

Cross-resistance to azole fungicides in *Zymoseptoria tritici* disease of wheat

by
Emily Sykes

A PROJECT

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Abstract approved:

Christopher Mundt

Fungicide resistance has become an increasing problem in North American populations of *Zymoseptoria tritici*, the causal agent of septoria leaf blotch of wheat. The goal of this experiment was to determine whether *Z. tritici*'s resistance to one azole fungicide would predict resistance in other fungicides within the same family. Cross-resistance could have implications on the fungicides available to treat the disease locally, as seen in Europe over the past decade. 178 isolates were harvested from leaves collected from two locations in the Willamette Valley. Isolates were grown in the presence of 12 serial dilutions of four different azole fungicides (cyproconazole, propiconazole, tebuconazole, and prothioconazole), their growth measured, and their respective EC50s calculated. In these assays strong cross-resistance was found between cyproconazole and propiconazole; weak cross resistance between cyproconazole and tebuconazole, propiconazole and tebuconazole, and prothioconazole and tebuconazole; and no cross-resistance between cyproconazole and prothioconazole or prothioconazole and propiconazole. These findings have significant implications for the Willamette Valley wheat industry; namely, growers can continue to use a diversity of azole fungicides to successfully control *Z. tritici*.

Key Words: *Zymoseptoria tritici*; septoria tritici blotch; azole; CYP51; cross-resistance

Corresponding email address: ems.sykes@gmail.com

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APPROVED:

Christopher Mundt, Mentor, representing Botany & Plant Pathology

Kara Ritzheimer, Committee Member, representing History

Kathryn Sackett, Committee Member, representing Botany & Plant Pathology

Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Emily Sykes, Author

Introduction

The wheat disease septoria tritici blotch (STB) is caused by the fungus *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola*, *Septoria tritici*). The disease is characterized by large populations, both asexual and sexual reproduction, and heavy gene flow (Zhan & McDonald, 2004) – all of which contribute to a high risk of fungicide resistance development (Cools & Fraaije 2013).

Fungicide use has increased in North America recently due to an increase in grain prices, lower fungicide costs, and a change in pathogens. In the Willamette Valley, wheat growers have typically used 1-2 applications of fungicide a year, starting with flag leaf appearance, to control STB. However, around the mid-2000s, fungicide use was increased to 3-4 applications a year because of stripe rust epidemics. Increased use of fungicides to control stripe rust has had the unintended consequence of increasing the selective pressure for resistance in all fungi, including *Z. tritici*. The most used azole fungicides in this region is propiconazole, followed closely by tebuconazole and occasionally metconazole or prothioconazole (C. Mundt, personal communication, 2015).

Azoles are demethylation inhibitors (DMIs) that target the sterol 14 α -demethylase encoded by the *CYP51* gene. They have been in use in Europe since the 1980s.

Cyproconazole, propiconazole, and tebuconazole fall within the triazole class of azoles, and are classified as a medium risk to resistance development (FRAC, 2009).

Prothioconazole is a triazolinthione azole, and its unique structure among the azole fungicides have made it highly effective in treating STB (Clark, 2006).

Over the past decade azole resistance has slowly increased in European populations of *Z. tritici*. This increase in sensitivity to azoles is due to three main

mechanisms: increase in *CYP51* point mutations (the most common mechanism), *CYP51* overexpression, and up-regulation of active efflux proteins such as ABC transporters (Leroux & Walker, 2011). Estep *et al.* (2015) reported recent resistance in Willamette Valley populations of *Z. tritici* is caused by point mutations, although only one was observed per isolate.

Cross-resistance, or resistance to similar substances, of *Z. tritici* to azoles was first identified in Europe in the 1990s. Some level of cross-resistance has been identified between all azole fungicides in European populations (Leroux *et al.*, 2007). However, cross-resistance has not yet been formally identified in North American populations of *Z. tritici*. Determining whether cross-resistance is present in the Willamette Valley is an important step in guiding future fungicide to lessen the impact of resistance.

Methods

Sampling

The pathogen isolates evaluated in this experiment were collected from two locations in the Willamette Valley of Oregon, USA. The southern collection site was Hyslop Crop Science Field Research Laboratory (Benton County – 44° 37'52.85" N, 123°11'55.19" W), and the northern collection site was a commercial wheat field near Forest Grove, Oregon North Valley Farm (Washington County - 45° 33'58.53" N, 123°00'11.78" W). Both collection sites were part of a spray trial using propiconazole (as the commercial product "Tilt") and a mixture of propiconazole and azoxystrobin (as the commercial product Quilt Xcel). Uppermost leaves infected with *Z. tritici* were collected

from these locations on two occasions: 14-15 April 2012 and 27-29 June 2012. Collected leaves were then dried and stored in a 4°C, low humidity room.

To isolate the pathogen, leaves were first removed from storage and placed in a Petri dish with a moistened filter paper. The next morning, extruded pycnidiospores were harvested off the leaves and transferred to yeast malt agar plates supplemented with gentamycin (YMAa, 10ml gentamycin/L), and allowed to grow for two days in a dark, 22°C incubator. Plugs were taken from the plates and stored in 50% glycerol solution at -80°C for later use.

Assays

The azole fungicides tested in these assays were cyproconazole (Syngenta, 95% purity), tebuconazole (Bayer, 95% purity), propiconazole (Syngenta, 95% purity), and prothioconazole (Bayer, 97% purity). The isolates were chosen based on previous assays (Estep *et al.*, 2015), and consisted of 178 isolates, split almost equally between the isolates with the highest and lowest propiconazole resistance EC50s (concentration of fungicide that limits growth by 50%).

Isolates were removed from freezer storage, thawed, and re-plated on YMAa plates. After three days of growth in a dark, 22°C incubator the plates were flooded, scraped to create a spore suspension, and adjusted to 500,000 spores/ml using a haemocytometer. The spore suspensions were then loaded into 96-well microtiter plate (VWR) wells with potato dextrose broth (PDB, 75 µl) and one of the twelve dilutions of the four azole fungicides. Each of the four fungicides was diluted to concentrations of 1.25, 0.25, 0.05, 0.01, 0.002, 0.0004, 0.00008, 1.6×10^{-5} , 3.2×10^{-6} , 6.4×10^{-7} ,

1.28×10^{-8} , and 0 mg/ml using 95% ethanol. Each microtiter plate was treated with the dilutions from one of the fungicides, and the ethanol was then allowed to evaporate in a sterile laminar flow hood. Afterwards 75 μ l of the spore suspension and 75 μ l of PDB broth were added to each well. The pathogen was found to be highly sensitive to prothioconazole. As a result, prothioconazole dilutions were changed to 0.002, 0.0004, 0.00008, 1.6×10^{-5} , 3.2×10^{-6} , 6.4×10^{-7} , 1.28×10^{-7} , 2.56×10^{-8} , 5.12×10^{-8} , 1.024×10^{-9} , and 2.04×10^{-10} mg/ml in later runs. Because of this sensitivity and change in dilutions only 55 isolates were tested for this fungicide, although a few failed assays for the other fungicides reduced this number even further in the pairwise comparisons (Fig. 2).

Each microtiter plate contained seven isolates and a PDB-water control, and was replicated three times for each of the four fungicides. Once filled, the plates were sealed with Parafilm and stacked together by fungicide. These stacks were placed on a microplate shaker (VWR, orbit size 3 mm), set to 250rpm in a dark incubator set to 22°C, and allowed to grow for five days. After the incubation period, the plates were removed and their optical density was measured at 595 nm (Spectra MAX 190 Microplate Spectrophotometer, Molecular Devices).

Optical densities of the isolates were averaged over the three replicates, and the average optical density for the control row was then subtracted from the averaged rows. For each isolate, the resulting data was used to estimate the upper asymptote (R_0) of a dose response curve. Using this asymptote, we fit a curve in SSasymp in R (R Core Team 2013; Fig. 1) and calculated the concentration of fungicide that reduced the amount of isolate growth by half ($R_0/2$), giving us the EC50 of the isolate.

Statistical Analyses

Pairwise comparison graphs of the log-transformed EC50 values for all of the fungicide treatments were created. Linear regression was completed for each of the six fungicide pairs to determine the correlation coefficient and significance level.

Results

The mean EC50 was -4.46, -4.51, -4.02, and -7.19 log(mg/ml) for cyproconazole, propiconazole, tebuconazole, and prothioconazole, respectively.

Plots of EC50s between pairs of fungicides showed a positive slope in all cases (Fig. 2). Sensitivity of the isolates to cyproconazole and propiconazole, cyproconazole and tebuconazole, propiconazole and tebuconazole, and prothioconazole and tebuconazole were significant ($P < 0.05$), with correlation coefficients ranging from $r = 0.72$ to $r = 0.32$. No significant correlation was observed between prothioconazole and propiconazole ($r = 0.2839$, $P = 0.1691$) or cyproconazole and prothioconazole ($r = 0.1130$, $P = 0.6258$). Some of the plots display two data clusters, high and low, which represent our selection of isolates that displayed high and low sensitivity in previous assays with propiconazole.

Discussion

Cross-resistance was found between four of the six pairwise comparisons of cyproconazole, propiconazole, tebuconazole, and prothioconazole; however, in all but one of the cases, the level of cross-resistance was weak.

EC50 values for the three triazole fungicides were similar, ranging from -4.02 to -4.51 log(mg/ml); however, the EC50 value for prothioconazole was much higher at -7.19 log(mg/ml). Isolates were more sensitive to prothioconazole than the other fungicides at the levels used in this experiment, which accounts for the limited data for this fungicide. Because of this lack of data it is difficult to draw accurate conclusions on the prothioconazole data in this experiment. The range of EC50s are variable in the three pairwise comparisons involving prothioconazole (unlike the other azole combinations), which shows that we may not have captured the full range of resistance. High sensitivity of the isolates to the original concentrations of prothioconazole is likely due to its different chemical structure, which causes it to interact with the target sterol differently than the triazoles (Parker *et al.*, 2013). Additionally, prothioconazole acts as a competitive inhibitor while triazoles are non-competitive inhibitors, which may explain the lack of cross resistance between the two types of azoles.

By using the lowest and highest isolates from a previous propiconazole assays as the baseline for this experiment, we may not have captured the average EC50 for all fungicides tested.

Willamette Valley growers commonly use either propiconazole or tebuconazole individually early in the season. A mixture of tebuconazole and prothioconazole is used later in the season because of resistance to strobilurin fungicides. The results from these assays are positive for growers in the Willamette Valley, as they show that there is not strong cross-resistance among the fungicides being used in the region. This means that a diversity of azole fungicides should continue to be used to treat STB.

Azole resistance began in European populations of *Z. tritici* as single resistance mutations per individual, but the general trend over the last decade has been the emergence of an increased number and complexity of *CYP51* mutations (Cools & Fraaije, 2013). These mutations have limited the efficacy of many azoles to the point that some older azole fungicides (cyproconazole, propiconazole, and tebuconazole) are basically no longer able to control the disease and growers have moved on to newer, more effective azoles like metconazole, prothioconazole, and epoxiconazole (Clark, 2006).

It is important to note that while not all of the isolates used in this collection were sequenced, the ones that were had only one resistance mutation per isolate (Estep *et al.*, 2015). However, it is possible that increased resistance will occur in the future as multiple mutations develop in individual isolates, as has happened in European populations of *Z. tritici* over the last 10-15 years (Leroux *et al.*, 2007)

There are different resistance management strategies that can be used to avoid increased fungicide resistance. Van den Bosch *et al.* (2014) reports that mixing at-risk fungicides can effectively decrease the selection pressure for resistance. Other studies have shown that using the lowest dose of a fungicide that still provides disease control decreases also decreases the selection pressure for resistance (Mikaberidze *et al.*, 2014).

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Figure Legends

Figure 1. Sample azole EC50 curve (Estep *et al.*, 2015). Dose-response curve fitted to the average absorbance over three replications of an isolate for each of the twelve fungicide concentrations. This curve is used to estimate the EC50 for each isolate (the amount of fungicide needed to limit fungal growth by half the amount of the control). In the figure, the open point represents the EC50 value, the solid line the fitted asymptote curve, and the closed points the amount of isolate growth for the twelve fungicide concentrations.

Figure 2. Correlation of sensitivity of 178 *Z. tritici* isolates between the azole fungicides cyproconazole, propiconazole, prothioconazole, and tebuconazole.

Figure 1

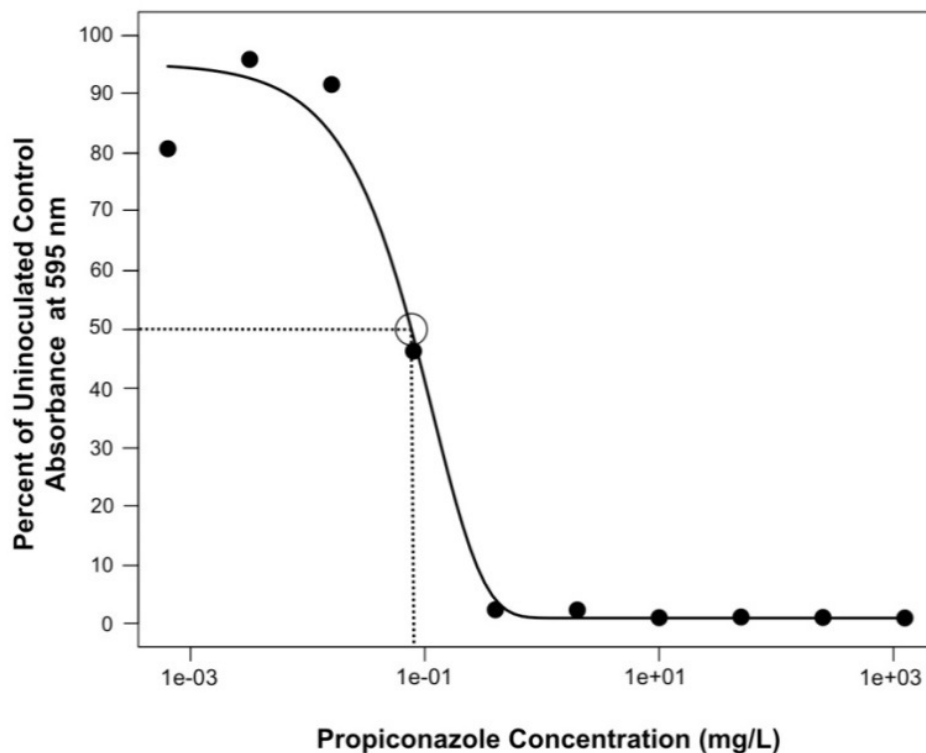
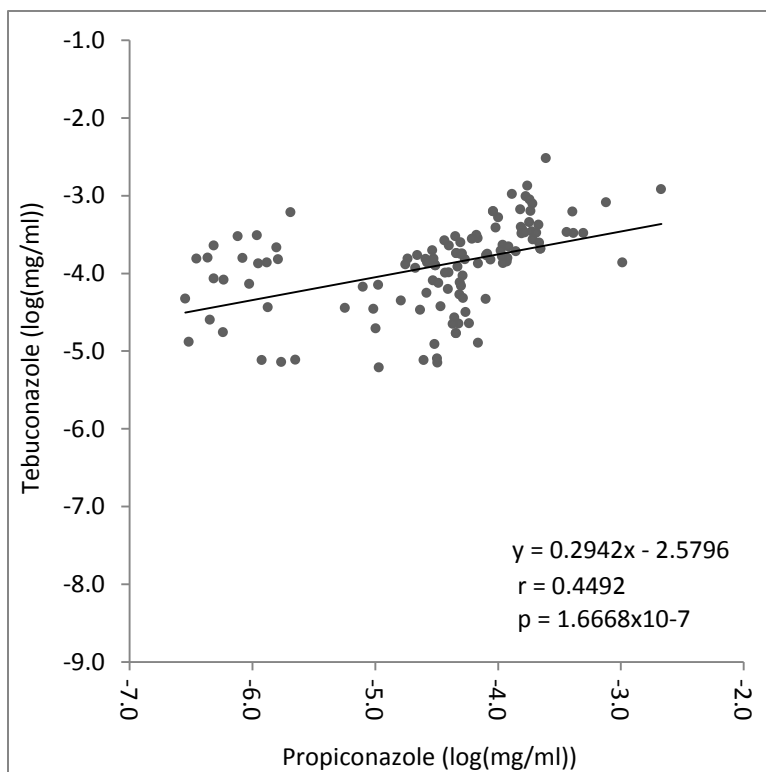
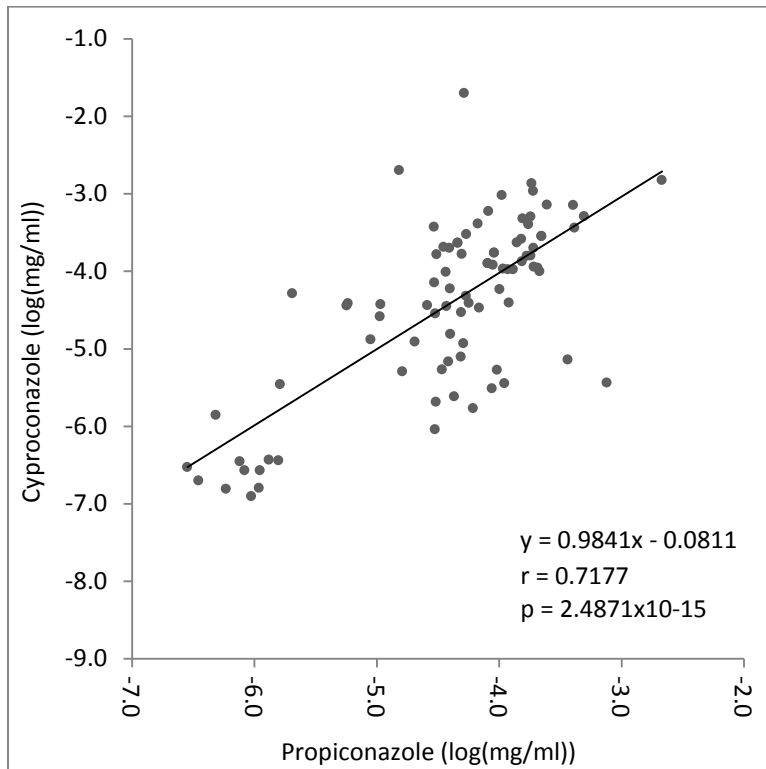
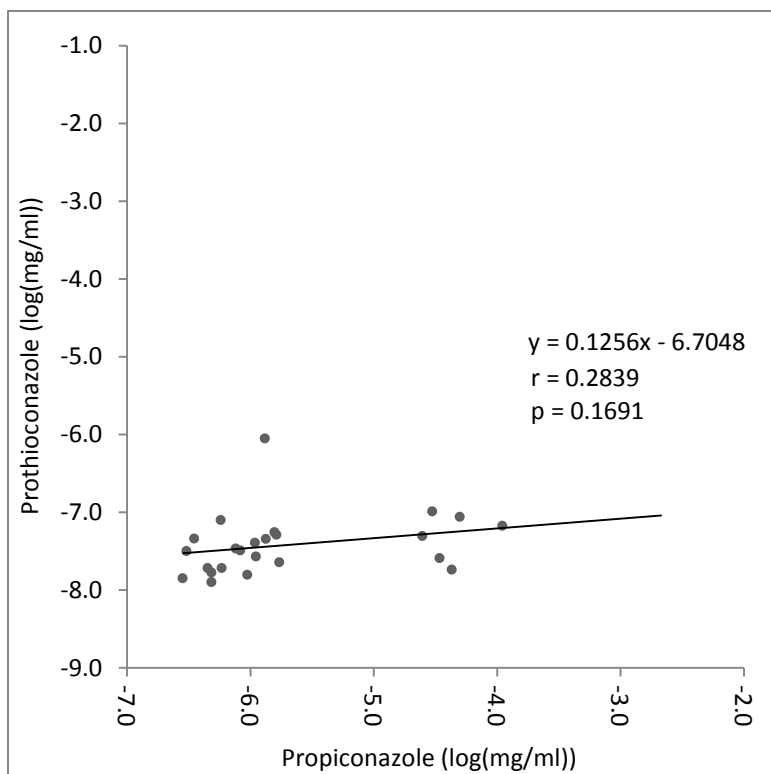
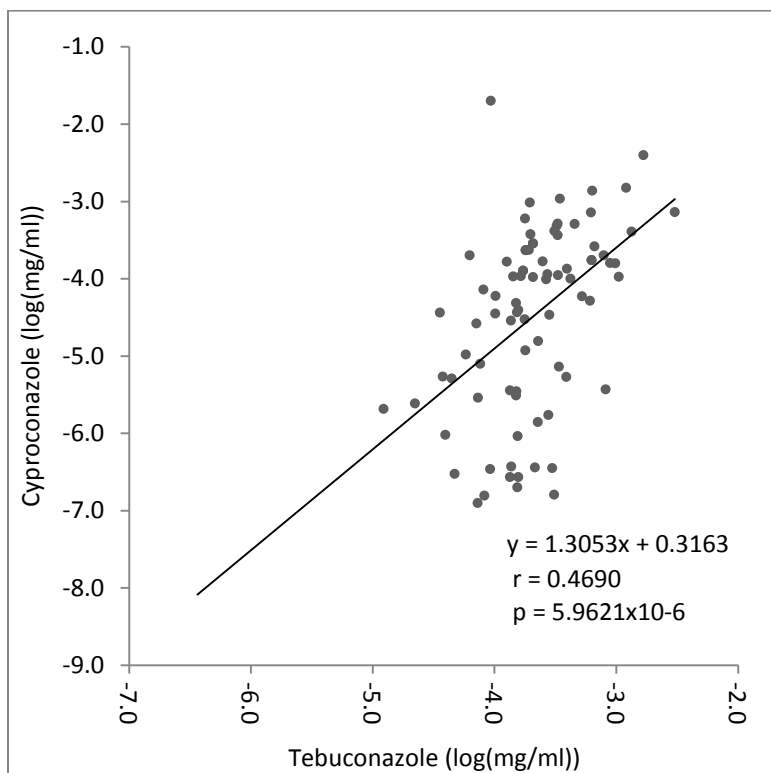
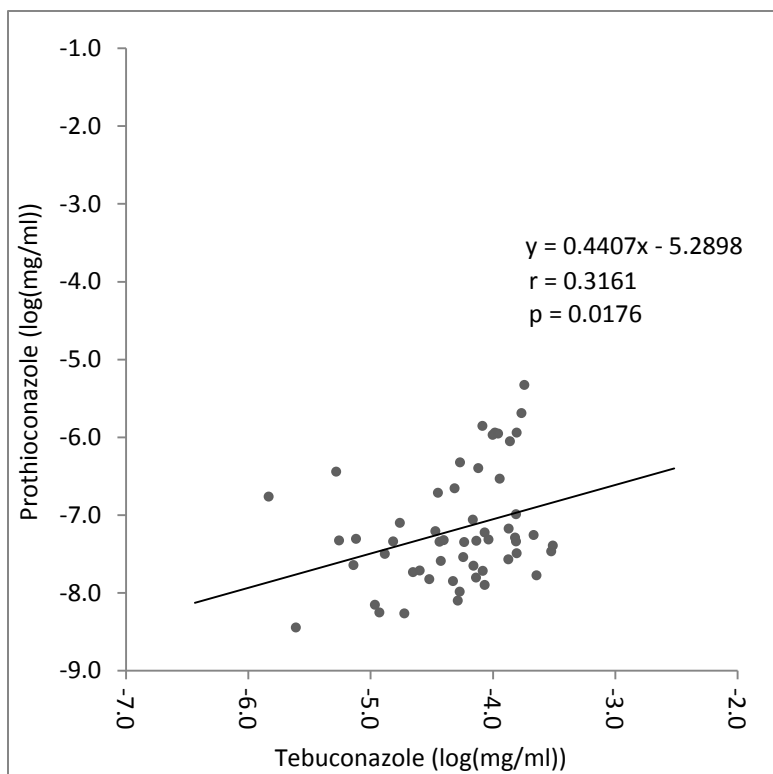
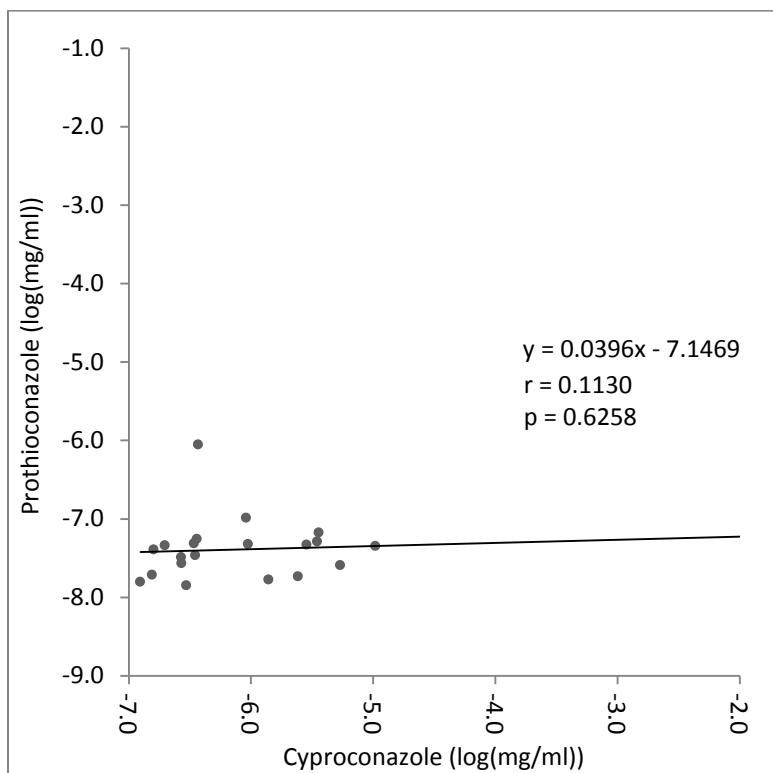


Figure 2







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