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CROP PROTECTION

In vivo sensitivity of *Phakopsora pachyrhizi* to fungicides

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ABSTRACT: *Asian soybean rust is one of the most destructive diseases that can be found in this crop. It can be largely controlled by fungicide application. The objective was to assess the sensitivity of P. pachyrhizi isolates to fungicides. The tests were performed in a completely randomized design, with six replicates. The sensitivity of twelve isolates to site-specific and multisite fungicides at concentrations of 0.1; 1.0; 10.0, and 100.0 mg L^{-1}, plus a control with absence of fungicide (0.0 mg L^{-1}) was assessed. Soybean leaflets were immersed in the appropriate fungicide solutions, disposed in wet chambers in plastic boxes, and inoculated using each uredinia suspension of P. pachyrhizi (5.0 x 10^4 uredospores mL^{-1}), separately. Boxes were incubated for 20 days at a temperature of 23°C and a 12-hour photoperiod. Next, the number of uredinia per cm^2 on the abaxial face of each leaflet was evaluated. The active ingredients prothioconazole, trifloxystrobin, fluxapiroxade, trifloxystrobin + prothioconazole, trifloxystrobin + bixafen + prothioconazole, azoxystrobin + benzovindiflupyr, and azoxystrobin + benzovindiflupyr + diphenoconazole were highly fungitoxic for the majority of the isolates, with EC_{50} lower than 1.0 mg L^{-1}. Diphenoconazole, azoxystrobin, and fenpropimorph were considered moderately fungitoxic for nine of the twelve isolates, with EC_{50} between 1 and 10 mg L^{-1}. The multisites mancozeb and copper oxychloride presented EC_{50} responses classified as low toxic for the twelve isolates and eight for chlorothalonil (EC_{50} between 10 mg L^{-1} and 50 mg L^{-1}). Site-specific fungicides showed high-to-moderate fungitoxicity to P. pachyrhizi isolates, even as the multisites presented moderate-to-less toxic activity.*
Key words: *Asian soybean rust, detached leaf test, effective concentration, multisite fungicide, soybean.*

Sensibilidade *in vivo* de *Phakopsora pachyrhizi* a fungicidas

RESUMO: *A ferrugem-asiática da soja é uma das doenças mais destrutivas que ocorre na cultura. Seu controle é baseado, principalmente, na aplicação de fungicidas. O objetivo foi avaliar a sensibilidade de isolados de P. pachyrhizi a fungicidas. Os ensaios foram realizados em delineamento inteiramente casualizado, com seis repetições. Por meio de teste de folíolos destacados de soja, foram avaliadas as sensibilidades de doze isolados do fungo a fungicidas sítio-específicos e multissítios, nas concentrações de 0,1; 1,0; 10,0 e 100,0 mg L^{-1}, mais uma testemunha sem fungicida (0,0 mg L^{-1}). Os folíolos de soja foram imersos nas devidas soluções fungicida, dispostos em câmaras úmidas em caixas gerbox e inoculados com as devidas suspensões de esporos de P. pachyrhizi (5,0x10^4 uredosporos mL^{-1}). As caixas foram incubadas durante 20 dias, em temperatura de 23 °C e fotoperíodo de 12 h. Em seguida, avaliou-se o número de urédias cm^{-2} da face abaxial de cada folíolo. Os ingredientes ativos protioconazol, trifloxistrobina, fluxapiroxade, trifloxistrobina + protioconazol, trifloxistrobina + bixafem + protioconazol, azoxistrobina + benzovindiflupir e azoxistrobina + benzovindiflupir + difenoconazol foram altamente fungitóxicos para a maioria dos isolados, com CE_{50} menor do que 1,0 mg L^{-1}. Difenoconazol, azoxistrobina e fenpropimorfe foram considerados medianamente fungitóxicos para nove dos doze isolados, com CE_{50} entre 1 e 10 mg L^{-1}. Os multissítios mancozebe e oxicloreto de cobre apresentaram respostas de CE_{50} classificadas como pouco tóxicas para os doze isolados do fungo e o clorotalonil para oito deles (CE_{50} entre 10 e 50 mg L^{-1}). Os fungicidas sítio-específico apresentaram alta a moderada fungitoxicidade aos isolados de P. pachyrhizi oriundos dos distintos locais, enquanto os multissítios apresentaram atividade moderada a pouco tóxica.*
Palavras-chave: *concentração efetiva, ferrugem-asiática, folíolos destacados, fungicida multissítio, soja.*

INTRODUCTION

The *P. pachyrhizi* fungus is the causal agent for Asian soybean rust (ASR) and it had been first reported in Brazil, at the end of the 2000/2001 season, as being a threat to the American continent (GODOY et al., 2006). The disease damage could reach more than 90%, but its impact on production depend on the presence of climatic conditions favorable to pathogen development and also on the characteristics related to the soybean cultivar used, such as, cycle, phenological stage, and architecture (YANG et al., 1991; DEBORTOLI et al., 2012; TORMEN et al., 2012). Among the disease management strategies used, the application of fungicides is the most significant for maintenance of disease severity below damage level (KLOSOWSKI et al., 2016). In Brazil, the application of fungicides for ASR control was

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initiated in the 2002–2003 soybean season, using products from the triazoles group (demethylation inhibitors - DMIs), followed by mixtures of triazoles and strobilurins (quinone outside inhibitors - QoIs) (REIS et al., 2014); and finally, mixtures containing carboxamides (succinate dehydrogenase inhibitors - SDHIs), with the use of multisites and morpholines as disease control reinforcement.

EDGINGTON et al. (1971) defined the criteria for associating a fungicidal substance with the level of fungitoxicity. In this regard, the authors elaborated a scale, based on the concentrations of active ingredients that inhibited 50% of spore germination (50% inhibitory concentration - IC_{50}) or on the ones that controlled 50% of the disease severity (effective concentration of 50% - EC_{50}). Concentrations less than 1 mg L^{-1} are considered highly toxic to the fungus, values between 1 and 10 mg L^{-1} are moderate, between 10 and 50 mg L^{-1} are slightly fungitoxic, and above 50 mg L^{-1}, have no toxic effect on the fungus.

One of the problems of fungicide application for disease control is the rise of resistant phytopathogenic fungi in the population (GHINI & KIMATI, 2002). When threatened, the fungus can develop mechanisms that make it resistant to products that were previously considered toxic. The great diversity of the fungus and its capacity of multiplication are characteristics that generate an opportunity for the selection of resistant races spontaneously (PARREIRA et al., 2009).

Authors such as DEKKER (1977) and GHINI & KIMATI (2002) explained that genetic changes that result in the pathogen resistance to fungicides occur more easily with compounds that act primarily on one or in a few action sites of the fungus than fungicides that act on multiple sites of the fungal metabolic process. The continuous use of the same active ingredient may promote the selection of resistant phytopathogenic fungi, which may lead to a reduction in disease control, due to the loss of fungal sensitivity to fungicides (GHINI & KIMATI, 2002).

The development of fungal resistance to fungicides is based on genetic and biochemical mechanisms. These include alteration of the target site due to mutation in the gene encoding it, reduction in absorption or increase of fungicide efflux (xenophobia), lack of conversion to the active compound, compensation through increased production of the target enzyme, by overexpression and development of alternative metabolic pathways that do not include the target site of the fungicide, such as the use of an alternative respiration pathway (BRENT & HOLLOMON, 2007; FERNÁNDEZ-ORTUÑO et al., 2010; LEROUX & WALKER, 2011).

The fungi resistance that is characterized by a rapid and marked loss of effectiveness of a fungicide, presenting different responses between sensitive and resistant populations, is classified as qualitative. When slower resistance occurs, affecting both susceptible and resistant populations, it is classified as quantitative and reversible (BRENT, 1995). The first is controlled by few genes, with a marked (oligogenic) effect. For many fungicides, mutation in a single gene is sufficient to acquire a high degree of resistance, regardless of the fungal species (BRENT & HOLLOMON, 2007). Conversely, quantitative resistance is caused by many genes, each of them being responsible for a small (polygenic) effect (GEORGOPOULOS, 1995). In the case of polygenic inheritance, for a high degree of resistance to occur, there is a need for mutations in many genes, each of them should have an additive effect (BRENT & HOLLOMON, 2007).

In recent years, a reduction in fungicide efficiency has been observed, which has resulted in a failure of ASR control. This has been confirmed by the detection of mutations in *P. pachyrhizi* isolates. Assays performed by SCHMITZ et al. (2014) have shown that in the tests with tebuconazole, a DMI fungicide, five different mutations in the fungus isolates have been detected in the *CYP51* gene (cytochrome P-450); they are F120L, Y131H / F, K142R, I145F, and I475T. Subsequently, for QoIs, KLOSOWSKI et al. (2016) investigated the occurrence of mutations in the *CYTB* (cytochrome-b) gene, and 100% of the isolates from Mato Grosso and Goiás presented the F129L mutation, conferring a reduction in fungal sensitivity to azoxystrobin. For the SDHIs, isolates of the fungus presented the mutation of I86F in subunit C of the *SDH* (succinate dehydrogenase) gene (KLAPPACH, 2017), reducing fungal sensitivity to benzovindiflupyr. The objective of this study was to assess *P. pachyrhizi* sensitivity to fungicides.

MATERIALS AND METHODS

The experimental design was completely randomized, with six replicates — each one represented by a soybean leaflet. For this, soybean seeds of the cultivar BMX Lança were sown in pots of 2000 mL containing the Garden Plus® sterilized substrate, composed of peat and limestone, activated with 0,02% of N, 0,08% of P_2O_5 and 0,04% of K_2O,

in a greenhouse. Plants received only water during their development.

Samples of soybean leaflets with Asian rust symptoms from different Brazilian locations (Table 1) were received at the Phytopathology Laboratory. The isolates obtained were from commercial crops or from experimental areas where fungicide applications had been conducted. The leaflets were harvested from soybean plants at the reproductive stage.

For inoculum multiplication and in order to prove the isolates' pathogenicity, suspensions of uredospores were prepared, wherein, the symptomatic leaflets from different sites were deposited separately in 500 mL Erlenmeyers, together with 200 mL of distilled water and one drop of spreader Tween20®. After stirring, the suspensions were filtered and an aliquot of 10.0 µL of each was obtained to be visualized in a hemacytometer under an optical microscope. The uredospore numbers were counted in triplicate and the concentrations were adjusted to 5 x 10^4 uredospores mL^{-1}.

With the aid of a 500 mL hand spray, the healthy soybean plants grown in a greenhouse (V5-V6 stage) were inoculated with the appropriate spore suspension of the isolates and covered with black plastic bags for 36 hours at a temperature of 23 ºC, in order to stimulate spore germination. Then plants were placed in transparent plastic boxes and placed in a growth chamber with a photoperiod of 12 hours, at the same temperature. When the ASR symptoms appeared on the leaves and it was verified that sporulation was occurring, these leaflets were used to prepare the uredospore suspensions used in the experiments.

Healthy leaflets of soybean plants, with a size close to 50 cm^2, were collected with the help of pruning shears, forty days after sowing. The base of each petiole was immersed in a container with water, to maintain hydration during transportation to the laboratory.

Fungicides (Table 2) were tested at concentrations of 0.1; 1.0; 10.0, and 100.0 mg L^{-1} of fungicide active ingredient, plus a control composed only of distilled water (0.0 mg L^{-1} of fungicide). Products were dosed in 400 mL plastic cups containing 200 mL of distilled water and the leaflets were immersed for five seconds in the respective solutions and allowed to dry at room temperature. The methodology utilized for all the trials was the detached leaf test described by Scherb & Mehl (2006), adapted by CHECHI et al. (2018). Humid chambers were prepared in plastic boxes, consisting of a box-size polyethylene foam unit (121 cm^2) and two sheets of filter paper of the same size. Chambers were moistened with distilled water, and leaflets were deposited on them after the fungicide treatment, with the abaxial side facing up. A piece of cotton was added to the petiole, which was saturated with distilled water in order to maintain the leaflet hydration.

Twenty-four hours after fungicide treatment, each leaflet was inoculated with uredospores suspension of *P. pachyrhizi* (5.0 x 10^4 uredospores mL^{-1}) from nine isolates obtained from the State of Rio Grande do Sul, one from Santa Catarina, one from São Paulo, and one from Mato

Table 1 - *Phakopsora pachyrhizi* isolates used in the effective concentration test to control 50% of the number of uredinia on soybean leaflets, Passo Fundo-UPF, 2019.

Isolate	City/State	Responsible for collecting	Year
1	Passo Fundo/RS	Amanda Chechi	2018
2	Panambi/RS	Carlos Alberto Forcelini	2017
3	Tupanciretã/RS	Carlos Alberto Forcelini	2017
4	Condor/RS	Carlos Alberto Forcelini	2017
5	Ibirubá/RS	Amanda Chechi	2018
6	Sertão/RS	Amanda Chechi	2017
7	Ipiranga do Sul/RS	Amanda Chechi	2018
8	Caseiros/RS	Bruna Piton	2018
9	Nonoai/RS	Elias Zuchelli	2018
10	Campos Novos/SC	Amanda Chechi	2018
11	Itaberá/SP	Bianca de Moura	2017
12	São Gabriel/MS	Valéria C. Ghissi-Mazetti	2018

Table 2 - Active ingredient, commercial name, concentration, formulation and mode of action of fungicides used in the test of effective concentration for the control of 50% of the uredinia number caused by different *Phakopsora pachyrhizi* isolates in soybean leaflets. Passo Fundo-UPF, 2019.

Active ingredient	Commercial name	Concentration (g or mL L^{-1})	Formulation	Action mode
Azoxystrobin	Priori®	250	CS[a]	QoI[1]
Trifloxystrobin	Flint®	500	WG[b]	QoI
Prothioconazole	Proline®	250	EC[c]	DMI[2]
Diphenoconazole	Score®	250	EC	DMI
Fenpropimorph	Versatilis®	750	EC	SBI[3]
Fluxapiroxade	-	250	CS	SDHI[4]
Mancozeb	Unizeb Gold®	750	WG	Multisite
Chlorothalonil	Bravonil®	500	CS	Multisite
Cooper oxichloride	Difere®	588	WP[d]	Multisite
Azoxystrobin + benzovindiflupyr	Elatus®	300 + 150	WG	QoI + SDHI
Azoxystrobin + benzovindiflupyr + diphenoconazole	Elatus Trio®	180 + 90 + 225	WG	QoI + SDHI + DMI
Trifloxystrobin + prothioconazole	Fox®	150 + 175	CS	QoI + DMI
Trifloxystrobin + bixafen + prothioconazole	Fox Xpro®	150 + 175 + 125	CS	QoI + SDHI + DMI

[a]Concentrated suspension; [b] Wet granules; [c] Emulsifiable concentrate; [d] Wet powder.
[1]Quinone outside inhibitor; [2]Sterol demethylation inhibitor; [3]Sterol biosynthesis inhibitor; [4]Succinate dehydrogenase inhibitor.

Grosso do Sul, which were prepared separately. The same methodology described previously was used, both for the preparation of the suspensions and for the leaflet inoculation.

For uredospore germination, the plastic boxes were left in the dark for 24 hours, in a temperature of 23 ºC. After this period, they were arranged in benches in a growth chamber, with a 12-hour photoperiod, at the same temperature. Every two days, distilled water (5 mL) was added to the cotton that held the petiole of the leaflet, with the help of a pissette, for moisture maintenance.

After 20 days of incubation, the uredinia number per cm^2 of each leaflet was assessed. For this, an area of 2.0 cm^2, being 1.0 cm^2 of each half of the abaxial face of the leaflet, was evaluated under a stereoscopic microscope. The data were expressed as control percentage in relation to the control, as per Abbott's formula (ABBOTT, 1925) and submitted to regression analysis by the sigmoidal model for the calculation of EC_{50}, using the SigmaPlot software. The sensitivity reduction factor of each fungicide in relation to EC_{50} of the isolate with the highest sensitivity to the fungicidal molecule(s) in question (reference sensitivity) was determined.

RESULTS AND DISCUSSION

All the tested *P. pachyhrizi* isolates showed a positive response to pathogenicity in the soybean plants, as symptoms began to appear between eight and twelve days after inoculation with uredospores, from different isolates in healthy plants. Different sensitivity values of *P. pachyrhizi* isolates were observed for the tested fungicides. Also, all the regressions obtained for the EC_{50} calculation were significant ($P<0.0042$).

Among the sterol demethylation inhibitors (Table 3), the fungicide prothioconazole showed differences up to 8.2 times in the effective concentration that controlled 50% of the uredinia caused by *P. pachyrhizi* isolates. The isolate from Caseiros-RS presented the lowest EC_{50} value (0.20 mg L^{-1}), while the Itaberá-SP isolate presented the highest value (1.64 mg L^{-1}). XAVIER et al. (2015) observed the EC_{50} values between 0.000001 and 0.39 mg L^{-1} for isolates from eight Brazilian states. JULIATTI et al. (2017) reported values between 0.0001 and 3.16 mg L^{-1} for isolates from Minas Gerais and Mato Grosso do Sul. In Brazilian areas cultivated with soybean, GODOY et al. (2017) reported reduced sensitivity of *P. pachyrhizi* to prothioconazole.

Table 3 - Effective concentration of isolated single-site and multisite fungicides for the control of 50% (EC_{50} - mg L^{-1}) of the number of uredinia caused by different isolates of *Phakopsora pachyrhizi* in soybean leaflets, confidence intervals (CI), and factor of sensitivity reduction (SRF). Passo Fundo-UPF, 2019.

Isolate (City/State)	EC_{50}[j]	CI EC_{50}	SRF	EC_{50}[j]	CI EC_{50}	SRF	EC_{50}[j]	CI EC_{50}	SRF
	---------Prothioconazole---------			----------Diphenoconazole----------			----------Fenpropimorph----------		
P. Fundo-RS	0.88	0.72-1.04	4.4	1.52	1.32-1.72	2.1	1.92	1.70-2.14	4.3
Panambi-RS	0.76	0.52-1.00	3.8	0.86	0.66-1.06	1.2	1.11	0.91-1.31	2.5
Tupaciretã-RS	1.03	0.92-1.14	5.2	1.20	1.04-1.36	1.6	1.03	0.86-1.20	2.3
Condor-RS	0.68	0.51-0.85	3.4	1.42	1.24-1.60	1.9	1.89	1.73-2.05	4.2
Ibirubá-RS[c]	0.35	0.15-0.55	1.8	1.23	1.13-1.33	1.7	0.45	0.31-0.59	-
Sertão-RS	0.93	0.75-1.11	4.7	1.89	1.73-2.05	2.6	2.24	2.10-2.38	4.9
Ip. do Sul-RS	0.78	0.60-0.96	3.9	1.21	1.09-1.33	1.6	3.74	3.48-4.00	8.3
Caseiros-RS[a]	0.20	0.01-0.41	-	0.99	0.80-1.18	1.3	1.61	1.361.86	3.6
Nonoai-RS	0.43	0.24-0.62	2.2	1.14	1.05-1.23	1.5	0.72	0.50-0.94	1.6
C. Novos-SC[b]	0.35	0.08-0.62	1.8	0.74	0.53-0.95	-	1.66	1.47-1.85	3.7
Itaberá-SP	1.64	1.44-1.84	8.2	1.72	1.44-2.00	2.3	1.47	1.25-169	3.3
S. Gabriel-MS	1.11	0.88-1.34	5.6	1.24	1.05-1.43	1.7	0.79	0.60-098	1.7
Average	0.76	0.57-0.96	4.1	1.26	1.09-1.44	1.8	1.55	1.35-1.75	3.7
	----------Azoxystrobin----------			----------Trifloxystrobin----------			----------Fluxapiroxade----------		
P. Fundo-RS	1.44	1.25-1.63	2.9	0.35	0.26-0.44	11.7	1.93	1.77-2.09	6.9
Panambi-RS[d]	0.49	0.34-0.64	-	0.05	0.006-0.16	1.7	3.45	3.26-3.64	12.3
Tupaciretã-RS	2.22	1.97-2.47	4.5	0.63	0.47-0.79	21.0	0.86	0.62-1.10	3.1
Condor-RS	1.05	0.77-1.33	2.1	0.24	0.08-0.40	8.0	3.90	3.73-4.07	13.9
Ibirubá-RS[f]	0.86	0.51-1.21	1.8	0.06	0.01-0.23	2.0	0.28	0.15-0.41	-
Sertão-RS	6.66	6.50-6.82	13.6	0.28	0.12-0.44	9.3	0.77	0.49-1.05	2.8
Ip. do Sul-RS	0.77	0.41-1.13	1.6	0.15	0.05-0.35	5.0	1.80	1.57-2.03	6.4
Caseiros-RS	1.24	1.05-1.43	2.5	0.08	0.03-0.19	2.7	0.91	0.76-1.06	3.3
Nonoai-RS	2.26	2.11-2.41	4.6	0.09	0.02-0.20	3.0	0.88	0.65-1.11	3.1
C. Novos-SC[e]	1.13	0.89-1.37	2.3	0.03	0.007-0.13	-	0.86	0.63-1.09	3.1
Itaberá-SP	1.85	1.69-2.01	3.8	0.29	0.02-0.56	9.7	0.38	0.09-0.67	1.4
S. Gabriel-MS	1.14	0.87-1.41	2.3	0.49	0.36-0.62	16.3	2.56	2.37-2.75	9.1
Average	1.76	1.53-1.99	3.8	0.23	0.12-0.37	8.2	1.55	1.36-1.75	5.9
	--------Copper oxychloride------			----------Mancozeb----------			----------Clorothalonil----------		
P. Fundo-RS	44.7	43.7-45.6	1.8	21.7	20.7-22.7	1.9	13.5	12.4-14.5	1.5
Panambi-RS	36.0	34.9-37.1	1.4	20.8	19.6-22.1	1.8	9.5	8.5-10.4	1.1
Tupaciretã-RS	44.7	43.1-46.3	1.8	26.6	24.9-28.4	2.3	15.9	14.8-16.9	1.8
Condor-RS	40.9	39.7-42.1	1.6	22.9	21.7-24.1	2.0	9.3	8.4-10.1	1.1
Ibirubá-RS	45.8	44.6-46.9	1.8	11.8	10.4-13.1	1.0	14.6	13.3-15.8	1.7
Sertão-RS	28.9	27.8-30.1	1.1	30.3	29.1-31.6	2.6	21.9	20.9-22.8	2.5
Ip. do Sul-RS	39.5	37.5-41.5	1.6	21.4	20.1-22.6	1.8	12.4	11.4-13.3	1.4
Caseiros-RS	26.2	25.1-27.2	1.0	25.8	24.7-26.8	2.2	9.6	8.4-10.8	1.1
Nonoai-RS[h,i]	38.7	37.0-40.4	1.5	11.7	10.4-12.9	-	8.7	7.7-9.6	-
C. Novos-SC[g]	25.3	24.1-26.6	-	14.0	12.6-15.4	1.2	20.5	19.1-21.8	2.4
Itaberá-SP	38.1	37.0-39.1	1.5	18.5	17.2-19.8	1.6	10.1	9.3-10.9	1.2
S. Gabriel-MS	27.1	26.1-27.2	1.1	21.2	20.0-22.3	1.8	15.5	14.5-16.6	1.8
Average	36.3	35.1-37.6	1.5	20.6	19.3-21.8	1.8	13.5	12.4-14.5	1.6

[a]Reference sensitivity for prothioconazole. [b]Reference sensitivity for diphenoconazole. [c]Reference sensitivity for fenpropimorph. [d]Reference sensitivity for azoxystrobin. [e]Reference sensitivity for trifloxystrobin. [f]Reference sensitivity for fluxapiroxade. [g]Reference sensitivity for copper oxychloride. [h]Reference sensitivity for mancozeb. [i]Reference sensitivity for chlorothalonil. [j]Values obtained through SigmaPlot regression with 95% confidence interval.

For diphenoconazole, the Sertão-RS isolate (1.89 mg L^{-1}) showed the highest EC_{50} value and Campos Novos-SC (0.74 mg L^{-1}) the lowest, with maximum EC_{50} differences between isolates being 2.6 times. In general, the fungicide prothioconazole obtained an EC_{50} value below 1.0 mg L^{-1} for nine of the twelve isolates tested; whereas, for difenoconazole, only three isolates had concentrations below 1.0 mg L^{-1}.

The EC_{50} value for the fungicide fenpropimorph, was determined for the first time in this study. The isolates that presented the greatest and least effective control of the disease were from Ibirubá-RS (0.45 mg L^{-1}) and Ipiranga do Sul-RS (3.74 mg L^{-1}), respectively. The EC_{50} value difference among isolates was up to 8.3 times for this chemical and only three isolates had EC_{50} values below 1.0 mg L^{-1}.

With regard to QoIs fungicides, the isolates showed a difference of up to 13.6 times for azoxystrobin, whereas, for trifloxystrobin, there was 21.0 times difference in magnitude. However, the fungicide trifloxystrobin was, in general, more effective in controlling the disease, as all the isolates had EC_{50} values below 1.0 mg L^{-1}, with values ranging from 0.03 mg L^{-1} (Campos Novos-SC) to 0.63 mg L^{-1} (Condor-RS). JULIATTI et al. (2017) reported concentrations lower than 0.007 mg L^{-1} in isolates obtained from Uberlândia-MG and Chapadão do Sul-MS.

EC_{50} values for azoxystrobin ranged from 0.49 mg L^{-1} (Panambi-RS) to 6.66 mg L^{-1} (Sertão-RS). On testing the IC_{50} of azoxystrobin *in vitro*, JULIATTI (2013) reported that the concentration of fungicide that inhibited spore germination ranged from 0.1 to 1.0 mg L^{-1}. In this study, values below 0.1 mg L^{-1} were reported only for three isolates (Ipiranga do Sul-RS, Ibirubá-RS, and Panambi-RS). SCHMITZ et al. (2014) reported EC_{50} value variations from 0.14 to 2.47 mg L^{-1} for the same active ingredient.

JULIATTI et al. (2017) reported that azoxystrobin-sensitive isolates had IC_{50} values lower than 5.0 mg L^{-1}. In this study, all values of EC_{50}, relative to the same molecule, were below 5.0 mg L^{-1}, except for the Sertão-RS isolate. This indicated a reduction in the sensitivity of this isolate to the active principle, which may have been caused by punctual mutations in cytochrome b in the mitochondria of the fungus, such as, the substitution of a leucine for phenylalanine at codon 129 (F129L), which had already been related to the *P. pachyrhizi* isolates from the soybean areas in Brazil (KLOSOWSKI et al., 2016). Factors such as alternative respiration or deviation of the active metabolic pathway may also occur, and have already been described for other pathogens (WOOD & HOLLOMON, 2003; FERNÁNDEZ-ORTUÑO et al., 2008).

The only SDHI fungicide tested alone was fluxapiroxade. EC_{50} values ranged from 0.28 mg L^{-1} (Ibirubá-RS) to 3.90 mg L^{-1} (Condor-RS), with up to a 13.9-fold difference in the active concentration, being only seven isolates with EC_{50} values lower than 1.0 mg L^{-1}. The study by JULIATTI et al. (2017) reported EC_{50} values between 0.05 and 0.35 mg L^{-1} for the same fungicide, for *P. pachyrhizi* isolates from Uberlândia and Chapadão do Sul, respectively.

For the commercial mixture of trifloxystrobin + prothioconazole, minimum values for EC_{50} of 0.39 mg L^{-1} (Campos Novos-SC) and maximum of 1.07 mg L^{-1} (Itaberá-SP) were observed (Table 4). The resistance factor among isolates for this mixture was 2.7 times. In the spore germination test, MOURA et al. (2016) verified IC_{50} values of 0.29 mg L^{-1} (Passo Fundo-RS), 0.27 mg L^{-1} (Ponta Grossa-PR), and 0.37 mg L^{-1} (Primavera do Leste-PR) for the same fungicide mixture. When the trifloxystrobin + bixafen + prothioconazole triple mixture was used, the EC_{50} values were lower when compared to those obtained with the previous fungicidal mixture, ranging from 0.01 mg L^{-1} (Nonoai-RS) to 0.53 mg L^{-1} (Itaberá-RS), showing greater efficacy in disease control.

For the fungicide composed of azoxystrobin + benzovindiflupir, the EC_{50} values ranged from 0.31 mg L^{-1} (Ibirubá-RS) to 1.28 mg L^{-1} (Sertão-RS). The latter isolate was previously reported in this study showing a reduction in sensitivity to azoxystrobin. The maximum difference of 4.1-fold was reported among the isolates for EC_{50} values. MOURA et al. (2016) reported IC_{50} variations between 0.16 mg L^{-1} and 1.39 mg L^{-1} for three different *P. pachyrhizi* isolates for the same compound. Azoxystrobin + benzovindiflupir + diphenoconazole showed, on an average, lower EC_{50} values between the isolates, ranging from 0.11 mg L^{-1} (Campos Novos-SC) to 0.61 mg L^{-1} (Tupanciretã-RS), when compared to the previous mixture.

The reduction in sensitivity of several fungi to azoxystrobin has been reported since the year of its commercial launch (SIEROTZKI et al., 2000). However, only in the year 2016, the first occurrence of F129L mutation in cytochrome b (*CYTB* gene) in the mitochondria of *P. pachyrhizi* isolates (KLOSOWSKI et al., 2016) was reported, associated with a reduction in the sensitivity of the fungus to the chemical. Regarding benzovindiflupyr, in the soybean

Table 4 - Effective concentration of commercial mixtures of fungicides for the control of 50% (EC_{50} - mg L^{-1}) of the number of uredinia caused by different isolates of *Phakopsora pachyrhizi* in soybean leaflets, confidence intervals (CI), and factor of sensitivity reduction (SRF). Passo Fundo-UPF, 2019.

Isolate (City/State)	EC_{50}[e]	CI EC_{50}	SRF	EC_{50}[e]	CI EC_{50}	SRF
	----------Trifloxystrobin + Prothioconazole-------			----------Azoxystrobin + Benzovindiflupyr----------		
P. Fundo-RS	0.81	0.62-1.00	2.1	0.95	0.77-1.13	3.1
Panambi-RS	0.76	0.53-0.99	1.9	0.56	0.24-0.88	1.8
Tupaciretã-RS	0.78	0.63-0.93	2.0	0.98	0.81-1.15	3.2
Condor-RS	0.69	0.50-0.88	1.8	0.84	0.66-1.02	2.7
Ibirubá-RS[b]	0.47	0.19-0.75	1.2	0.31	0.22-0.40	-
Sertão-RS	0.91	0.79-1.03	2.3	1.28	1.10-1.46	4.1
Ip. do Sul-RS	0.53	0.39-0.67	1.4	0.43	0.23-0.63	1.4
Caseiros-RS	0.44	0.20-0.68	1.1	0.36	0.10-0.62	1.2
Nonoai-RS	0.73	0.56-0.90	1.9	0.47	0.25-0.69	1.5
C. Novos-SC[a]	0.39	0.21-0.57	-	0.40	0.27-0.53	1.3
Itaberá-SP	1.07	0.94-1.20	2.7	0.99	0.86-1.12	3.2
S. Gabriel-MS	0.99	0.85-1.13	2.5	0.65	0.48-0.82	2.1
Average	0.71	0.53-0.89	1.9	0.68	0.50-0.87	2.3
	Trifloxystrobin + Bixafen + Prothioconazole			Azoxystrobin + Benzovindiflupyr + Diphenoconazole		
P. Fundo-RS	0.50	0.34-0.66	50	0.51	0.37-0.65	4.6
Panambi-RS	0.44	0.30-0.58	44	0.34	0.19-0.49	3.1
Tupaciretã-RS	0.42	0.24-0.60	42	0.61	0.52-0.70	5.5
Condor-RS	0.33	0.12-0.54	33	0.33	0.14-0.52	3.0
Ibirubá-RS	0.25	0.10-0.40	25	0.16	0.008-0.40	1.5
Sertão-RS	0.51	0.39-0.63	51	0.55	0.39-0.71	5.0
Ip. do Sul-RS	0.24	0.04-0.44	24	0.39	0.14-0.64	3.5
Caseiros-RS	0.02	0.005-0.18	2.0	0.15	0.005-0.35	1.4
Nonoai-RS[c]	0.01	0.002-0.20	-	0.34	0.10-0.58	3.1
C. Novos-SC[d]	0.05	0.007-0.17	5.0	0.11	0.01-0.35	-
Itaberá-SP	0.53	0.35-0.71	53	0.43	0.22-0.64	3.9
S. Gabriel-MS	0.31	0.12-0.50	31	0.19	0.03-0.35	1.7
Average	0.30	0.17-0.46	32.7	0.34	0.18-0.53	3,3

[a]Reference sensitivity for Trifloxystrobin + Prothioconazole. [b]Reference sensitivity for Azoxystrobin + Benzovindiflupyr. [c]Reference sensitivity for Trifloxystrobin + Bixafen + Prothioconazole. [d]Reference sensitivity for Azoxystrobin + Benzovindiflupyr + Diphenoconazole. [e]Values obtained through SigmaPlot regression with 95% confidence interval.

crop 2016–2017, there were cases of reduction in the performance of the product in a field in Brazil, and a mutation was detected in position 86 of subunit C of the *SDH* gene (succinate dehydrogenase) of the mitochondria, with a substitution of an isoleucine by phenylalanine (I86F) (KLAPPACH, 2017).

The fungicides considered as protective multisite presented higher EC_{50} values than the site-specific ones tested in this study. For the active ingredient mancozeb, the values varied between 11.7 mg L^{-1} (Nonoai-RS) and 30.3 mg L^{-1} (Sertão-RS) (concentration 2.6 times greater). The Nonoai-RS isolate presented the highest sensitivity to the active chlorothalonil (8.7 mg L^{-1}) and the lowest was reported for the Sertão-RS isolate (21.9 mg L^{-1}), this concentration being 2.5 times higher.

The highest values of EC_{50} were reported for the copper oxychloride fungicide, which varied between 25.3 mg L^{-1} (Campos Novos-SC) and 44.7 mg L^{-1} (Tupanciretã-RS), with up to 1.8 times

variation in effective concentration (50%) between isolates. JULIATTI et al. (2017) reported the values of 19.88 and 22.78 mg L^{-1} for mancozeb, 1.97 and 23.74 mg L^{-1} for chlorothalonil, and 2.93 and 69.31 mg L^{-1} for copper oxychloride in the soybean detached leaf test, with two *P. pachyrhizi* isolates.

According to EDGINGTON et al. (1971), the defining criteria that fit a fungicidal substance with respect to its fungitoxicity *in vivo,* the fungicides prothioconazole, trifloxystrobin, fluxapiroxade, prothioconazole + trifloxystrobin, prothioconazole + bixafen + trifloxystrobin, azoxystrobin + benzovindiflupyr, and difenoconazole + azoxystrobin + benzovindiflupyr would be classified as substances that are highly fungitoxic, because they presented EC_{50} values less than 1.0 mg L^{-1} for a majority of the isolates. According to the same criteria, difenoconazole, azoxystrobin, and fenpropimorph would be considered moderately fungitoxic (EC_{50} between 1 and 10 mg L^{-1}) (Table 5). The multisites mancozeb, chlorothalonil, and copper oxychloride presented moderate-to-less toxic responses to the fungus; therefore, it explains the need for the use of higher doses of these products to control *P. pachyrhizi*.

The development of resistance in phytopathogenic fungi has a greater impact when the repeated use of site-specific fungicides is involved. In this context, the application of mixtures of fungicides of different chemical groups constitutes a fundamental strategy in the management of resistance (SIEROTZKI & SCALLIET, 2013). Studies have shown that fungicide mixtures delay the evolution of fungal resistance to the active ingredients (BRENT & HOLLOMON 2007; VAN DEN BOSCH et al., 2014). The multisites, for example, mancozeb, chlorothalonil, and copper oxychloride, are fungicides that present minimal risks of reducing the sensitivity of fungi to them, and should be used in mixtures with site-specific fungicides, constituting an anti-resistance strategy (HOLLOMON, 2015). These strategies to manage the evolution of resistance should not only reduce the population of the resistant phenotypes in relation to the sensitive ones, but also improve the general levels of disease control (HOLLOMON, 2015).

The use of subdoses or superdoses, repeated sprays with the same fungicide, curative applications, use of reduced application rates, incorrect choice of spray tips, and spraying in adverse atmospheric conditions can also select resistant organisms (ROESE, 2011).

The recommendations for use of fungicides should follow the indicated guidelines regarding the applied dose, validity of the product used, number and interval between applications per crop, together with the use of good cultural practices, such as sowing at the beginning of the recommended season, use of early maturing cultivars, elimination of voluntary plants, with respect to the period of vazio sanitário (period without soybean cultivation) and the use of appropriate application technology. The use of resistant cultivars is another important tool that can contribute to ASR management and in reducing the resistance of fungus to fungicides (GODOY & MEYER, 2014). The orientation of anti-resistance strategies discourages curative applications in favor of preventive ones, which is equivalent to an early fungicide treatment when populations of the pathogen

Table 5 - Classification of the effective concentration of the fungicides tested in this study for the control of 50% (EC_{50}) of the number of uredinia in soybean leaflets caused by twelve isolates of *Phakopsora pachyrhizi* from different areas, according to EDGINGTON et al. 1971. Passo Fundo-UPF, 2019.

Highly fungitoxic	Moderately fungitoxic	Little fungitoxic
EC_{50}<1 mg L^{-1}	EC_{50} between 1 and 10 mg L^{-1}	EC_{50} between 10 and 50 mg L^{-1}
Prothioconazole (9 isolates)	Diphenoconazole (9 isolates)	Mancozeb (12 isolates)
Trifloxystrobin (12 isolates)	Azoxystrobin (9 isolates)	Chlorothalonil (8 isolates)
Fluxapiroxade (7 isolates)	Fenpropimorph (9 isolates)	Cooper oxichloride (12 isolates)
Trifloxystrobin + prothioconazole (11 isolates)		
Trifloxystrobin + bixafen + prothioconazole (12 isolates)		
Azoxystrobin + benzovindiflupyr (11 isolates)		
Azoxystrobin + benzovindiflupyr + diphenoconazole (12 isolates)		

are still small; although, this does not guarantee that the selection of resistant individuals does not occur (HOLLOMON, 2015).

CONCLUSION

The fungicides present different levels of fungitoxicity to the tested *Phakopsora pachyrhizi* isolates, indicating that the variation in the sensitivity of the isolates occurs due to differences in the management and chemical control of the disease, in the different locations, from where the isolates were obtained.

For the 12 isolates of *P. pachyrhizi* tested, the active ingredients prothioconazole (9 isolates), trifloxystrobin (12 isolates), fluxapiroxade (7 isolates), trifloxystrobin + prothioconazole (11 isolates), trifloxystrobin + bixafen + prothioconazole (12 isolates), azoxystrobin + benzovindiflupyr (11 isolates), and azoxystrobin + benzovindiflupyr + diphenoconazole (12 isolates) are highly fungitoxic, with EC_{50} values less than 1.0 mg L^{-1}.

Diphenoconazole, azoxystrobin, and fenpropimorph are considered moderately fungitoxic for nine of the twelve isolates, with EC_{50} values between 1 and 10 mg L^{-1}.

The multisite mancozeb and copper oxychloride presented EC_{50} responses classified as low toxic for the twelve isolates and chlorothalonil for eight of them (EC_{50} values between 10 mg L^{-1} and 50 mg L^{-1}).

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work and manuscript preparation.

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