IN VITRO MYCELIAL SENSITIVITY REDUCTION OF *Drechslera tritici-repentis*, ISOLATES FROM WHEAT, TO TRIAZOLE FUNGICIDES

Erlei Melo Reis[1,2] & Rosane Baldiga Tonin[1]

(1) Laboratório de Fitopatologia - Micologia, Faculdade de Agronomia e Medicina Veterinária, Universidade de Passo Fundo, 99001-970, Passo Fundo, RS, Brasil. (2) Bolsista do CNPq

(2) Autor para correspondência: Erlei Melo Reis, e-mail: erleireis@upf.br

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RESUMO

Reis, E. M.; Tonin, R. B. Redução da sensibilidade miceliana de *Drechslera tritici-repentis*, isolados de trigo, a fungicidas triazóis, *in vitro*. ***Summa Phytopathologica,*** n.00, p.00, 2016.

A mancha-amarela da folha do trigo, causada por *Drechslera tritici-repentis*, causa danos elevados na cultura. Nas últimas safras, produtores e técnicos observaram falha no controle da doença com os fungicidas tradicionalmente eficazes para a doença. Procurou-se esclarecer o fato, por meio da determinação da sensibilidade miceliana de cinco isolados de Dtr de trigo, aos fungicidas inibidores da desmetilação (IDMs). A concentração inibitória (CI_{50}) foi determinada para cinco fungicidas: ciproconazol, epoxiconazol, propiconazol, protioconazol e tebuconazol. Sete concentrações dos fungicidas foram testadas no bioensaio 0,00; 0,01; 0,10; 1,00; 10,00; 20,00 e 40,00 mg/L de ingrediente ativo (i.a.) suplementadas ao meio agarizado. O diâmetro das colônias foi medido com um paquímetro digital quando o crescimento do fungo no tratamento testemunha atingiu a borda da placa. O delineamento experimental foi inteiramente casualizado com quatro repetições e o trabalho repetido duas vezes. Os dados da porcentagem de inibição do crescimento miceliano, foram submetidos à análise de regressão logarítmica e calculada a CI_{50}. A menor sensibilidade, dos cinco isolados, foi detectada para o ciproconazol com CI_{50} de 34,08 a > 40 mg/L. A CI_{50} para o epoxiconazol situou-se entre 0,35 a 1,37 mg/L, para o propiconazol de 0,49 a 1,28 mg/L e de 1,41 a 2,34 mg/L para tebuconazol.

O protioconazol foi o mais potente, com CI_{50} entre 0,09 a 0,21 mg/L. Confirmou-se a hipótese de que a falha de controle pode ser atribuída a redução da sensibilidade de Dtr aos fungicidas IDMs.

Palavras-chave*:* *Triticum aestivum*, mancha foliar, fungitoxicidade, CI_{50}.

ABSTRACT

Reis, E. M.; Tonin, R. B. *In vitro* mycelial sensitivity reduction of *Drechslera tritici-repentis* isolates from wheat, to triazole fungicides. ***Summa Phytopathologica****,* n.00, p.00, 2016.

Wheat yellow leaf blotch incited by *Drechslera tritici-repentis* (Dtr), causes high damage to the crop. In recent growing seasons, producers and technicians observed failure in disease control with fungicides traditionally effective. We sought to clarify the fact, by determining the mycelial sensitivity of five Dtr isolates from wheat to demethylation inhibitor (IDMs) fungicides. The inhibitory concentration (IC_{50}) was determined for five IDMs fungicides: cyproconazole, epoxiconazole, propiconazole, prothioconazole and tebuconazole. Seven concentrations of fungicides were tested in the bioassay 0.00, 0.01, 0.10, 1.00, 10.00, 20.00 and 40.00 mg/L of active ingredient (a.i.) supplemented to the agar medium. The mycelial colony diameter was measured with a caliper when fungal growth, in the control treatment, reached the plate edge. The experimental design was a completely randomized with four replications and the work repeated twice. The percent mycelial growth inhibition was analyzed by logarithmic regression and the IC_{50} calculated. The lowest sensitivity, of the five isolates, was shown for cyproconazole 38.08 to > 40.0 mg/L. The IC_{50} for epoxiconazole was 0.35 to 1.37 mg/L, for propiconazole 0.49 to 1.28 mg/L and for tebuconazole 1.41 to 2.34 mg/L. Prothioconazole was the most potent fungicide, with the lowest IC_{50}, between 0.09 to 0.21 mg/L. The hypothesis that the control failure could be attributed to Dtr sensitivity reduction to DMI fungicides was confirmed.

Keywords: *Triticum aestivum*, yellow leaf spot, fungitoxicity, IC_{50}.

In wheat (*Triticum aestivum* L.) crop, there are diseases caused by fungi, bacteria and viruses. The main fungal diseases are leaf spots, root rots, powdery mildew, rusts and fusarium head blight (18). The principal leaf diseases of wheat in southern Brazil, are yellow leaf spot (YLS) caused by *D. tritici-repentis*, brown spot caused by *Bipolaris sorokiniana* (Sacc.) Shoem., and glume blotch, caused by *Stagonospora nodorum* (Berk.) Cast. & Germ. (15). Among the diseases caused by fungi, YLS is the most frequent in occurrence and damage, and has been considered difficult to be controlled.

The YLS is caused by *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem. (18). Damage caused by the disease may reach 13-48% (Rees & Platz, 1983) and can be estimated by the normalized function Y = 1000 - 5.7 I (where Y = grain yield, and I = leaf incidence) (15).

The integrated disease management strategies include the use of healthy seeds, seed treatment with efficient fungicide, crop rotation, and chemical control by spraying the aerial organs (15). The use of resistant cultivars is the main control strategy, however, there are not in Brazil cultivars with sufficient resistance to avoid damage caused by YLS (15).

As a further control measure, fungicides are important tools to manage major plant diseases in intensive production systems. However, their constant use can promote the selection of fungal resistant strains, putting at risk the control efficiency (4, 5).

Two groups of fungicides are used to control wheat diseases, the triazoles and strobilurins. Among the demethylation inhibitors (DMI) or triazoles, cyproconazole, epoxiconazole, and tebuconazole are intensively used by farmers to control the pathogen, recommended alone or in mixture to quinone outside inhibitors (QoI) or strobilurins (15). The fungicide propiconazole was first used in the 1986 growing season (11), tebuconazole in the

1991 (12), cyproconazole in the 1993 (13), and epoxiconazole in the 2000 (14). After fifteen years of triazoles use, growers start to complaint of leaf rust *Puccinia triticina* Eriks. Control failure (2).

From the 2005/06 growing season, producers and technicians have observed and complained the YLS control failure in several wheat fields where IDM were used alone. A hypothesis was raised that the control inefficiency could be attributed to the occurrence of Dtr sensitivity shift to DMI fungicides.

The detection of sensitivity reduction can be achieved by comparing sensitivity data among fungal populations suspect of resistant to a sensitive isolate measured by the inhibitory concentration (IC_{50}) (9). Few studies, concerning the monitoring of Dtr sensitivity to fungicide recommended by the Brazilian research in disease control in wheat, have been reported (17).

The objectives of this study were to determine the *in vitro* mycelial IC_{50} for Dtr isolates from wheat, to compare the fungitoxicity among the DMIs fungicide used to YLS control, to determine the isolates sensitivity, and to use the sensitivity reduction factor as a measure of Dtr sensitivity reduction degree to fungicides.

Material and methods

The experiments were conducted at the Laboratory of Plant Pathology - Mycology, Faculty of Agronomy and Veterinary Medicine, University of Passo Fundo, Passo Fundo/RS. The Dtr mycelial sensitivity to fungicide was determined in agar medium supplemented with fungicide, as proposed by Russel (16).

Isolates of *Drechslera tritici-repentis.* The fungal strains were isolated from leaves with YLS symptoms; in samples taken from wheat fields in the states of Parana and Rio Grande do

Sul. Five monosporic isolates were selected and stored in test tubes containing potato dextrose agar (Merck PDA, 39 g/L water) in the refrigerator at 5°C (Table 1).

Fungicides and concentrations. The following DMI fungicides were used: cyproconazole (Alto 100, 100 SL), epoxiconazole (Opus 125 SC), propiconazole (Tilt 250 EC), prothioconazole (Proline 250 EC) and tebuconazole (Folicur 200 EC). These fungicides are recommended in mixture to QoI to control YLS in wheat crop (Recommendations, 2011). Prothioconazole, a novel fungicide, was used to compare its performance with the traditionally used fungicides.

Seven concentrations of each fungicide were assessed: 0.00; 0.01; 0.10; 1.00; 10.00; 20.00 and 40.00 mg/L a.i.

***In vitro* mycelial sensitivity of *Drechslera tritici-repentis* to fungicides.** For the dilution, fungicide aliquots were transferred with a micropipette to flask containing sterile distilled water (SDW), resulting in a final volume of 100 ml (base 1 suspension). One mL from this first suspension was transferred to 99.0 mL SDW in a volumetric flask, the second dilution solution (base 2 suspension). Required volumes of the base 2 suspension, was added to wheat leaf extract agar medium (WLEA) (3 g wheat leaf, 20 g dehydrated PDA, 8 g agar, 1.0 L distilled) to obtain the desired concentrations. The flasks were carefully shaken and the supplemented medium poured in sterilized petry dishes (90 diameter x 15 mm high), in a laminar flow hood.

Five mm diameter discs containing the fungus mycelium, taken from the edge of colonies within seven days old were transferred to the Petry dishes containing WLEA supplemented with the fungicides concentrations. The plates were incubated in a growth chamber at 25 ± 2°C, 12 h photoperiod, provided by three fluorescent lamps, 40 W, 50 cm above the plates, and randomly distributed on shelves.

Assessment. The evaluation was performed by measuring with a digital caliper (Mitutoyo) the radial growth of colonies in two perpendicular lines, when the fungal growth in the control treatment, reached the plate edge.

The experimental design was a complete randomized factorial (5 fungicides x 5 isolates) with four replications; each experimental unit was represented by a Petry dish. The experiments were repeated twice and the means used in the statistical analysis. The mycelial growth data in millimeters were converted to percent, analyzed and, the concentration which inhibits 50% of mycelium growth (IC_{50}), calculated by logarithmic regression using the statistical program Costat.

To detect the fungus shift in sensitivity to fungicides, the tool used was the sensitivity reduction factor (SRF) calculated by dividing the IC_{50} of the suspected isolate by the IC_{50} of the most sensitive one. Base line CI_{50} of 0.32 mg/L was taken from Stolte (17). When the SRF value was equal to 1, there was no shift in sensitivity and, when > 1 there was sensitivity reduction.

Results

Regarding the effects of cyproconazole, epoxyconazole, propiconazole, prothioconazole and tebuconazole, none inhibited 100% the fungus mycelial growth at concentrations of 0.01, 0.1 and 1.0 mg/L (Figures 1-5).

The fungal isolates sensitivity was classified by the standard Edgington´s et al. (1971) criterion. After analyzing our data a modified classification was proposed: $IC_{50} > 40$ mg/L insensitive; IC_{50} between 10 and 40 mg/L, low sensitivity; IC_{50} between 1 and 10 mg/L moderately sensitive, and $IC_{50} < 1$ mg/L, highly sensitive isolate.

01/QTZ isolate. In the two experiments overall mean, the coefficient of determination (R^2) between 0.91 to 0.98 and IC_{50} from 0.18 to > 40 mg/L were determined.

This strain was classified as sensitive according to our classification, with IC_{50} values less than 1.0 mg/L, for prothioconazole and propiconazole. IC_{50} ranged from 0.86 mg/L for propiconazole to 0.18 mg/L for prothioconazole. The strain was considered moderately sensitive to fungicides epoxiconazole, with IC_{50} values 1.37 mg/L and to tebuconazole 1.84 mg/L. The strain was considered insensitive to the active ingredient cyproconazole with IC_{50} value > 40 mg/L (Figure 1).

02/ONX isolate. Coefficient of determination (R^2) varied from 0.89 to 0.96 and IC_{50} from 0.16 to 28.11 mg/L. This isolate was highly sensitive to epoxiconazole and prothioconazole. IC_{50} ranged from 0.35 mg/L for epoxiconazole (Figure 3) and 0.16 mg/L for prothioconazole (Figure 5). Values greatest than 1.0 mg/L were observed for propiconazole 1.28 mg/L and 2.34 mg tebuconazole/L, and the isolate considered moderately sensitive for these active ingredients and classified as low sensitivity to cyproconazole (IC_{50} 28.11 mg/L).

03/HTZ isolate. IC_{50} ranged from 0.21 to >40 mg/L. The coefficient of determination (R^2) ranged from 0.81 to 0.99. This isolate was classified as insensitive to cyproconazole with IC_{50} value > 40 mg/L, indicating the lowest sensitivity to this fungicide. Similar results were also found for the other strains. IC_{50} values ranged from 0.49 mg/L for propiconazole (Figure 4) and 0.21 mg/L for prothioconazole (Figure 5). This strain was considered moderately sensitive to fungicides epoxiconazole (IC_{50} = 1.11 mg/L) and tebuconazole (IC_{50} =1.82 mg/L) (Figure 3 and 2).

04/GUA isolate. The coefficient of determination (R^2) for ranged from 0.90 to 0.98, and IC_{50} < 1 mg/L for epoxiconazole 0.63 mg/L, for propiconazole 0.50 mg/L and for prothioconazole 0.09 mg/L. The isolate was considered sensitive to these fungicides. At 10 mg/L mycelium, growth inhibition was 100% for prothioconazole (Figure 5). The strain was considered moderately sensitive to tebuconazole with of IC_{50} 1.41 mg/L and insensitive to cyproconazole with IC_{50} of 40 mg/L (Figure 1).

05/CD isolate. The coefficient of determination (R^2) for this strain ranged from 0.92 to 0.98. The IC_{50} ranged from 0.17 to 34.08 mg/L for the five fungicides. The strain was classified as sensitive having values less than 1mg/L for epoxiconazole (0.45 mg/L) and prothioconazole (0.17 mg/L). Values greater than 1.0 mg/L were determined for propiconazole, tebuconazole and cyproconazole (Figure 4, 2 and 1). The IC_{50} were 1.17 mg/L (propiconazole) and 2.21 mg/L (tebuconazole), and the isolate considered moderately sensitive to these active ingredient. Low sensitivity was detected for cyproconazole with IC_{50} 34.08 mg/L, respectively (Figure 1).

The interaction between isolates and fungicides was significant (p <0.05) (Table 2). Among the five IDMs fungicides tested *in vitro*, prothioconazole showed the lowest IC_{50} for the five isolates, thus the most efficient in inhibiting mycelium growth of Dtr. The values found varied from 0.09 to 0.21 mg/L (Table 2). At 10 mg/L the mycelial growth of the five isolates was completely inhibited (Figure 5).

The lowest IC_{50} was observed for strain 04/GUA to prothioconazole (0.09 mg/L). This fungicide was the most fungitoxic presenting IC_{50} value of 0.09 mg/L (not statistically different from epoxiconazole and propiconazole). In the literature no report was found on the *in vitro* fungitoxicity and IC_{50} values for prothioconazole to Dtr isolated from wheat, however, this chemical can be highly promising in controlling the disease, according to our results.

The highest IC_{50} were determined for cyproconazole, with values between 28.11 mg/L to > 40.0, showing that *in vitro* the isolates were less sensitive to this fungicide. There was fungal mycelium growth, expressed as the colony diameter, for all concentrations tested, and for the five isolates (Figure 1).

The active ingredient tebuconazole was classified as moderately fungitoxic to all tested isolates, statistically different from the other fungicides except for 01/QTZ isolate to epoxiconazole (Table 2) (Figure 2).

On the overall mean, prothioconazole was the most potent fungicide, followed by epoxiconazole, propiconazole and tebuconazole (Table 2).

Comparing the isolates sensitivity, in the overall mean for the five fungicides, the 01/QTZ isolate, coming from Ventania, PR, was the least sensitive, but did not differ statistically from 03/HTZ from Julio de Castilhos, RS. The 02/ONX isolate from St. Augusto, RS was the most sensitive (Table 2).

The SRF is a useful tool to quantify the shift in sensitivity of a fungus to a fungicide (Ghini & Kimati, 2000). Considering the SRF (Table 3), there was reduction in sensitivity for the isolates studied. In the average among the isolates, the 02/ONX, from Santo Augusto, RS, showed the highest SRS (3.19) and the 04/GUA, from Coxilha, RS the lowest (2.05).

In the general mean the SRF ranged from 1.09 to 7.31 i.e., the isolates require a fungicide concentration from 1.09 to 7.31 times higher than the most sensitive to obtain a 50% reduction in the fungal mycelial growth (Table 3).

Discussion

The development of fungal sensitivity shift to DMI is gradual and slow due to the polygenic or quantitative resistance type (4). According to the same author, fungi sensitivity reduction to DMI fungicides can be reversed to a condition of a higher fungal sensitivity when these fungicides are less extensively used and/or when alternative fungicides are used to control the disease.

In the case of Dtr, no references in the literature were found regarding the fungicide IC_{50} sensitivity reference. Therefore, it becomes difficult to conclude whether or not there was sensitivity shift of Dtr isolates to fungicides. Retrieving data reported in the past on the Dtr sensitivity (IC_{50}) to fungicide, could be useful to compare our results with those early reported.

Data reported by Stolte (17), for tebuconazole $IC_{50} < 0.1$ to 0.32mg/L, and < 0.1 to 0.53 mg/L for cyproconazole, differed from our data, where the IC_{50} were higher than those reported by that author, suggesting the occurrence of Dtr sensitive reduction to DMI fungicides. Stolte (17), conducting *in vivo* tests to monitor the Dtr mycelial sensitivity, isolates from wheat to fungicides, in the 2005, determined $IC_{50} < 1$mg/L for cyproconazole, epoxiconazole, propiconazole and tebuconazole. She considered the isolates sensitive to these active ingredients. Beard et al. (3), in a work conducted with Dtr reported IC_{50} 0.19 mg/L for epoxiconazole, 0.39 mg/L for propiconazole, and 0.25 mg/L for tebuconazole. Hunger & Brown (10), working with Dtr, the IC_{50} for propiconazole was 0.04 mg/L and 0.19 mg/L for tebuconazole. IC_{50} for propiconazole found in our study ranged from 0.49 to 1.28 mg/L and for tebuconazole 1.41 to 2.34 mg/L. Comparing our values with those reported by Hunger & Brown (10), IC_{50} has increased 12-32 fold for propiconazole and 7-12 times for tebuconazole. The IC_{50} determined in our work were superior to the other authors indicating reduction in sensitivity of Dtr to DMI.

Several factors can contribute to the reduced sensitivity onset, the product used, intensity of use and the fungal genetics (5).

The use of the fungicide must be managed according to anti-resistance strategies. These strategies are based on the principle that when there is fungicide application, a selection pressure is exerted on the pathogen population, and can in the long or short time, depending on the genetic mechanisms involved, result in the selection and predominance of the less sensitive individuals in fungal population (9).

According to our results, it was found sensitivity reduction of some Dtr isolates to triazole fungicides but with different degrees among them. The fungicides tested in our study belong to the same family of triazoles and with the same mechanism of action, DMI (7). However, their potency differed among the representatives of the group. Just for belonging to the same family was not assured that they had the same fungitoxicity.

The fungicide prothioconazole was the most potent in Dtr mycelial inhibition. cyproconazole showed the highest IC_{50} among the tested triazoles, being considered the least fungitoxic.

Our results confirm the hypothesis that the failure to control YLS in wheat, observed in the last seasons, can be attributed to the fungal sensitivity reduction to fungicides used to control the disease.

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Table 1. Origin and *Drechslera tritici-repentis* isolates identification

Isolate	Wheat cultivar	County/state	Identification
01	Quartzo	Ventania – PR	01/QTZ
02	Onix	Santo Augusto – RS	02/ONX
03	Horizonte	Júlio de Castilhos – RS	03/HZT
04	Guamirim	Coxilha – RS	04/GUA
05	CD 104	Pitangueiras – PR	05/CD

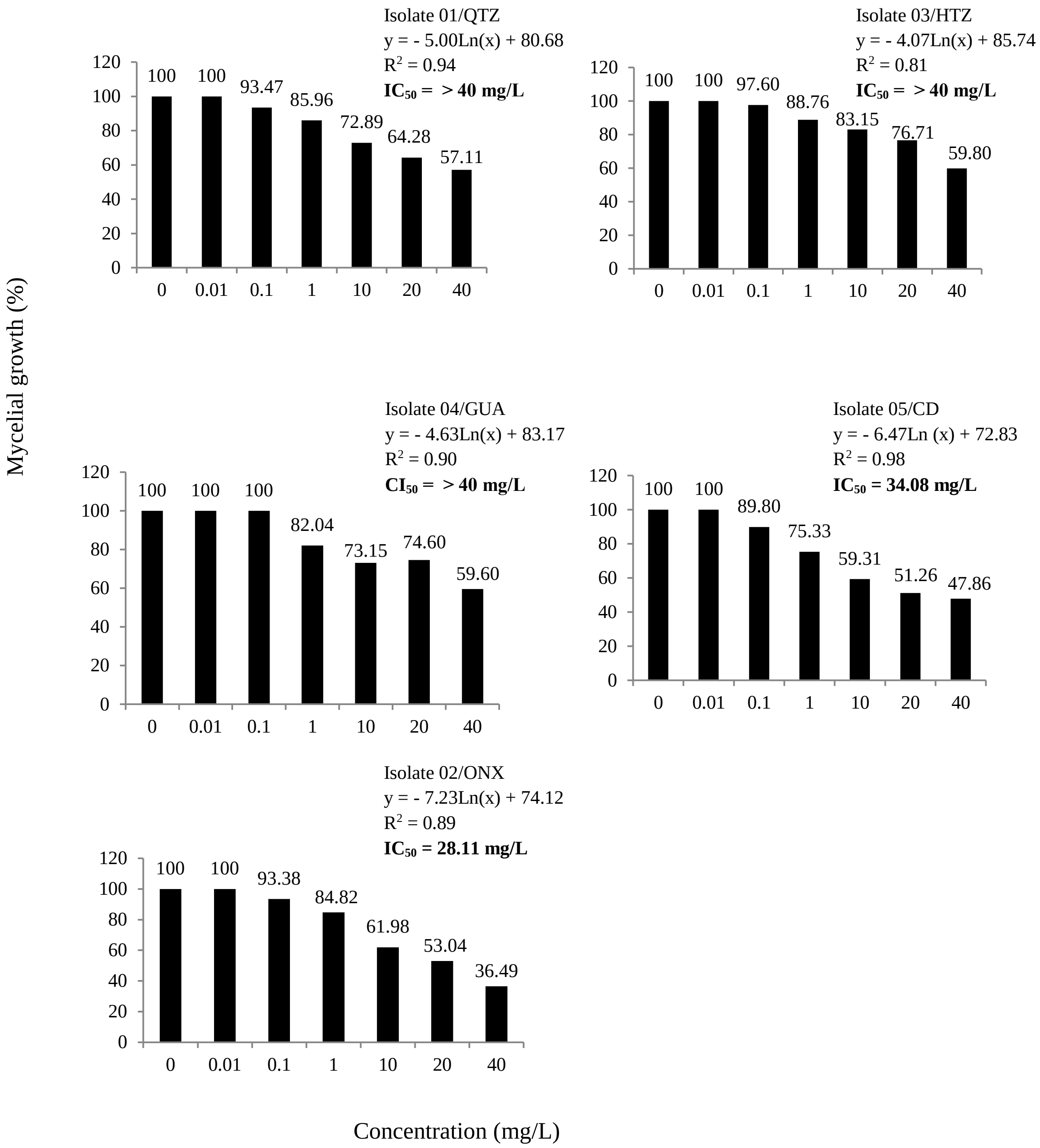

Figure 1. *In vitro* mycelial growth (%) of five *Drechslera tritici-repentis* isolates from wheat, in seven concentrations of cyproconazole fungicide.

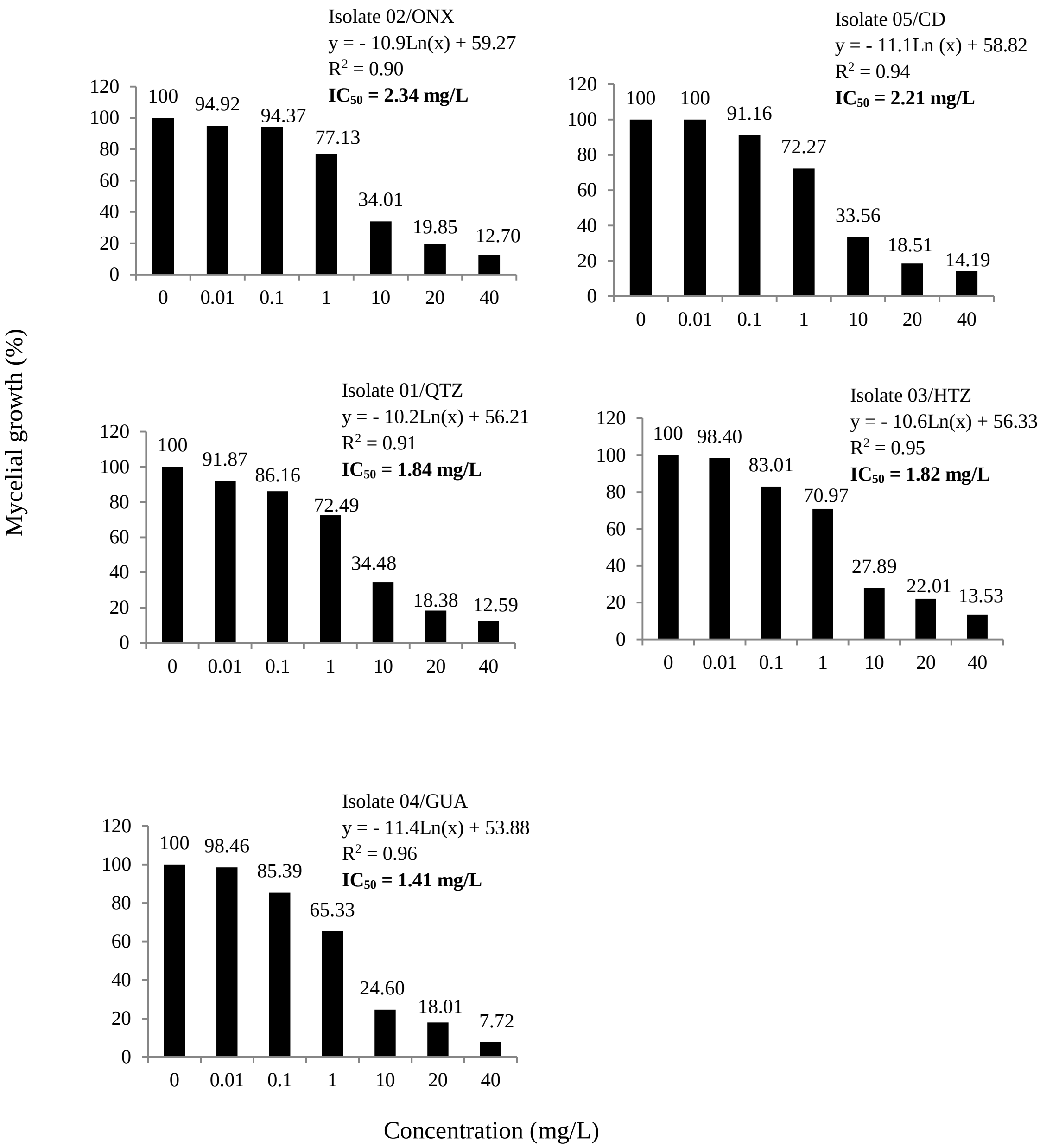

Figure 2. *In vitro* mycelial growth (%) of five *Drechslera tritici-repentis* isolates from wheat, in seven concentrations of tebuconazole fungicide.

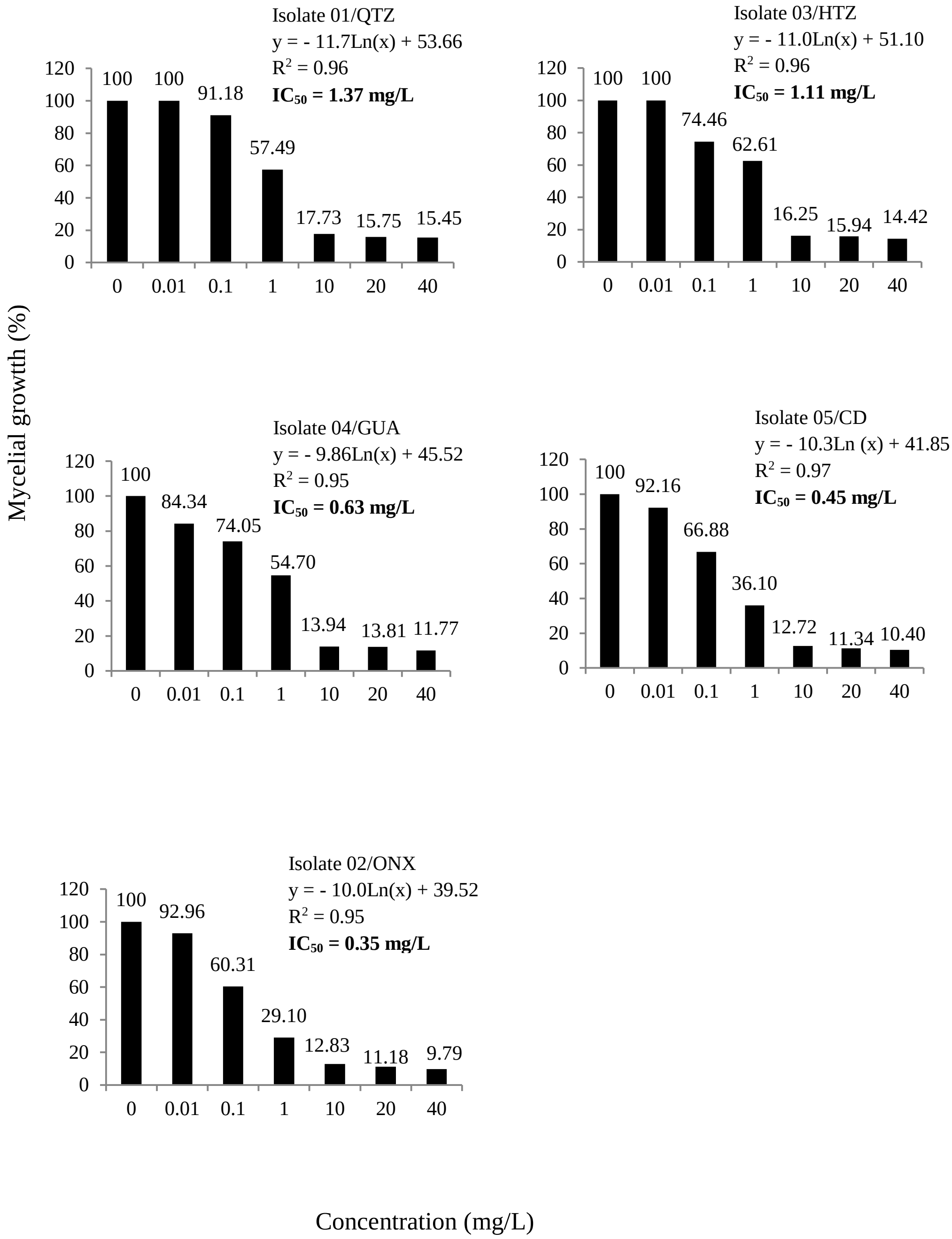

Figure 3. *In vitro* mycelial growth (%) of five *Drechslera tritici-repentis* isolates from wheat, in seven concentrations of epoxyconazole fungicide.

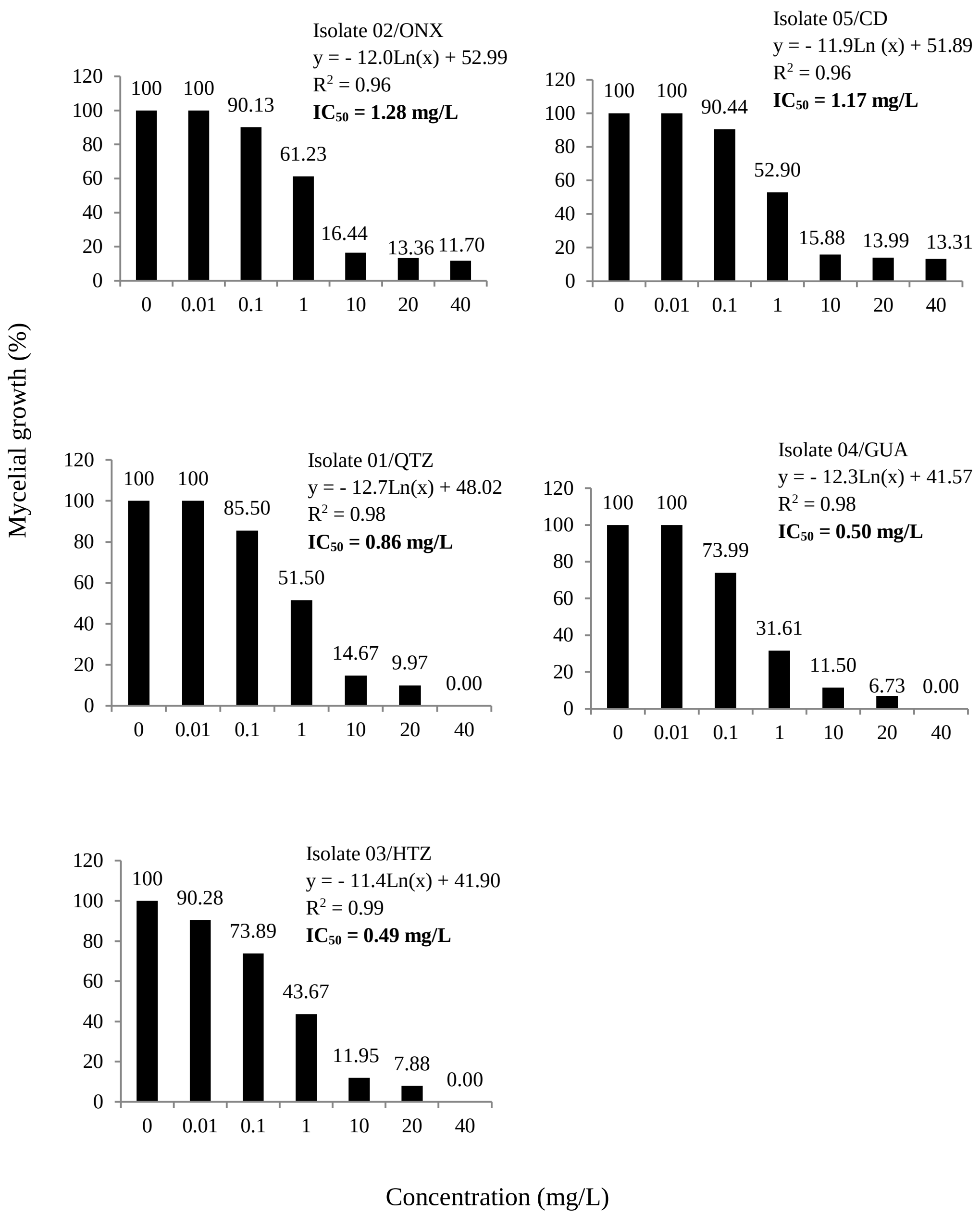

Figure 4. *In vitro* mycelial growth (%) of five *Drechslera tritici-repentis* isolates from wheat, in seven concentrations of propiconazole fungicide.

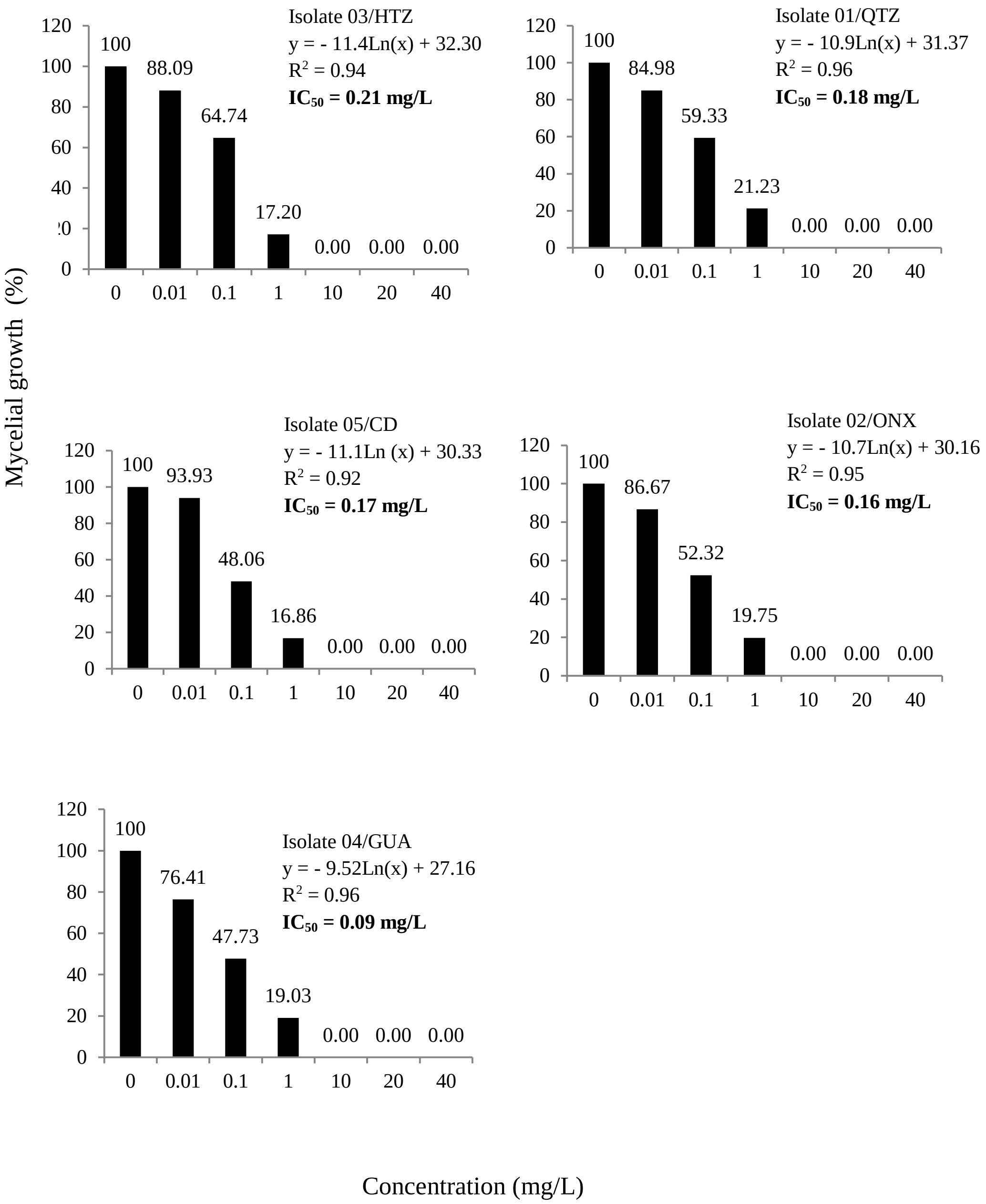

Figure 5. *In vitro* mycelial growth (%) of five *Drechslera tritici-repentis* isolates from wheat, in seven concentrations of prothioconazole fungicide.

Table 2. DMI fungicide concentration (mg/L) to inhibit 50% mycelial growth (IC_{50}) of *Drechslera tritici-repentis* isolates

Fungicide	Isolates					Mean
	01/QTZ	02/ONX	03/HZT	04/GUA	05/CD	
Cyproconazole	A > 40.0 a	C 28.11a	A > 40.0 a	A > 40.0 a	B 34.08 a	> 36.44 a
Epoxiconazole	A 1.37 bc	C 0.35 d	AB 1.11 c	BC 0.63 c	C 0.45 d	0.78 c
Propiconazole	AB 0.86 c	A 1.28 c	B 0.49 cd	B 0.50 c	A 1.17 c	0.86 c
Prothioconazole	A 0.18 d	A 0.16 d	A 0.21 d	A 0.09 c	A 0.17 d	0.16 d
Tebuconazole	AB 1.84 b	A 2.34 b	AB 1.82 b	B 1.41 b	A 2.21b	1.94 b
Mean	> 8,85 a	6,45 d	> 8,72 ab	> 8,52 b	7,42 c	
CV (%)	2,77					

Means followed by same letter do not differ by Tukey test at 5%. Capital letters compare means in the column and lower case in the line. Average of two experiments.

Table 3. Sensitivity reduction factor (SRF) of *Drechslera tritici-repentis*, wheat isolates, to IDM fungicides

Fungicide	Isolates					Mean
	01/QTZ	02/ONX	03/HZT	04/GUA	05/CD	
Cyproconazole	-^{z}	-	-	-	-	-
Epoxiconazole	4.28	1.09	3,46	1.98	1.40	2.44
Propiconazole	2,68	4,00	1.53	1.56	3.65	2.68
Prothioconazole	0,56	0.37	0,65	0.28	0,53	0.47
Tebuconazole	3.23	7.31	5.68	4.40	6,90	5.50
Mean	2,68	3.19	2.83	2.05	3.12	

^{z} - Insensitive. Base line of 0.32 mg/L for IDMs taken from Stolte (2006).