UNIVERSIDADE FEDERAL DO PARANÁ

MÔNICA ANGHINONI MÜLLER

# RESISTÊNCIA MÚLTIPLA, SELEÇÃO À CAMPO E ESTABILIDADE DAS MUTAÇÕES EM ISOLADOS DE *Phakopsora pachyrhizi* AOS FUNGICIDAS IDM, IQe E ISDH

CURITIBA

2019

MÔNICA ANGHINONI MÜLLER

# RESISTÊNCIA MÚLTIPLA, SELEÇÃO À CAMPO E ESTABILIDADE DAS MUTAÇÕES EM ISOLADOS DE *Phakopsora pachyrhizi* AOS FUNGICIDAS IDM, IQe E ISDH

Tese apresentada ao Programa de Pós-Graduação em Agronomia – Produção Vegetal, Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutor em Ciências.

Orientadora: Drª Louise Larissa May De Mio.

CURITIBA

2019

M958r

Müller, Mônica Anghinoni
Resistência múltipla, seleção à campo e estabilidade das mutações em isolados de Phakopsora pachyrhizi aos fungicidas IDM, IQe e ISDH / Mônica Anghinoni Müller. - Curitiba, 2019.
72 p.: il.

Tese (Doutorado) - Universidade Federal do Paraná. Setor de Ciências Agrárias, Programa de Pós-Graduação em Agronomia - (Produção Vegetal).
Orientadora: Louise Larissa May De Mio

1. Soja. 2. Soja - doenças e pragas. 3. Fungicidas. 4. Mutação (Biologia). I. Mio, Louise Larissa May De (Orientadora). II. Título. III. Universidade federal do Paraná.

CDU 633.34:632.952

Sistema de Bibliotecas/UFPR, Biblioteca de Ciências Agrárias
Paula Carina de Araújo - CRB9/1562

MINISTÉRIO DA EDUCAÇÃO
SETOR SETOR DE CIENCIAS AGRARIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO AGRONOMIA
(PRODUÇÃO VEGETAL) - 40001016031P6

# TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MÔNICA ANGHINONI MÜLLER** intitulada: **RESISTÊNCIA MÚLTIPLA, SELEÇÃO À CAMPO E ESTABILIDADE DAS MUTAÇÕES EM ISOLADOS DE *Phakopsora pachyrhizi* AOS FUNGICIDAS IDM, IQe E ISDH**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 25 de Fevereiro de 2019.

LOUISE LARISSA MAY-DE-MIO
Presidente da Banca Examinadora

CHIRLEI GLIENKE
Avaliador Externo (UFPR)

KELLY SIMÕES
Avaliador Externo (BASF)

ANA CLAUDIA KLOSOWSKI
Avaliador Externo (BAYER, AG)

WAGNER VICENTE PEREIRA
Avaliador Externo (UFPR)

Dedico esta tese aos meus pais Valmor e Melania, à minha irmã Camila e à minha avó Zelinda.

## AGRADECIMENTOS

Agradeço à Deus primeiramente, pela dádiva da vida.

À minha base que são meus pais Melania e Valmor, e minha irmã Camila, vocês são tudo para mim, assim como à minha avó Zelinda e à toda minha família que são um bem precioso.

Ao Felipe Fuchs, que me apoiou e foi paciente mesmo diante de todas as minhas ausências.

À minha orientadora Louise Larissa May De Mio que mesmo sem me conhecer confiou a mim uma vaga de doutorado, e me despertou admiração por sua busca incessante por possibilidades, que abre portas e nos permite traçar caminhos mais brilhantes do que podíamos esperar, sou muito grata.

Aos mestres que compuseram minha educação desde o ensino básico, um pouco do ensino de cada um foi necessário durante o doutorado, principalmente do Prof. José Renato Stangarlin que me inspirou para a seguir os caminhos da fitopatologia.

Aos amigos queridos, os antigos e os novos, vocês sempre serão essenciais para que eu seja feliz na caminhada que escolher.

A todos aqueles que fazem ou fizeram parte do time Lemid, professores, técnicos, estagiários, colegas e amigos, foi muito importante o apoio e troca de conhecimento que tivemos desde o início, e foi muito bom compartilhar com vocês as conquistas do dia a dia.

Aos que fizeram parte do período de doutoramento sanduíche, em especial ao pesquisador Gerd Stammler e toda sua equipe, Angelika Hawlik, Birgit Wieja, Alexandra Rehfus, Gabriele Berthold, Kerstin Röschl e Anna Huf, pelo auxílio pessoal e profissional de grande competência, aos pesquisadores Kelly Simões e Fernando Gava e todo o time envolvido pelo treinamento e envio de amostras.

Ao professor Sérgio Mazaro e sua equipe de orientados que me auxiliaram sem medir esforços para treinamento e análises na UTFPR.

À professora Maristella Dalla Pria e equipe de técnicos e orientados e à equipe da Fazenda Escola Capão da onça- UEPG, pela disponibilidade e auxílio.

À Universidade Federal do Paraná e ao Curso de Pós Graduação em Produção Vegetal, aos professores e funcionários, em especial à secretária Lucimara Antunes por tamanha eficiência.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos e concessão de bolsa no exterior, que valoriza o ensino e a pesquisa em um momento de delicado cenário político no país.

*“Ame a vida que você tem enquanto cria a vida dos seus sonhos”.*

*Hal Elrod*

## RESUMO

O Brasil é o segundo maior produtor de soja do mundo, representando um terço da produção mundial. A ferrugem-asiática da soja (FAS) causada pelo fungo *Phakopsora pachyrhizi* é a principal doença que acomete a cultura da soja na América Latina, por ocasionar severas perdas de produtividade em decorrência da desfolha precoce. O controle da doença está baseado principalmente no controle cultural e químico, pela implantação dos métodos de vazio sanitário, calendarização de semeadura e aplicação de fungicidas. Apesar disto, a eficiência dos fungicidas a campo vem sendo reduzida pela seleção de isolados menos sensíveis aos fungicidas, resultante de mutações pontuais nos genes alvo dos fungicidas. Os principais fungicidas para controle da doença pertencem aos grupos químicos: inibidores da desmetilação (IDMs), inibidores da quinona externa (IQes), inibidores da succinato desidrogenase (ISDHs) e os multissítios. Para elucidar o que vem acontecendo no campo, foram delineados estudos visando: i) Avaliar resistência múltipla aos fungicidas IDM, IQe e ISDH em isolados monouredíniais e populacionais através de ensaios de sensibilidade aos fungicidas ($CE_{50}$) e detecção de mutações pontuais nos genes citocromo P450 lanosterol C-14α-demethylase (*CYP51*), citocromo *b* (*CYTB*) e succinato desidrogenase (*SDH)*, provenientes dos estados brasileiros, Paraná, Rondônia, Mato Grosso e São Paulo; ii) Verificar a seleção de indivíduos a campo a partir de aplicações sequenciais de fungicidas comerciais dos grupos químicos IDM, IQe e ISDH, em diferentes municípios do estado de São Paulo e Paraná; iii) Verificar se a ausência de aplicação de fungicidas durante 50 ciclos da doença acarreta perda das mutações nos genes *CYP51* e *CYTB* e se a aplicação de diferentes doses de protioconazol (IDM) aumenta a porcentagem de mutação no gene *CYP51*. Como resultados foi obtido que a mutação combinada F120L+Y131H que confere menor sensibilidade aos DMIs, prevaleceu em todas as localidades analisadas e se mostrou mais relacionada com a menor sensibilidade de protioconazol do que demais mutações pontuais encontradas no gene *CYP51*, que também foram menos frequentes e mais comumente encontradas no estado de São Paulo. Dos isolados analisados, 90% apresentaram mutação F129L, que confere menor sensibilidade aos IQes, ocorrendo em todas as regiões analisadas, porém com menor ocorrência no estado de São Paulo. Indicando menor pressão de seleção de fungicidas. Isolados com a mutação C-I86F conferiram menor sensibilidade aos fungicidas SDHIs e apesar de ainda pouco frequentes foram detectados em Rondônia, Paraná e São Paulo, demonstrando a rápida disseminação ou seleção destes genótipos a campo. Isolado monouredinial apresentando múltipla resistência nos genes alvo dos fungicidas IDM, IQe e ISDH foi detectado e sua disseminação e impacto deve ser futuramente avaliado. Quatro aplicações do mesmo fungicida comercial a campo é capaz de selecionar isolados mutados para os genes *CYP51*, *CYTB* e *SDH* em apenas um ciclo da cultura. Cinquenta ciclos da doença na ausência de fungicidas não ocasionaram perda de mutações nos genes *CYP51* e *CYTB* de isolados monouredíniais, assim como a aplicação de diferentes doses de protioconazol (IDM) não aumentou a porcentagem de mutação no gene *CYP51,* confirmando a estabilidade das mutações pontuais nos isolados analisados de *P. pachyrhizi*.



Palavras chave: Mutações pontuais. Múltipla resistência. Controle químico. Ferrugem-asiática da soja.

## ABSTRACT

Brazil is the second largest producer of soybeans in the world, accounting for one-third of world production of this commodity. Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* is the main disease that affects soybean cultivation in Latin America, as it causes severe productivity losses due to early defoliation. The control of the disease is based mainly on the cultural and chemical control, the implantation of the methods of free host period, sowing scheduling and application of fungicides. Despite this, field efficacy of fungicides has been reduced by the selection of isolates less sensitive to fungicides and with point mutations in fungicide target genes. The main fungicides used belong to the groups demethylation inhibitor (DMIs), quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs) and multisite fungicides. Therefore, this study aimed to: i) Evaluate multiple resistance to DMI, QoI and SDHI fungicides in monouredinial and populational isolates from the Brazilian states, Paraná, Rondônia, Mato Grosso and São Paulo, through fungicide sensitivity tests ($EC_{50}$) and detection of point mutations in the *CYP51*, *CYTB* and *SDH* genes; ii) Verify the selection of individuals in the field from sequential applications of commercial DMI, QoI and SDHI fungicides, in different municipalities of the state of São Paulo and Paraná; iii) Verify if the absence of fungicide application during 50 cycles of the disease leads to loss of mutations in the *CYP51* and *CYTB* genes and whether the application of different doses of prothioconazole (DMI) increases the percentage of mutation in the *CYP51* gene. The F120L + Y131H combined mutation, which confers less sensitivity to DMIs, prevailed in all the analyzed locations and was more related to the lower sensitivity of prothioconazole than other point mutations found in the *CYP51* gene, which were also less frequent and more commonly found in the state of São Paulo. From the isolates evaluated, 90% presented the F129L mutation, which confers lower sensitivity to QoIs, being observed in all analyzed regions, but with lower occurrence in the state of São Paulo, indicating a lower fungicide selection pressure in this state. Isolates with the C-I86F mutation showed lower sensitivity to SDHI fungicides and, although still uncommon, were detected in Rondônia, Paraná and São Paulo, demonstrating the rapid dissemination or selection of these genotypes in the field. Genotype exhibiting multiple resistance in the fungicides DMI, QoI and SDHI target genes was detected and its dissemination and impact should be evaluated in the future. Three field applications of the same commercial fungicide can select mutated isolates for the *CYP51*, *CYTB* and *SDH* genes in only one crop cycle. Fifty cycles of the disease did not cause loss of mutations in the *CYP51* and *CYTB* genes. In addition, the application of different doses of prothioconazole (DMI) did not increase the percentage of mutation in the *CYP51* gene, confirming stability of point mutations in *P. pachyrhizi* analysed isolates.



Key words: Point mutations. Chemical control. Multiple resistance. Asian soybean Rust.

## LISTA DE FIGURAS

**CAPÍTULO I: BRAZILIAN *Phakopsora pachyrhizi* ISOLATES ADAPTED TO DMI, QOI AND SDHI FUNGICIDES**



**CAPÍTULO II: ASIAN SOYBEAN RUST: ONE SEASON SELECTION OF *Phakopsora pachyrhizi* RESISTANT ISOLATES BY FUNGICIDE APPLICATION IN THE FIELD**

## APÊNDICES

## LISTA DE TABELAS

## LISTA DE ACRÔNIMOS

ASR – Asian soybean Rust

CYP51 – Sterol 14α-demethylase

CYTB – Cytochrome b

DMI – Demethylation inhibitors

EUA – Estados Unidos da América

FRAC – Fungicide Resistance Action Committee

MAPA – Ministério da Agricultura Pecuária e Abastecimento

MT – Mato Grosso

PR – Paraná

QoI – Quinone outside inhibitors

R – Resistant

RO – Rondônia

S – Sensitive

SDH – Succinate dehydrogenase

SDHI – Succinate dehidrogenase inhibitors

SP – São Paulo

**SUMÁRIO**

# 1 INTRODUÇÃO

O cultivo da soja (*Glycine max* (L.) Merr.) ocupa aproximadamente 55% das áreas agricultáveis no Brasil, com 35,2 milhões de hectares semeados (CONAB 2018). A princípio o que era uma opção de ocupação de terras ociosas no período de verão no estado do Rio Grande do Sul em meados de 1960 (FUGANTI; CARVALHO, 2015), tomou proporções suficientes para tornar o país o segundo maior produtor mundial e o maior exportador mundial desta *commodity*. A produção brasileira é de 119,8 milhões de toneladas, ficando atrás apenas dos Estados Unidos da América (EUA) com produção de 120,04 milhões de toneladas na safra 2017/18 (USDA, 2018). A relevância da oleaginosa é explicada pelo destino do grão, utilizado principalmente para alimentação animal, mas também humana através de seus derivados, como tofu e óleo de soja (ISLAS-RUBIO; HIGUERA-CIAPARA, 2002; HIRAKURI; LAZZAROTO, 2014).

Dentre os estados brasileiros, o Mato Grosso é o maior produtor, produzindo 31,9 milhões de toneladas por ano, com área plantada de 9,519 milhões de há. O estado do Paraná ocupa a segunda posição no ranking, com produção de 19,1 milhões de toneladas por ano e aproximadamente 5,444 milhões de ha de plantados na safra 2017/18 (CONAB, 2018).

A ferrugem-asiática da soja (FAS), causada pelo fungo *Phakopsora pachyrhizi* Syd & P. Syd., é capaz de causar danos devastadores na cultura da soja e por isso é a doença de maior importância no cenário atual. Ocorre em todos os estados brasileiros produtores e também nos demais países produtores da América do Latina. Sua presença no início do período reprodutivo da cultura é crítica, podendo atingir danos de até 75% na produção (DALLA LANA et al., 2015). É uma doença de desenvolvimento rápido, com período de latência de 8 a 11 dias, para isolados monouredíniais sob condições ambientais favoráveis, podendo desta forma realizar vários ciclos durante uma mesma safra da cultura (KLOSOWSKI et al., 2018). O desenvolvimento do patógeno é favorecido por temperaturas entre 15°C e 25°C (ALVES, 2007; ZAMBENEDETTI et al., 2007) e chuvas bem distribuídas e longo período de molhamento foliar (DEL PONTE et al., 2006). É disseminado facilmente pelo vento, podendo percorrer longas distâncias (YORINORI et al., 2005).

Desde o descobrimento na América Latina em 2002, esta doença foi combatida principalmente através do controle químico, pelo uso de fungicidas

(YORINORI et al., 2005; GODOY et al., 2016). Alguns anos depois, foi iniciado o estabelecimento de controle cultural no manejo desta doença, pelo uso do vazio sanitário e calendarização de semeadura. As medidas citadas do controle cultural estão sendo implementadas de acordo com a legislação de cada estado (EMBRAPA, 2019). Além do controle químico e cultural, o controle genético com o uso de cultivares tolerantes também é empregado, porém poucos produtores optam pelo seu cultivo, pelo fato de que nem todas as regiões produtoras possuem cultivares tolerantes adaptadas.

Os fungicidas do grupo dos inibidores da desmetilação (IDMs), que atuam inibindo processos importantes na membrana do fungo, foram os primeiros fungicidas utilizados no controle da doença. Em seguida foram incorporados ao manejo os fungicidas inibidores da quinona externa (IQes), que atuam em processos respiratórios do fungo. Por muitos anos foram utilizadas no controle da FAS misturas de fungicidas do grupo dos IDM e IQes, conhecida popularmente como a dupla triazol + estrobilurina. O uso destes fungicidas é ainda essencial no controle desta doença, em misturas com fungicidas de outros grupos químicos ou com ingredientes ativos atuais dos mesmos grupos, como o IDM protioconazol (REIS; REIS; ZANATTA, 2018).

Atualmente, aproximadamente 60% dos produtos comerciais possuem triazóis (IDMs) e estrobilurinas (IQes) em sua formulação. Em 2013 foram incluídos os fungicidas do grupo dos inibidores da succinato desidrogenase (ISDHs) no controle de FAS, popularmente chamado de carboxamidas, e neste grupo estão incluídos os ingredientes ativos bixafen, fluxapiroxade e benzovindiflupir, que estão presentes em 18% dos produtos comerciais. Além dos ISDHs, foram incorporados no manejo os fungicidas protetores, com ação multíssítio, indicados como estratégia no manejo anti-resistência por atuarem em vários sítios ativos do patógeno, estando presentes em aproximadamente 50% dos produtos comerciais registrados para ferrugem-asiática da soja (MAPA, 2019; MAY DE MIO et al., 2018). No Brasil, podem ser encontradas misturas comerciais duplas ou triplas de fungicidas sítio-específicos dos grupos dos IDMs, IQes, ISDHs e fungicidas multissítios (MAPA, 2019).

Ao longo das safras, foi possível observar a redução da eficiência destes fungicidas a campo, que está relacionado com o aumento de indivíduos resistentes aos fungicidas dentro de uma população. Existem diversos mecanismos de resistência, dentre eles a alteração do sítio alvo devido à mutação pontual no gene, ou seja, alterações nas bases nitrogenadas do DNA em uma única posição do gene,

levando a mudanças na proteína por ele codificada; a compensação por meio do aumento da produção da enzima alvo, também chamado de superexpressão do gene; o desenvolvimento de vias metabólicas alternativas que não incluem o sítio alvo do fungicida, dentre outras (BRENT; HOLLOMON, 2007a).

Para *P. pachyrhizi,* mutações pontuais no gene *CYP51*, assim como a superexpressão do gene, foram descritas por reduzir a eficiência dos IDMs no controle de ferrugem asiática da soja (SCHMITZ et al., 2014). Já para IQes, a mutação pontual F129L no gene *CYTB* está associada à menor eficiência dos fungicidas deste grupo (KLOSOWSKI et al., 2016). A partir da detecção de redução da eficiência e do descobrimento das mutações, se intensificou a busca por novas moléculas, e os fungicidas do grupo dos inibidores da succinato desidrogenase (ISDHs), que atuam no processo respiratório do fungo, foram incorporados ao manejo da doença a partir da safra 2013/14. Apenas duas safras após a introdução dos fungicidas ISDHs ao manejo, observou-se a mutação C-I86F no gene *SDH,* capaz de reduzir a eficiência destes fungicidas (SIMÕES et al., 2018).

Isolados monourediniais de *P. pachyrhizi* com resistência múltipla aos fungicidas IDMs e IQes, por apresentar mutações nos genes *CYTB* e *CYP51* simultaneamente, já foram relatados para as safras de 2012/13 e 2013/14 (KLOSOWSKI et al., 2018). Isolados com resistência múltipla, apresentando mutações nos genes *CYP51*, *CYTB* e *SDH*, genes alvo dos fungicidas sítio-específicos IDM, IQe e ISDH respectivamente, não foram detectados. Com a pressão de seleção destes fungicidas em diferentes localidades brasileiras é possível que isolados com resistência múltipla já tenham sido selecionados a campo. Para confirmar tal hipótese, mutações pontuais nos genes alvo dos fungicidas sítio-específicos devem ser avaliados em isolados monourediniais de diferentes localidades, principalmente localidades com grande pressão de seleção por fungicidas.

A princípio, para que populações do fungo se tornem resistentes, indivíduos mutados que estão presentes em pequeno número na população são selecionados a partir da aplicação de fungicidas, e caso não tenham nenhuma penalidade em adaptabilidade ou competitividade se tornarão cada vez mais predominantes dentro da população. O número de aplicações de fungicidas do mesmo grupo durante uma mesma safra da cultura, também é um fator que pode levar à seleção de isolados resistentes (BRENT; HOLLOMON, 2007a).

A partir do descobrimento de FAS e início do controle pela aplicação de fungicidas, apenas duas a três aplicações eram realizadas por safra. Com o passar dos anos e a redução da eficiência das misturas comerciais triazóis+estrobilurinas, eram realizadas em média 4 a 5 aplicações do fungicida por safra. Com a incorporação dos SDHIs em 2013, e multissítios no manejo, houve um aumento da eficiência dos fungicidas a campo, porém não houve redução do número de aplicações (GODOY et al., 2017; TISOT, 2016). Não existem trabalhos que mostrem a seleção de isolados resistentes de *P. pachyrhizi* a campo por diferentes fungicidas e número de aplicações dos mesmos. Diante disso, é possível que áreas com aplicações frequentes de fungicidas selecionem maior porcentagem de mutantes de *P. pachyrhizi* do que áreas sem aplicação de fungicidas. Estudos em estados com diferentes níveis de produção de soja e consequentemente diferente pressão de seleção de fungicidas, bem como testes a campo com e sem aplicação de diferentes fungicidas, confirmariam essa hipótese.

O surgimento de isolados resistentes fazem parte de um processo evolutivo e o melhor entendimento envolvendo estes mecanismos, podem atenuar estratégias de manejo e informar a melhor maneira de avaliar os riscos de resistência. O conceito de seleção de isolados resistentes pelo uso de fungicidas já está bem estabelecido, e neste caso as mutação são estáveis, ou seja, indivíduos mutados nunca irão perder esta mutação, pelo fato dos genótipos resistentes serem pré-existentes em uma população, sendo apenas selecionados pela pressão de seleção dos fungicidas (BRENT; HOLLOMON, 2007a).

Porém, Hawkins et al. (2019) relataram que além deste conceito há um possível surgimento de indivíduos resistentes a partir de uma mudança ambiental, tornando essa mutação seletivamente vantajosa, chamando esse conceito de mutações *de novo*. Os autores afirmam que por alguma necessidade evolutiva ou através de estímulos, como a sobrevivência à aplicação de fungicidas ou condições ambientais desfavoráveis, indivíduos sejam capazes de desenvolver as mutações que conferem resistência aos fungicidas, e neste caso as mutações são seriam estáveis e poderiam também revertidas. Reversões já foram relatadas, como para o vírus da imunodeficiência em humanos (HIV), vírus da imunodeficiência símia (SIV) que ocasiona infecções em macacos, e reversão de forma da bactéria Escherichia coli a partir da síntese *de novo* (FRIEDRICH et al., 2004; KOBAYASHI et al., 2005; TADA; YAMAGUCHI, 1983). A partir do conceito de *de novo*, é levantada a hipótese de que

a falta de estímulos provocaria a reversão das mutações presentes em *P. pachyrhzi*, ou seja, mutações pontuais poderiam ser perdidas após vários ciclos da doença isolados sem estímulos de competitividade e aplicação de fungicidas.

Com base no que foi exposto, foram elaborados três capítulos que têm como objetivos: i) Avaliar resistência múltipla aos fungicidas IDM, IQe e ISDH em isolados e populações monouredíniais brasileiros, através de ensaios de sensibilidade aos fungicidas e detecção de mutações pontuais nos genes *CYP51*, *CYTB* e *SDH*; ii) Verificar quais indivíduos são selecionados a campo a partir de aplicações sequenciais de fungicidas comerciais dos grupos químicos IDM, IQe e ISDH, em diferentes municípios dos estados de São Paulo e Paraná; iii) Verificar se a ausência de aplicação de fungicidas durante 50 ciclos da doença leva à perda das mutações nos genes *CYP51* e *CYTB* e se a aplicação de diferentes doses de protioconazol (IDM) aumenta a porcentagem de mutação no gene *CYP51*.

# 2 CAPÍTULO I: BRAZILIAN *Phakopsora pachyrhizi* ISOLATES ADAPTED TO DMI, QOI AND SDHI FUNGICIDES[1]

**Short running title: Multifungicide resistance of *Phakopsora pachyrhizi***


## ABSTRACT

**BACKGROUND:**

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* Syd & P. Syd is the main disease that affects soybean crop since it emerged in South America in 2001. Chemical and cultural control, based on fungicides and on absence of live soybean plants, are the most important methods for disease management. Continuous and intensive use of fungicides has been selected isolates of *P. pachyrhizi*, with reduced sensitivity to DMIs, QoIs and SDHIs. This work includes sensitivity studies and genetic analysis of target site mutations related to sensitivity reduction to DMIs, QoIs and SDHIs.

**RESULTS:**

Different *CYP51* genotypes, with distinct point mutations to DMIs are present in Brazilian isolates. Combined mutation F120L+Y131H on *CYP51* gene was most frequent among Brazilian isolates. While wild type isolates showed $EC_{50}$ values of 0.8 - 1.5 µg $mL^{-1}$, mutants with F120L+Y131H on *CYP51* gene showed $EC_{50}$ values of 2.1 – 29.7 µg $mL^{-1}$ to the DMI prothioconazole. Almost all Brazilian isolates carried the mutation F129L in the cytochrome *b* gene, which is known to influence QoI sensitivity. Wild type of the SDH gene were the most frequent genotype to SDHI fungicides, but the mutation leading to the C-I86F amino acid exchange has also been detected. Such isolates showed higher $EC_{50}$ values to the SDHIs bixafen, benzovindiflupyr and fluxapyroxad. A monouredinial isolate with mutations in all three target genes was found in our studies and such isolates are here described for the first time.

**CONCLUSION:**

The most frequent genotype in our collection presented target site mutations in the *CYP51* and cytochrome *b* genes. The monouredinial isolate with mutations in all three target genes have also been detected. Its current and further spread, and their impact


---

[1] Prepared in accordance with the standards of Pest Management Science.

on field performance of fungicide products with these modes of action need further evaluation.

**Keywords:** Asian soybean rust; demethylation-inhibitors; quinone-outside-inhibitors; succinate-dehydrogenase inhibitors, fungicide resistance.

## 2.1 INTRODUCTION

Soybean is one of the most produced crop in the world, and Brazil is responsible for a third of the world production, being the second largest producer of this *commodity* [1,2]. It is a significant source of protein for livestock feeding and is also used for industrial purposes [3,4].

Asian soybean rust (ASR) is the main soybean disease in South America since its identification in 2001 [5], and it is caused by the fungus *Phakopsora pachyrhizi* Syd & P. Syd. Its infection in the leaves can lead to complete defoliation and yield losses up to 80% [6–8].

Chemical control is one of the main methods to manage pathogens and it is performed by fungicide application that aims to prevent entrance of the pathogen and the secondary cycle of diseases [9,10]. Another very important measure is the establishment of the free host period, a period where no soybeans are grown with the aim to reduce the inoculum potential in between the seasons. This leads to a delay of disease onset and a reduced selection pressure on the various modes of action and is therefore an effective and important tool in disease and resistance management [11].

Site-specific fungicides belonging to the demethylation inhibitors (DMI) and quinone outside inhibitors (QoIs) have effectively controlled the disease in the crop. However, efficacy fungicides with these modes of action has decreased over the years [12] and the search for new molecules have become pronounced. Succinate dehydrogenase inhibitors (SDHIs) fungicides were incorporated in the management of ASR, with the release and registration of fluxapyroxad in 2013 and of benzovindiflupyr in 2014. A reduction of SDHIs efficacy in the field has been observed and reported by the annual monitoring of Embrapa 2-3 seasons after market introduction [13]. Multisite fungicides were also incorporated in the management of ASR, in order to control the disease and limit the increase of resistant populations to site-specific fungicides [14,15].

The DMI target site is the C14 demethylase in the ergosterol biosynthesis pathway, which is encoded by the *CYP51* gene [16]. The target protein of the QoIs, cytochrome bc1-ubiquinol oxidase, is encoded by the mitochondrial gene *CYTB* [17,18]. The SDHI target site is the succinate dehydrogenase (SDH) complex in the respiratory chain [19]. This enzyme complex is codified by *SDH* genes, composed by subunits A, B, C and D [20–22].. Decrease of fungicide efficacy in the field can be conferred by different mechanisms, whereas the main resistance mechanisms in phytopathogenic fungi are point mutations in fungicide target genes [23].

For *P. pachyrhizi* there are reports of isolates with point mutations in the above mentioned genes conferring reduction of sensitivity to DMIs [24], QoIs [25,26] or SDHIs [27] independently. Multiple resistance for DMI and QoI in the same isolate was already described in monouredinial isolates, collected and established from fields before the launch of SDHIs [25,28]. However, there are no reports on combined SDHI, DMI and QoI resistance in individual isolates so far available. In Brazil with the high selection pressure of those fungicides in soybean production areas, isolates may present multiple resistance to the three main groups used to control ASR.

It was the objective of this work to evaluate if isolates with multiple adaptation to SDHIs, DMIs and QoIs can already be detected in Brazil. Therefore, sensitivity studies were performed, and target genes were analyzed for published target site mutations.

## 2.2 MATERIALS AND METHODS

### 2.2.1 *Phakopsora pachyrhizi* isolates

Twenty-one isolates of *P. pachyrhizi* collected from soybean leaves infected by ASR were used in the experiments, of which 7 were named “populational isolates” and 14 “monouredinial isolates”, according to the collection methodology. Isolates made from spores collected directly from samples of infected leaves from the field were called populational isolates. Monouredinial isolates were made from spores collected directly from one isolated uredia on the leaf. In order to obtain an isolated uredia, spores collected from the field were inoculated on healthy soybean leaves from plants grown in the greenhouse. After 15 days spores that grew in one uredia on the detached leaf were collected with a sterile needle and passed to a new detached leaf for the

establishment of the monouredinial isolate. The spores produced from the monouredinial isolate are assumed to be genetically identical since most probably a single spore gave rise to the collected uredia.

Samples of *P. pachyrhizi* were collected from soybean fields from different locations and seasons (Fig. 1). Both kind of isolates were transferred to healthy detached leaves every two weeks and in this way the isolates were maintained and multiplied for the experiments of $EC_{50}$ and genotyping.

### 2.2.2 Sensitivity test /$EC_{50}$ assays

*Ex vivo* assays on detached leaves were performed to calculate the effective concentration to inhibit 50% of the pathogen activity ($EC_{50}$), for the DMI prothioconazole and the SDHIs bixafen, benzovindiflupyr and fluxapyroxad according to the leaf methodology proposed by FRAC (Fungicide Resistance Action Committee) [29]. Resistance factors (RFs) were calculated by dividing the $EC_{50}$ value for each isolate with around 50% of C-I86F mutation by the mean $EC_{50}$ value of all the sensitive ones (WT). Commercially available formulated products were used for DMI prothioconazole (Proline® Bayer AG, Leverkusen, Germany) and for the SDHIs: benzovindiflupyr (Solatenol®, Syngenta AG, Basel, Switzerland), bixafen (Thore® Bayer AG, Leverkusen, Germany) and fluxapyroxad (Xemium®, BASF SE, Ludwigshafen, Germany).

Approximately 7 days old plants of the soybean cultivar 'ES Mentor' soybean (Saatbau, Linz, Austria), cultivated in the greenhouse were treated with different fungicide concentrations (DMI: 0, 0.03, 0.1, 0.3, 1, 3, 10, 30 µg $mL^{-1}$; SDHIs: 0, 0.1, 0.3, 1, 3, 10, 30, 100 µg $mL^{-1}$). The plants were treated in a spray chamber and the leaves were evenly covered with the fungicide solutions. Afterwards the plants were placed for 24 hours in growth chambers at 23°C for the superficial drying of the leaves. Then, each unifoliolate leaf was removed and packaged in disposable petri dishes containing agar-water medium (1%) with streptomycin (30 mg $L^{-1}$).

A spore suspension of each *P. pachyrhizi* isolate was inoculated in the abaxial part of the unifoliolate leaf. The spore suspension was prepared in distilled water + Tween 20 (0.01%) with $10^5$ spores $mL^{-1}$. Petri dishes containing treated and inoculated leaves were incubated for 12 hours in the dark at 23 ° C and thereafter for 15 days at the same temperature with 12 hours photoperiod. Then disease severity

was evaluated by diagrammatic scale [30] and from the severity the $EC_{50}$ was calculated. For each concentration of the fungicide there were three Petri dishes containing an unifoliolate leaf, each plate represented a repetition.

### 2.2.3 Point mutation analysis

#### 2.2.3.1 DNA extraction

*P. pachyrhizi* DNA was extracted from spores using the NucleoSpin DNA Plant II extraction Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions.

#### 2.2.3.2 Pyrosequencing

The Pyrosequencing technique was performed using PyroMark Gold 96 Reagents on a PSQ 96MA machine (Qiagen, Hilden, Germany), for quantitative detection of F129L mutations in the *CYTB* gene [26] and for the mutations F120L, Y131H/F, K142R, I145F, I475T in the *CYP51* gene [24].

#### 2.2.3.3 Real time qPCR

A specific quantitative real-time PCR was performed for *P. pachyrhizi* for quantitative detection of the C-I86F mutation in the ‘c’ subunit of the *SDH* gene on a Rotor-Gene Q cycler (Qiagen) as described by Simões et al. 2018.

## 2.3 RESULTS

### 2.3.1 Sensitivity of *P. pachyrhizi* to DMI fungicides and *CYP51* gene analysis

The $EC_{50}$ values for the DMI prothioconazole ranged from 0.8 – 29.7 μg $mL^{-1}$ for the 21 *P. pachyrhizi* isolates analyzed. The monouredinial isolates of the Mid West region of Brazil presented higher values of $EC_{50}$ than the other regions. Most of the isolates showed mutations in the *CYP51* gene. Genotypes with the combined mutation (F120L+Y131H) were most frequent, corresponding to 85% of the isolates analyzed.

Isolates with this combined mutation showed frequencies up to ~50% of the mutation F120L and Y131H in the DNA (Table 1). Other genotypes were also found: monouredinial isolate 191 with the Y131F and I475T mutation combination and the populational isolate 193 with F120L and Y131H combination and low frequency of the I475T mutation. The populational isolates GWH and 149/17 were the only isolates that did not show mutations in the *CYP51* gene. Isolates with mutations in the *CYP51* gene had higher $EC_{50}$ values to the DMI prothioconazole (2.1-29.7 μg $mL^{-1}$) than the wild type genotypes (0.8 and 1.5 μg mL-1).

### 2.3.2 *CYTB* gene analysis

Nearly all monouredinial and populational isolates sampled from different seasons and Brazilian regions showed very high frequencies (up to 100%) of the F129L mutation in the *CYTB* target gene of QoIs (Table 2). Only the monouredinal isolates 109 and 191 from the south and southeast were wild type in the *CYTB* gene and the populational isolate 193 from the southeast presented a lower percentage (58%) of the F129L mutation.

### 2.3.3 Sensitivity of *P. pachyrhizi* to SDHI fungicides and *SDH-c* gene analysis

The $EC_{50}$ values of *P. pachyrhizi* isolates to fungicides of the SDHI group ranged from 6.4 - 63.0 μg $mL^{-1}$ for bixafen; 0.2 - 71.4 μg $mL^{-1}$ for benzovindiflupyr; and 1.6 - 20.6 for fluxapyroxad. The sensitivity of the isolates to the fungicides varied without any specific pattern between the regions and seasons sampled (Table 2).

Isolates with approximately 50% of the mutation C-I86F presented $EC_{50}$ values higher than the wild type isolates (Fig. 2). The mean $EC_{50}$ for WT isolates was 11.8 μg $mL^{-1}$ for bixafen; 2.4 μg $mL^{-1}$ for benzovindiflupyr and 3.8 μg $mL^{-1}$ for fluxapyroxad, while for isolates with C-I86F mutation (198, 203, CV17), the mean $EC_{50}$ values were 34.1 μg $mL^{-1}$ for bixafen; 43.8 μg $mL^{-1}$ for benzovindiflupyr; and 14.0 μg $mL^{-1}$ for fluxapyroxad (Table 2; Fig. 2). The $EC_{50}$ values for the monouredinial isolates 198 and 203 with around 50% C-I86F were higher than for the populational isolate CV17 with only 11% C-I86F.

## 2.4 DISCUSSION

Multiple selection of resistant isolate for DMIs, QoIs and SDHIs fungicide groups was detected by point mutations in one monouredinial isolate of *P. pachyrhizi*, collected in the 2016/17 season, in the north region of Brazil. The genotype with mutations on target genes of DMI and QoI fungicide groups was the most frequent, representing 76 % of the isolates. Sensitivity tests with *P. pachyrhizi* showed a reduction of DMI sensitivity in isolates with F120L + Y131H combined mutation on gene *CYP51*. Isolates with the C-I86F mutation were less sensitive to SDHIs benzovindiflupyr, bixafen and fluxapyroxad, however such were present in low frequency within Brazilian isolates (14 %) in our study. On the opposite the F129L mutation, was present with high frequency in all isolates analysed.

Since the 2009/10 season, F120L + Y131H mutant isolates were detected in all soybean production areas in Brazil [24,25]. This spread of mutated isolates makes sense since fungicides of DMI group were the first to be used for control of *P. pachyrhizi* since season 2002/03. Besides that, DMI application was not interrupted at any time [31]. With high selection pressure of DMI fungicides over the years, those mutant individuals emerged and remain present within the population. Most isolates with the combined mutation F120L + Y131H presented less sensitivity to the DMI prothioconazole then the isolate with Y131F + I145T combined mutation. The isolate with Y131F + I145T combined mutation was collected in São Paulo state that does not have as many soybean production areas as other Brazilian states[1], which may lead to a reduced source of inoculum of diseases and consequently lower selection pressure of fungicides, avoiding or delaying the selection of resistant isolates.

In other pathosystems not only point mutations on the *CYP51* gene, but also overexpression of the gene as in *Blumeriella jaapii* [32], *Monilinia fructicola* [33], *Puccinia triticina* [34], *Venturia inaequalis* [35] and the up-regulation of efflux-transporter as in *Botrytis cinereal* [36], *Penicillium digitatum* [37], *Mycosphaerella graminicola* [38] are resistance mechanisms that have been described by reducing sensitivity to DMIs. For *P. pachyrhizi* sensitivity to fungicides of the DMIs group, was described to be related to overexpression and point mutations on the *CYP51* gene, the point mutations F120L, Y131H, Y131F, K142R, I145F and I475T was already found occurring independently or combined with each other in this pathosystem [24].

In previous studies all isolates of *P. pachyrhizi* containing the F120L + Y131H showed overexpression of the *CYP51* gene, and those containing the Y131F + I145T showed no overexpression of the gene [24]. This suggests that in the present work, although the overexpression has not been evaluated, it may be present in those isolates that presented F120L + Y131H combined mutation and may had helped to reduce fungicide sensitivity of some isolates. In the same way, the isolate 191, with Y131F+I145T, may have presented higher sensitivity for DMI prothioconazole due to the lack of overexpression. Although these indicate that the combined mutation F120L+Y131H is more related to the reduction of sensitivity to prothioconazole, other isolates with the Y131F+I145T combined mutation need to be investigated to confirm this relationship.

Another point to be noticed about the results of *CYP51* gene is that the isolates from 2015/16 and 2016/17 seasons showed higher frequencies of the mutation F120L+Y131H than isolates from previous seasons. The *CYP51* gene has several copies in the genome and the number of copies that presented the mutation can influence the percentage of mutation within an isolate [24]. Isolates analyzed from 2009/2010 presented a frequency of these mutations around 30% in each isolate [24], while in this work from 2015/16 season the isolates showed around 50% of these mutations. The percentage of the mutation within the isolate appeared not be related with lower sensitivity to prothioconazole, but studies with other fungicides of DMI groups could clarify if this change in frequency of mutations can interfere in fungicide sensitivity.

Point mutations located on subunits B, C and D of the *SDH* gene are most commonly found and were described as responsible for a decrease of SDHI efficacy in plant pathogens [39–42]. So far the mutation C-I86F located in the subunit C of the *SDH* gene, was only found in *P. pachyrhizi* on season 2015/16 in Rio Grande do Sul, the southernmost state of Brazil (Simões et al. 2018). In this work one monouredinial isolate and a populational isolate with the C-I86F mutation were found on season 2016/17 and both isolates also presented mutations on target genes of DMI and QoI fungicides. The monouredinial isolate with multiple resistance to the three groups of fungicides, was found in the north region of Brazil in the state of Rondônia, that borders Bolivia. Interestingly, both allow soybean-soybean cultivation. Selection pressure of fungicides in the case of successive soybean cultivation is high and can be the reason that this multiple resistant isolate was selected for the three modes of action. Although

not frequently found in the field, these mutant isolates can be present in other regions, since they were found in states that are geographically distant.

Isolates with the C-I86F mutation were less efficient controlled by the SDHIs bixafen, benzovindiflupyr and fluxapyroxade than the wild type isolates. Between the SDHI fungicides analyzed, benzovindiflupyr seemed to be more affected by this mutation, since the average of $EC_{50}$ of this fungicide were higher than fluxapyroxad and bixafen. For Simões et al. 2018, $EC_{50}$ values of C-I86F isolates were higher for bixafen followed by benzovindiflupyr and fluxapyroxad. However, a dose response were detected for all SDHIs and resistance factors are rather low for all SDHIs.

The F129L single mutation is the only mutation in the *CYTB* gene described in *P. pachyrhizi* isolates [25]. Other plant pathogens presentthe mutations F129L, G137R and G143A in the *CYTB* gene [43]. The G143A mutation is described as the most common and the most related to the reduction of plant pathogens sensitivity to QoI fungicides, while F129L is described as the mutation with less influence in the reduction of sensitivity to QoI fungicides [43,44]. Despite this, for *P. pachyrhizi* G143A mutation did not occur [18,24], and the G137R mutation was also not found so far.

The detection of F129L in the *CYTB* gene first occurred for *P. pachyrhizi* isolates from 2013/14 season, being responsible for reduced QoI sensitivity; on that time, only 35% of the Brazilian isolates presented this mutation [25]. In the present study almost all monouredinial and populational isolates presented this mutation. Corroborating with these data, competitiveness and fitness work predicted an increase of frequency of these mutants, since mutated isolates were as competitive as wild type genotypes [28]. Competitiveness and fitness might be reduced by the presence of resistance mechanisms. The emergence and spread of resistance pathogen isolates depend on whether they are able to outcompete pre-existing isolates. Competitivity assays with *P. pachyrhizi* resistant isolates to DMIs and QoIs and wild type isolates showed that fitness might be reduced by point mutations for DMIs, but not for F129L mutations on QoIs target gene [28].

For SDHIs *P. pachyrhizi* mutant isolates as for multiple resistant isolates, competitiveness and fitness are not yet investigated. The mutation C-I86F is still rarely detected in the field and dynamic of these mutant isolates on season 2017/18 showed a stabilization in the frequency of mutants found in Brazil, compared to previous seasons (data not shown). Therefore, it is possible that this mutation may be connected to fitness penalties. Studies performed with other pathogens indicated fitness penalties

at least for some mutations. *Botrytis cinerea* resistant genotypes were not as competitive as wild type isolates, in this order the alternation of groups of fungicides application should reduce the frequency of SDHI resistant isolates [45,46]. Studies in this sense with *P. pachyrhizi* specifically are important to elucidate the increase of mutant isolates in the population and will be performed.

When there is a fitness penalty, management strategies such as limiting the use of the fungicide of the same group per season should be adopted. Thus, the selection pressure caused by the application of fungicides is reduced and mutated isolates will be less selected within the population also avoiding the spread of resistant isolates to other regions [23]. In this order it is recommended to implement anti-resistance measures, as a limitation to two applications of SDHIs per season, use of mixed active ingredients, alternation of active ingredients, adequate the timing and frequencies of applications using preventive applications of fungicides and cultural strategies as free host period to reduce inoculum source and early sowing of soybeans to avoid disease-favorable weather (FRAC, 2017; Embrapa 2017).

Although resistant isolates to three chemical fungicide groups are still rarely observed, the results of this study suggest that mixing or alternating different chemical classes of fungicides can select for isolates with multiple resistance. Therefore, among the anti-resistance recommendations, the following control measures are essential to avoid further multiple resistance isolates selection: i) Implementation of a limited soybean sowing schedule, aiming to prohibit more than one cycle of the crop during the season, reducing fungicide selection pressure and the number of applications of single-site fungicides per season [11]; ii) Incorporation of multisite fungicides on management, since they affect different metabolic processes of the fungus and present low risk of resistance [47] and; iii) to use cultivars with lower susceptibility to ASR, in order to reduce the amount of inoculum produced.

## 2.5 ACKNOWLEDGMENTS

This study was financed in part by the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” - Brazil (CAPES) - Finance Code 001. The last author received research fellowships from National Council for Scientific and Technological Development (CNPq)/Brazil. The authors would like to thank Gerd Stammler lab team

for excellent technical assistance, Kelly Simões and Fernando Gava for training and sending samples.

## REFERENCES

1 *CONAB*, Acompanhamento da safra brasileira de grãos. *Monit agrícola* **5**:1–178 (2018).

2 *USDA*, World Agricultural Production. *Circular Series* 31 p (2018).

3 Hirakuri MH and Lazzaroto JJ, O agronegócio da soja nos contextos mundial e brasileiro. *Doc Embrapa* **349**:70 p (2014).

4 USB, Soy Products Guide 2017 [Online]. Available: https://unitedsoybean.org/farmer-resources/tools/soy-products-guide/ [13 July 2018].

5 Yorinori JT, Soja E, Paiva WM, Miranda C, Frederick RD, Ars U, et al., Epidemics of Soybean Rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. *Am Phytopathol Soc* **89**:2005 (2005).

6 Yang XB, Tschanz a T, Dowler WM, and Wang TC, Development of Yield Loss Models in Relation to reductions of components of soybean infected with *Phakopsora pachyrhizi*. *Phytopathology* **81**:1420–1426 (1991).

7 Hirano M, Hikishima M, José Da Silva A, Xavier SA, and Canteri MG, Validação de escala diagramática para estimativa de desfolha provocada pela ferrugem-asiática em soja. *Summa Phytopathol* **36**:248–250 (2010).

8 Dalla Lana F, Ziegelmann PK, de N Maia AH, Godoy CV, and Del Ponte EM, Meta-analysis of the relationship between crop yield and soybean rust severity. *Phytopathology* **105**:307–315 (2015).

9 Van Der Plank JE, Plant diseases: Epidemics and Control. Acad Press: 349 (1963).

10 Mayton H, Forbes GA, Mizubuti ESG, and Fry WE, The Roles of Three Fungicides in the Epidemiology of Potato Late Blight. *Plant Disease* **85**:1006-1012 (2001).

11 Martin Neto L and Farias JRB, Alertas da Embrapa sobre a soja safrinha. *Nota Técnica- Embrapa* (2014).

12 Godoy CV, Utiamada CM, Jaccoud Filho D, de Andrade Junior, Edson R, MV S, Siqueri F, et al., Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2013/14: resultados sumarizados dos ensaios cooperativos. *Circ Técnica* **103**:1-7 (2014).

13 Godoy CV, Utiamada CM, Meyer MC, Campos HD, Forcelini CA, Pimenta CB, et al., Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2016/17: Resultados sumarizados dos ensaios cooperativos. *Circ Técnica* **129**:1–10 (2017).

14 Godoy CV, Utiamada CM, Meyer MC, Campos HD, Lopes I de ON, Forcelini CA, et al., Eficiência de fungicidas multissítios e produto biológico no controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2016/17: Resultados sumarizados dos ensaios cooperativos. *Circ Técnica* **131**:1-8 (2017).

15 *FRAC*, Succinate Dehydrogenase Inhibitor (SDHI) Working Group. [Online]. Available: http://www.frac.info/docs/default-source/working-groups/sdhi-fungicides/archiv/minutes-of-2010-sdhi-meeting-recommendations-for-2011.pdf?sfvrsn=fb94419a_6 [05 February 2019]

16 Gisi U, Chin KM, Knapova G, Kuk Ng Fak R, Mohr U, Parisi S, et al., Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides, *Crop Prot* **19**:863–872 (2000).

17 Gisi U, Sierotzki H, Cook A, and McCaffery A, Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides, *Pest Manag Sci* **58**:859–867 (2002).

18 Grasso V, Palermo S, Sierotzki H, Garibaldi A, and Gisi U, Cytochrome *b* gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag Sci* **62**:465–472 (2006).

19 Yankovskaya V, Horsefield R, Törnroth S, Luna-Chavez C, Miyoshi H, Léger C, et al., Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* **299**:700–704 (2003).

20 Stammler G, Glättli A, Koch A, and Schlehuber S, Mutations in the target protein conferring resistance to sdhi fungicides, in *Modern Fungicides and Antifungal Compounds VI*, ed. by Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H. Deutsche Phytomedizinische Gesellschaft. Friedrichroda, Germany (2011).

21 Glättli A, Stammler G, and Schlehuber S, Mutations in the target proteins of succinate-dehydrogenase inhibitors (SDHI) and 14α-demethylase inhibitors (DMI) conferring changes in the sensitivity - structural insights from molecular modelling, in *9ème Conférence Int sur les Mal des plantes*, Association Française de Protection des Plantes. Tours, France pp. 670–681 (2009).

22 Stammler G, Brix HD, Nave B, Gold R, and Schoefl U, Studies on the biological performance of boscalid and its Mode of Action, in *Modern Fungicides and Antifungal Compounds V*, ed. by Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H. Deutsche Phytomedizinische Gesellschaft. Friedrichroda, Germany, pp. 45-51 (2008).

23 Brent KJ and Hollomon DW, Fungicide resistance: the assessment of risk, 2nd ed., *Fungic Resist Action Comm*, 2nd ed. 53p (2007).

24 Schmitz HK, Medeiros C-A, Craig IR, and Stammler G, Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. *Pest Manag Sci* **70**:378–388 (2014).

25 Klosowski AC, Castellar C, Stammler G, and May De Mio LL, Fungicide sensitivity and monocyclic parameters related to the *Phakopsora pachyrhizi* -soybean pathosystem from organic and conventional soybean production systems. *Plant Pathol* **67**:1–9 (2018).

26 Klosowski AC, May De Mio LL, Miessner S, Rodrigues R, and Stammler G, Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi*. *Pest Manag Sci* **72**:1211–1215 (2016).

27 Simões K, Hawlik A, Rehfus A, Gava F, and Stammler G, First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi*. *J Plant Dis Prot* **125**:21–26 (2018).

28 Klosowski AC, Brahm L, Stammler G, and May De Mio LL, Competitive fitness of *Phakopsora pachyrhizi* isolates with mutations in the *CYP51* and *CYTB* genes. *Phytopathology* **106**:1278–1284 (2016).

29 Scherb CTS and Mehl A, Detached leaf test (2006). Accessed: http://www.frac.info/monitoring-methods [20 January 2019].

30 Godoy C V, Koga LJ, and Canteri MG, Diagrammatic scale for assessment of soybean rust severity. *Fitopatol Bras* **31**:63–68 (2006).

31 Reis EM, Camargo A, and Zanatta M, The future of site-specific fungicides to control Asian soybean rust: past, present and future. *Inst Agris* pp. 1-17 (2018).

32 Ma Z, Tyre JP, Jacobs JL, and Sundin GW, Overexpression of the 14-Demethylase target gene (*CYP51*) mediates fungicide resistance in *Blumeriella jaapii*. *Appl Environ Microbiol* **72**:2581–2585 (2006).

33 Luo C-X and Schnabel G, The Cytochrome P450 lanosterol 14-Demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. *Appl Environ Microbiol* **74**:359–366 (2008).

34 Stammler G, Cordero J, Koch A, Semar M, and Schlehuber S, Role of the Y134F mutation in *cyp51* and overexpression of *cyp51* in the sensitivity response of *Puccinia triticina* to epoxiconazole. *Crop Prot* **28**:891–897 (2009).

35 Schnabel G and Jones AL, Genetics and Resistance The 14α−Demethylase (*CYP51*A1) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. *Phytopathology* **91**:103–110 (2001).

36 Hayashi K, Schoonbeek H-J, and De Waard MA, Expression of the ABC transporter BcatrD from *Botrytis cinerea* reduces sensitivity to sterol demethylation inhibitor fungicides. *Pestic Biochem Physiol* **73**:110–121 (2002).

37 Nakaune R, Adachi K, Nawata O, Tomiyama M, Akutsu K, and Hibi T, A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. *Appl Environ Microb* **64**:3983-3988(1998).

38 Zwiers L-H, Stergiopoulos I, Van Nistelrooy JGM, and De Waard MA, ABC Transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. *Antimicrob Agents Chemother* **46**:3900–3906 (2002).

39 Sierotzki H and Scalliet G, A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* **103**:880–887 (2013).

40 Avenot HF, Thomas A, Gitaitis RD, Langston DB, and Stevenson KL, Molecular characterization of boscalid- and penthiopyrad-resistant isolates of *Didymella bryoniae* and assessment of their sensitivity to fluopyram. *Pest Manag Sci* **68**:645–651 (2012).

41 Veloukas T, Leroch M, and Hahn M, Detection and molecular characterization of boscalid-resistant *Botrytis cinerea* isolates from strawberry. *Plant Dis* **95**:1032–1037 (2011).

42 Rehfus A, Strobel D, Bryson R, and Stammler G, Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathol* **67**:175–180 (2018).

43 *FRAC*, Mutations Associated with QoI-Resistance. [Online]. Available: http://www.frac.info/docs/default-source/about-frac/mutations-associated-with-qoi-resistance.pdf [05 February 2019]

44 Kim Y-S, Dixon EW, Vincelli P, and Farman ML, Genetics and resistance field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome *b* gene. *Phythopathology* **93**:891–900 (2003).

45 Veloukas T, Kalogeropoulou P, Markoglou AN, and Karaoglanidis GS, Fitness and competitive ability of *Botrytis cinerea* field isolates with dual resistance to SDHI and QoI fungicides, associated with several *sdh*B and the *cytb* G143A mutations. *Phytopathology* **347**:347–356 (2014).

46 Lalève A, Fillinger S, and Walker A-S, Fitness measurement reveals contrasting costs in homologous recombinant mutants of *Botrytis cinerea* resistant to succinate dehydrogenase inhibitors. *Fungal Genetics and Biology* **67**:24–36 (2014).

47 McGrath MT, What are Fungicides? [Online]. Available: https://www.apsnet.org/edcenter/intropp/topics/Pages/fungicides.aspx [09 February 2019].

TABLE 1 - Sensitivity of monouredinial and populational isolates of *Phakopsora pachyrhizi* to prothioconazole and respective mutations in *CYP51* gene.

| Isolate/ Region | Season | $EC_{50}$ (µg $mL^{-1}$) | Mutations in *CYP51* gene (%) | | | |
|---|---|---|---|---|---|---|
| | | **Prothioconazole** | **F120L** | **Y131H** | **Y131F** | **I475T** |
| *North (Vilhena -RO)* | | | | | | |
| 202 | 16/17 | 3.2 | **42** | **40** | 0 | 0 |
| 203 | 16/17 | 11.0 | **29** | **28** | 0 | 0 |
| *Mid West (Diamantino -MT)* | | | | | | |
| 161 | 15/16 | 29.7 | **45** | **47** | 0 | 0 |
| 163 | 15/16 | 12.4 | **41** | **41** | 0 | 0 |
| 164 | 15/16 | 20.2 | **45** | **43** | 0 | 0 |
| 165 | 15/16 | 13.2 | NA ‡ | NA | NA | NA |
| *South* (*Cascavel -PR*) | | | | | | |
| 174 | 16/17 | 7.3 | **45** | **43** | 0 | 0 |
| CV 17† | 16/17 | 14.7 | **35** | **33** | 0 | 0 |
| *South* (*Toledo -PR*) | | | | | | |
| 205 | 16/17 | 5.9 | **43** | **43** | 0 | 0 |
| TL 16† | 15/16 | 3.9 | **45** | **45** | 0 | 0 |
| TL 17† | 16/17 | 11.9 | **28** | **25** | 0 | 0 |
| *South* (*Ponta Grossa – PR*) | | | | | | |
| 29 | 13/14 | 6.8 | **30** | **28** | 0 | 0 |
| 95 | 13/14 | 2.1 | **49** § | **52** | 0 | 0 |
| 104 | 14/15 | 8.5 | **25** | **31** | 0 | 0 |
| 109 | 14/15 | 8.5 | **22** | **30** | 0 | 0 |
| *South East* (*Santo Antônio de Posse – SP*) | | | | | | |
| 191 | 16/17 | 3.6 | 0 | 0 | **47** | **43** |
| 193† | 16/17 | 5.6 | **25** | **13** | 0 | **16** |
| *Reference isolates* | | | | | | |
| 198† | | 19.9 | **35** | **33** | 0 | 0 |
| 149/17† | | 0.8 | 0 | 0 | 0 | 0 |
| GWH† | | 1.5 | 0 | 0 | 0 | 0 |
| 138 m3 | | 2.8 | **49** § | **50** | 0 | 0 |

†Populational isolate. ‡Not Analyzed (NA). Between brackets the city and state abbreviation. §Means that the nitrogenous base replacement was different from the others (Unlike the other isolates that replace the nitrogenous base from A to C, these isolates replace A to G, both changes encoding to the same amino acid).

TABLE 2 - Sensitivity of monouredinial and populational isolates of *Phakopsora pachyrhizi* to fungicides of succinate dehydrogenase inhibitors (SDHIs) group and respective mutation in two different genes.

| Isolate | Season | $EC_{50}$ of SDHI fungicides (µg $mL^{-1}$) | | | Mutation in *sdh*-c gene (%) | Mutation in *CYTB* gene (%) |
|---|---|---|---|---|---|---|
| | | **Bixafen** | **Benzovindiflupyr** | **Fluxapyroxad** | **C-I86F** | **F129L** |
| *North* (*Vilhena -RO*) | | | | | | |
| 202 | 16/17 | 11.4 | 3.1 | 3.7 | WT ‡ | **98** |
| 203 | 16/17 | 31.1 | 71.4 | 20.6 | **50** | **95** |
| *Mid West* (*Diamantino -MT*) | | | | | | |
| 161 | 15/16 | 17.1 | 3.5 | 1.6 | WT | **97** |
| 163 | 15/16 | 18.7 | 2.5 | 5.6 | WT | **98** |
| 164 | 15/16 | 13.3 | 3.9 | 5.8 | WT | **96** |
| 165 | 15/16 | 12.6 | 1.4 | 2.9 | WT | NA |
| *South* (*Cascavel -PR*) | | | | | | |
| 174 | 16/17 | 10.2 | 2.5 | 2.6 | WT | **94** |
| CV17† | 16/17 | 8.1 | 9.3 | 10.0 | **11** | **94** |
| *South* (*Toledo -PR*) | | | | | | |
| 205 | 16/17 | 3.1 | 1.4 | 1.7 | WT | **93** |
| TL 16† | 15/16 | 8.8 | 2.5 | 4.2 | WT | **100** |
| TL 17† | 16/17 | 10.6 | 1.5 | 2.3 | WT | **94** |
| *South* (*Ponta Grossa – PR*) | | | | | | |
| 29 | 13/14 | 11.2 | 2.8 | 8.4 | WT | **96** |
| 95 | 13/14 | 9.8 | 0.2 | 2.1 | WT | **96** |
| 104 | 14/15 | 11.2 | 3.2 | 5.1 | WT | **96** |
| 109 | 14/15 | 17.6 | 3.1 | 3.2 | WT | WT |
| *South East* (*Santo Antônio de Posse – SP*) | | | | | | |
| 191 | 16/17 | 8.5 | 1.1 | 3.9 | WT | WT |
| 193† | 16/17 | 8.8 | 2.6 | 4.2 | WT | **58** |
| *Reference isolates* | | | | | | |
| 198† | | 63.0 | 50.7 | 11.4 | **47** | **52** |
| 149/17 † | | 13.0 | 1.7 | 3.6 | WT | **93** |
| 138 m3 | | 6.4 | 2.5 | 1.8 | WT | **91** |
| GWH[1] | | 19.2 | 2.1 | 4.7 | WT | **99** |

† Populational isolate. ‡ Wild Type (WT). Between brackets: the city and state abbreviation.

FIGURE 1 - Brazilian map municipalities (origin of the isolates) and states (abbreviation in the map) where leaves samples with Asian-soybean rust were collected.

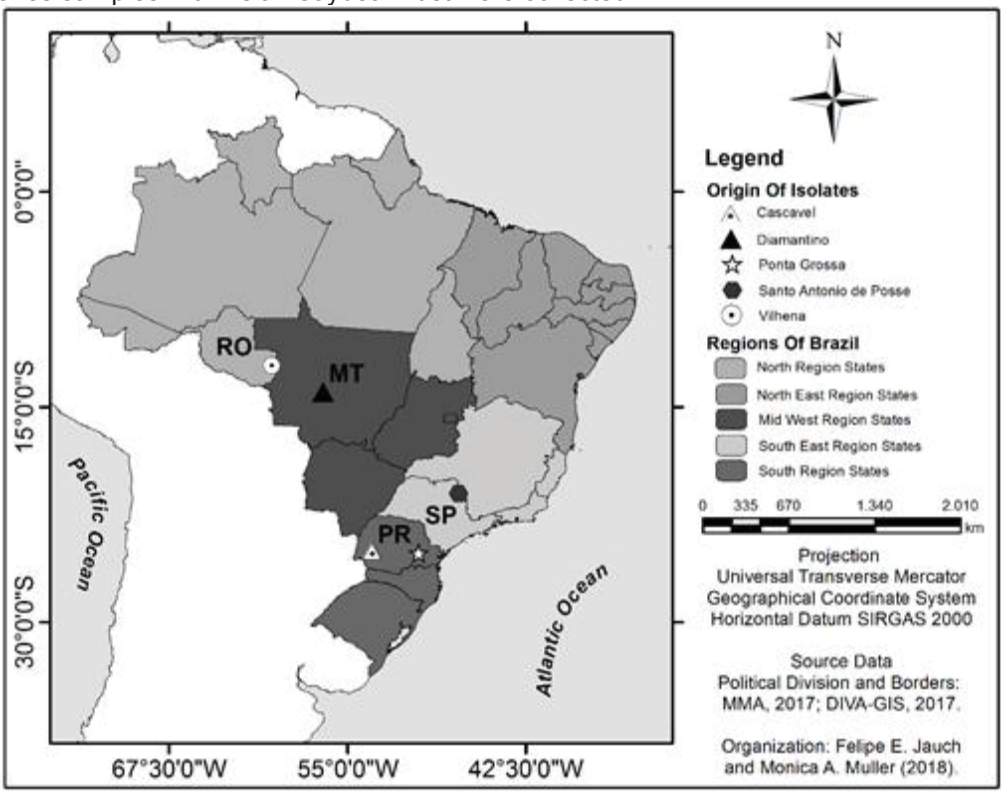
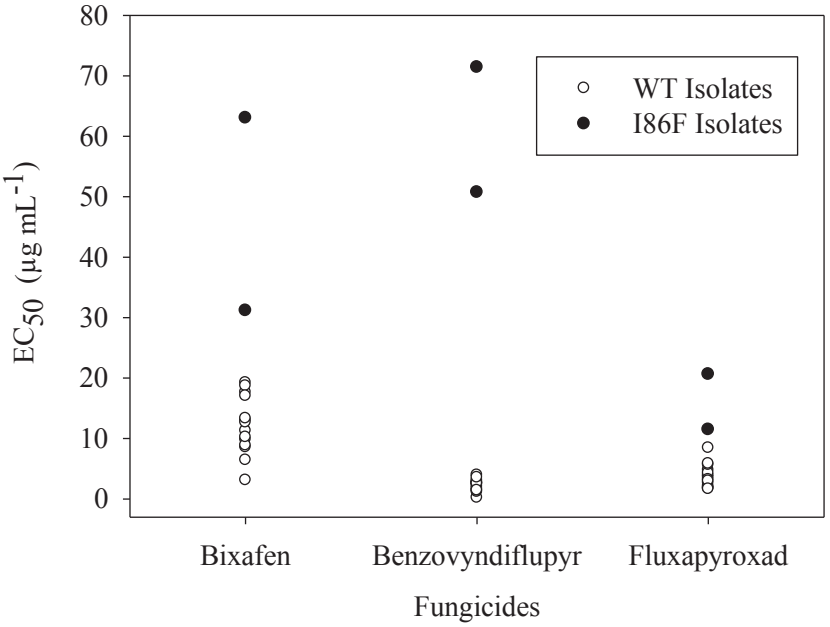
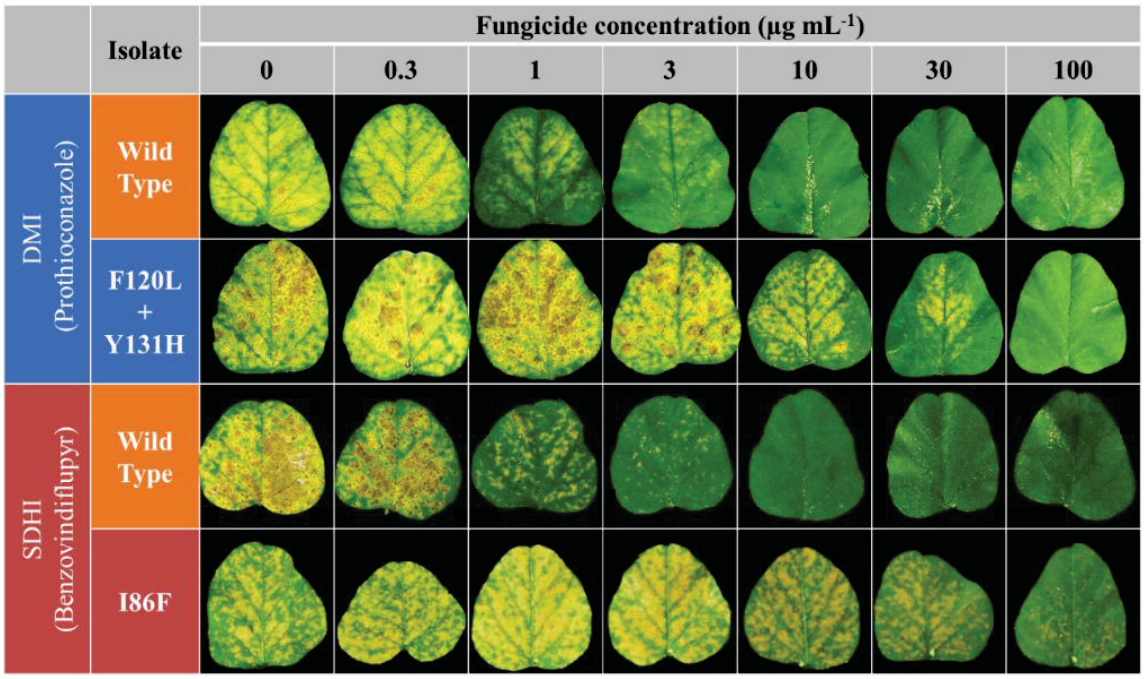


FIGURE 2 - Sensitivity of monouredinial isolates of *Phakopsora pachyrhizi* to fungicides of succinate dehydrogenase inhibitors (SDHIs) group by effective concentration to inhibit 50% of the pathogen ($EC_{50}$), value to wild type (WT) or 50% mutant (C-I86F) isolates (n=21 isolates in total).



## Graphic abstract



# 3 CAPÍTULO II: ASIAN SOYBEAN RUST: ONE SEASON SELECTION OF *Phakopsora pachyrhizi* RESISTANT ISOLATES BY FUNGICIDE APPLICATION IN THE FIELD[1]


## Abstract

Asian soybean rust (ASR) causes severe yield losses on soybean, the most cultivated grain in Brazil. The control of this foliar disease consists on cultural and chemical control, by the use of fungicides. The efficacy decrease of site-specific fungicides in the field is related to *Phakopsora pachyrhizi* point mutations on the *CYP51*, *CYTB* and *SDH* genes. Those mutant isolates are selected in the field by fungicide application, and currently an average of five fungicide applications are performed per season. Therefore, the objective of this study was to evaluate which mutated isolates are selected in the field by four fungicide application of commercial mixtures belonging to DMI, QoI and SDHI chemical groups in the Brazilian cities of Arapoti, Castro, Ponta Grossa and Itaberá. Mutations on *CYP51* gene, target gene of DMIs, were selected by all fungicides application. The F129L mutation on *CYTB* gene, target gene of QoIs, was selected by all fungicides application in the city of Arapoti. The cities of Castro and Ponta Grossa presented almost 100% of F129L mutation. The C-I86F mutation on *SDH* gene, was detected in all localities, but was less frequent, and was selected by SDHI application. All fungicides selected mutated isolates in four applications in only one soybean season.


## Introduction

Soybeans are one of the main commodities cultivated on a global scale, with more than 124 million hectares planted. Brazil was responsible for producing approximately 119 of the 337 million tons produced worldwide in the 2017/2018 season, being the second largest producer in the world. In addition to expressive production, Brazil is the world's largest grain exporter (USDA 2018). Since the 2006/07 season there was an increase of approximately 70% of the area cultivated in the country, and in the season 2017/18 35.2 million of hectares were seeded. Paraná is one of the states that stands out in the production with 19,170.5 thousand tons per

---

[1] Prepared in accordance with the standards of Brief Communication of European Journal of Plant Pathology.

year, and approximately 5,464.8 thousand ha of cultivation in the 2017/18 season. The state of SP produces only 3,409.8 thousand tons per year with a cultivation area of 961.6 thousand ha (CONAB 2018), since this state directs its agricultural area for the cultivation of sugar cane and citrus (SEAB-SP 2018).

The most important disease affecting soybean cultivation is Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi* Syd & P. Syd. of occurrence in all Brazilian producing states. According to the simulation performed by Cepea/ESALQ (2018) in the 2016/17 crop, approximately 15% of the production is destined to cover the costs of fungicide applications for the control of ASR in some producing regions of Brazil. Production damages of up to 75% can be reached (Dalla Lana et al. 2015) since ASR reduces the leaf foliar area and can cause complete defoliation (Hirano et al. 2010).

This disease was mainly controlled by chemical control by the use of fungicides, when it was detected in South America in 2002 (Godoy et al. 2017). Some years later, the establishment of cultural control in the management of this disease was initiated (Yorinori et al. 2005), with the use of free host period. Since 2016, the cultural control based on sowing scheduling is being implemented gradually by some Brazilian states. In addition to chemical and cultural control, genetic control with the use of tolerant cultivars is also employed, although it is adopted by few producers and the cultivars are adapted to specific regions and states.

The fungicides of the demethylation inhibitor group (DMIs), which inhibit important processes in the ergosterol biosynthesis of the fungus membrane, were the first fungicides used to control the disease. Then, the quinone outside inhibitors (QoIs) fungicides, which act on respiratory processes of the fungus, were incorporated in the control. For many years this fungicide mixture popularly known as the double triazoles + strobilurins was used to control rust, and their use is still essential in the control of this disease, but in mixtures with modern fungicides (Reis et al. 2018). In 2013, the fungicide chemical group succinate dehydrogenase inhibitors (SDHIs), in which carboxamides are included, were incorporated in the disease management. In Brazil, double or triple commercial mixtures of site-specific fungicides of the QoIs, IDMs and ISDHs and the multisite fungicides are registered (MAPA 2019) and currently used for ASR control.

Throughout the seasons, with the intensive use of the fungicides, it was observed an efficacy reduction of those fungicide groups in the field (Dalla Lana et al.

2018; Godoy et al. 2014). Point mutations in the *CYP51* gene of *P. pachyrhizi*, as well as overexpression of the gene, were described as reducing the efficacy of the DMIs efficiency in the control of ASR (Schmitz et al. 2014). As for QoIs, the F129L point mutation in the *CYTB* gene is associated with the lower efficacy of fungicides in this group (Klosowski et al. 2018). The C-I86F mutation in the *SDH* gene was first detected in isolates of 2016/17 season reducing the efficacy of SDHI fungicides (Simões et al. 2018).

The increasing of individuals with a mutation, that are present in a small number in the population occurs manly by the selection of this individuals from the application of fungicides, and if they have no fitness or competitiveness penalties, they will become prevalent within the population. Although the control efficacy of diseases can be improved by a higher number of fungicide applications, it also speed the selection of resistant individuals (Brent and Hollomon 2007). Studies with *Blumeria graminis* f.sp. *hordei* showed an increase of the selection ratio of resistant strains with increasing of the fungicide application number (Hobbelen et al. 2011). Despite the fact that there are no data of the number of fungicides used for ASR control in each Brazilian state, it is possible to infer that localities that produce more soybean and have favorable conditions for the occurrence of the disease, will have a higher occurrence of the disease. Therefore, a higher number of fungicide applications will be made, compared to areas that have low soybean production and consequently low inoculum. In this order, localities with greater selection pressure of fungicides consequently have greater selection of mutant individuals. From this, it is important to know which mutations are being selected in different locations. It is also fundamental to understand if the selection of resistant individuals varies according to the active ingredient applied to the field and with intrinsic characteristics of the pathogen related to the environmental conditions of each region, therefore the management of fungicides for ASR can be more precisely recommended.

Thus, in this work it was verified which genotypes are selected in different municipalities of the state of Paraná and São Paulo according to application of commercial fungicides mixtures of the groups DMI, QoI and SDHI.

## Materials and methods

Four field trials were conducted in areas belonging to an agricultural experimental institution (Fundação ABC). Three of four trials were conducted in the state of Paraná, in the cities of Arapoti, Castro and Ponta Grossa and one trial was conducted in the state of São Paulo in the city of Itaberá. Therefore, the same soybean cultivar, M5917 IPRO (Monsoy®, São Paulo, Brazil) was planted in all trials on December 15, 2016. Each trial was divided on six plots, comprehending one treatment per plot, without repetition. Each trial had the same treatments and number of plots. Adding up the plots of all trials, were in total 24. The size of the plots was 15 $m^2$, 3 x 5 m, with 50 cm between rows and 25 between plants, 50 cm of each side of the plot was considered as border and no sample of leaves were collected from the border, only from the useful area of the plot. The treatments consist of plots with untreated plants and plots with four applications of the same commercial fungicides, described in Table 1. The fungicide applications started 40 days after soybean emergence and were applied at 14-day intervals. No other fungicide treatment was applied during the season, only insecticides and herbicides as needed for the crop.

For molecular analysis, leaf samples infected with soybean rust were collected. A sample consists of 12 trifoliolate leaves, in order to obtain those, four soybean plants were randomly chosen within a useful area of the plot and three trifoliolate leaves were collected from each soybean plant. A sample of each plot was collected, totaling 6 samples per municipality and 24 samples in total. The collection was performed when the plants were already at the end of the cycle, just before the leaves fall, at the phenological stage R7. From each sample, *P. pachyrhizi* spores were collected from the leaves with the aid of a brush and stored in a freezer at -80ºC until the moment of DNA extraction and analysis of the point mutations. The amount of urediniospores collected from each sample, was called isolate.

The DNA was extracted from urediniospores of *P. pachyrhizi* isolates using the NucleoSpin DNA Plant II Kit following the instructions of the manufacturer for cetyltrimethyl ammonium bromide-based DNA extraction (Macherey- Nagel GmbH & Co. KG, Düren, Germany).

To analyze the presence of the mutations F120L, Y131F, Y131H, K142R, and I475T on the *CYP51* gene of the isolates, a pyrosequencing assay was carried out using the primers and methods described by Schmitz et al. (2014) and to analyze point the point mutation F129L on the *CYTB* gene a pyrosequencing assay was carried out using the primers and methods described by Klosowski et al. (2016).

A specific quantitative real-time PCR was performed for *P. pachyrhizi* for quantitative detection of the C-I86F mutation in the 'c' subunit of the SDH gene on a Rotor-Gene Q cycler (Qiagen) as described by Simões et al. (2018).

For data analysis, percentage of mutated isolates from fungicide treated leaves were compared with percentage of mutated isolates from the treatment without fungicide application (Untreated leaves) by the One-Sample Student' t test using the statistical software R (R Development Core Team, Vienna).

**Results**

On the cities of Arapoti-PR, Castro-PR and Ponta Grossa-PR it was observed that the isolates with the F120L+Y131H combined mutation in the *CYP51* gene were selected by all fungicides applied in the field, independently if they contain or not DMIs on their formulation. This was verified when compared the percentage of the F120L + Y131H mutation of untreated leaves, with the percentage of this combined mutation of other treatments ($P \leq 0.05$) (Figure 1). The trial in the city of Castro-PR did not present the same pattern as the other trials in other cities of the state of Paraná, since all treatments of Castro, including the treatment without fungicide application, presented higher frequency mutation, around 35%.

Paraná trials, seems to be unlike the trial in the city of Itaberá in São Paulo state. Samples of this trial almost did not present the F120L + Y131H combined mutation, but presented the mutations Y131F, K142R and I475T. Interestingly, the mutation that prevailed for this location did not even appear in the others. Only the pyraclostrobin+fluxapyroxade treatment from the city of Itaberá presented the F120L + Y131H combined mutation, but at a low frequency (15%) compared to the cities of Paraná State (32% ± 4%), and also no statistical difference was observed between treatments of this city. With the appearance of this mutation in this treatment, the frequency of Y131F, K142R and I475T was lower than in the other treatments. The other treatments had a higher frequency of the Y131F, K142R and I475T mutations than the control, indicating that fungicide application selects individuals with mutations in the *CYP51* gene (Figure 2).

For the F129L mutation in the *CYTB* gene, it was observed that the cities of the Paraná state presented almost 100% of frequency of the mutation F129L in the treatments with fungicide applications, and for the city of Castro and Ponta Grossa

even the untreated plot presented this frequency of mutation (Figure 3). Only the city of Arapoti-PR presented frequency of mutation for the untreated leaves well below the frequency of the other treatments ($P \leq 0.05$), indicating that the fungicides with QoI in their formulation select individuals with the F129L mutation, when the population is not completely mutated. The city of Itaberá in the state of São Paulo had a different behavior from the cities of the state of Paraná even for this gene, presenting low frequency of the F129L mutation for most treatments (around 10%). Coincidentally, the pyraclostrobin+fluxapyroxade treatment was an exception, as it was for the *CYP51* gene, and presented 60% of F129L mutation frequency (Figure 1, 2 and 3).

When evaluating the C-I86F mutation of the SDH gene, it was observed that the city of Arapoti-PR was the one with the highest frequency of this mutation, with the mean frequency between the treatments being 33% and the control 13% (Figure 4). All the fungicide treatments selected this mutation, compared to untreated leaves for the city of Arapoti-PR ($P \leq 0.05$), and also comparing only the treatments with fluxapyroxad and benzovyndiflupyr (SDHIs), with the the average of the other treatments, it was observed that this mutation was more selected by SDHI treatments ($P \leq 0.05$). For the other cities, although it was not possible to evaluate them statistically because of the missing samples and some 0% of frequency, the behavior was similar to treatments of the city of Arapoti-PR. It was observed that in city of Ponta Grossa-PR, the treatment that most selected this mutation was azoxystribin+benzovyndiflupyr, which contains the benzovyndiflupyr component belonging to the SDHI group in the mixture. The cities of Castro-PR and Itaberá-SP showed that fungicides containing fluxapyroxade or benxovyndiflupyr, both SDHIs, selected a higher frequency of individuals with the C-I86F mutation than the treatments without the presence of SDHI fungicides.

## Discussion

In this study, even though the first three applications were not evaluated, four applications of the same commercial fungicide with different mixture combinations of the DMI, QoI and SDHI fungicides, were enough to select resistant *P. pachyrhizi* isolates with point mutations on *CYP51*, *CYTB* and *SDH* genes. This corroborates with anti-resistance strategies proposed by the Fungicide Resistance Action Committee

(FRAC) that does not recommend more than two applications of fungicide of the same chemical group per season.

Historically, four commercial fungicide applications of the DMI + QoI mixture were commonly performed in order to control ASR up to 2012/13 seasons (Godoy et al. 2016). SDHIs fungicides and multisite fungicides were incorporated in the management since 2013/14 season (MAPA 2019) allowing a better rotation of active ingredient, minimizing the fungicide pressure of DMIs and QoIs and increasing the disease control levels. Currently, five to six commercial fungicides applications per soybean season are used for this purpose in the field (Tisot 2016), linking this information with the results of this work, it is possible to affirm that mutant individuals are being highly selected in soybean fields.

In the state of São Paulo, a diversity of genotypes in the *CYP51* gene was detected for the samples assessed. The genotypes were wild type, or presented the mutations F120L+Y131H, Y131F, K142R and I475T mutations in the *CYP51* gene. Since there is a lack of a sexual stage on *Phakopsora pachyrhizi* cycle at least in field conditions, this diversity cannot be explained by sexual recombination, as it occurs in other plant pathogenic fungi (Burdon & Silk 1997). Although, there is already one case of anastomosis occurring between *P. pachyrhizi* hyphae that enables the exchange of genetic material and could justify the diversity of genotypes found (Vittal et al. 2016).

The diversity of genotypes is reduced with application of fungicides since resistant isolates are selected in the field (Dekker 1976; Hawkins et al. 2019). The state of Paraná is the second larger producer of soybean (CONAB 2018), which lead us to presume that this lack of diversity and higher number of F129L mutated in the state of Paraná is due to intensive cultivation of soybean and consequently higher inoculum and fungicide pressure. On the opposite, a higher diversity of genotypes in the *CYP51* gene in the state of São Paulo also associated with low frequency of F129L mutation in the *CYTB* gene. São Paulo state stands out for the production of tomato and maize being one of the largest producers of the state (SEAB-SP 2018) and only few soybean fields are cultivated.

The mutation C-I86F on the *SDH* gene was detected in São Paulo state on season 2016/17. Recent studies also report this mutation in the states of Paraná and Rondônia at the same season (Müller et al. 2018). The mutation C-I86F on the *SDH* gene was described by the first time in the state of Rio Grande do Sul in *P. pachyrhizi* samples of 2015/16 season. At that moment, the monitoring study could not detect this

mutation in any other Brazilian soybean producer state (Simões et al. 2017), which means that C-I86F mutated isolates could spread or be selected very quickly by the application of fungicides.

Polycyclic pathogens with short disease cycles per season tend to rapidly increase the number of resistant isolates within a population (FRAC 2014). That is the case of *P. Pachyrhizi*, showing a latent period (time between inoculation and sporulation) of 6 days (Klosowski et al. 2018). This characteristic makes *P. pachyrhizi* able to complete several cycles during a crop of soybean which lasts approximately four months, varying according to cultivar and Photoperiod (Trentin et al. 2013). With selection pressure of fungicides application, wild type isolates are eliminated, and resistant isolates are selected. The higher is the number of fungicide applications during the season, the greater is the selection of resistant isolates.

The growth of soybean after soybean in the same season is an agricultural practice that increase the number of fungicide application of the same chemical group on a population of *P. pachyrhizi*. A second soybean growth is affected by higher amounts of secondary inoculum and requires more frequent fungicide applications compared to the first soybean growth, starting even earlier in the crop cycle. This practice occurs in Paraguay and Bolivia countries. In Brazil, the sowing period is regulated by state laws, but if this period is longer than the soybean cycle it gives a breach to the practice of soybean after soybean growth. Researchers from several institutions have been opposed to state laws that try to increase the sowing period (ADAPAR 2018), and this work provides scientific support so that practices that practices that increase the number of fungicide application in only one season, should not be performed.

In conclusion, *P. pachyrhizi* mutated isolates in *CYP51*, *CYTB* and *SDH* are selected in the field by four applications of the same commercial fungicide in just one season. Fields with greater selection pressure of fungicides in the surrounding area, such as the areas of Paraná, have a higher frequency of mutations F120L+Y131H, F129L and C-I86F on *CYP51*, *CYTB* and *SDH* genes respectively, than isolates of São Paulo State. These results reinforce the importance of anti-resistance management strategies proposed by FRAC that recommend the use of the same fungicide not more than twice per season (FRAC 2018).

**References**

ADAPAR: Agência de Defesa Agropecuária do Paraná. Portaria N° 264 de 17 de setembro de 2018 (2018). http://www.adapar.pr.gov.br/arquivos/File/GABINETE/PORTARIAS/2018/264_18.pdf

Brent, K. J., & Hollomon, D. W. (2007). Fungicide resistance in crop pathogens: how can it be managed? Fungicide Resistance Action Committee. http://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf.

Burdon, J. J., & Silk, J. (1997) Sources and patterns of diversity in plant-pathogenic Fungi. *Phytopathology, 87*, 664-669.

CONAB, C. N. de A. (2018). Acompanhamento da safra brasileira de grãos. *Monitoramento agrícola, 5*, 1–178.

Dalla Lana, F., Paul, P. A., Godoy, C., Utiamada, C. M., Silva, L. H. C. P., Siqueri, F. V., et al. (2018). Meta-analytic modeling of the decline in performance of fungicides for managing soybean rust after a decade of use in Brazil. *Plant Disease, 102*, 807–817.

Dalla Lana, F., Ziegelmann, P. K., de N Maia, A. H., Godoy, C. V, & Del Ponte, E. M. (2015). Meta-Analysis of the relationship between crop yield and soybean rust severity. *Phytopathology, 105*, 307-315.

Dekker, J. (1976). Acquired resistance to fungicides. *Annual Rev. Phytopathology, 14*, 405–428.

FRAC. (2014). *Pathogen risk list*. http://www.frac.info/docs/default-source/publications/pathogen-risk/pathogen-risk-list.pdf.

Godoy, C. V., Seixas, C. D. S., Soares, R. M., Marcelino-Guimarães, F. C., Meyer, M. C., Costamilan, L. M., et al. (2016). Asian soybean rust in Brazil: past, present and future. *Pesquisa Agropecuária Brasileira, 51,* 407–421.

Godoy, C. V., Utiamada, C. M., Meyer, M. C., Campos, H. D., Forcelini, C. A., Pimenta, C. B., et al. (2014). Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2013/14: resultados sumarizados dos ensaios cooperativos. *Circular Técnica, 103*, 1-7.

Godoy, C. V., Utiamada, C. M., Meyer, M. C., Campos, H. D., Forcelini, C. A., Pimenta, C. B., et al. (2017). Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2016/17: resultados sumarizados dos ensaios cooperativos. *Circular técnica, 129*, 1–10.

Hawkins, N. J., Bass, C., Dixon, A., & Neve, P. (2019). The evolutionary origins of pesticide resistance. *Biological Reviews, 94,* 135–155.

Hirakuri, M. H., & Lazzaroto, J. J. (2014). O agronegócio da soja nos contextos mundial e brasileiro. *Documentos Embrapa, 349*, 70 p.

Hirano, M., Hikishima, M., José Da Silva, A., Xavier, S. A., & Canteri, M. G. (2010). Validação de escala diagramática para estimativa de desfolha provocada pela ferrugem asiática em soja. *Summa Phytopathol, 36*, 248–250.

Hobbelen, P. H. F., Paveley, N. D., Fraaije, B. A., Lucas, J. A., & Van Den Bosch, F. (2011). Derivation and testing of a model to predict selection for fungicide resistance. *Plant Pathology, 60*, 304–313.

Klosowski, A. C., Castellar, C., Stammler, G., & May De Mio, L. L. (2018). Fungicide sensitivity and monocyclic parameters related to the *Phakopsora pachyrhizi* -soybean pathosystem from organic and conventional soybean production systems. *Plant Pathology, 67*, 1–9.

MAPA, Ministério da Aricultura Pecuária e Abastecimento (2019). Agrofit - Sistemas de Agrotóxicos Fitossanitários.
http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons

Müller, M. A., Stammler, G., & May De Mio, L. L. (2018). Adaptação de *Phakopsora pachyrhizi* aos fungicidas dos grupos IDM, IQE e ISDH. In *Annals of Inovação, tecnologias digitais e sustentabilidade da soja*, A. de Oliveira, R. M. V. B. de Campos Leite, A. J. Cattelan (Eds.), VIII Congresso Brasileiro de Soja (pp. 523–526). Goiânia.

Reis, E. M., Camargo, A., & Zanatta, M. (2018). The future of site-specific fungicides to control Asian soybean rust: past, present and future, How to reduce the fungus directional selection ?. *Instituto Agris, 1*, 1-7.

Schmitz, H. K., Medeiros, C.-A., Craig, I. R., & Stammler, G. (2014). Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. *Pest Management Science, 70*, 378–388.

SEAB-SP: Secretaria da Agricultura e Abastecimento. (2018). Estatísticas da Produção Paulista. São Paulo. http://ciagri.iea.sp.gov.br/nia1/subjetiva.aspx?cod_sis=1&idioma=1.

Simões, K., Hawlik, A., Rehfus, A., Gava, F., & Stammler, G. (2018). First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi*. *Journal Plant Disease Protection, 125*, 21-26.

Tisot, B. (2016). O comportamento da ferrugem asiática na soja ao longo dos anos, dificuldades de controle e seus custos. *Artigos DuPont Pioneer*. http://www.pioneersementes.com.br/media-center/artigos/193/o-comportamento-da-ferrugem-asiatica-na-soja-ao-longo-dos-anos-dificuldades-de-controle-e-seus-custos.

Trentin, R., Heldwein, B., Streck, N. A., Trentin, G., & Cordeiro Da Silva, J. (2013). Subperíodos fenológicos e ciclo da soja conforme grupos de maturidade e datas de semeadura. *Pesq. agropec. bras, 7,* 703–713.

Vittal, R., Yang, H.-C., & Hartman, G. L. (2012). Anastomosis of germ tubes and migration of nuclei in germ tube networks of the soybean rust pathogen, *Phakopsora pachyrhizi*. *European Journal of Plant Pathology, 132*, 163–167.

Yorinori, J. T., Soja, E., Paiva, W. M., Miranda, C., Frederick, R. D., Ars, U., et al. (2005). Epidemics of Soybean Rust (*Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. *Plant Disease, 89,* 676-677.

TABLE 1 - Fungicides applied in soybean plants during the 2016/17 season, discriminating the active ingredients used, chemical group and commercial name of the products.

| Treatments/Active ingredient | Chemical Group | Commercial product |
|---|---|---|
| Untreated leaves | - | - |
| Picoxystrobin + Cyproconazol | IQe+IDM | Approach® prima |
| Azoxystrobin + Benzovindiflupyr | IQe + ISDH | Elatus™ |
| Trifloxystrobin + Prothioconazole | IQe + IDM | Fox® |
| Pyraclostrobin +Fluxapyroxad | IQe + ISDH | Orkestra® |
| Fluxapyroxad + Pyraclostrobin+ Epoxiconazole | IQe+IDM+ISDH | Ativum® |

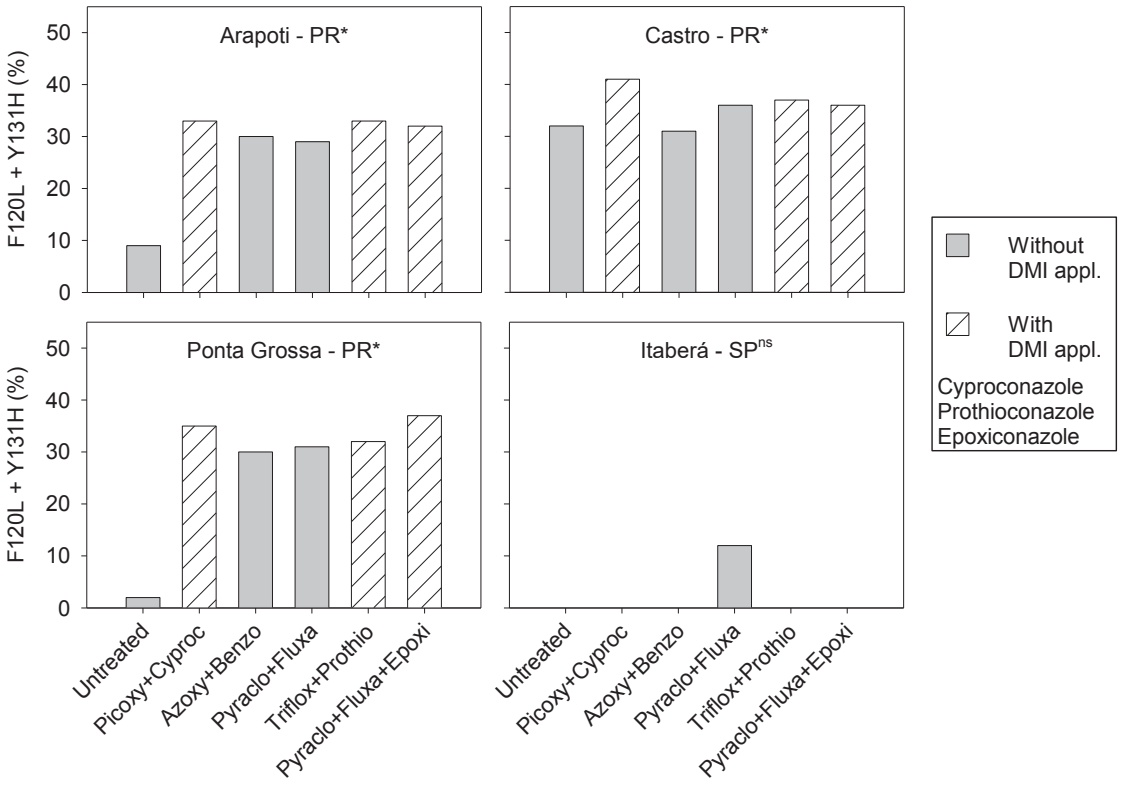



FIGURE 1 - Percentage of the F120L + Y131H combined mutation on the *CYP51* gene in *Phakopsora pachyrhizi* samples collected from soybean leaves infected with Asian soybean rust, without fungicide application and with four applications of commercial fungicides in the field (Picoxystrobin+ cyproconazole (Approach® prima); Azoxystrobin + Benzovindiflupyr (Elatus™); Fluxapyroxad + Pyraclostrobin (Orkestra®), Trifloxystrobin + Prothioconazole (Fox®); Fluxapyroxad + Pyraclostrobin+ Epoxiconazole (Ativum®) belonging to demethylation inhibitors (DMI) and other groups of fungicides, from season 2016/17 and different Brazilian locations.*Treatments differs by Untreated leaves by the One-sample t-test. nsNot significant.

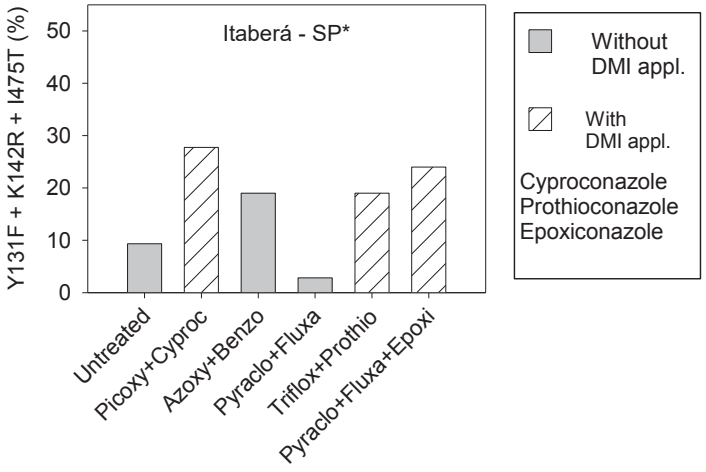



FIGURE 2 - Percentage of the Y131F, K142R and I475T mutations on the *CYP51* gene in *Phakopsora pachyrhizi* from samples collected from soybean leaves infected with Asian soybean rust, without fungicide application and with four applications of commercial fungicides in the field (Picoxystrobin+ cyproconazole (Approach® prima); Azoxystrobin + Benzovindiflupyr (Elatus™); Fluxapyroxad + Pyraclostrobin (Orkestra®), Trifloxystrobin + Prothioconazole (Fox®); Fluxapyroxad + Pyraclostrobin+ Epoxiconazole (Ativum®) belonging to demethylation inhibitors (DMI) and other groups of fungicides, from season 2016/17 and different Brazilian locations. *Treatments differs by Untreated leaves by the One-sample t-test. $^{ns}$Not significant.

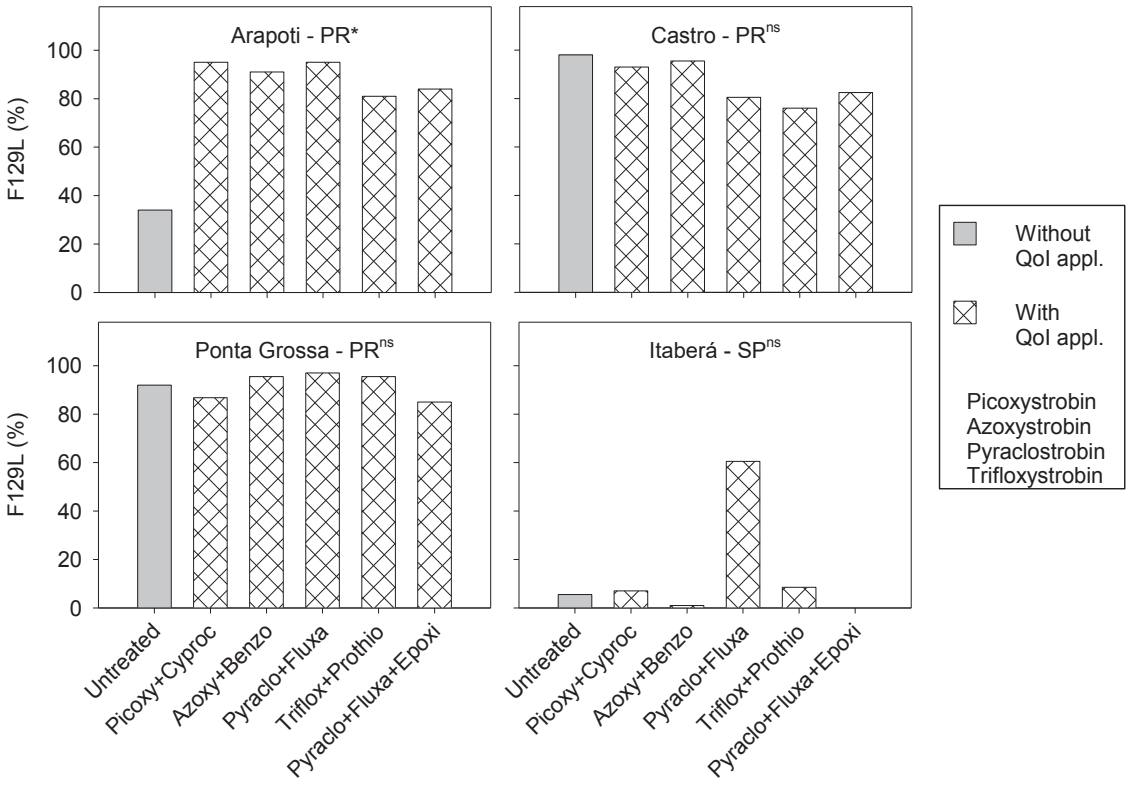



FIGURE 3 - Percentage of the F129L mutation on the *CYTB* gene in *Phakopsora pachyrhizi* samples collected from soybean leaves infected with Asian soybean rust, without fungicide application and with four applications of commercial fungicides in the field (Picoxystrobin+ cyproconazole (Approach® prima); Azoxystrobin + Benzovindiflupyr (Elatus™); Fluxapyroxad + Pyraclostrobin (Orkestra®), Trifloxystrobin + Prothioconazole (Fox®); Fluxapyroxad + Pyraclostrobin+ Epoxiconazole (Ativum®) belonging to quinone-outside inhibitors (QoI) and other groups of fungicides, from season 2016/17 and different Brazilian locations. *Treatments differs by Untreated leaves by the One-sample t-test. $^{ns}$Not significant.

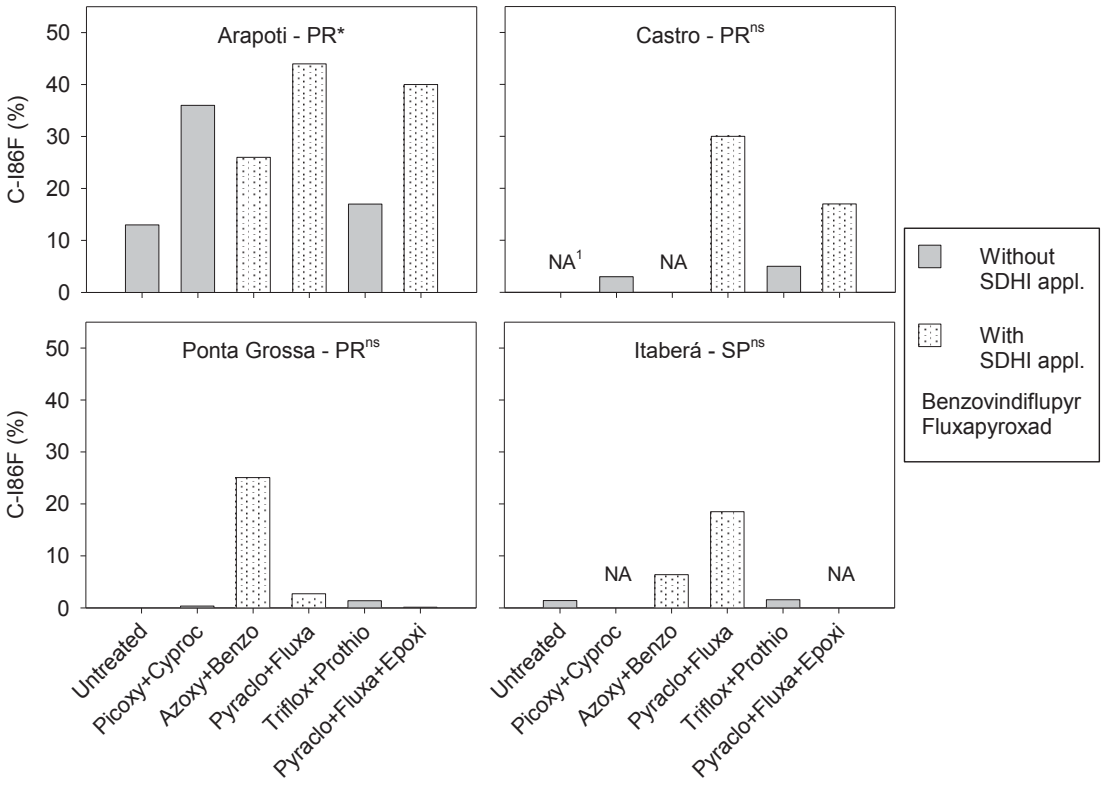



FIGURE 4 - Percentage of the mutation C-I86F on the *SDH*-c gene in *Phakopsora pachyrhizi* samples collected from soybean leaves infected with Asian soybean rust, with four field applications of commercial fungicides (Picoxystrobin+ cyproconazole (Approach® prima); Azoxystrobin + Benzovindiflupyr (Elatus™); Fluxapyroxad + Pyraclostrobin (Orkestra®), Trifloxystrobin + Prothioconazole (Fox®); Fluxapyroxad + Pyraclostrobin+ Epoxiconazole (Ativum®) belonging to succinate-dehydrogenase inhibitors (SDHI) and other groups of fungicides, from season 2016/17 and different Brazilian locations. [1]Not analyzed samples (NA), low DNA quality. *Treatments differs by Untreated leaves and treatments With SDHI appl. differ of Untreated by the One-sample t-test [ns]Not significant.

# 4 CAPÍTULO III: POINT MUTATIONS STABILITY ON THE *CYP51* AND *CYTB* GENES IN *Phakopsora pachyrhizi*, CAUSAL AGENT OF ASIAN SOYBEAN RUST[1]


## Abstract

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*, is the most important soybean disease in Brazil and most regions of Latin America. Combined mutation F120L+Y131H on the *CYP51* gene is related to decrease of DMI fungicides efficacy in the field and the point mutation F129L on the *CYTB* gene is related to decrease of QoI fungicides efficacy in the field. The objective of this work was to verify the stability of those mutation in monouredinial isolates and the impact of the DMI prothioconazole treatment in *CYP51* mutated and wild type isolates. Monouredinial isolates did not present loss of mutations F120L+Y131H and F129L on the *CYP51* and *CYTB* genes after being cultivated without fungicide application for two years, by transfers to new leaves. Prothioconazole treated leaves did not increase the percentage of mutations on *CYP51* gene. Mutations of *P. pachyrhizi CYP51* and *CYTB* genes were stable and not induced by fungicide application.


## Introduction

Soybean (*Glycine max* (L.) Merr) is a commodity of worldwide importance. Its grains are used mainly for animal feed and its derivatives for human consumption (Hirakuri and Lazzaroto 2014). Brazil is the second largest producer in the world with production of just over 119 million tons per year (CONAB 2018).

Damage on the crop and economic losses can be related to unfavorable environmental conditions, inadequate soil fertilization, pest, weeds, and disease incidence (Mattos 1987). Among diseases that occur in the soybean crop, Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* Syd & P. Syd, is the most important in Latin America. Its presence at the beginning of the reproductive period of the crop is critical and can reach production damages of up to 75% (Dalla Lana et al. 2015). This disease is fast developing, with latency period from 8 to 11 days under

---

[1] Prepared in accordance with the standards of Brieff communication of European Journal of Plant Pathology.

favorable environmental conditions (Klosowski et al. 2018) and can thus have several cycles during the soybean growing season, as a typical polycyclic disease (Zadoks and Schein 1979).

Since the detection in Latin America in 2002, this disease was mainly controlled using fungicides (Godoy et al. 2018). The fungicides of the demethylation inhibitor group (DMIs), which inhibit important processes in the fungus membrane, were the first fungicides used to control ASR. Then, the quinone outside inhibitors (QoIs) fungicides, which act on respiratory processes of the fungus, were incorporated in the control (Reis et al., 2018). For many years this fungicide mixture has been popularly known as the double triazoles + strobilurins was used to control ASR, and their use is still essential in the control of this disease, but in mixtures with modern fungicides (Reis et al. 2018). In 2013, succinate dehydrogenase inhibitors (SDHIs) fungicides, the carboxamides, were incorporated in the management of ASR. In Brazil, commercial double or triple mixtures of site-specific fungicides of the QoIs, DMIs and SDHIs and multisite fungicides are registered for ASR control (MAPA 2018).

The intensive use of these fungicides has contributed with the reduction of fungicide efficacy in the field over the crop cycle, reported by a field monitoring efficacy on season 2013/14 (Godoy et al. 2014). Concomitantly, point mutations in the *CYP51* gene of *P. pachyrhizi*, as well as overexpression of the gene, were described as reducing DMIs efficacy in ASR control (Schmitz et al. 2014). As for QoIs, the F129L point mutation in the *CYTB* gene is associated with the lower efficacy of fungicides in this group (Klosowski et al. 2018).

For populations of the fungus to become resistant, mutant individuals that are present in a small number within the population are selected after the application of fungicides, and if they have no fitness or competitiveness penalty compared to sensitive isolates, they will become increasingly prevalent within the population (Brent and Hollomon, 2007). However, Hawkins et al. (2019) reported that beyond this this selection by standing variation, there is a possible emergence of resistant individuals from environmental change, making this mutation selectively advantageous, calling this concept *de novo*. In addition, the authors affirm that because of some evolutionary need, such as survival to the application of fungicides(stimuli), individuals can develop *de novo* mutation.

It is not known if mutations on *CYP51* and *CYTB* genes of *P. pachyrhizi* were originated by *de novo* mutations or standing variation, although the sequential accumulation of *CYP51* mutations indicates *de novo* origins (Hawkins et al., 2019).

Mutated individuals, originated by standing variation, will never lose its mutation (Barrett and Schluter 2008). The lost of mutations within a population mostly occur through genetic drift, which mutant individuals are eliminated from populations over time, regardless of whether the origin of the mutation is *de novo* or standing variation.

The theory about *de novo* on plant pathogens (Hawkins et al. 2019), leads to think that if the isolates have evolved and become resistant from stimuli, this mutation may not be stable and may disappear in mutated individuals. If this is possible, mutated isolates without application of fungicides (stimuli) would lose the mutation, thus different resistance management strategies could be incorporated, as taking out of one group of active ingredient fungicide for a period. In addition, application of higher doses of the fungicide would increase the frequency of the mutation in each individual, or else select individuals with a higher frequency of mutation within a population, in cases of genes that probably has more than one copy of the gene, for example *CYP51* gene (Schmitz et al. 2014).

Thus, it was verified if the absence of fungicide application during several cycles of *P. pachyrhizi* lead to loss of the mutations on *CYP51* and *CYTB* genes and if the application of different doses of DMI prothioconazole may increase the percentage of mutation on the *CYP51* gene or select individuals with greater mutation percentage.

**Materials and methods**

An experiment of subsequent transfers of *P. pachyrhizi* isolates to new leaves was conducted for two years with eight Brazilian isolates from historic collection belonging to the Laboratory of Epidemiology for Integrated Disease Management (LEMID) of the Federal University of Paraná (UFPR). Those Brazilian isolates were collected from Planalto and Ponta Grossa, both cities of Paraná State (PR), in the 2013/14 season. The isolates from Planalto-PR were collected from leaves under organic soybean cultivation, without application of pesticides, and those from Ponta Grossa-PR were collected from leaves under conventional soybean cultivation, with

application of pesticides and genetically modified soybean. After collecting the *P. pachyrhizi* populations from the leaves, monouredinial isolates were obtained collecting spores from an isolate uredia. In order to obtain monouredinial isolates, the populational isolates were inoculated with fresh and healthy uniofoliolate leaves from a suspension of $1x10^2$ spores per mL of water + tween 20 (0.1%) solution. The inoculation was made intentionally with spore concentration below that recommended for *P. pachyrhizi*, in order to obtain few uredia per leaf. The inoculated leaves were placed in Petri dishes containing 1.5% water agar medium and incubated in BOD with photoperiod of 12 hours and temperature of 23 °C. After 15 days of incubation, spores grown in a single uredia was collected with an aid of a needle. To avoid the contamination of spores that had different genetic characteristics, the spores were collected from a uredia isolated at least 1 cm from the others. For multiplication of these monouredinial isolates, transfers of the isolates to new unifoliolate leaves were made every bi-weekly until the desired quantity of spores were obtained. Then part of these spores was collected from three leaves, constituting a sample and frozen for the first molecular analysis, and the other part was maintained by leaf transfers every 15 days for a period of two years. Each transfer represented one cycle of the pathogen, since colonization until sporulation, so a total of 50 cycles of the pathogen occurred without fungicide application.

The molecular analysis of the isolates was performed in two moments of collection, the first in 2015 (Klosowski et al. 2018), and the second analysis was performed in 2017, after the 50 transfers. Therefore, the DNA was extracted from urediniospores of *P. pachyrhizi* isolates using the NucleoSpin DNA Plant II Kit following the instructions of the manufacturer for cetyltrimethyl ammonium bromide-based DNA extraction (Macherey- Nagel GmbH & Co. KG, Düren, Germany). To confirm the presence of the mutations F120L, Y131H, on the *CYP51* gene in the isolates, a pyrosequencing assay was carried out using the primers and methods described by Schmitz et al. (2014) and to analyze the point mutation F129L on the *CYTB* gene a pyrosequencing assay was carried out using the primers and methods described by Klosowski et al. (2016). Each sample was analyzed once, and pyrosequencing analyzes were done in duplicate.

Another experiment was performed to analyze the frequency of mutations of 11 isolates of *P. pachyrhizi* inoculated at leaves with and without prothioconazole treatment. Nine of those isolates belong to a collection of the Laboratory of

Epidemiology for Integrated Disease Management (LEMID) of the Federal University of Paraná and the other two were from BASF SE collection. These isolates came from different Brazilian locations and from different areas. Seven of these isolates were monouredinial isolates and four were populational isolates (Table 3). Those isolates were inoculated in leaves pretreated with the doses 0 and 10 or 30 μg $mL^{-1}$ of the fungicide DMI prothioconazole and the percentage of mutation of each isolate at the different concentrations of the fungicide was analyzed. The methodology of treatment and inoculation was carried out with modifications based on the methodology proposed by the FRAC (Fungicide Resistance Action Committee) (Scherb and Mehl 2006). Leaf fungicide application was performed by a spray chamber develop by BASF in 7-day plants presenting two unifoliolated leaves using the commercial fungicide Proline® (Bayer CropScience, Levverkusen, Germany). After treatment the plants remained for 24 hours in growth greenhouses for drying the fungicide. After drying the leaves were detached from the plants and conditioned in Petri dishes containing agar medium and inoculated in the abaxial surface of the leaf. Fifteen days after inoculation, the spores were collected for molecular analysis. The point mutations F120L and Y131H of the *CYP51* gene were analyzed by the same techniques used for the experiment of subsequent transfers and in duplicate as well.

Both experiments were analyzed by the pairwise Student's t test. The data analysis was performed using the statistical software R (R Development Core Team, Vienna).

**Results**

The frequency of the F129L mutation on the *CYTB* gene after 50 transfers to new leaves without fungicide application was not different ($P \geq 0.01$) from the frequency of this mutation in the first analysis before the transfers for all monouredinial isolates analyzed (Table 1). This means that resistant isolates that presented around 100% of the mutation in the first evaluation in 2015 continue resistant in the second evaluation in 2017 and the sensitive isolate 99 that did not present the mutation in 2015 continue as wild type in 2017.

The frequency of the F120L+Y131H combined mutation on the *CYP51* gene after 50 transfers to new leaves without fungicide application was not different ($P \geq 0.01$) from the frequency of this mutation in the first analysis before the transfers (Table

2). This means that all resistant monouredinial isolates that presented around 30% of the mutation in the first evaluation in 2015, characterizing them as resistant genotype, and continued resistant in the second evaluation in 2017. No wild type isolate was found for *CYP51* gene.

The frequency of the F120L+Y131H combined mutation on the *CYP51* gene did not increase in isolates of *P. pachyrhizi* treated with different doses of the fungicide prothioconazole ($P \geq 0.01$) (Table 3). Those isolates that did not present the mutation in untreated leaves did not present the mutation when the leaf was treated with prothioconazole.

## Discussion

The mutations in both *CYP51* and *CYTB* genes were stable over 50 disease cycle. The frequency of the F120L+Y131H combined mutation on *CYP51* gene was also stable with different doses of prothioconazole fungicide application.

The reproduction of *P. pachyrhizi* occurs basically by the spores called urediniospores. This type of reproduction is called anamorphic or clonal, so the genetic characteristics will be the same for the next generation (Bromfield 1984) which means that the mutation is transmitted to the next generations. Although there is a report of reversion of transmitted mutations in human virus (Schneidewind et al. 2009), for *P. pachyrhizi* this reversion was not observed with the absence of fungicides in this work after 50 cycles of the disease, and also after four cycles of the disease (Klosowski et al. 2018).

The frequencies of the F129L mutation in *P. pachyrhizi* presented for most isolates 100% of mutation for resistant isolates or 0% of the mutation for wild type isolates. Interestingly, the isolate 94 presented approximately 35% of F129L mutation on *CYTB* gene in both analyses, before and after 50 cycles of the disease. Mutant monouredinial isolates of *P. pachyrhizi* already described by Klosowski et al. (2016), presented 100% of F129L mutation, and lower percentage of this mutation just occurred in populational isolates. It is possible that the isolate 94 is a mixture, that is a populational isolate instead of a monouredinial isolate. Also, there are several mitochondria inside the cell with their own DNA, where the *CYTB* gene is located, and they are not necessarily identical, therefore some mitochondria can be mutated, and some can be not mutated, so a different percentage of F129L mutation is possible

within a monouredinial isolate that is genetically identical (Chial and Craig 2008; Gisi et al. 2002).

Considering that the isolate 94 is a monouredinial isolate and just presented mitochondrial DNA genotypically distinct, which lead to only 35% of mutation, is possible that this isolate is less fit than a completely mutated isolate. This can be corroborated because the same isolate used in Klosowski et al. 2018 work, showed high sensitivity to the QoI azoxystrobin but presented lower number of pustules compared to the isolate 95 that presented 100% of the mutation F129L on the *CYTB* gene. In a competitivity and fitness work, monouredinial isolates of *P. pachyrhizi* presenting 100% mutated did not present loss of fitness when compared to wild type isolates (Klosowski et al. 2016).

The frequencies of the CYP51 mutations in *P. pachyrhizi* are not 100% as the frequency of the F129L mutation in the *CYTB* gene, 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 (Schmitz et al. 2014). One application of the DMI prothioconazole did not increase the percentage of the mutation found in the *CYP51* gene, indicating that probably the other copies of the gene did not suffer an amino acid exchange in the genome with the fungicide pressure. Since there was no change on genotype of the isolates after 50 disease cycle without fungicide application, it was not possible to infer if those mutations were originated by *de novo* (Hawkins et al. 2019). Considering the lack of a known sexual stage of *P. pachyrhizi* (Bromfield 1984) and the absence of reversal of mutations described in this work, the genetic diversity found in the field can be depend on the fitness and competitiveness of each isolate, it means that in the absence of the selection pressure of the fungicides the populations of the fungus would become sensitive as the baseline only if mutated isolates are less fitness than wild type isolates.

## References

Barrett, R. D. H., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in Ecology and Evolution, 23*, 38–44.

Brent, K. J., & Hollomon, D. W. (2007). Fungicide resistance in crop pathogens: how can it be managed? Fungicide Resistance Action Committee. http://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf.

Bromfield, K. R. (1984). Soybean Rust Monograph. St. Paul, MN: *American Phytopathological Society.*

Chial, H., & Craig, J. (2008). mtDNA and Mitochondrial Diseases. *Nature education, 1,* 1–7.

CONAB, Companhia Nacional de Abastecimento. (2018). Acompanhamento da safra brasileira de grãos. *Monitoramento agrícola, 5,* 1–178.

Dalla Lana, F., Ziegelmann, P. K., Maia, A. D. H. N., Godoy, C. V, & Del Ponte, E. M. (2015). Meta-Analysis of the Relationship Between Crop Yield and Soybean Rust Severity. *Phytopathology, 105,* 307–315.

Gisi, U., Sierotzki, H., Cook, A., & McCaffery, A. (2002). Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science, 58,* 859-867.

Godoy, C. V., Utiamada, C. M., Meyer, M. C., Campos, H. D., Forcelini, C. A., Pimenta, C. B., et al. (2014). Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2013/14: resultados sumarizados dos ensaios cooperativos. *Circular Técnica, 103*, 1-7.

Godoy, C. V, Utiamada, C. M., Meyer, M. C., Campos, H. D., Lopes, I. de O. N., Dias, A. R., et al. (2018). Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *P. pachyrhizi*, na safra 2017/2018. *Circular Técnica, 138,* 1–8.

Hawkins, N. J., Bass, C., Dixon, A., & Neve, P. (2019). The evolutionary origins of pesticide resistance. Biological Reviews, 94, 135–155.

Hirakuri, M. H., & Lazzaroto, J. J. (2014). O agronegócio da soja nos contextos mundial e brasileiro. *Documentos Embrapa, 349,* 70 p.

Klosowski, A. C., Brahm, L., Stammler, G., & MayDe Mio, L. L. M. (2016). Competitive Fitness of *P. pachyrhizi* Isolates with Mutations in the CYP51 and CYTB Genes. *Phytopathology, 106,* 1278–1284.

Klosowski, A. C., Castellar, C., Stammler, G., & May De Mio, L. L. (2018). Fungicide sensitivity and monocyclic parameters related to the *P. pachyrhizi* -soybean pathosystem from organic and conventional soybean production systems. *Plant Pathology, 67,* 1–9.

Klosowski, A. C., May De Mio, L. L., Miessner, S., Rodrigues, R., & Stammler, G. (2016). Detection of the F129L mutation in the cytochrome b gene in *P. pachyrhizi*. *Pest Management Science, 72*, 1211-1215.

MAPA, Ministério da Aricultura Pecuária e Abastecimento (2019). Agrofit - Sistemas de Agrotóxicos Fitossanitários. http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons.

Reis, E. M., Camargo, A., & Zanatta, M. (2018). The future of site-specific fungicides to control Asian soybean rust: past, present and future, how to reduce the fungus directional selection ?. *Instituto Agris, 1*, 1-7.

Scherb, C. T. S., & Mehl, A. (2006). Detached leaf test. Available: http://www.frac.info/monitoring-methods.

Schmitz, H. K., Medeiros, C.-A., Craig, I. R., & Stammler, G. (2014). Sensitivity of *P. pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. *Pest Management Science, 70,* 378–388

Schneidewind, A., Brumme, Z. L., Brumme, C. J., Power, K. A., Reyor, L. L., O'sullivan, K., et al. (2009). Transmission and Long-Term stability of compensated CD8 escape mutations. *Journal of virology, 83,* 3993–3997.

Zadoks, J. C. & Schein, R. D. (1979), Epidemiology and plant disease management. *Oxford University Press*.

TABLE 1 - Stability of F129L mutation on the *CYTB* gene from monouredinial isolates of *Phakopsora pachyrhizi* after 50 transfers to new soybean leaves since the first evaluation (2015) until the last (2017), and its genotype resistance (R) or sensitive (S).

| Isolate *P. pachyrhizi* | Season | Brazilian location | First evaluation (2015) | | Evaluation after 50 transfers (2017) | |
|---|---|---|---|---|---|---|
| | | | F129L % | Genotype | F129L% | Genotype |
| 29 | 2013/14 | Planalto-PR | 100 | R | 96 | R |
| 40 | 2013/14 | Planalto-PR | 100 | R | 99 | R |
| 71 | 2013/14 | Ponta Grossa -PR | 95 | R | 100 | R |
| 82 | 2013/14 | Ponta Grossa -PR | 100 | R | 100 | R |
| 87 | 2013/14 | Ponta Grossa -PR | 100 | R | 100 | R |
| 94 | 2013/14 | Ponta Grossa -PR | 40 | R | 31 | R |
| 95 | 2013/14 | Ponta Grossa -PR | 90 | R | 96 | R |
| 99 | 2013/14 | Ponta Grossa -PR | 0 | S | 0 | S |

TABLE 2 - Stability of F120L + Y131H mutation on the *CYP51* gene from monouredinial isolates of *Phakopsora pachyrhizi* after 50 transfers to new soybean leaves since the first evaluation (2015) until the last (2017), and its genotype resistance (R) or sensitive (S).

| Isolate | Season | Brazilian location | First evaluation (2015) | | Evaluation after 50 transfers (2017) | |
|---|---|---|---|---|---|---|
| | | | F120L+ Y131H % | Genotype | F120L+ Y131H % | Genotype |
| 29 | 2013/14 | Planalto-PR | 30 | R | 30 | R |
| 40 | 2013/14 | Planalto-PR | 34 | R | 27 | R |
| 71 | 2013/14 | Ponta Grossa -PR | 32 | R | 32 | R |
| 82 | 2013/14 | Ponta Grossa -PR | 50 | R | 30 | R |
| 87 | 2013/14 | Ponta Grossa -PR | 46 | R | 42 | R |
| 94 | 2013/14 | Ponta Grossa -PR | 33 | R | 27 | R |
| 95 | 2013/14 | Ponta Grossa -PR | 37 | R | 37 | R |

TABLE 3 - Stability of the F120L + Y131H combined mutation on the *CYP51* gene of *Phakopsora pachyrhizi* spores from monouredinial and populational isolates grown in pretreated leaves with different concentrations of prothioconazole fungicide.

| *P. pachyrhizi* Isolates | Brazilian location[2] | Season | Untreated leaves | Prothioconazole pretreated leaves | |
|---|---|---|---|---|---|
| | | | | 10 µg mL$^{-1}$ | 30 µg mL$^{-1}$ |
| | | | **F120L + Y131H (%)** | | |
| GWH[1] | BASF SE | - | 0 | 0 | - |
| 191 | Sto Antonio de Posse-SP | 2016/17 | 0 | 0 | - |
| 193 | Sto Antonio de Posse-SP | 2016/17 | 7 | 5 | - |
| 29 | Planalto-PR | 2013/14 | 28 | 23 | - |
| 109 | Ponta Grossa-PR | 2016/17 | 30 | 25 | - |
| 202 | Vilhena-RO | 2016/17 | 40 | 43 | - |
| 161 | Diamantino-MT | 2015/16 | 47 | 46 | - |
| 164 | Diamantino-MT | 2015/16 | 43 | - | 39 |
| TL17[1] | Toledo-PR | 2016/17 | 25 | - | 21 |
| CV17[1] | Cascavel-PR | 2016/17 | 33 | - | 33 |
| 198[1] | BASF SE | - | 33 | - | 30 |

[1] Not monouredinial isolate. [2] City and State.

# 5 CONCLUSÕES GERAIS

Durante o estudo realizado monitorando amostras de ferrugem-asiática em vários estados do Brasil, foi encontrado um isolado monouredinial de *Phakopsora pachyrhizi* originário do estado de Rondônia apresentando resistência múltipla aos fungicidas sítio-específicos dos grupos químicos IDMs, IQes e ISDHs simultaneamente, caracterizada por mutações pontuais nos genes *CYP51*, *CYTB* e *SDH*-C. Foi encontrado também um isolado populacional, o que é representado por uma coleta de urediniósporos de folhas de uma amostra com a doença, originário do Paraná, apresentando mutações pontuais nos genes acima citados. Ambos isolados localizados em áreas próximas ao Paraguai e Bolívia, que realizam plantio de soja sobre soja, prática que intensifica o número de aplicações de fungicidas, selecionando isolados resistentes.

Aplicações sequenciais de fungicidas comerciais sítio-específicos a campo selecionaram indivíduos com mutações pontuais nos genes *CYP51*, *CYTB* e *SDH*-C durante uma safra da cultura, comprovando o risco potencial de aplicações repetidas dentro de uma única safra. As amostras de *P. pachyrhizi* dos municípios do Paraná, apresentaram maior porcentagem da mutação F129L no gene *CYTB* e a mutação F120L+Y131H no gene *CYP51*, possivelmente devido ao intenso cultivo de soja e consequentemente maior pressão de inóculo de *P. pachyrhizi* e de pressão de seleção de fungicidas no estado do Paraná.

Mutações pontuais nos genes *CYP51* e *CYTB*, que codificam a proteína alvo dos fungicidas IDMs e IQes, não foram perdidas após 50 ciclos da doença sem a aplicação de fungicidas em ensaios *ex vivo* conduzidos em laboratório. Além disso, o aumento da dose do fungicida protioconazol não alterou a porcentagem da mutação F120L+Y131H no gene *CYP51* em isolados de *P. pachyrhizi*.

# REFERÊNCIAS


ALVES, S. A. M. **Quantificação de parâmetros da pré-penetração e monocíclicos relacionados ao patossistema** *Phakopsora pachyrhizi* - soja. 2007. Universidade de São Paulo Escola Superior de Agricultura “Luiz de Queiroz”, 2007.

BRENT, K. J.; HOLLOMON, D. Fungicide resistance: the assessment of risk. 2. ed. Brussels: **Fungicide Resistance Action Commitee**, 2007a.

BRENT, K. J.; HOLLOMON, D. Fungicide resistance in crop pathogens: How can it be managed? 2. ed. Brussels: **Fungicide Resistance Action Committee**, 2007b.

CONAB, Companhia Nacional de Abastecimento. Acompanhamento da safra brasileira de grãos. **Monitoramento agrícola**, v. 5, n. 10, p. 1–178, 2018.

DALLA LANA, F.; ZIEGELMANN, P. K.; MAIA, A. H.; GODOY, C. V.; DEL PONTE, E. M. Meta-analysis of the relationship between crop yield and soybean rust severity. **Phytopathology**, v. 105, n. 3, p. 307–315, 2015.

DALLA LANA, F.; PAUL, P. A.; GODOY, C.; UTIAMADA, C. M.; SILVA, L. H. C. P. et al. Meta-analytic modeling of the decline in performance of fungicides for managing soybean rust after a decade of use in Brazil. **Plant Disease**, v. 102, n. 4, p. 807–817, 2018.

EMBRAPA, Empresa Brasileira de Pesquisa Agropecuária. **Vazio sanitário e calendarização de semeadura da soja**. Disponível em: <https://www.embrapa.br/soja/ferrugem/>. Acesso em: 20 jan. 2019.

FRIEDRICH, T. C.; DODDS, E. J.; YANT, L. J.; VOJNOV, L.; RUDERSDORF, R. et al. Reversion of CTL escape–variant immunodeficiency viruses *in vivo*. **Nature Medicine**, v. 10, p. 275, 2004.

FUGANTI, E. N.; CARVALHO, L. C. Caracterização da cadeia produtiva da soja em Santa Catarina. **Revista Cadernos de Economia**, v. 19, n. 35, p. 5–19, 2015.

GODOY, C. V.; SEIXAS, C. D. S; SOARES, R. F.; MARCELINO-GUIMARÃES, F. C.; MEYER, M.C.; COSTAMILAN, L. M. Asian soybean rust in Brazil: past, present and future. **Pesquisa Agropecuária Brasileira**, v. 51, n. 5, p. 407–421, 2016.

GODOY, C. V.; UTIAMADA, C. M.; MEYER, M. C.; CAMPOS, H. D.; FORCELINI, C. A. et al. Eficiência de fungicidas para o controle da ferrugem-asiática da soja, *Phakopsora pachyrhizi*, na safra 2016/17: resultados sumarizados dos ensaios cooperativos. **Circular técnica**, v. 129, p. 1–10, 2017.

GODOY, C. V.; UTIAMADA, C. M.; MEYER, M. C.; CAMPOS, H. D.; LOPES, I. O. N. et al. Eficiência de fungicidas multissítios e produto biológico no controle da ferrugemasiática da soja, *Phakopsora pachyrhizi*, na safra 2016/17: resultados sumarizados dos ensaios cooperativos. **Circular Técnica**, v. 131, p. 1–8, 2017.

HAWKINS, N. J.; BASS, C.; DIXON, A.; NEVE, P. The evolutionary origins of pesticide resistance. **Biological Reviews**, v. 94, p. 135–155, 2019.

HIRAKURI, M. H.; LAZZAROTO, J. J. O agronegócio da soja nos contextos mundial e brasileiro. **Documentos Embrapa**, v. 349, p. 70, 2014.

ISLAS-RUBIO, A. R.; HIGUERA-CIAPARA, I. **Soybeans: Post-harvest Compendium**. Editores: AGSI/FAO: Danilo Mejia Beverly Lewis. Post-harvest, p. 94, 2002.

KLOSOWSKI, A. C.; CASTELLAR, C.; STAMMLER, G.; MAY DE MIO, L. L. Fungicide sensitivity and monocyclic parameters related to the *Phakopsora pachyrhizi* -soybean pathosystem from organic and conventional soybean production systems. **Plant Pathology**, v. 67, n. 8, p. 1–9, 2018.

KLOSOWSKI, A. C.; MAY DE MIO, L. L.; MIESSNER, S.; RODRIGUES, R.; STAMMLER, G. Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi.* **Pest Management Science**, v. 72, n. 6, p. 1211–1215, 2016.

KOBAYASHI, M.; IGARASHI, H.; TAKEDA, A.; KATO, M.; MATANO, T. Reversion *In Vivo* after inoculation of a molecular proviral DNA clone of simian immunodeficiency virus with a Cytotoxic-T-Lymphocyte escape mutation. **Journal of virology**, v. 79, n. 17, p. 11529–11532, 2005.

MAPA, Ministério da Agricultura Pecuária e Abastecimento. **Agrofit - Sistemas de Agrotóxicos Fitossanitários**. Disponível em: <http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons>. Acesso em: 20 jan 2019.

MAY DE MIO, L. L.; KLOSOWSKI, A. C.; MÜLLER, M. A. Ferrugem-asiática da soja: estudo sobre sensibilidade de *Phakopsora pachyrhizi* a fungicidas. [**Anais do**] XVIII Simpósio de Manejo de Doenças de Plantas: biotecnologia aplicada à fitopatologia. Lavras, p. 32-62. 2018.

DEL PONTE, E. M.; GODOY, C. V.; LI, X.;YANG, X. B. Predicting severity of Asian soybean rust epidemics with empirical rainfall models. **Phytopathology**, v. 96, n. 7, p. 797, 2006.

REIS, E. M.; REIS, A. C.; ZANATTA, M. Reflexo econômico e desenvolvimento da resistência de Phakopsora pachyrhizi a fungicidas em função do número de aplicações. **Summa Phytopathologica**, v. 44, n. 3, p. 289–292, 2018.

SCHMITZ, H. K.; MEDEIROS, C.-A.; CRAIG, I. R.; STAMMLER, G. Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. **Pest Management Science**, v. 70, n. 3, p. 378–388, 2014.

SIMÕES, K.; HAWLIK, A.; REHFUS, A.; GAVA, F.; STAMMLER, G. First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi*. **Journal Plant Disease Protection**, v. 125, n. 1, p. 21–26, 2018.

TADA, Y.; YAMAGUCHI, U. Necessity for *de Novo* DNA synthesis for reversion of *Escherichia coli* spheroplasts to bacillary forms. **Microbiology Immunology**, v. 27, n. 10, p. 893-896, 1983.

TISOT, B. **O comportamento da ferrugem asiática na soja ao longo dos anos, dificuldades de controle e seus custos.** Disponível em: <http://www.pioneersementes.com.br/media-center/artigos/193/o-comportamento-da-ferrugem-asiatica-na-soja-ao-longo-dos-anos-dificuldades-de-controle-e-seus-custos>. Acesso em: 19 jan 2019.

USDA, United States Department of Agriculture. **World Agricultural Production 2018.** Disponível em: <https://apps.fas.usda.gov/psdonline/circulars/production.pdf>. Acesso em: 22 dez. 2018.

YORINORI, J. T.; FREDERICK, R. D.; COSTAMILAN, L. M.; BERTAGNOLLI, P. F.; HARTMAN, G. E. Epidemics of Soybean Rust *(Phakopsora pachyrhizi*) in Brazil and Paraguay from 2001 to 2003. **The American Phytopathological Society**, v. 89, n. June, p. 2005, 2005.

ZAMBENEDETTI, E. B.; ALVES, E.; POZZA, E. A.; ARAÚJO, D. V. Germinação de urediniósporos de *Phakopsora pachyrhizi* em diferentes métodos de armazenamento. **Summa Phytopathol**, v. 33, n. 1, p. 83–85, 2007.

# APÊNDICES

FIGURA 1 - Distribuição da produção de soja no Brasil por estados (siglas) na safra de 2017-2018. Adaptado por Mônica A. Müller e Felipe Jauch de: CONAB, 2018.

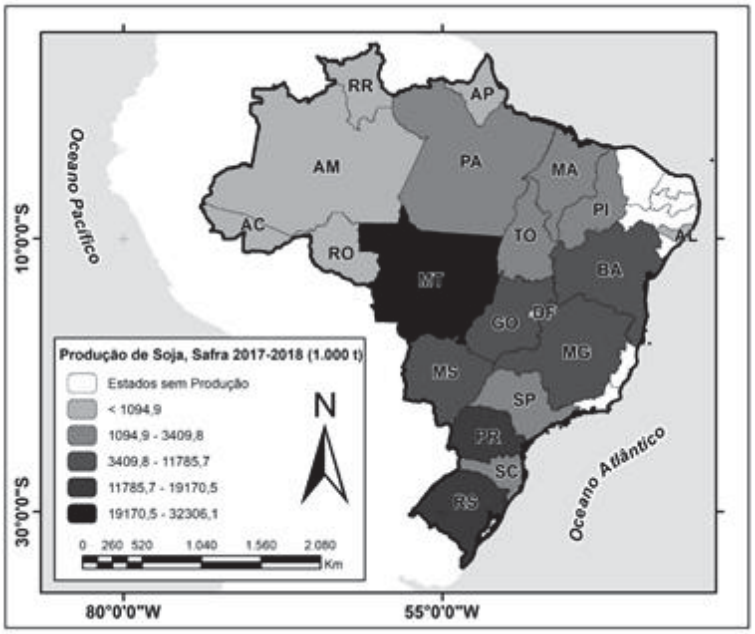


FIGURA 2 - Sintomas (A, B, C e D) e sinais (E, F, G e H) da ferrugem-asiática da soja (*Glycine max*), causada por *Phakopsora pachyrhizi*; urédias iniciando a liberação de urediniósporos de *P. pachyrhizi* em folha de soja. Fotos: Autor.

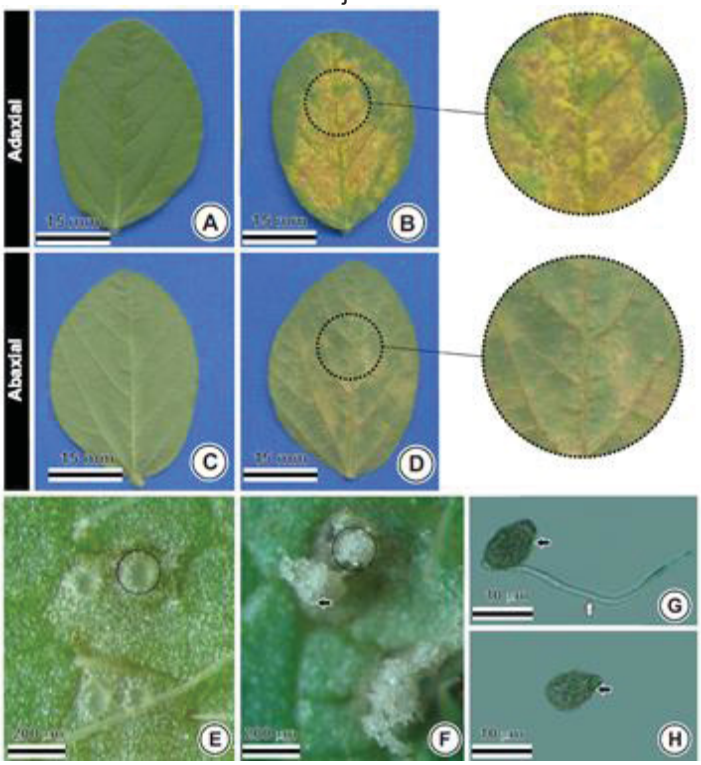


FIGURA 3 - Evolução dos sintomas de Ferrugem-asiática da soja em campo experimental de soja, sem inoculação artificial de *P. pachyrhizi*, safra 2015-2016, em intervalos de 15 dias, início dos sintomas (A), coloração marrom das folhas (B) desfolha prece total (C).