and 63; M3 = mutated isolate in CYP51 gene (Y131F+I475T) = 27 and 28; M4 = mutated isolate in CYP51 (F120L+Y131H) and CYTB genes (F129L) = GWH-B.

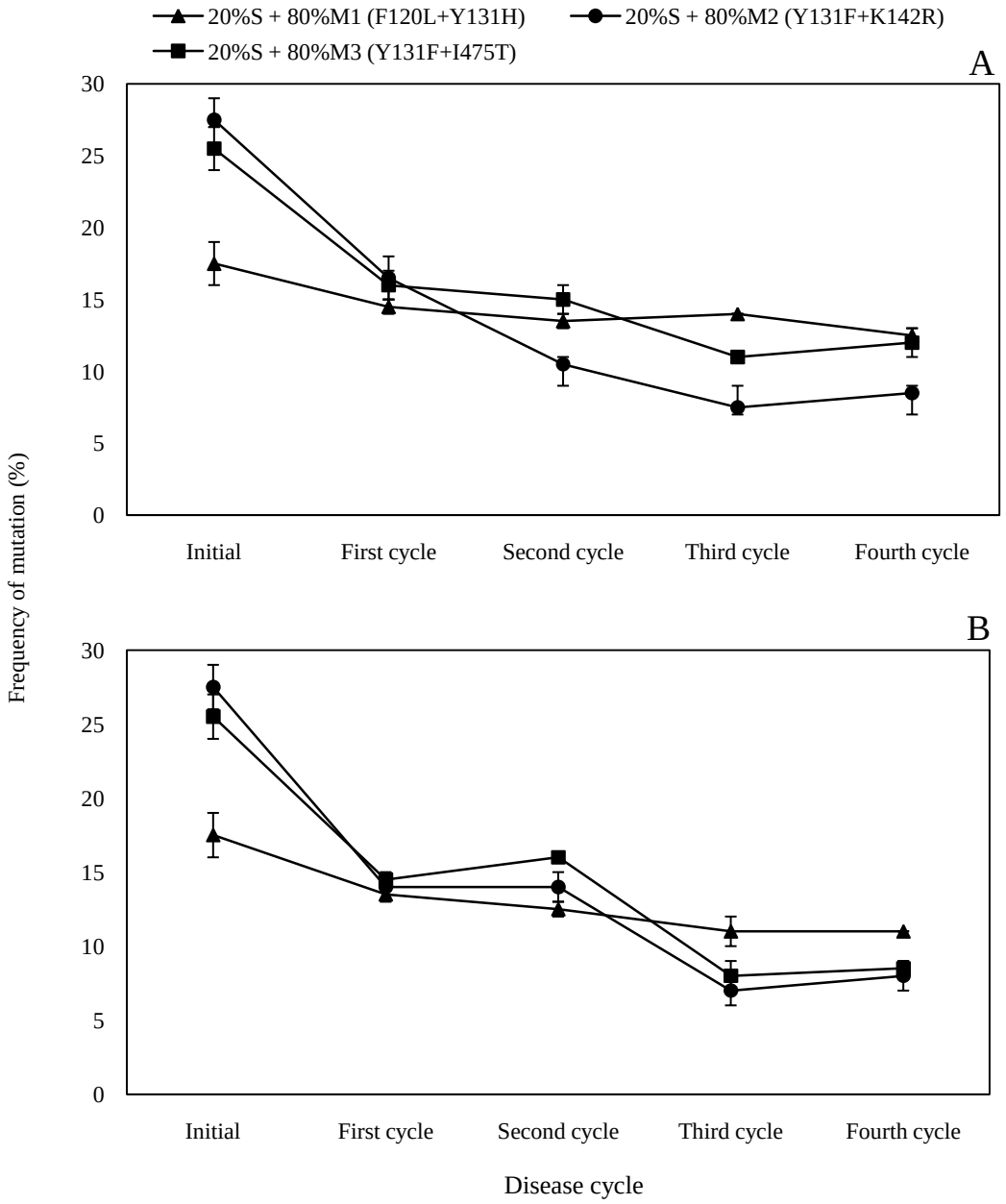

FIGURE 2. Frequency of mutations in cytochrome P450 14α-sterol demethylase (CYP51) gene in competition assays between sensitive (S=wild type) and mutated isolates (M1=F120L+Y131H, M2=Y131F+K142R and M3=Y131F+I475T) of *Phakopsora pachyrhizi* during four disease cycles on non-treated (A) and mancozeb-pretreated-soybean leaves (B). Vertical lines indicate the standard error of the mean.

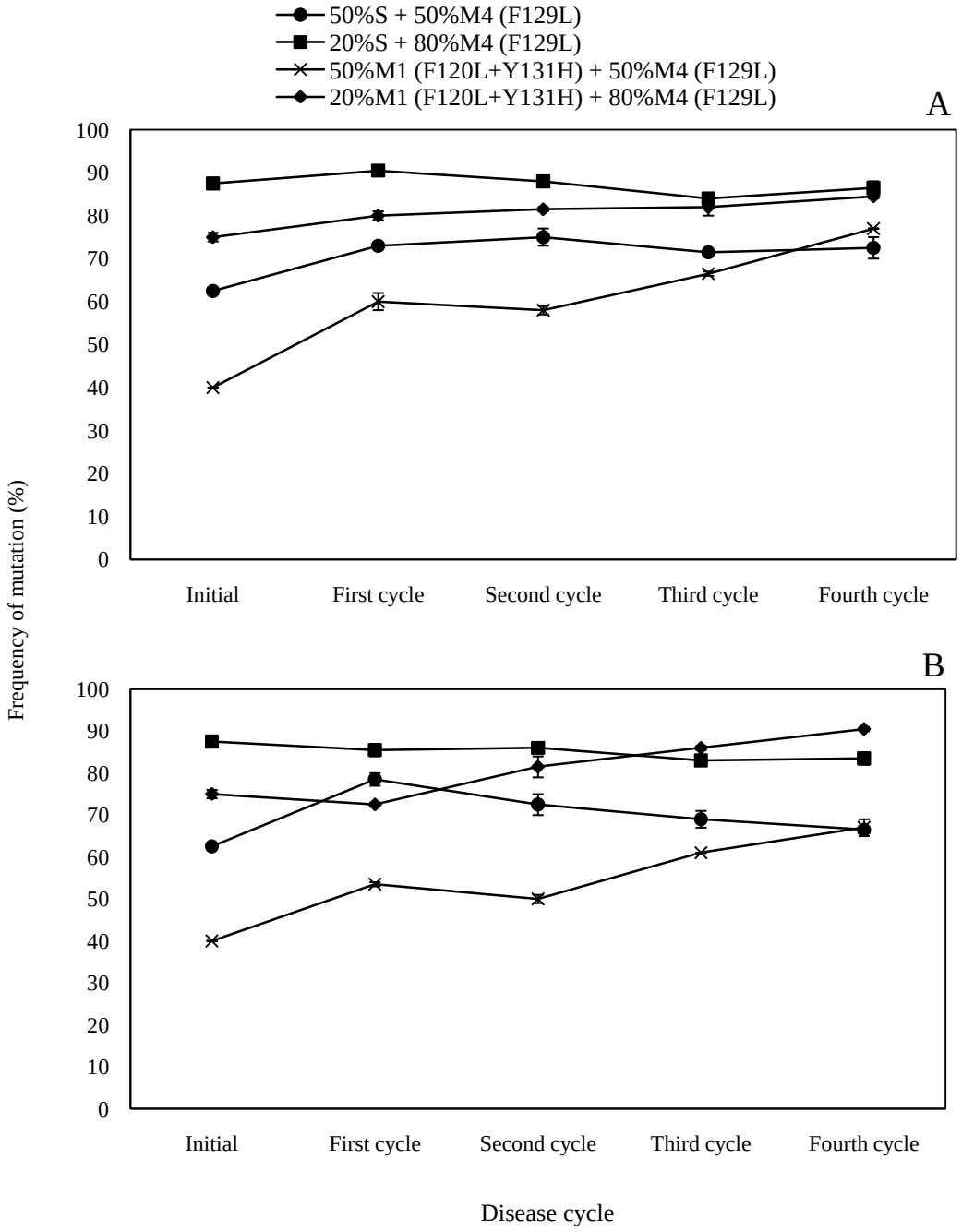

FIGURE 3. Frequency of mutations in cytochrome *b* (CYTB) gene in competition assays between sensitive (S=wild type) and mutated isolates (M1=F120L+Y131H in cytochrome P450 14α-sterol demethylase gene (CYP51) and F129L in CYTB gene and M4=F120L+Y131H in CYP51) of *Phakopsora pachyrhizi* during four disease cycles on non-treated (A) and mancozeb-pretreated-soybean leaves (B). Vertical lines indicate the standard error of the mean.

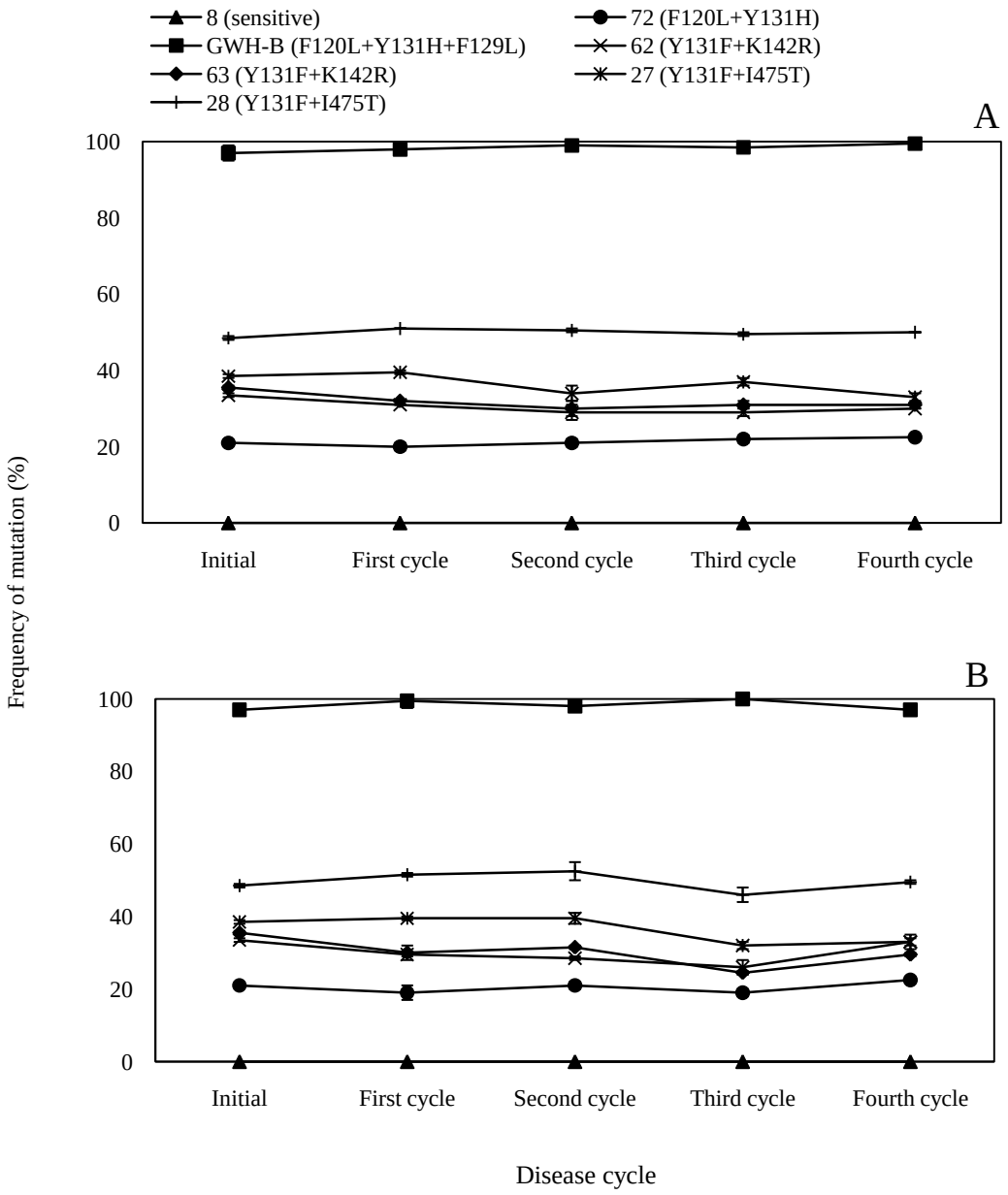

FIGURE 4. Frequency of mutations in cytochrome *b* (CYTB) (isolate GWH-B) and in cytochrome P450 14α-sterol demethylase (CYP51) gene (other isolates) in isolates of *Phakopsora pachyrhizi* during four disease cycles on non-treated (A) and mancozeb-pretreated-soybean leaves (B). Vertical lines indicate the standard error of the mean. Values shown are means from two independent experiments.

6 CONCLUSÕES GERAIS

Isolados de *P. pachyrhizi* provenientes de áreas de produção de soja convencional se mostraram menos sensíveis ao tebuconazol (IDM) e menos adaptados, com base nos parâmetros monocíclicos, do que isolados provenientes de área de produção de soja orgânica, sem aplicação de fungicidas. Este resultado indica que o uso de fungicidas na área de produção de soja convencional selecionou isolados menos sensíveis e que a perda de sensibilidade pode estar associada a custos adaptativos.

Mais de 96% dos isolados analisados apresentaram mutações no gene CYP51, que estão relacionadas à menor sensibilidade aos IDMs, e a combinação F120L+Y131H prevaleceu, especialmente na segunda safra (2013-2014), quando também foi detectada pela primeira vez uma mutação tripla (F120L+Y131F+I475T) no gene CYP51 de *P. pachyrhizi*, indicando que estas mutações podem estar ligadas à adaptação evolutiva dos isolados no campo.

A mutação F129L no gene CYTB foi relatada pela primeira vez para *P. pachyrhizi* e foi detectada em mais da metade dos isolados analisados, aumentando consideravelmente da safra 2012-2013 (9%) para a safra 2013-2014 (47%). O pirosequenciamento se revelou um método rápido para detectá-la e quantificá-la.

Aproximadamente 58% dos isolados apresentaram mutações em ambos os genes (CYP51 e CYTB), caracterizando resistência múltipla.

Os genótipos de *P. pachyrhizi* contendo mutações nos genes CYP51 e CYTB permaneceram estáveis durante quatro ciclos da ferrugem-asiática, na ausência de fungicidas e em folhas tratadas com um fungicida multi-sítio.

Isolados com mutações no gene CYP51 tiveram desvantagens competitivas quando co-cultivados com isolado sensível (sem mutação), sugerindo que estas mutações estão associadas a custos adaptativos, como também ficou sugerido no ensaio do monociclo. O isolado com a mutação F129L no gene CYTB competiu igualmente bem com o isolado sensível (sem mutação), no entanto estudos envolvendo um maior número de isolados devem ser realizados para se concluir sobre a relação desta mutação com a adaptabilidade.

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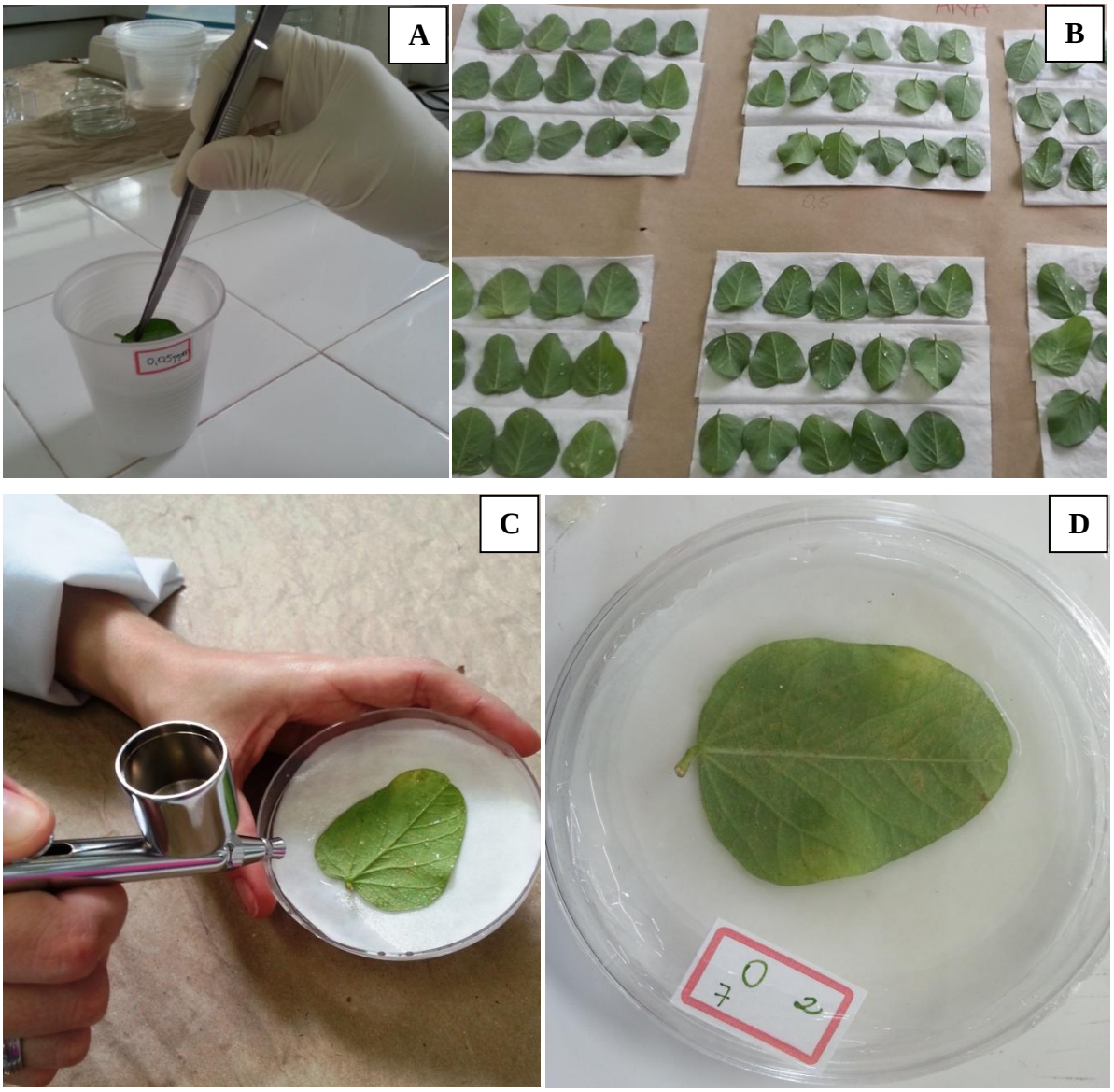
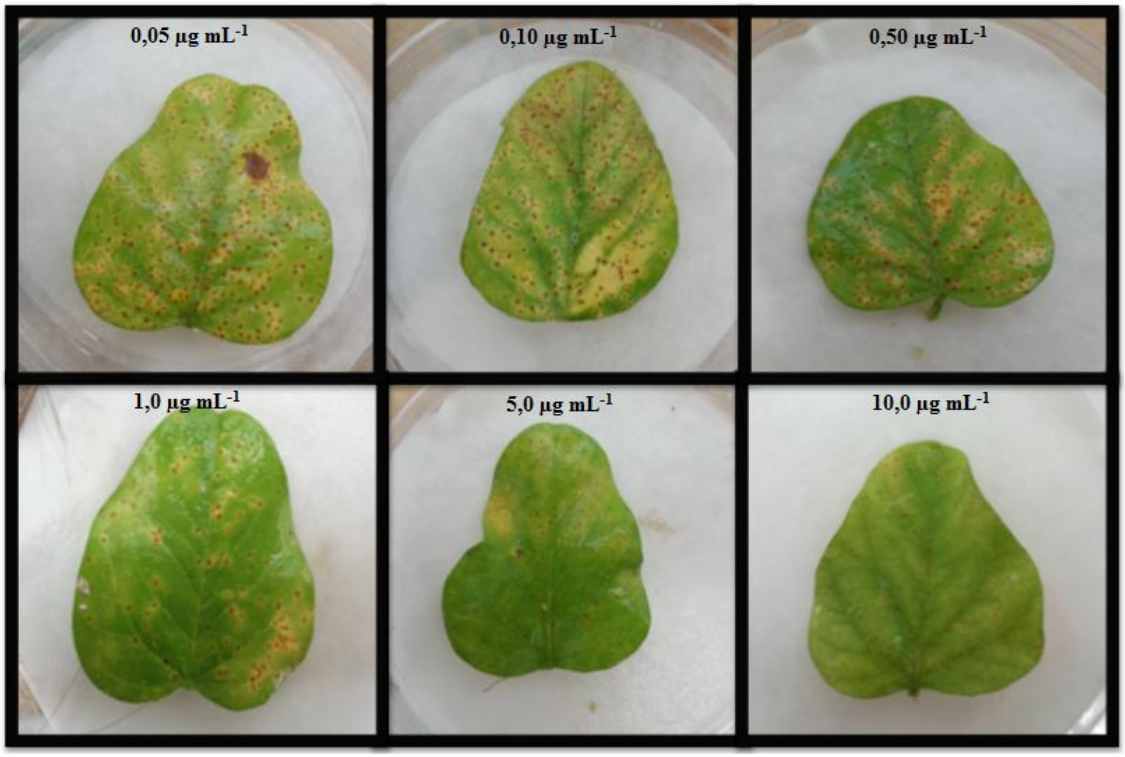
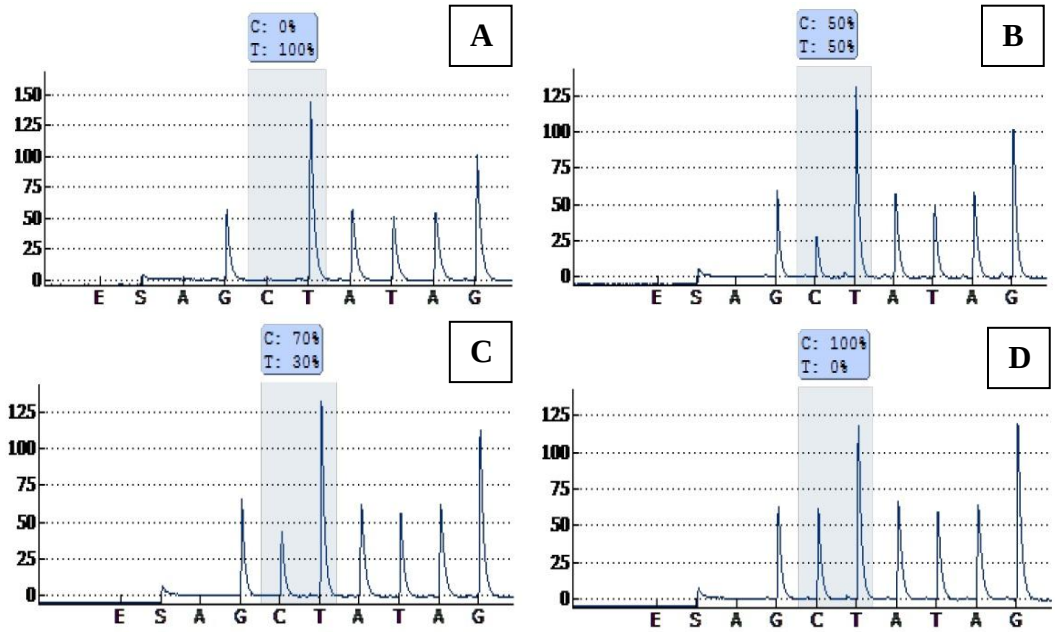
ANEXOS



ANEXO 1. Passos para instalação dos ensaios de sensibilidade de *Phakopsora pachyrhizi* a fungicidas em folhas unifolioladas destacadas de soja (*Glycine max*). Tratamento das folhas em soluções de fungicida (A); secagem das folhas (B); inoculação de folhas com suspensão de urediniósporos de *P. pachyrhizi* (C); folhas tratadas com fungicida e inoculadas com *P. pachyrhizi* incubadas em placas de Petri (D). Fotos: O autor (2013)



ANEXO 2. Resultado do ensaio de sensibilidade de *Phakopsora pachyrhizi* ao tebuzonazol em folhas unifolioladas destacadas de soja (*Glycine Max*). Folhas tratadas com diferentes concentrações de tebuconazol 15 dias após a inoculação com urediniósporos de *P. pachyrhizi*. Fotos: O autor (2013)



ANEXO 3. Pirogramas gerados pelo método do pirosequenciamento mostrando a detecção quantitativa da mutação F129L no gene do citrocromo *b* (CYTB) de isolados monouredíniais (A e D) e de mistura entre isolados mutados e não mutados (B e C) de *Phakopsora pachyrhizi* do Brasil. O programa de avaliação quantitativa calculou a porcentagem das bases alélicas C e T. A enzima (E) e o substrato (S) contendo APS e luciferina são adicionados antes da primeira adição de nucleótido. Fonte: O autor (2015)