

Multiple resistance to DMI, QoI and SDHI fungicides in field isolates of *Phakopsora pachyrhizi*

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ABSTRACT

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* Syd & P. Syd, is the main disease that affects soybean crop in South America. Disease control is based on the use of site-specific fungicides (QoIs, DMIs and SDHIs), multisite fungicides and host-free period. Continuous and intensive use of fungicides has selected isolates of *P. pachyrhizi*, with reduced sensitivity to DMIs, QoIs and SDHIs. This work includes sensitivity studies for prothioconazole and SDHIs and genetic analysis of target site mutations in *P. pachyrhizi* related to sensitivity reduction to DMIs, QoIs and SDHIs collected from different regions of soybean production areas. Different *CYP51* genotypes with distinct point mutations known to influence DMI sensitivity are present in Brazilian isolates. Combined mutation F120L + Y131H on *CYP51* gene was the most frequent among Brazilian isolates. Mutant isolates showed higher EC₅₀ values to the DMI prothioconazole compared to wild type isolates. Regarding QoI, almost all Brazilian isolates carried the mutation F129L in the *CYTb* gene. Wild type of the *SdhC* gene was the most frequent genotype to SDHI fungicides, but the mutation leading to the I86F amino acid exchange has also been detected. Such isolates showed higher EC₅₀ values to the SDHIs bixafen, benzovindiflupyr and fluxapyroxad compared to wild type isolates. The most frequent genotype in our collection presented target site mutations in the *CYP51* and *CYTb* genes. A monouredinal isolate with mutations in all three target genes was detected and is here described for the first time. Its current and further spread, and the impact on field performance of fungicides with these modes of action needs further evaluation.

1. Introduction

Soybean is one of the most produced crop in the world, and Brazil is responsible for a third of the global production, being the second largest producer of this commodity (CONAB, 2018; USDA, 2018). It is a significant source of protein for livestock feeding and is also used for industrial purposes (Hirakuri and Lazzaroto, 2014; USB, 2017).

Asian soybean rust (ASR) is the main soybean disease in South America since its identification in 2001 (Yorinori et al., 2005), 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% (Dalla Lana et al., 2015; Hirano et al., 2010; Yang et al., 1991).

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 (Mayton et al., 2001; Van Der Plank, 1963). Another very important method is the establishment of the host-free period, when no soybeans are grown

to reduce the inoculum potential between growing seasons. This leads to a delay of disease onset and a reduced selection pressure by the various modes of action and is therefore an effective and important tool in disease and resistance management (Martin Neto and Farias, 2014).

Site-specific fungicides belonging to the demethylation inhibitors (DMIs) and quinone outside inhibitors (QoIs) effectively controlled the disease in the crop for years. However, efficacy of fungicides with these modes of action has decreased over time (Godoy et al., 2014) and the search for new molecules was intensified. 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. However, a reduction of SDHIs efficacy in the field was observed and reported by the annual monitoring of Embrapa 2–3 seasons after market introduction (Cláudia V. Godoy et al., 2017a). Multisite fungicides were also incorporated in the management of ASR, in order to control the disease and limit the increase of resistant strains within populations to site-specific fungicides (FRAC, 2018; Cláudia V.

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Godoy et al., 2017b).

Decrease of fungicide efficacy in the field can be conferred by different mechanisms, as efflux, overexpression, detoxification, and target site mutations whereas the main resistance mechanisms in phytopathogenic fungi are point mutations in fungicide target genes (Brent and Hollomon, 2007). The DMI target site is the 14- α demethylase in the ergosterol biosynthesis pathway, which is encoded by the *CYP51* gene (Gisi et al., 2000). The target protein of the QoIs, cytochrome *bc1*-ubiquinol oxidase, is encoded by the mitochondrial gene *CYTB* (Gisi et al., 2002; Grasso et al., 2006). The SDHI target site is the succinate dehydrogenase (SDH) complex in the respiratory chain (Yankovskaya et al., 2003). This enzyme complex is codified by *Sdh* gene, composed by subunits A, B, C and D (Glättli et al., 2009; Stammler et al., 2008, 2011).

For *P. pachyrhizi* there are reports of isolates with point mutations in the above mentioned genes conferring reduction of sensitivity to DMIs (Schmitz et al., 2014), QoIs (Kłosowski et al., 2016b, 2018) or SDHIs (Simões et al., 2018) 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 (Kłosowski et al., 2018). However, there are no reports on combined SDHI, DMI and QoI resistance in individual isolates so far. In Brazil, with the high selection pressure of these fungicides in soybean production areas, isolates may present multiple resistance to the three main groups used to control ASR.

In this work it was evaluated if isolates with multiple resistance to SDHIs, DMIs and QoIs can already be detected in Brazil. Therefore, sensitivity studies were performed, and genes were analysed for known target site mutations in isolates from four Brazilian states.

2. Materials and methods

2.1. *Phakopsora pachyrhizi* isolates

Samples of *P. pachyrhizi* isolates were collected from soybean fields from different locations (Cascavel and Ponta Grossa municipalities in Paraná State, Santo Antônio da Posse in São Paulo State, Diamantino in Mato Grosso State, Vilhena in Rondonia State) and seasons (2013/14, 2014/15, 2015/16 and 2016/17) (Fig. 1). Twenty-one isolates of *P. pachyrhizi* collected from soybean leaves infected by ASR were used in

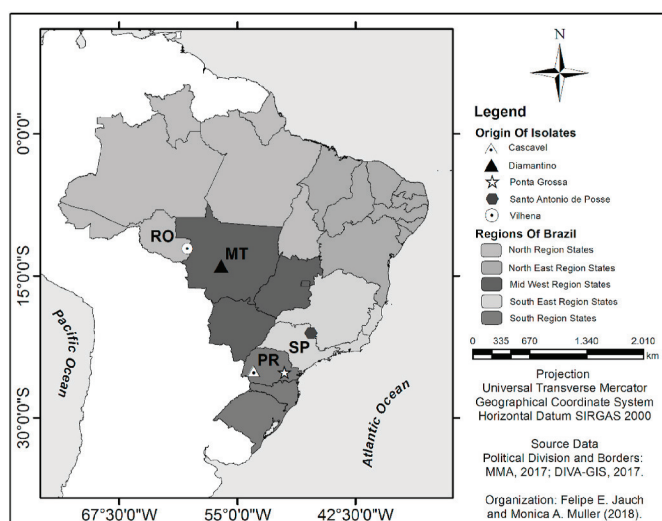


Fig. 1. Brazilian map municipalities (origin of the isolates) and states where leaves samples infected with Asian-soybean rust were collected. Brazilian States: RO – Rondonia, MT – Mato Grosso, SP – Sao Paulo and PR- Parana. Municipalities: Cascavel. Ponta Grossa, Santo Antonio da Posse, Diamantina e Vilhena.

the experiments, of which 7 were named “population” and 14 “monouredinial isolates”, according to the collection methodology. Population were composed of spores collected directly from field soybean leaves infected with ASR of different locations with the aid of a brush. Monouredinial isolates were composed of spores collected from the field but prepared from one single uredinia. So, from this single uredinia each monouredinial isolate was transferred several times to new soybean leaves until reaching the necessary amount to perform the experiments. In order to obtain an uredinia isolated, 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 uredinia 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 uredinia.

Monouredinial isolates and populations were transferred to healthy detached leaves every two weeks so the isolates were maintained and multiplied for the experiments for EC₅₀ detection and for genotyping of the target genes.

2.2. Sensitivity test/EC₅₀ assays/resistance factor

Detached leaves assays were performed to calculate the effective concentration to inhibit 50% of the pathogen activity (EC₅₀), for the DMI prothioconazole and the SDHIs bixafen, benzovindiflupyr and fluxapyroxad according to the leaf methodology proposed by FRAC (Fungicide Resistance Action Committee) (Scherb and Mehl, 2006). Resistance factors (RFs) were calculated by dividing the EC₅₀ value for each isolate with around 50% of I86F mutation by the mean EC₅₀ value of all the sensitive ones (WT). Commercially available formulated products were used for the 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’ (Saatbau, Linz, Austria), grown in the greenhouse, were treated with different fungicide concentrations (DMI: 0, 0.03, 0.1, 0.3, 1, 3, 10, 30 $\mu\text{g mL}^{-1}$; SDHIs: 0, 0.1, 0.3, 1, 3, 10, 30, 100 $\mu\text{g mL}^{-1}$). 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 h in growth chambers at 23 °C for the superficial drying of the leaves. Then, each unifoliolate leaf was removed and placed in disposable petri dishes containing water agar medium (1%) with streptomycin (30 mg L⁻¹) and kinetin (0.2 mg L⁻¹). For each concentration of the fungicide there were three Petri dishes containing a unifoliolate leaf, each plate represented a repetition.

A spore suspension of each *P. pachyrhizi* isolate was inoculated in the abaxial side of the unifoliolate leaf. The spore suspension was prepared in distilled water + Tween 20 (0.01%) with 10⁵ spores mL⁻¹. Petri dishes containing treated and inoculated leaves were incubated for 12 h in the dark at 23 °C and thereafter for 15 days at the same temperature with 12 h photoperiod. Then disease severity was evaluated by diagrammatic scale (Godoy et al., 2006) with six severity levels, varying from 0.6 to 78.5% of the leaf area with lesions. EC₅₀ was estimated from severity data at different fungicide concentrations.

2.3. Point mutation analysis

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 for CTAB-based DNA extraction (Schmitz et al., 2014).

2.3.2. Pyrosequencing

The Pyrosequencing technique was performed using PyroMark Gold 96 Reagents on a PSQ 96 MA machine (Qiagen, Hilden, Germany), for quantitative detection of F129L mutations in the *CYTB* gene (Kłosowski et al., 2016b) and for the mutations F120L, Y131 H/F, K142R, I145F, I475T in the *CYP51* gene (Schmitz et al., 2014). This procedure was performed in the Fungicide Resistance Laboratory of BASF SE (Limburgerhof, Germany).

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. Takyon qPCR MasterMix Plus dTTP No Rox reagents (Eurogentec, Köln, Germany) were used for the amplification, and the reactions were performed on a Rotor-Gene Q cycler (Qiagen, Hilden, Germany). The following conditions were applied: 5 min at 95 °C and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min (Simões et al., 2017). The relative allele frequencies were calculated based on the method described by Germer et al. (2000).

3. Results

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

The EC₅₀ values for the DMI prothioconazole ranged from 0.8 to 29.7 µg mL⁻¹ for the 21 *P. pachyrhizi* isolates analysed (Table 1). The monouredinal isolates of the Mid West region of Brazil presented higher

values of EC₅₀ than isolates from the other regions. Most of the isolates showed mutations in the *CYP51* gene, only the reference population GWH and 149/17 were the only samples that did not show mutations in the *CYP51* gene.

Within the *CYP51* gene, genotypes with the combined mutation (F120L + Y131H) were most frequent, corresponding to 85% of the isolates analysed. Monouredinal isolates with this combined mutation showed frequencies up to ~50% of the mutation F120L and Y131H. Other genotypes were also found: monouredinal isolate 191 with the Y131F and I475T mutations and the population named 193 with F120L, Y131H and I475T mutations. Isolates with mutations in the *CYP51* gene had higher EC₅₀ 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).

3.2. *CYTB* gene analysis

Nearly all monouredinal isolates and population 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 population 193 from the southeast presented a lower percentage (58%) of the F129L mutation.

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

The EC₅₀ values of *P. pachyrhizi* isolates to fungicides of the SDHI group ranged from 6.4 to 63.0 µg mL⁻¹ for bixafen; 0.2–71.4 µg mL⁻¹ for benzovindiflupyr; and 1.6–20.6 for fluxapyroxad (Table 2). The isolates sensitivity to fungicides varied without any specific pattern between the regions and seasons sampled.

Among the samples analysed, two of them (198 and 203) were considered completely mutated with around 50% I86F, the population CV17 presented 11% of the I86F mutation and the other 18 samples did not present the mutation I86F on the *SDH-C* gene. Isolates with approximately 50% of the mutation C-I86F (198, 203) presented higher EC₅₀ values than the wild type isolates and the population sample CV17 with only 11% I86F (Fig. 2).

The mean EC₅₀ for WT isolates was 11.75 µg mL⁻¹ for bixafen; 2.31 µg mL⁻¹ for benzovindiflupyr and 3.74 µg mL⁻¹ for fluxapyroxad, while for isolates with ~50% I86F mutation (198, 203), the mean EC₅₀ values were 47.05 µg mL⁻¹ for bixafen; 61.05 µg mL⁻¹ for benzovindiflupyr; and 16.0 µg mL⁻¹ for fluxapyroxad. Resistance factors for the population 198 were 5.36; 21.94 and 4.93 and for the monouredinal isolate 203 were 2.64; 30.90 and 5.50 for bixafen, benzovindiflupyr and fluxapyroxad, respectively.

The monouredinal isolate 203 and the populations 198 and CV17 that presented the mutation I86F on the *SdhC* gene also presented mutations on the genes *CYP51* and *CYTB* (Table 1; Table 2).

4. Discussion

Multiple selection of resistance to DMIs, QoIs and SDHIs fungicide groups was detected in one monouredinal isolate of *P. pachyrhizi*, collected in the 2016/17 season, in the north region of Brazil. Genotypes with mutations in the target genes of DMIs and QoIs were the most frequent ones, representing 76% of the isolates. Sensitivity tests showed a reduction of DMI sensitivity in isolates with the F120L + Y131H combined mutation in the *CYP51* gene. Isolates with the *SdhC* I86F mutation were less sensitive to SDHIs, however they were present in low frequency within Brazilian isolates (14%) in our study. On the other hand, the F129L mutation, which causes lower QoI sensitivity, was present in high frequency in all isolates analysed.

Since the 2009/10 season, F120L + Y131H mutant isolates were detected in all soybean production areas in Brazil (Kłosowski et al.,

Table 1
Sensitivity of monouredinal isolates and population 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
<i>North (Vilhena -RO)</i>						
202	16/17	3.2	42	40	0	0
203	16/17	11.0	29	28	0	0
Average		7.1				
<i>Mid West (Diamantino -MT)</i>						
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 ^b	NA	NA	NA
Average		18.9				
<i>South (Cascavel -PR)</i>						
174	16/17	7.3	45	43	0	0
CV 17 ^a	16/17	14.7	35	33	0	0
Average		11				
<i>South (Toledo -PR)</i>						
205	16/17	5.9	43	43	0	0
TL 16 ^a	15/16	3.9	45	45	0	0
TL 17 ^a	16/17	11.9	28	25	0	0
Average		7.2				
<i>South (Ponta Grossa – PR)</i>						
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
Average		6.5				
<i>South East (Santo Antônio de Posse – SP)</i>						
191	16/17	3.6	0	0	47	43
193 ^a	16/17	5.6	25	13	0	16
Average		4.6				
<i>Reference isolates</i>						
198 ^a		19.9	35	33	0	0
149/17 ^a		0.8	0	0	0	0
GWH ^a		1.5	0	0	0	0
138 m ³		2.8	49	50	0	0

^a Population.

^b Not Analysed (NA).

Table 2

Sensitivity of monouredinal isolates and population 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 <i>SdhC</i> gene (%)	Mutation in <i>CYTb</i> gene (%)
		Bixafen	Benzovindiflupyr	Fluxapyroxad	I86F	F129L
<i>North (Vilhena -RO)</i>						
202	16/17	11.4	3.1	3.7	WT ^b	98
203	16/17	31.1	71.4	20.6	50	95
Average		21.2	37.2	12.1		
<i>Mid West (Diamantino -MT)</i>						
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
Average		15.4	2.8	4.0		
<i>South (Cascavel -PR)</i>						
174	16/17	10.2	2.5	2.6	WT	94
CV17 ^a	16/17	8.1	9.3	10.0	11	94
Average		9.1	5.9	6.3		
<i>South (Toledo -PR)</i>						
205	16/17	3.1	1.4	1.7	WT	93
TL 16 ^a	15/16	8.8	2.5	4.2	WT	100
TL 17 ^a	16/17	10.6	1.5	2.3	WT	94
Average		7.5	1.8	2.7		
<i>South (Ponta Grossa – PR)</i>						
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
Average		12.4	2.3	4.7		
<i>South East (Santo Antônio de Posse – SP)</i>						
191	16/17	8.5	1.1	3.9	WT	WT
193 ^a	16/17	8.8	2.6	4.2	WT	58
Average		8.6	1.8	4.0		
<i>Reference isolates</i>						
198 ^a		63.0	50.7	11.4	47	52
149/17 ^a		13.0	1.7	3.6	WT	93
138 m ³		6.4	2.5	1.8	WT	91
GWH ¹		19.2	2.1	4.7	WT	99

^a Population.

^b Wild Type (WT).

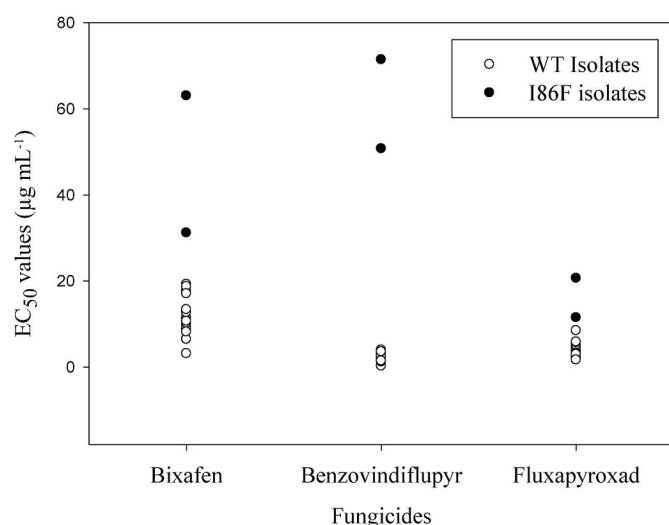


Fig. 2. Sensitivity of isolates of *Phakopsora pachyrhizi* to fungicides of succinate dehydrogenase inhibitors (SDHIs) group by effective concentration to inhibit 50% of the pathogen (EC₅₀) value, to wild type (WT) or 50% mutant (I86F) isolates (n = 21 isolates in total) in the *SdhC* gene.

2018; Schmitz et al., 2014). This spread of mutated isolates is the result of the long time and continuous use of different DMI fungicides for control of *P. pachyrhizi* since season 2002/03 (Reis et al., 2018). With such a high selection pressure of DMI fungicides over the years, less

sensitive individuals emerged and remain present within the population. Isolates with mutations in the *CYP51* gene were less sensitive to the DMI used in our study, prothioconazole, which is in line with previous reports on DMI adaptation (Kłosowski et al., 2016a; Schmitz et al., 2014).

Not only point mutations in the *CYP51* gene, but also overexpression of this gene was described for *Blumeriella jaapii* (Ma et al., 2006), *Monilinia fructicola* (Luo and Schnabel, 2008), *Puccinia triticina* (Stammler et al., 2009), *Venturia inaequalis* (Schnabel and Jones, 2001) and also the up-regulation of efflux-transporter in *Botrytis cinerea* (Hayashi et al., 2002), *Penicillium digitatum* (Nakaune et al., 1998), *Mycosphaerella graminicola* (Zwiers et al., 2002) have been described as resistance mechanisms which reduce DMI sensitivity. For *P. pachyrhizi*, *CYP51* overexpression and target site mutations (F120L, Y131H, Y131F, K142R, I145F and I475T) were identified as DMI resistance mechanisms (Schmitz et al., 2014), while no reports are so far available on a potential influence of enhanced efflux activity.

In previous studies all isolates of *P. pachyrhizi* containing the F120L + Y131H showed overexpression of the *CYP51* gene, and those containing the Y131F + I145T did not (Schmitz et al., 2014). Overexpression of *CYP51* can also compensate for reduced enzyme function as a result of particular target site alterations (alone or in combination). This suggests that in the present work, although the overexpression has not been evaluated, it may be present in those isolates with F120L + Y131H and may be responsible for the stronger DMI adaptation of some isolates, such as 161 or 164. In the same way, the isolate 191, with Y131F + I145T, may have presented higher sensitivity for DMI prothioconazole due to the lack of overexpression. Because of the interaction of target gene overexpression on DMI sensitivity, it is not clear so far

if the F120L + Y131H cause stronger DMI adaptation than other *CYP51* genotypes such as Y131F + I145T.

Interestingly, the *CYP51* gene of 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 (Schmitz et al., 2014). Monouredinal isolates analysed from 2009/2010 season by Schmitz et al. (2014) showed a mean frequency of 33% for both, F120L and Y131H, over all isolates containing these mutations included in their study, while in the present study the isolates from the 2015/2016 season contained around 50% of these mutations. However, which alleles are expressed is fundamental and Schmitz et al. showed that the mutated ones were overexpressed compared to the wild types, which consequently led to higher mutation frequencies in the cDNA (Schmitz et al., 2014). That might explain why the higher percentage of the F120L + Y131H in DNA in some of our isolates appeared not to be related with lower DMI 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 (Avenot et al., 2012; Rehfus et al., 2018; Sierotzki and Scalliet, 2013; Veloukas et al., 2011). So far, the mutation I86F located in the subunit C of the *Sdh* gene, was only found in *P. pachyrhizi* in season 2015/16 in Rio Grande do Sul, the southernmost state of Brazil (Simões et al., 2018). In this work one monouredinal isolate and a population with the I86F mutation were found in season 2016/17 and both isolates also presented mutations in the target genes of DMI and QoI fungicides. The monouredinal 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 locations allow two soybean crop cycles in one season. 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 I86F mutation were less efficiently controlled in our assays by the three SDHIs included in this study than the wild type isolates. Calculation of resistance factors (RF) was done by division of mean EC_{50} of the 2 isolates with ~50% I86F by mean EC_{50} values of all wild type isolates. These were 4, 26 and 4 for bixafen, benzovindiflupyr and fluxapyroxad (supplemental table 1). Our RF values for benzo-vindiflupyr and fluxapyroxad were similar to those of Simões et al. (2017), but lower for bixafen. This might be caused by the mean EC_{50} wild type value to bixafen, which is lower in the study of Simões et al. (2017) than in ours (1.2 vs. 11.8). Both studies show that all three SDHIs were affected, but a dose response was demonstrated for each and the resistance factors are rather limited. Compared with different SDHI resistance cases in other pathogens e.g. in *Botrytis cinerea* caused by different mutations (Veloukas et al., 2011), the resistance factors found in *Phakopsora pachyrhizi* caused by the I86F mutation in the *SDH-C* gene are lower.

The detection of F129L in the *CYTB* gene first occurred for *P. pachyrhizi* isolates in samples from the 2013/14 season, where 35% of the Brazilian isolates presented this mutation (Kłosowski et al., 2018). In the present study almost all monouredinal isolates and populations presented this mutation with high frequency. The F129L single mutation is so far the only mutation in the *CYTB* gene described in *P. pachyrhizi* isolates (Kłosowski et al., 2018). In other plant pathogenic fungi, the G143A and rarely the G137R mutation in the *CYTB* gene have been detected (FRAC, 2006). The G143A is described as the most common mutation and the mutation which causes high resistance factors, while F129L is described as having less effect on QoI sensitivity reduction (FRAC, 2006; Kim et al., 2003). Because of the gene structure (presence of intron after codon 143), the occurrence of the G143A mutation seems rather unlikely to occur in rust fungi, including *P. pachyrhizi* (Grasso

et al., 2006; Schmitz et al., 2014).

Competitiveness and fitness of isolates might be reduced by the presence of resistance mechanisms. The emergence and spread of resistance depend on whether they are able to outcompete pre-existing sensitive isolates under selection pressure and under non-selection pressure. Competitivity assays with *P. pachyrhizi* resistant isolates to DMIs and QoIs and wild type isolates showed that the fitness might be reduced by DMI resistance mechanisms (target site mutations in the *CYP51* and/or *CYP51* overexpression), but not for the F129L mutation in the *CYTB* gene (Kłosowski et al., 2016a). No reports on competitiveness and fitness are so far available for *SdhC* I86F mutants of *P. pachyrhizi*.

The mutation I86F is still detected at low levels in the field and dynamic of these mutation in the season 2017/18 showed a stabilization in the frequency in Brazil compared to previous seasons (FRAC, 2019). Therefore, it is rather likely that this mutation is connected to fitness penalties. Studies performed with other pathogens indicated fitness penalties at least for some mutations. SDHI-resistant *Botrytis cinerea* were less competitive than wild type isolates and, therefore, the alternation of groups of fungicides application should reduce the frequency of SDHI resistant isolates (Lalève et al., 2014; Veloukas et al., 2014). Studies on this subject with *P. pachyrhizi* are important to elucidate the dynamic of mutant isolates in the population and will be performed. When there are fitness costs connected with specific resistance mechanisms, resistance management strategies can be efficient.

5. Conclusion

Although isolates with mutations on genes that confers reduced sensitivity to three chemical fungicide groups (DMI, QoI, SDHI) are still rarely observed, the results of this study suggest that the continuous use of these three modes of action bear the risk for development of multiple resistance. Therefore, the following control measures are suggestions to avoid further ASR multiple resistance isolates selection: i) implementation of a limited soybean sowing schedule, 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; ii) incorporation of multisite fungicides on management, since they affect different metabolic processes of the fungus and present low risk of resistance and; iii) use of cultivars with lower susceptibility to ASR, in order to reduce the amount of inoculum produced.

CRedit authorship contribution statement

Mônica Anghinoni Müller: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Investigation. **Gerd Stammler:** Conceptualization, Methodology, Visualization, Writing – review & editing. **Louise Larissa May De Mio:** Conceptualization, Supervision, Project administration, Visualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Table

Mean EC_{50} values of wild type (WT) and I86F mutant isolates (with ~50% I86F) of *Phakopsora pachyrhizi* in the *SdhC* gene to SDHIs.

Mutation in <i>SdhC</i> gene (%)	Mean EC_{50} of SDHI fungicides ($\mu\text{g mL}^{-1}$)		
	Bixafen	Benzovindiflupyr	Fluxapyroxad
WT [†]	11.75	2.31	3.74
I86F [‡]	47.05	61.05	16.00
RF value	4	26	4

[†]18 isolates.

[‡]2 isolates.

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