Klosowski et *al.*, Detection of Mutations in CYP51 and CYTB Genes of *Phakopsora pachyrhizi* Isolates and Competitive Fitness of Mutated and Wild Type Isolates. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 233-238. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Detection of Mutations in CYP51 and CYTB Genes of *Phakopsora pachyrhizi* Isolates and Competitive Fitness of Mutated and Wild Type Isolates

Klosowski AC[1], Brahm L[2], Rodrigues R[3], Stammler G[2], May De Mio LL[1]

[1]*Universidade Federal do Paraná, Rua dos Funcionários 1540, 80035-050 Curitiba, Brazil.*
[2]*BASF SE, Speyerer Straße 2, 67117 Limburgerhof, Germany.*
[3]*BASF SA Estação Experimental Agrícola, Rodovia SP 340 – Km 144, 13830-000 Santo Antonio de Posse, Brazil.*
Email: ana_clkl@yahoo.com.br

ABSTRACT

The Asian soybean rust, caused by *Phakopsora pachyrhizi*, is controlled by sterol demethylation inhibitor (DMI) and quinone outside-inhibiting (QoI) fungicides in Brazil. Mutations in *CYP51* (sterol 14α-demethylase) and *CYTB* (cytochrome *b*) genes can lead to pathogen resistance to DMIs and QoIs, respectively. The occurrence of the mutations in both genes was investigated in 41 Brazilian isolates of *P. pachyrhizi* sampled during the 2012-2013 and 2013-2014 seasons. Additionally, we investigated if fitness costs are associated with mutations in the *CYP51*, and/or in the *CYTB* gene, in competition trials. For *CYP51* and *CYTB* analysis, the DNA of *P. pachyrhizi* spores was extracted and pyrosequencing assays applied to detect and quantify mutations. For competition assays, seven isolates from the BASF SE collection with different *CYP51* and/or *CYTB* haplotypes were used. Spores of sensitive wild type isolate and isolates with different *CYP51* and/or *CYTB* haplotypes were mixed and inoculated on detached soybean leaves. Frequency of relevant target site mutations were measured over four disease cycles using pyrosequencing. In the analysis of *CYP51* gene, only one *P. pachyrhizi* isolate was wild type, whereas most isolates showed the mutation combination F120L+Y131H and two isolates showed a triple combination (F120L+Y131F+I475T). The analysis of *CYTB* gene showed the presence of the F129L mutation in approximately 50% of isolates. In the competition assays, isolates with lower DMI sensitivity and different *CYP51* haplotypes had competitive disadvantages compared with sensitive *CYP51* wild type isolates. The isolate with the F129L CYTB competed equally well with the QoI sensitive and *CYTB* wild type isolate, under the conditions of this experiment.

INTRODUCTION

Asian soybean rust, caused by *Phakopsora pachyrhizi*, is a widespread foliar disease and has potential for great damage due to premature defoliation interfering directly in the grain formation and filling. Yield loss up to 80% has been reported in the absence of control measures (Hartman *et al*. 1991; Yang *et al*. 1991).

In Brazil, the disease is mostly controlled by two fungicide groups: sterol demethylation inhibitor (DMI) and quinone outside-inhibiting (QoI) fungicides. Reduced efficiency of fungicides for soybean rust control in Brazil has been reported (Godoy *et al*. 2014; Godoy *et al*. 2015) and associated with a lower DMI and QoI sensitivity (Schmitz *et al*. 2014; Klosowski *et al*. 2015; FRAC 2015). Mutations in *CYP51* and *CYTB* genes can lead to resistance to DMIs and QoIs, respectively. Resistance conferring mutations can reduce the efficiency of important physiological and biochemical processes in the pathogen, leading to lower fitness. A study of the population sensitivity to fungicides and knowledge of potential fitness costs linked with less sensitive strains are needed to understand the dynamics of resistance in the field and therefore recommend a suitable resistance management program to preserve and to retain the efficacy of the products.

The aims of this work were: to monitor mutations in the *CYP51* and *CYTB* genes in *P. pachyrhizi* isolates from Brazil using pyrosequencing assays, and to investigate if fitness costs are associated with mutations F120L, Y131F/H, K142R, I475T, in the *CYP51*, and/or F129L, in *CYTB* gene.

MATERIAL AND METHODS

P. pachyrhizi isolates

Molecular analysis of CYP51 and CYTB genes

Isolates of *P. pachyrhizi* were obtained by transferring spores from single uredia of infected leaves from the 2012-2013 and 2013-2014 seasons to healthy unifoliate leaves using an inoculation needle. The isolates were multiplied by transferring the spores to healthy leaves every two weeks by inoculation of a spore suspension in water with Tween (0.01%) to the abaxial surface of leaves, using an airbrush (0.3 mm nozzle; alpha Arprex® 3). The leaves were kept in Petri dishes with water agar (1%), including streptomycin sulfate (30 mg L^{-1}) and kinetin (0.2 mg L^{-1}) and were incubated at 23^{o}C with a photoperiod of 12 hours.

Competition assay

Isolates of *P. pachyrhizi* were obtained from the BASF SE (Limburgerhof, Germany) culture collection (Table 1). The isolates with mutations in the *CYP51* gene were previously characterized by Schmitz et *al*. (2014) and the isolate with a mutation in the *CYTB* gene was characterized by the BASF SE group.

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-39-5

Pyrosequencing for mutations in *CYP51* and *CYTB* genes

The point mutations F120L, Y131F, Y131H, K142R and I475T in *CYP51* gene and F129L in *CYTB* gene were analyzed for 31 and 41 Brazilian isolates, respectively. PCR amplifications and pyrosequencing assay were carried out using the primers described by Schmitz et *al*. (2014) and Klosowski et *al*. (2015) and following procedures and conditions previously published by them. For the isolates used in the competition assay, pyrosequencing was performed to confirm the presence and to quantify the mutations described previously by Schmitz et *al*. (2014) and the BASF SE group.

Competition assay

Mancozeb was sprayed on soybean plants one day before inoculation at 50 mg L^{-1} to verify the behavior of *P. pachyrhizi* isolates under stress conditions caused by the multi-site fungicide. The suspensions of spores were prepared in water with Tween (0.01%) for each isolate and were adjusted to 2.5 x 10^4 urediniospores mL^{-1}. "S" means wild type for *CYP51* and *CYTB*. "M" means mutation in *CYP51* and/or *CYTB*. M1, M2, M3 and M4 describe different *CYP51* and/or *CYTB* haplotypes. A detailed description of isolates and their mutations is given in Table 1. Mixtures of the sensitive isolate (8) with mutated isolates (72, 62, 63, 27 and 28) were made as follows: 20% S + 80% M1, 20% S + 80% M2, 20% S + 80% M3. For the mutations M2 and M3, a mixture of two isolates (62 and 63; 27 and 28, respectively) were used. For mutation in *CYTB* gene, the experiment involved three isolates, 8, GWH-B and 72. Mixtures of 20% S + 80% M4 and 20% M1 + 80% M4 were made.

Table 1 Isolates of *Phakopsora pachyrhizi* of Brazil and their *CYP51* and *CYTB* mutations

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

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

The spore suspension was inoculated using the same procedure as for inoculum multiplication described earlier. For each mixture, six non-treated leaves and six mancozeb-pretreated leaves were inoculated. After 21 days of incubation, the sporulating lesions were placed in 5 mL of water with Tween (0.01%) and shaken to release the spores. The resulting suspension was used to inoculate new six non-treated leaves and six mancozeb-pretreated leaves (6 mL), starting a new disease cycle, and to extract DNA (2 mL) for each subsequent pyrosequencing assay. The procedure was repeated after every disease cycle and the experiment was concluded after four cycles. To quantify the frequency of mutations in the mixtures, the

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-39-5

pyrosequencing assay was done at the outset of the experiment and after every disease cycle, following the procedures described by Schmitz et *al*. (2014) and Klosowski et *al*. (2015). The frequency of resistant isolates in the last cycle was compared with the initial frequency by the pairwise Student's *t* test. The data analysis was performed using the statistical software R (R Development Core Team, Vienna, Austria).

RESULTS AND DISCUSSION

Molecular analysis of CYP51 and CYTB genes

Most *P. pachyrhizi* isolates (81 %) showed the mutation combination F120L+Y131H in the CYP51 gene, about 10 % showed Y131F+I475T and a triple combination (F120L+Y131F+I475T) was found in two out of the 31 isolates tested (6 %). One isolate carried wild type *CYP51* and *CYTB* genes. The F129L mutation was found in the *CYTB* gene in 21 of 41 isolates (51%, Figure 1). Multiple resistance, i.e. resistance to both DMI and QoI fungicides, due to target site mutations, was observed for 18 isolates (Figure 1).

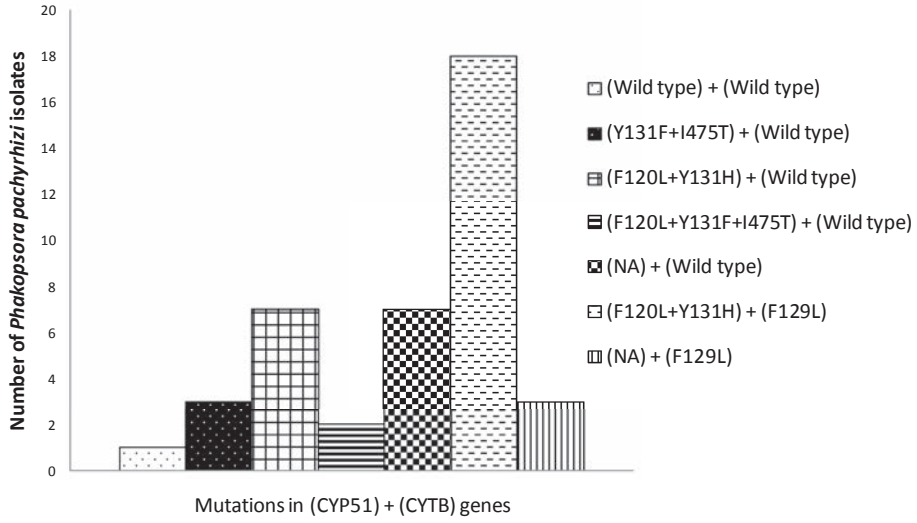

Figure 1 Presence of mutations in cytochrome P450 14α-sterol demethylase (CYP51) and cytochrome b (CYTB) genes of Brazilian monouredinial isolates of *Phakopsora pachyrhizi*. CYP51 and CYTB genes were assessed for 31 and 41 isolates, respectively. Wild type: mutation not detected; NA: CYP51 not assessed.

Isolates showing triple-point mutations in *CYP51*, F120L+Y131F+I475T, are reported for the first time for *P. pachyrhizi*. The prevalence of the F120L+Y131H mutation combination and the appearance of isolates showing a triple combination suggest that these mutations might be related to the evolutionary adaptability of isolates, which may be associated with a lower fitness cost relative to other mutations or combination of mutations. Just one isolate was wild type, i.e., no mutations tested in *CYP51* gene were found, whereas in Schmitz *et al*. (2014)

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-39-5

work, with isolates collected in 2009-2010 season, 22% of isolates (20 of 88) were wild type. This means that from 2009-2010 to 2013-2014 season, the proportion of mutated isolates increased and currently most isolates have mutations related with a lower sensitivity to DMIs in Brazil. The F129L mutation in *CYTB* gene was found for *P. pachyrhizi* isolates since the 2012-2013 season (Klosowski *et al*. 2015) and the dynamic of this mutation in *P. pachyrhizi* populations in Brazil and the impact on field efficacy of QoI fungicides should be monitored in future seasons.

The relatively high frequency of mutated isolates, especially the isolates with multiple resistance, indicates that the continued use of DMIs and QoIs to control soybean rust has led to the selection of mutated individuals.

Competition assay

The frequency of isolates with *CYP51* mutations decreased in the mixtures with wild type isolate after four disease cycles ($p \leq 0.01$) both on non-treated leaves and mancozeb-pretreated leaves. The frequency of isolate with F129L mutation,"M4", in the mixture with sensitive isolate "S" after four disease cycles was not different from the initial frequency ($p \leq 0.01$) both on non-treated and mancozeb-pretreated leaves. In the mixture of isolates "M4" and "M1", the frequency of the first increased during the four disease cycles and the results were similar for non-treated and treated leaves (Table 2).

The use of multi-site fungicide mancozeb did not have effect on the dynamics of competition among wild type isolate and isolates with mutation in *CYP51* and *CYTB* genes (Table 2).

Table 2 Frequency of *CYP51* and *CYTB* mutations in competition assays between sensitive (S=wild type) and mutated isolates (M1, M2, M3 and M4 in *CYP51* and F129L in *CYTB* gene) of *Phakopsora pachyrhizi* during four disease cycles on non-treated and mancozeb-pretreated-soybean leaves.

Gene	Multi-site fungicide treatment	Mixture	% of mutation				
			Initial	1° cycle[a]	2° cycle	3° cycle	4° cycle
CYP51	Non-treated leaves	S+M1 (F120L+Y131H)[b]	17.5	14.5	13.5	14.0	12.5*
		S+M2(Y131F+K142R)	27.5	16.5	10.5	7.5	8.5*
		S+M3(Y131F+I475T)	25.5	16.0	15.0	11.0	12.0*
	Mancozeb-treated leaves	S+M1 (F120L+Y131H)	17.5	13.5	12.5	11.0	11.0*
		S+M2(Y131F+K142R)	27.5	14.0	14.0	7.0	8.0*
		S+M3(Y131F+I475T)	25.5	14.5	16.0	8.0	8.5*
CYTB	Non-treated leaves	S+M4 (F129L)[b]	87.5	90.5	88.0	84.0	86.5
		M1+M4 (F129L)	75.0	80.0	81.5	82.0	84.5*
	Mancozeb-treated leaves	S+M4 (F129L)	87.5	85.5	86.0	83.0	83.5
		M1+M4 (F129L)	75.0	72.5	81.5	86.0	90.5*

[a] Soybean rust cycles

[b] Mutation(s) analyzed by pyrosequencing

* Means are significantly different from the initial value according the pairwise Student's *t* test ($p \leq 0.01$).

Note: All mixtures contain 80% of mutated isolates + 20% of sensitive isolates but lower than expected frequencies were detected for the CYP51 mutations as reported previously by Schmitz *et al*. (2014).

Isolates with lower DMI sensitivity and three different *CYP51* haplotypes had competitive disadvantages compared with the sensitive *CYP51* wild type isolate. This competitive disadvantage might be used for resistance management strategies. Tools that reduce the selection pressure, such as limitation of number of applications, alternation and mixing with different modes of action, should be implemented in disease control strategies. The isolate with the F129L mutation in *CYTB* competed equally well with the sensitive, *CYTB* wild type isolate, under the conditions of this experiment. However, our studies on F129L fitness costs were performed with only one mutated isolate and one host plant cultivar. Therefore, more extensive competition studies, involving a larger number of resistant and sensitive isolates and host cultivars would be useful.

REFERENCES

FRAC 2014. Mutations associated with QoI-resistance. Online. http://www.frac.info/docs/default-source/about-frac/mutations-associated-with-qoi-resistance.pdf

Godoy CV; Utiamada CM; Meyer MC; Campos HD; Pimenta CB; Cassetari Neto D. *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. Embrapa, Londrina, Brazil.

Godoy CV; Utiamada CM; Meyer MC; Campos HD; Forcelini CA; Pimenta CB; Borges EP; de Andrade junior ER; Siqueri FV; Juliatti FC; Favero F; Feksa HR; Grigolli JFJ; Nunes, junior J; Carneiro LC; da Silva LHCP; Sato LN; Canteri MG; Volf MR; Debortoli MP; Goussain M; Martins MC; Balardin RS; Furlan SH; Madalosso T; Carlin VJ; Venancio WS (2015). *Eficiência de fungicidas para o controle da ferrugem-asiática da soja, Phakopsora pachyrhizi, na safra 2014/15*: resultados sumarizados dos ensaios cooperativos. *Circular Técnica* 111. Embrapa, Londrina, Brazil.

Hartman GL; Wang TC; Tschanz AT (1991). Soybean rust development and the quantitative relationship between rust severity and soybean yield. *Plant Disease* 75, 596-600.

Klosowki AC; May De Mio LL; Miessner S; Rodrigues R; Stammler G (2015). Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi. Pest Management Science.* http://onlinelibrary.wiley.com/doi/10.1002/ps.4099/abstract, DOI: 10.1002/ps.4099

Schmitz HK; Medeiros CA; Craig IR; Stammler G (2014). Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. *Pest Management Science* 70, 378-388.

Yang XB; Tschanz AT; Dowler WM; Wang TC (1991). Development of yield loss models in relation to reductions of components of soybean infected with *Phakopsora pachyrhizi. Phytopathology* 81, 1420–1426.