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 (CI_{50}) 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.21-24, 2015.

In *in vivo* experiments the sensitivity of 18 isolates of *Phakopsora pachyrhizi* from several regions of Brazil to IDM fungicides (cyproconazole, epoxiconazole and tebuconazole and an IQE (pyraclostrobin) were evaluated. The assessments were based on leaflet uredia density. Inhibitory concentration (IC_{50}) and sensitivity reduction factor were determined for all fungicide x strain interactions. Tebuconazole sensitivity reduction was detected for most fungus isolates. In contrast, there was no fungicide shift in sensitivity of the fungus to pyraclostrobin. We conclude that the control failure of soybean rust found in some farms is due to the reduced sensitivity of the fungus to the IDM fungicide and that it remains sensitive to pyraclostrobin.

Additional keywords: Soybean rust, fungitoxicity, resistance, triazol and strobilurin fungicides.

In the 2013/14 growing season, soybean acreage in Brazil reached 30.17 million ha, 86.1 million tons 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 earlier as 2002/03 growing season and in the next season, an area of about 20 million hectares was sprayed with fungicides. Mean number of sprays per hectare has been three (4). As early as the 2007/08 growing seasons, at five seasons after the beginning of fungicide use, initially in Mato Grosso state, farmers started to complaine about the control efficiency of demethylation inhibitors (DMI) fungicides (8, 18). Tebuconazole and flutriafol fungicides 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 reduction or loss.

In vivo assays are needed for biotrophic pathogens, in which *in vitro* procedures are not compatible with their objectives. *In vivo* tests can also be used for necrotrophic pathogens, when the *in vitro* techniques are considered inappropriate (1, 15, and 16). Several methodologies are available and their 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 for sensitivity tests of *P. pachyrhizi* to fungicides, especially for DMIs.

Parameters such as ED_{50} (the effective dose that promotes a desired effect in 50% of microorganism 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.

We hypothesized that such reduction in ASR control noticed in several fields (2007/08 growing season) was due to the reduction in the fungus sensitivity to the DMI fungicides used for six seasons (from 2002/03 to 207/08 seasons).

The aimof this study was to determine *in vivo* the fungitoxicity

of DMI and QoI fungicides for suspected isolates of *P. pachyrhizi*, in samples taken from several locations in Brazil and one from Paraguay.

MATERIAL AND METHODS

The experiments to quantify the *in vivo* sensitivity of *P. pachyrhizi* to fungicide, 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 one farm in Paraguay in 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 in soybean plants grown in for 1L-pots (CD 219 soybean cultivars RR, low susceptible to powdery mildew, *Erysiphe diffusa* Cooke & Peck), protected inside individual plastic acrylic boxes (30 x 40 x 60 cm high) 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 a two layeres 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 formulations: The used commercial formulation were: Pyraclostrobin - Comet (250), Tebuconazole - Folicur (200 EC), Flutriafol - Impact (125 SC), Epoxiconazole – Opus (125 SC) and Cyproconazole - 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, as well as six concentrations of QoI: 0.001; 0.01; 0.1; 1; 10.0 mg/L of active ingredient were used in the tests.

Fungicide application. Fungicide suspensions were prepared in distilled water added of 6.0 µL/L of Tween 20 in a 250 mL-volume Becker. Central leaflets detached from soybean in V2 – V3 growing stage were immersed for three seconds in each suspension by holding the petiole with a tweezers and shaken three times to eliminate excess suspension. After soaking, the leaflets were placed inside the boxes, with the adaxial side down, and distributed four leaflets per box totalizing sixteen leaflets per treatment.

Leaflets inoculation. On the followingd day when fungicide suspension had dried, boxes were open 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 under dark for 24 hs for spore germination and penetration, and later at 22ºC and 12 h photoperiod, until to fungus sporulation.

During the incubation period, care was taken to keep the filter paper saturated with distilled water.

Disease assessment. The disease was evaluated at 15 to 20 days after inoculation by counting the uredinium/cm^2. Counts were done in a selected area of the lealet with uniform uredia density in a 0.9 mm circle 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 four soybean leaflets.

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

Table 1. Soybeans samples with *Phakopsora pachyrhizi* maintained in growth chamber at the Universidade de Passo Fundo. 2007 2008

Isolate	Collected 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

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 amount of sensitivity reduction for a fungicide (10, 15).

IC_{50} and the 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 by 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 mantained 18 samples here called isolates. No monosporic isolation was done (Table 1).

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

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

There was a large variation of *P. pachyrhizi* sensitivity for tebuconazole among the isolates from samples collected in several regions of Brazil (Table 2). The magnitude of in sensitivity can be calculated by the SRF. A 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 the IC_{50}s ≤ 0.11 mg/L (mean = 0.053) were adopted to calcularte SRF. The sensitivity reduction was not general for all samples. Only five out of 18 isolates showed sensitivity reduction.

Several genes command sensitivity shift for DMIs 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 IC_{50} mean of 0.053 mg/L. This difference may be due to sensitivity difference for the tested isolates.

For the isolate 1 (Table 1), considered sensitive to *P. pachyrhizi*, 0.61 for cyproconazole, 2.16, for cyproconazol., 0.87 for epoxyconazole, 2.50 for metconazole , and 0.192 mg/L for pyraclostrobin.

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

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 DMI fungicides tested. This fact did not occur with pyraclostrobin (Table 4). The largest change was for tebuconazole with SRF of 7.62

O isolate 24 (Table 1) showed sensitivity shift to the tested DMI fungicides. This did not occur with pyraclostrobin (Table 5). The greatest changes was for the tebconazole with SRF of 20.44

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

Sensitivity reduction was shown for *P. pachyrhizi* isolates towards DMIs 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 the QoI fungicides and therefore DMIs should not be used alone to prevent control failure. On the other hand, QoI 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* CI_{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 for 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 the methodology used in our work, which could explain the variations in results, depending on the sensitivity of pathometric method. The authors used FRAC International and Brazil methodology, i.e. visual assessment of the disease using a grading scale.

Furlan and Scherb (9) determined the IC_{50} for four commercial formulations of tebuconazole in Brazil for *P. pachyrhizi*. IC_{50} values varied from 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

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

Isolates (n°)	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.

Table 3. *In vivo* inhibitory concentrations (IC50) for fungicides to isolate 01 (Sensitive) of *Phakopsora pachyrhizi* related to the number of uredia/cm^2

Fungicides	IC	Error
Tebuconazole	0.39	0.23
Cyproconazole	1.27	0.69
Epoxiconazole.	0.20	0.47
Pyraclostrobina	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 urredia/cm^2

Fungicides	IC	Error	Baseline[z]	SRF
Tebuconazole	4.69	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.

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

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