Short communication

In vivo sensitivity reduction of *Phakopsora pachyrhizi* to strobilurins fungicides

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Abstract

Blum, M. M.C. e Reis, E. M. *In vivo* sensitivity reduction of *Phakopsora pachyrhizi* to strobilurins fungicides. **Summa Phytopathologica.**

Experiments were conducted *in vivo* to determine the concentration (mg/L) of the mitochondrial quinone outside inhibitors (QoI, or strobilurins), which control 50% (IC_{50}) two strains of *Phakopsora pachyrhizi*. The work was performed in the laboratory with detached 'Potência RR' soybean leaflets testing the fungicides at 0.0, 0.001, 0.01, 0.1, 1, 10.0 and 100.0 mg of active ingredient/L water. Azoxystrobin, picoxystrobin, pyraclostrobin, and trifloxystrobin were preventatively applied by dipping the leaflet for five seconds in the fungicide suspension 24 hours before the spore inoculation with 2 x 10^3 spore/mL water. Inoculated leaflets were incubated 8 h under dark and later for 15 days at 22ºC and 12 h photoperiod. The effect on rust control was assessed based on the (IC_{50}) the number of lesions and uredia/cm^2. The strain from Primavera do Leste (MT) was less sensitive than the one from Passo Fundo (RS state). The sensitivity reduction factor for azoxystrobin ranged from 365.7 to 891.1, for pyraclostrobin from 5.6 to 245.9, for trifloxystrobin from 12.2 to 17.5, and for picoxystrobin from 10.2 to 12.12. We confirmed the hypothesis of *P. pachyrhizi* sensitivity reduction to QoI fungicides. This is the first report on sensitivity reduction of *P. pachyrhizi* towards QoI fungicides.

Additional key words - Asian soybean rust, resistance, QoI fungicides.

Resumo

Blum, M. M.C. e Reis, E. M. Redução *in vivo* da sensibilidade de *Phakopsora pachyrhizi* à fungicidas estrobilurinas. **Summa Phytopathologica.**

Experimentos foram conduzidos *in vivo* para determinar a concentração (mg/L) dos inibidores da quinona externa da mitocôndria (IQe, ou estrobilurinas), que controle 50% (CI_{50}) duas linhagens de *Phakopsora pachyrhizi*. O trabalho foi conduzido em laboratório com folíolos destacados da cultivar de soja 'Potência RR'. Os fungicidas azoxistrobina, picoxistrobina, piraclostrobina e trifloxistrobina foram testados nas concentrações de 0,0; 0,001; 0,01; 0,1; 1,0; 10,0 e 100,0 mg de ingrediente ativo/L de água. Esses produtos, nas soluções aquosas, foram preventivamente aplicados pela imersão do folíolo por cinco segundos 24 horas antes da inoculação com uma concentração de 2 x 10^3 uredosporos/mL. Os folíolos inoculados foram incubados no escuro por 8h e após, por 15 dias a 22^{o}C e 12 h de fotoperíodo. O efeito dos tratamentos sobre o controle da ferrugem, foi determinado baseando-se (CI_{50}) no número de lesões e de urédias/cm^2. A linhagem de Primavera do Leste (MT) apresentou menor sensibilidade do que a de Passo Fundo (RS). O fator de redução da sensibilidade para a azoxistrobina variou de 365,7 a 891,1, para a piraclostrobina de 5,6 a 245,9, para a trifloxistrobina de 12,2 a 17,5, e para a picoxistrobina de 10,2 a 12,1. Esse é o primeiro relato da redução da sensibilidade de *P. pachyrhizi* aos fungicidas estrobilurinas, no Brasil.

Palavras-chave - IQes, ferrugem asiática da soja, resistência de fungos.

Introduction

Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi* Sydow and Sydow, has been considered the most important crop disease causing damage of up to 80% (8, 17).

The main strategy for ASR control has been the development of cultivars with genetic resistance but the resistance has been not durable due to the high fungal variability. Hence, chemical control of ASR has been the most feasible tactic for the epidemic management and reducing damages. Basically, ASR control has been done by

the application of fungicides in 35 million ha sprayed three times during the soybean cycle as in the 2014/15 growing season (17). However, in the case of a first followed by a second soybean annual crop under center pivot irrigation, up to 11 sprayings have been performed in the same area.

In Brazil, ASR chemical control started in the 2002/03 growing season and after four seasons, in 2007/08, growers complaint about the control failure with demethylation inhibition fungicides (DMI) in the state of Mato Grosso. Further work showed that there was a shift in sensitivity of *P. pachyrhizi* towards DMI fungicides (1, 13, 15, and 16). The *P. pachyrhizi* cross sensitivity reduction towards DMI was determined for cyproconazole, epoxiconazole and tebuconazole the most commonly used (7, 13, and 16). After 2007/08 growing seasons the control efficacy reduction by the DMI + QoI, co-formulations were also detected. Control shift from 80 – 90% up to 37% has been reported in field experiments (7, 8).

We hypothesized that the control reduction noticed in several fields (2012/13 growing season) could be to the sensitivity reduction of the fungus to the QoI fungicides.

To test this hypothesis, the present study was conducted to compare, based on the IC_{50}, the sensitivity of two strains of *P. pachyrhizi* to QoIs. The main objective was to determine (*in vivo*) the inhibitory concentration (CI_{50}) of QoI to two *P. pachyrhizi* isolates suspect of sensitivity reduction and calculate the sensitivity reduction factor.

The experiments were conducted at the Laboratory of Plant Pathology, Agenda Fitossanidade - Pesquisa e Consultoria Agrícola in Passo Fundo, RS.

The inoculum came from a uredospore population collected in naturally infected volunteer soybean plants, in the 2012/13 growing season in Passo Fundo, RS and in Primavera do Leste , MT, here designated as "suspect isolates".

Seedlings of 'Potência' soybean cultivar were grown in 300-mL volume pots containing vermiculite and compost at 3:1 proportion; five seedlings were grown in each pot. Thirty-five days old unifoliate leaves were detached and maintained inside acrylic boxes (12.0 x 12 x 2.5 cm high) containing at the bottom a nylon foam 5 mm thick, covered with two filter paper layers, and saturated with sterile distilled water.

The detached soybean leaflets method was used to measure the isolates sensitivity based on IC_{50} (inhibitory concentration of fungicide that reduces by 50% the lesion and uredia number/cm^2 determined in *in vivo* tests)(5, 6, 14).

QoI azoxystrobin (Priori F 25), picoxystrobin (Acapela SC 25), pyraclostrobin (Comet 25), and trifloxystrobin (Flint WG 50) at concentrations in mg/L active ingredient, 0.0, 0.01, 0.1, 1.0, 10, and 100 mg/L water were applied preventively on detached soybean leaflets, 24 hours before inoculation. Four treated leaflets were kept in each acrylic box and left lid opened up to surface has dried.

Spore suspension (2 x 10^3) was sprayed onto the leaflets inside de boxes and immediately closed and kept under dark for 48 h and after, at 22ºC and 12 h photoperiod, for 15 days up to the evaluation. Assessment was performed under a dissecting stereo microscope (40 X) by counting the lesions and uredia number/cm^2 in a delimited circle (0.8 cm^2) marked with a cork borer in each side of the leaflet mid rib.

The experimental design was a completely randomized treatments with five replications. Each experiment was repeated twice for picoxystrobin, pyraclostrobin and trifloxystrobin and for azoxystrobin five times with the concentration increase up to 200 mg/L.

The IC_{50} was estimated by the Tinn R program (10).

The lesions density on leaflets is a consequence of spore germination and subsequent penetration of the germ tube in the leaflet. Therefore, the effect of treatment on germination was considered, as fungicides were applied preventively, before spores deposition. Moreover, the number of lesions on soybean leaves due to rust is directly proportional to the spores germination and fungus penetration. Those that did not germinate (killed by the fungicide) of course did not penetrate (protective action). The lesions density was a result of the germination/penetration.

The reduction control of soybean rust by fungicides, in Brazil, was first noted by growers which complained the control failure, then the cooperative fungicide trials (number of ± 20 in several sites of Brazil) showed a gradual reduction control by DMIs, QoIs and mixtures in all growing seasons, reaching values below 50% (7, 8).

Therefore, the methodology of detached soybean leaflets reproduced in the laboratory the pathogen sensitivity reduction to DMIS, QoIs alone or in mixtures thereof proving data from field experiments. The control failure observed in soybean fields in various sites of Brazil, were confirmed by laboratory tests. As important as the molecular analysis are the experiments involving the host in the field and in the laboratory.

The sensitivity reduction factor (SRF) was calculated by dividing the IC_{50} of the strain collected in the field by the IC_{50}-value of the baseline sensitivity. Reference concentrations for lesion/cm^2 were azoxystrobin 0.137, picoxystrobin 0.131, trifloxystrobin 0.117 mg/L, and for uredia/cm^2 azoxystrobin 0.113, picoxystrobin 0.112, trifloxystrobin 0.116 mg/L (1). When there was no shift in sensitivity, the SRF value for the isolate was 1, and when >1 there was shift with different magnitudes (13).

IC_{50} and SRF were higher than in the earliest Blum and Reis's work (1) for the three tested QoI fungicides.

(Insert Table 1).

IC_{50}-values for the Passo Fundo isolate considering lesion/cm^2 ranged from 1.4 (picoxystrobin) to 50.1 mg/L (azoxystrobin) and the SRF ranged from 10.2 (picoxystrobin) to 365.7 (azoxystrobin). The lowest SRF was for picoxystrobin followed by trifloxystrobin and the highest for azoxystrobin (Table 1).

IC_{50} values for the Passo Fundo isolate considering uredia/cm^2 ranged from 0.3 (pyraclostrobin) to 91.3 mg/L (azoxystrobin) and the SRF ranged from 5.6 (pyraclostrobin) to 807.9 (azoxystrobin). IC50-values for the Primavera do Leste isolate considering lesion/cm^2 ranged from 1.6 (picoxystrobin) to > 95.4 mg/L (azoxystrobin) and the SRF ranged from 11.6 (picoxystrobin) to > 696.5(azoxystrobin). IC_{50} values for the Primavera do Leste isolate considering uredia/cm^2 ranged from 2.1 (picoxystrobin) to 100.7 mg/L (azoxystrobin) and the SRF ranged from 12.2 (picoxystrobin) to 891.1 (azoxystrobin) (Table 1).

It is likely that the *Pp* shift towards sensitivity reduction to QoI found in Brazil involve a new resistance mechanism. Up to the present, it has been accepted, based on Grasso et al. (3, 9) work, that *P. pachyrhizi*, could not develop a mechanism to resist the QoI fungicide action. The mutation to achieve resistance would be lethal for the fungus like a suicide theory.

In species carrying an intron directly after codon 143, it cannot be ruled out that, mechanisms other than the G143A mutation will arise, conferring resistance to QoIs, as observed in *A. solani*. In wheat rusts, no resistance to QoI fungicides has been detected so far (2, 1).

A change from phenylalanine to leucine at position 129, F129L, also confers resistance to QoIs, although the level of resistance is lower than that conferred by the

G143A substitution. There are no indications so far that F129L might be involve in rusts, but it cannot be ruled out. On 9 December 2014, the FRAC-International announced the official results of its annual meeting for the working group of IQes fungicides, which identified the mutation in position F129L to *P. pachyrhizi* Brazilian isolates. This result is related to work carried out by companies participating in the FRAC-International and FRAC-Brazil. It is likely that the *P. pachyrhizi* sensitivity reduction towards QoI, in Brazil, is due to this mutation.

According to Oliver (12), some pathogenic fungi have in a short period of time become resistant and thus they are regarded as high-risk species. Rust fungi have been classified as being low risk despite sharing many relevant features with high-risk pathogens. An examination of the evidence suggests that rust fungi, such as *P. pachyrhizi* should be classified as high risk.

The low ASR control by fungicides co-formulation in the two last seasons can be attributed to sensitivity reduction towards DMI and QoI to Brazilian isolates a multiple resistance case.

Analyzing Schmitz et al. (15) work, the sensitivity of the reference strain showed IC_{50}-values ranged from 0.24 to 1.11 mg/L. For azoxystrobin, they found the least 0.14 to the highest 2.47 mg/L. The concentration 2.47 mg/L is much higher than those found in Blum and Reis's work (1) 0.112 to 0.137 mg/L for a wild strain. Thus the SRF can be calculate for Schmitz et al. (115) CI_{50} of 2.47 mg/L divided by 0.12 mg/L reference concentration determined by Blum and Reis (2). Thus, Schmitz's et al (15) isolate sensitivity was 20.5 fold higher than for Blum and Reis (1).

Has been reported an effectiveness gradual reduction of DMIs fungitoxicity to *P. pachyrhizi* over the years of use (7, 8, 13). This reduction from 90% to 24% in 10 years is scary. Similar phenomenon has been observed with the performance of QoIs. In 2004/05 season the effectiveness of QoIs was 80-90% and in the experiments net conducted in 2012/13 season, control efficacy of azoxystrobin was 47% (7, 8) and in 2013/14 was just 13% (8).This is a strong evidence of sensitivity reduction towards *P. pachyrhizi*.

The SRF showed a shift in sensitivity of the two tested *P. pachyrhizi* isolates towards strobilurins. In spite of belonging to the same mode of action group, among the tested QoIs there was different fungitoxicity to *P. pachyrhizi*.

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Table 1. *In vivo* concentration (mg/L) of QoI fungicides to inhibit 50% (IC_{50}) the lesion and uredia number/cm^2 and sensitive reduction factor (SRF) for two isolates of *Phakopsora pachyrhizi* in detached soybean leaflets

Fungicide	Lesions/cm^2			
	Passo Fundo		Primavera do Leste	
	IC_{50}[z]	SRF	IC_{50}	SRF
Azoxystrobin	50.1	365.7	95.4	696.5
Picoxystrobin	1.4	10.2	1.6	11.6
Pyraclostrobin	3.1	22.6	28.5	208.0
Trifloxystrobin	2.0	14.5	2.4	17.5
	Uredia/cm^2			
Azoxystrobin	91,3	807.9	100.7	891.1
Picoxystrobin	1.8	10.5	2.1	12.2
Pyraclostrobin	0.3	5.6	42.3	245.9
Trifloxystrobin	2.1	12.2	2.7	15.7

([z]) Determined by Tinn R procedure**.** Reference concentrations for lesion/cm^2: azoxystrobin 0.137, picoxystrobin 0.131, trifloxystrobin 0.117 mg/L and for uredia/cm^2: azoxystrobin 0.113, picoxystrobin 0.112, trifloxystrobin 0.116 mg/L (2). Means of two experiments and for azoxystrobin of five.

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