In vivo sensitivity of *Phakopsora pachyrhizi* to DMI and QoI fungicides

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RESUMO

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Em experimentos conduzidos *in vivo* avaliou-se a sensibilidade de 15 isolados de *Phakopsora pachyrhizi* procedentes de várias regiões do Brasil. Foram testados fungicidas IDMs (ciproconazol, epoxiconazol e tebuconazol e um IQe (piraclostrobina). As avaliações foram baseadas na densidade foliolar de urédias. Determinou-se a concentração inibitória (CI50) e o fator de redução da sensibilidade para todos os isolados. Demonstrou-se a ocorrência de redução da sensibilidade de *P. pachyrhizi* ao fungicida tebuconazol. Contrariamente, não se detectou alteração na sensibilidade do fungo à piraclostrobina. Conclui-se que a falha de controle da ferrugem observadas em algumas lavouras de soja se deve a redução da sensibilidade do fungo ao fungicida IDM.

Palavras-chave adicionais: Ferrugem da soja, fungitoxicidade, resistência, fungicidas IDM e IQE.

ABSTRACT

Reis, E. M.; Deuner, E.; Zanatta, M. *In vivo* sensitivity of *Phakopsora pachyrhizi* to DMI and QoI fungicides.***Summa Phytopathologica,*** v.41, n.1, p.xx-xx, 2015.

In vivo experiments were conducted to evaluate the sensitivity of 15 *Phakopsora pachyrhizi* isolates from several regions of Brazil. DMI fungicides (cyproconazole, epoxiconazole and tebuconazole) and a QoI fungicide (pyraclostrobin) were tested. Assessments were based on the leaf density of uredia. Inhibitory concentration (IC50) and sensitivity reduction factor were determined for all isolates. A reduction in *Phakopsora pachyrhizi* sensitivity to tebuconazole was detected for most fungal isolates. In contrast, there was no shift in the fungus sensitivity to pyraclostrobin. We concluded that the rust control failure found for some soybean crops is due to the reduced sensitivity of the fungus to DMI fungicide.

Additional keywords: Soybean rust, fungitoxicity, resistance, DMI and QoI fungicides.

In the 2013/14 growing season, soybean crop in Brazil reached 30.17 million ha, 86.1 million tons of production and mean yield of 2.8 t/ha (3).

Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi* Sydow., was first reported in South America during the 2001 growing season in Paraguay (12). Chemical control of ASR in Brazil started as early as the 2003/04 growing season, when an area of approximately 20 million hectares was sprayed with fungicides. The mean number of fungicide applications per hectare used to be three (4). As early as the 2005/06 growing season, at five seasons after the beginning of fungicide use, initially in Mato Grosso State, farmers started to complain about the control efficiency of DMI fungicides (8, 18). Tebuconazole and flutriafol were the most efficient fungicides and largely used at that time (4). Flutriafol was even adopted as effectiveness pattern in fungicide trials.

A large number of methods have been described to measure the fungitoxicity of a chemical (1, 2, 5, 6, 11, 15, 16) or the sensitivity of a fungus to a given fungicide, or even to monitor the sensitivity reduction or loss.

In vivo assays are needed for biotrophic pathogens, since *in vitro* procedures are not compatible with the objectives for them. *In vivo* tests can also be used for necrotrophic pathogens when *in vitro* techniques are considered inappropriate (1, 15, and 16). Several methodologies are available and the choice will depend on the target pathogen and the properties of the fungicide. *In vivo* tests typically include detached plant parts, mostly leaves, leaf discs or segments deposited on a culture medium containing the fungicide in suspension or solution, or even whole seedlings (1, 2, 6, 15, and 17).

Scherb and Mehl (17) suggested a similar methodology to test the sensitivity of *P. pachyrhizi* to fungicides, especially demethylation inhibitors (DMIs).

Parameters such as ED_{50} (the effective dose that promotes a desired effect in 50% of the microorganisms subjected to the test), LD_{50} (lethal dose), LC_{50} (lethal concentration), EC_{50} (effective concentration), GI_{50} (growth inhibition), IC_{50} (inhibitory concentration) or MIC (minimum inhibitory concentration) have been used to define the fungitoxicity of a chemical (6, 9, 10, 15). IC_{50} values determined *in vivo* for different fungicides, specifically against *P. pachyrhizi* on soybean plants, are scarce in the literature, although they are useful in studies to monitor the sensitivity of the fungus, especially in regions where fungicides are largely used in soybean crops.

In Brazil, the chemical control of ASR started in the 2002/03 growing season and, after four seasons, in the 2007/08 growing season, farmers started to complain about control failure with the use of

demethylation inhibition fungicides (DMI) in the states of Mato Grosso and Goias. We hypothesized that such reduction in ASR control noticed in several fields (2012/13 growing season) was due to the reduction in the fungus sensitivity to the DMI fungicides that had been used for six seasons (from 2002/03 to 207/08 seasons).

The aim of this study was to determine *in vivo* the fungitoxicity of DMI and QoI fungicides for suspected isolates of *P. pachyrhizi* in samples from several locations in Brazil and one sample from Paraguay.

MATERIAL AND METHODS

The experiments to quantify the *in vivo* sensitivity of *P. pachyrhizi* to the fungicides were conducted in a growth chamber in the Laboratory of Plant Pathology (Mycology), Faculty of Agronomy and Veterinary Medicine, University of Passo Fundo - UPF, in 2008/09.

Soybean rust inoculum originated from uredospore samples obtained from naturally infected leaves, collected from several farms in the country and from one farm in Paraguay in the 2007/08 growing season (Table 1). The initial inocula, as uredospores, were removed by manually shaking soybean leaflets into an Erlenmeyer containing sterile distilled water and two drops/L water of tensoactive polyoxyethylene sorbitane monolaurate (Tween 20 Synth Laboratory).

Each inoculum sample was continuously maintained and multiplied on soybean plants grown in four 1L-pots (CD 219 soybean cultivars RR, low susceptibility to powdery mildew, *Erysiphe diffusa* Cooke & Peck), protected inside individual plastic acrylic boxes (30 x 40 x 60 cm height) under controlled environment (22 ± 2°C and 14 h photoperiod) to avoid mixture of isolates.

Plant inoculation. Spores were removed from the surface of leaves by introducing leaflets in a plastic bottle (500 mL volume) containing 200 mL distilled water added of two drops of polyoxyethylene sorbitane monolaurate (Tween 20, Synth Laboratory). The bottle was manually shaken for three minutes for spore removal and passed through two layers of cheesecloth. The inoculum was sprayed on the leaves in V3 growth stage and plants were kept in a moisture chamber for 24 h, in the dark, at 22ºC.

Fungicide concentrations and formulations: The used commercial formulations were: Pyraclostrobin - Comet (250), Tebuconazole - Folicur (200 EC), Flutriafol - Impact (125 SC), Epoxiconazole - Opus (125 SC) and Ciproconazole - Alto 100 (100 CE).

Fungicide concentrations. Seven concentrations of DMIs were used in the tests: 0.0; 0.02; 0.2; 2.0; 20.0; 50.0 and 100.0 $mg.L^{-1}$, as well as six concentrations of QoI: 0.001; 0.01; 0.1; 1; 10.0 $mg.L^{-1}$ of active ingredient.

Fungicide suspensions were prepared in distilled water added of 6.0 $\mu L.L^{-1}$ of Tween 20 in a 250mL-volume Becker. Central leaflets detached from soybean plants in V2–V3 growing stage were immersed for three seconds in each suspension by holding the petiole with tweezers and shaken three times to eliminate excess suspension. Treated leaflets were laid inside crystal acrylic boxes (11.0 x 11.0 x 3.27cm height) containing at the bottom nylon foam (0.5cm thick layer) and at the top two layers of filter paper saturated with water, and covered with a lid.

The methodology based on the detached leaflet technique proposed by the Fungicide Resistance Action Committee (4, 12) was used with the modification of counting the uredinium density instead of assessing the severity.

The assay for each isolate was run individually, as well as the concentrations of the treatments.

Previously, nylon foam (5.0 mm thick) was deposited at the bottom of boxes (crystal polystyrene boxes) and covered with two layers of filter paper (Whatman No. 6), which were soaked with distilled water until saturation.

After early expansion of the second trifoliate, the petioles of leaflets of the first trifoliate plants were cut with scissors, at 0.5 cm from the

Table 1. Soybean samples with *Phakopsora pachyrhizi* maintained in a growth chamber at University of Passo Fundo. 2007, 2008.

Isolate	Collection date	Sender	Location
01	01/23/2007	Tiago/Elaine	Passo Fundo/RS
02	03/03/2008	Elder Diniz	Rio Verde/GO
03	03/03/08	Miguel	Santa Helena/GO
04	03/03/08	Miguel	CEFET/GO
07	03/03/08	Miguel	Jataí/GO
09	-/- /08	Tatiana Dalla Nora	Primavera do Leste/MT
19	03/25/08	Nilceli F. Buzzerio	Holambra/SP
20	03//3108	Weber Barrinha	Rio Verde/GO
21	-/- /08	Rafael R. Gonçalves	Chapadão do Sul/MS
22	04/04/08	Marco T. Fujino	Aral Moreira/MS
24	-/- /08	Jairo dos Santos	Rondonópolis/MT
26	04/12/08	Erlei M. Reis	Paraguai
27	04/14/08	Vitor T. Igarashi	Rio Verde/GO
29	04/15/08	João Cason	Mogi Mirim/SP
31	06/05/08	Nilda Santos	Paulínia/SP
35	05/28//08	Márcia K.Pala	Sorocaba/SP
36	05/27/08	Reinaldo Bonnecarrere	Santo A. de Posse/SP
37	-/-/08	Nilda Santos	Paulínia/SP

leaf base, and fungicide concentrations were applied by immersing the leaflets, held with tweezers, in the fungicide suspension contained in a beaker for three seconds. After soaking, the leaflets were placed inside the boxes, with the adaxial side down, and distributed as four leaflets per box, totaling sixteen leaflets per treatment. Boxes were kept closed at room temperature and without photoperiod control for the next 24 hours. Inoculation was performed with uredospores of *Pp* (concentration of 4 x 10^4/mL) removed from the leaves of soybean plants as previously described. For inoculation, a hand sprayer (Sprayer Ultrajet 500 mL, Guarany) was used to deliver a volume of 5.0 ml per box. Boxes were closed and kept in the dark for 12 hours at 22 ± 2°C and then kept in a growth chamber at the same temperature, under 10-14h photoperiod, for an additional period, totaling 17 days from inoculation. At this time, signs of the pathogen were clearly seen in the leaflets of the control treatment. During the incubation period, care was taken to keep the filter paper saturated with distilled water.

On the following day, when fungicide suspension had dried, boxes were opened and inoculated by spraying a spore suspension containing > 2 x 10^4 spores/mL. The boxes were covered and kept in a growth chamber, initially in the dark for 24 hours for spore germination and penetration, and later at 22ºC and 12 h photoperiod, until fungal sporulation.

Disease assessment. The disease was evaluated at 15 to 20 days after inoculation by counting uredia/cm^2. Counts were done in a selected area with uniform uredinium density, using leaflets of 0.9 mm diameter marked with a hole borer. Data were presented as uredinium density per square centimeter.

A complete randomized block design with four replicates was used, adopting as experimental units a plastic box with three soybean leaflets. Each experiment was repeated twice.

IC_{50} and IC_{90} (inhibitory concentration) were calculated based on Weilbull's model, using the equation ($y = d \exp\{- \exp [b(\log x – e]\}$), described by Knezevic et al. (12).

The sensitivity reduction factor (SRF) was calculated by dividing the actual IC_{50} value for the isolate by that for the sensitive fungal isolate. Baseline values were taken from Blum (1). This shift indicates the value of sensitivity reduction for a fungicide (10, 15).

IC_{50} and SRF are shown in Table 1; for two times the experiments were conducted in relation to the number of uredia/cm^2 of *Pp*.

Scherb and Mehl (17) described the methodology proposed by FRAC, in which the disease is measured based on the estimated severity (visual assessment using a scale).

Each experiment, for every isolate, was replicated twice per concentration of the fungicide.

RESULTS AND DISCUSSION

Sampling was directed to those farms where fungicides had been sprayed for several growing seasons. We received and maintained 18 samples, here called isolates. No monosporic isolation was done (Table 1).

The *in vivo* toxicity of fungicides is shown in Tables 2 to 6; for two times the experiments were conducted as the number of lesions/cm^2 of *P. pachyrhizi*.

Blum (1) showed that either lesion or uredinium density may be used to assess *P. pachyrhizi* sensitivity.

There was a great variation in *P. pachyrhizi* sensitivity to tebuconazole among the isolates from samples collected in several regions of Brazil (Table 2). The magnitude of the shift in sensitivity can be calculated by the SRF. A value of 1.0 indicates no change, value <1.0 indicates lower sensitivity than the baseline, and value > 1.0 indicates reduction in the isolate sensitivity (10). Seven isolates showed SRF < 2.0, while eight showed SRF > 2.0 mg/L. The greatest shift occurred for isolates 7, 26, 27, 31 and 37 (Table 2). In this experiment, the baseline mean values of IC_{50}s ≤ 0.11 mg/L (mean = 0.053) were adopted to calculate SRF. The sensitivity reduction was not general for all samples. Only five out of 18 isolates showed sensitivity reduction.

Table 2. *In vivo* inhibitory concentrations (IC_{50}) (mg.L^{-1}) and sensitivity reduction factor (SRF) for tebuconazole to different isolates of *Phakopsora pachyrhizi* related to the number of uredia.cm^2

Isolates (no.)	IC_{50}	Error	SRF
Isolate 2	0.076	0.0205	1.43[z]
Isolate 3	0.025	0.0198	0.47
Isolate7	0.725	0.2369	13.67
Isolate 9	0.11	0.0384	2.07
Isolate 19	0.114	0.0052	2.15
Isolate 20	0.054	0.0873	1.01
Isolate 21	0.099	0.0314	1.86
Isolate 22	0.207	0.0500	3.9
Isolate 26	5.102	6.6700	96.26
Isolate 27	0.455	0.2370	8.58
Isolate 29	0.093	0.0582	1.75
Isolate 31	0.343	0.1251	6.47
Isolate 35	0.013	0.0057	0.27
Isolate 36	0.011	0.0074	0.207
Isolado 37	3.772	0.9048	71.16

Baseline mean values of IC_{50}s ≤ 0.11 mg.L^{-1}, mean = 0.053.
([z]) SRF for isolate 2, 0.076/0.53 = 1.43.

Several genes command the sensitivity shift for DMI fungicides and the response is dose-dependent (10).

Blum (1) determined, *in vitro* and *in vivo*, the IC_{50} of DMI and QoI fungicides for a sensitive isolate of *P. pachyrhizi*. For tebuconazole, IC_{50} was 0.61 and in the present study we used the mean IC_{50} of 0.053 mg/L. This difference may be due to the sensitivity difference for the tested isolates.

For isolate 1 (Table 1), considered sensitive to *P. pachyrhizi*, IC_{50} values ranged from 0.03 to 1.3 (Table 3). Blum (1) found IC_{50} of 0.61 for tebuconazole, 2.16 for cyproconazole, 0.87 for epoxyconazole, 2.50 for metconazole, and 0.192 mg/L for pyraclostrobin.

Regarding the IC_{50} values obtained in the present study, the tested DMI fungicides had a different behavior. In addition, SRF was not similar among them. The greatest shift in values occurred for tebuconazole. Although they have been reported to have the same biochemical mode of action, i.e., demethylation inhibitors (DMI), IC_{50} values greatly differed among them (Tables 2 to 6). For instance, SRF for tebuconazole was 96.26 (Table 2) and for cyproconazole, 1.24 (Table 4). This may be due to the ingredients of commercial formulation, as pointed out by Blum (1) and Furlan and Scherb (9).

Table 3. *In vivo* inhibitory concentrations (IC_{50}) for fungicides to isolate 01 (sensitive) of *Phakopsora pachyrhizi* related to the number of uredia.cm^{-2}

Fungicides	IC_{50}	Error
Tebuconazole	0.33	0.23
Cyproconazole	1.27	0.69
Epoxiconazole	0.20	0.47
Pyraclostrobin	0.03	0.06
Prothioconazole	0.11	0.17

Table 4. *In vivo* inhibitory concentrations (IC_{50}) for fungicides to isolate 21 (Chapadão do Sul) of *Phakopsora pachyrhizi* related to the number of uredia.cm^{-2}

Fungicides	IC_{50}	Error	Baseline[z]	SRF
Tebuconazole	4.65	5.80	0.61	6.62
Cyproconazole	6.14	2.90	2.16	2.84
Epoxiconazole.	1.43	1.81	0.87	1.67
Pyraclostrobina	0.20	0.03	0.192	1.01
Prothioconazole	0.18	0.04	z	z

([x]) Baseline IC_{50} values for each fungicide taken from Blum (2009).
([z]) Baseline not determined.

Table 5. *In vivo* inhibitory concentrations (IC_{50}) and sensitivity reduction factor (SRF) for fungicides to isolate 24 (Rondonópolis, MT 2008) of *Phakopsora pachyrhizi* related to the number of uredia.cm^{-2}

Fungicides	IC_{50}	Error	Baseline[x]	SRF
Tebuconazole	12.47	2.85	0.61	20.44
Cyproconazole	2.68	0.60	2.16	1.24
Epoxiconazole	1.14	0.80	0.87	1.42
Pyraclostrobin	0.11	0.13	0.192	0.57
Prothioconazole	0.27	0.33	z	z

([x]) Baseline IC_{50} values for each fungicide taken from Blum (2009).
([z]) Baseline not determined.

Table 6. *In vivo* inhibitory concentrations (IC_{50}) and sensitivity reduction factor (SRF) for fungicides to isolate 26 (Paraguay) of *Phakopsora pachyrhizi* related to the number of uredia.cm^{-2}

Fungicides	IC_{50}	Error	Baseline[y]	SRF
Tebuconazole	8.90	2.11	0.61	14.59
Cyproconazole	5.91	2.16	2.16	2.73
Epoxiconazole	4.49	2.14	0.87	5.16
Pyraclostrobin	0.16	0.04	0.192	0.83
Prothioconazole	0.10	0.04	[z]	[z]

([y]) Baseline IC_{50} values for each fungicide taken from Blum (2009).
([z]) Baseline not determined.

Testing a sensitive isolate, the lowest IC_{50} was 0.03 mg/L for pyraclostrobin and 1.27mg/L for cyproconazole.

Isolate 21 (Table 1) showed sensitivity shift to the tested DMI fungicides. This did not occur for pyraclostrobin (Table 4). The greatest change was for tebuconazole, SRF of 7.62

Isolate 24 (Table 1) showed sensitivity shift to the tested DMI fungicides. This did not occur for pyraclostrobin (Table 5). The greatest changes were found for tebuconazole, SRF of 20.44

Isolate 26 (Table 2) showed sensitivity shift to the tested DMI fungicides. This did not occur for pyraclostrobin. The greatest changes were found for tebuconazole and epoxiconazole, SRF of 14.59 and 5.16, respectively (Table 6)

Sensitivity reduction was shown for *P. pachyrhizi* isolates towards DMI fungicides. It was also shown that the fungus is still sensitivity to pyraclostrobin (2007/08 growing season). In some farms, rust control has been achieved by QoI fungicides and therefore DMIs should not be used alone to prevent control failure. On the other hand, QoIs should not be used alone to prevent selection pressure towards shift in their sensitivity loss.

Junqueira (11), working on the chemical control of *P. pachyrhizi* (latter determined as *P. meibomiae*), obtained *in vivo* IC_{50} (number of lesions/cm^2 in non-detached leaflets), for benomyl (7.5 mg/L), triadimefon (38.3 mg/L), triforine (18.3 mg/L), copper oxychloride (296.2 mg/L), chlorothalonil (5.7 mg/L) and maneb (0.75 mg/L).

Buzzerio et al. (2) monitored *in vivo* the sensitivity of *P. pachyrhizi* for cyproconazole fungicide and reported IC_{90}s in the range of 0.0934 to 0.5007 mg/L. However, the methodology used by Buzzerio et al. (2) differs from that used in our study, which could explain the variations in results, depending on the sensitivity of the pathometric method. Those authors used FRAC International and Brazilian methodology, i.e., visual assessment of the disease severity using a grading scale.

Furlan and Scherb (9) determined the IC_{50} of four commercial formulations of tebuconazole in Brazil for *P. pachyrhizi*. IC_{50} values varied between 0.54 for Folicur (200 CE), 0.81 for Orius (250 CE), 1.5 for Rival (200 EC) and 1.6 mg/L for Tebuconazole Nortox, demonstrating what Russell (15) warned about possible variations in IC_{50} for different commercial formulations of a given fungicide. The values reported for the fungicide tebuconazole in our experiments (Folicur 200 EC formulation) considering the uredinium number/cm^2 of *P. pachyrhizi* are similar and confirm the values reported in the literature, IC_{50} of 0.32 and 0.77 mg/L for Experiments 1 and 2, respectively.

The IC_{50} determined by Blum (1) can be used as a baseline for future studies monitoring the sensitivity of *P. pachyrhizi* to tebuconazole fungicides in soybean plants.

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