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Baseline Sensitivity to Demethylation Inhibitors Fungicides In Cercospora spp. and Corynespora spp. in Arkansas Soybeans

Evan Buckner
University of Arkansas, Fayetteville, ebuck101@gmail.com

Alejandro Rojas
University of Arkansas, Fayetteville, jarojas@uark.edu

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Cover Page Footnote

Evan Buckner is a May 2021 honors program graduate, with a major in Crop Science. Alejandro Rojas, the faculty mentor, is an Assistant Professor in the University of Arkansas System Division of Agriculture's Department of Entomology and Plant Pathology.

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Meet the Student-Author

Evan Buckner

Evan processing Cercospora Leaf Blight symptomatic soybean tissue to induce sporulation of *Cercospora* spp. in a moist chamber for isolation.

Growing up in the delta region of Pine Bluff, Arkansas, and frequently visiting my family's farm in Winterville, Mississippi, I was always surrounded by agriculture. Whether it was visiting the family farm or just walking in my backyard, my passion for plants was growing rapidly. In high school, I had the opportunity to present at the southeast regional and state science fairs, where I was introduced to the State Soybean Science challenge, which awarded prizes to soybean-related projects. Through competing and placing in these competitions, I chose to apply to the University of Arkansas for crop sciences in Bumpers College. This program has helped me see not only my potential in agriculture but the far-reaching benefits of plant pathology research on communities, states, and nations. I've been privileged to participate in 3 research internships at Michigan and Kansas State Universities and presented research at several regional and national conferences. In addition, I received numerous awards, including the Deans and Chancellor's List, Travel Award to the Emerging Researchers National Conference in Washington, D.C., and most recently 1st place in the Division I Undergraduate Oral Presentation for the Minorities in Agriculture and Natural Resources and Related Sciences Conference. I plan to continue my studies in a Ph.D. Program in the Department of Plant Pathology and Environmental Microbiology at Pennsylvania State University. For continued mentorship, time, and dedication to this research project, I thank Drs. J. Alejandro Rojas and Martin J. Egan who have been a significant influence in my academic career.

Research at a Glance

- Target Spot, *Cercospora* Leaf Blight, and Frogeye Leafspot show varying signs of sensitivity to Demethylation Inhibitors (DMI).
- Target Spot is at greater risk for becoming resistant to DMI fungicides used and should be used in an integrated pest management (IPM) approach with other Fungicide Resistance Action Committee (FRAC) groups.
- Continual applications of the same amount of fungicide favor disease resistance in soybeans, and in addition to general IPM, field history, and previous infection of pathogens should also be taken into account.

Baseline Sensitivity to Demethylation Inhibitor Fungicides in *Cercospora* spp. and *Corynespora* spp. in Arkansas Soybeans

Evan Buckner* and Alejandro Rojas†

Abstract

Cercospora spp. and *Corynespora* spp. are two common foliar fungal pathogens in Arkansas and other soybean-producing areas. Two primary diseases caused by *Cercospora* spp. are Cercospora Leaf Blight (CLB, caused mainly by *C. kikuchii*) and Frogeye Leaf Spot (*C. sojina*). Over time, many fungicides used to combat these diseases have become ineffective as the pathogens have developed a resistance to them. The class of the fungicide in question is Triazoles [Demethylation Inhibitors (DMI)–FRAC 3]. Fifteen isolates consisting of *Corynespora cassiicola*, *Cercospora sojina*, and *Cercospora flagellaris* were tested to determine baseline sensitivities using serial dilutions (0, 0.01, 1, 10, 50 mg/L) of the fungicide Tilt (active ingredient: propiconazole). From the three species tested, *Corynespora cassiicola* (isolate 1601) showed the greatest EC_{50} value (10 mg/L). Sensitivity levels for *Cercospora sojina* were close to 1.00 mg/L, and EC_{50} were evenly distributed across all samples of this isolate. *Cercospora flagellaris* samples had the lowest EC_{50} values out of the three species tested as no growth was reported after 1 mg/L. Based on the results of this study, *C. cassiicola* is at greatest risk of resistance to the DMI fungicide Tilt, followed by *Cercospora sojina* and *C. flagellaris*, respectively.

* Evan Buckner is a May 2021 honors program graduate, with a major in Crop Science.
† Alejandro Rojas, the faculty mentor, is an Assistant Professor in the University of Arkansas System Division of Agriculture's Department of Entomology and Plant Pathology.

Introduction

The United States currently ranks number one in the world for soybean production, with one of the leading states, Arkansas, ranking 10th in the nation. Soybean pathogens in Arkansas currently cause yield losses up to 10% (USDA-NASS, 2017; Allen et al., 2017). Two of the primary diseases in soybean in Arkansas are Cercospora Leaf Blight (*Cercospora kikuchii*) and Frogeye Leaf Spot (*Cercospora sojina*). Cercospora Leaf Blight is also caused by the *Cercospora flagellaris* due to varying cercosporin production in *Cercospora* species (Albu et al., 2016; Cochran and Thiessen, 2021). The causal agent of Cercospora Leaf Blight also causes Purple Seed Stain in soybean. Cercospora Leaf Blight and Frogeye Leaf Spot are foliar fungal diseases usually occurring in the latter half of the growing season. These diseases usually originate from infected seeds or from diseased debris from a prior soybean crop. Favorable climates include warm and wet weather conditions. The initial symptoms of this disease cause a faint purple color on the upper surface of the leaf. Throughout the disease stages for Cercospora Leaf Blight, the color and texture may turn to leathery and dark purple, with bronze highlights. Similarly for Frogeye Leaf Spot, once these diseases have infected the plant, leaf spots with reddish to purple color and necrotic centers occur, and spots could coalesce causing more extensive damage. As the disease progresses, it results in defoliation around the upper canopy. The reduced number of leaves from the plant hinders the production of photosynthesis, ultimately killing the soybean crop (Albu et al., 2016; Faske et al., 2014; Crop Protection Network, 2021).

Corynespora cassiicola is an ascomycete pathogen in the order Pleosporales, first described by Berkeley and Curtis in 1868. This pathogen is common in the tropic regions and in greenhouses, also existing as a saprophyte or an endophyte, and as a pathogen infects over 500 species of plants (Schlub et al., 2009; MacKenzie et al., 2018). Symptoms of the pathogen can be present on the stems and leaves, showing the typical lesion in a target pattern and has been reported by some Arkansas farmers to have caused at least a 1.01 to 1.35 tons/ha loss of soybeans (Faske et al., 2014; Berkeley and Curtis, 1868).

In tropic regions, this disease has caused yield loss in the world's second-largest soybean-producing country, Brazil, and in Argentina. Target Spot in Brazil was first identified in 1976, and for many years, the disease was not a persistent pest (Molina et al., 2019). However, the usage of agronomic practices, such as continuous no till, can allow for the fungus to resurface (Molina et al., 2019).

Cercospora spp. (*C. flagellaris* and *C. sojina*) and *Corynespora cassiicola* cause significant harm to soybeans, with the latter being more widespread. Previous control of these fungal pathogens in the southern United States included fungicides within Fungicide Resistance Action Committee (FRAC) groups 11 (Quinone Outside Inhibitors (QoI)), 1 (Benzimidizole (MBC)), and 3 (Demethylation Inhibitors (DMI)). With continuous use of fungicides in FRAC group 3, there is a threat of future fungicide resistance in *Cercospora* spp. and *Corynespora* to the chemical family propiconazole. The primary objective of this research was to determine fungicide resistance levels in *Cercospora*. spp. (*C. flagellaris* and *C. sojina*) and *Corynespora cassiicola* when challenged with the fungicide propiconazole (Faske et al., 2014; Almeida et al., 1976; Avozani, 2011; Rojas, pers. comm., January 2020).

Materials and Methods

Foliage samples were collected during the fall semester of 2019 from fields exhibiting symptoms associated with *Cercospora flagellaris*, *Cercospora sojina*, and *Corynespora cassiicola*. Plant tissue samples were collected from four Arkansas locations, the University of Arkansas System Division of Agriculture's Vegetable Research Station, Kibler; the Lon Mann Cotton Research Station, Marianna; the Jackson County Extension Center, Newport; and the Rohwer Research Station, Rohwer. Sample collection was limited to the time in the planting season in which the fungi heavily infect and reproduce on the soybean plants.

The initial step was to collect a minimum of 10 to 15 infected foliage/leaf samples from each location and place them in sealed plastic bags (Ziploc) for each separate location and for each pathogen. A minimum of 10 to 15 infected pods (to identify infected seeds) was taken from infected plants. Once the leaves and seeds were collected, fungal isolations were conducted to generate spores from infected tissue. For fungal isolations, moist chambers were set up using a moist towel paper placed at the bottom of a 0.102 × 0.102 × 0.305-m plastic container, and leaves were placed inside and sealed. Containers were incubated at room temperature for 48 hours, tracking spore development twice daily. After this, in a secure biological hood, growth from leaf samples was taken from plastic containers, and specimens with abundant fungal growth were chosen for plating. A metal needle was used to lightly scrape the surface of the infected leaf area and then was poked into potato dextrose agar (PDA). Spores were picked using a needle and transferred to PDA with antibiotics (Danitol, ampicillin, streptomycin, and rifampicin). Another method performed was taking a scraper with a circular end and wrapping the fungal growth around it by rubbing the scraper in a round pattern on the leaf surfaces; then the scraper was rubbed on the PDA. If the same metal scraper was used for multiple extractions, then close attention was paid to disinfect it of unwanted fungi by placing the needle in an open flame followed by rinsing with ethanol (70%).

Seed isolates from pods (for growing purple seed stain (*Cercospora flagellaris*)) were recovered directly from seed

after sterilizing the seeds in 70% ethanol and drying them in a sterile laminar flow hood. Seeds were plated on PDA media plus antibiotics (Danitol, ampicillin, streptomycin, and rifampicin), and plates were incubated at room temperature for 4 to 7 days (Cochran et al., 2021). Media plates with antibiotics were placed in sealed plastic containers until fungi reached growth in diameter of 40 to 50 mm. The specified growth allows for ample sample collection for fungicide trials. In addition to plates in the container, 3 mothballs in a paper slip were placed in the containers to minimize mite contamination.

After fungal growth reached a colony of roughly 40 mm, plugs were taken and transferred to fungicide-amended plates and an unamended control plate. Three replications each for treatment level of 0, 0.01. 0.1, 1, 10, and 50 mg/L of DMI fungicide were prepared. For each isolate of each pathogen tested, there were 3 replications for each of the treatment levels totaling 18 total plates per isolate. Potato Dextrose Agar was the base of the media, with corresponding treatment levels added for final levels of the fungicide concentrations. Each of these levels was used for the DMI fungicide for each of the 3 isolates, *C. flagellaris*, *C. sojina*, and *C. cassiicola*. A 5-mm diameter metal corkborer was pushed around the media to extract 18 separate plugs. Each fungal sample was placed on the media with the separate fungicide mixtures and allowed to grow over a period of 7 days. No antibiotics were used in addition to the fungicide media plates. Plates were placed in sealed plastic containers away from windows and incubated at room temperature, after which growth was measured in mm using a digital caliper of the colony in two perpendicular directions and averaged for analysis. Growth was recorded in a spreadsheet in Microsoft Excel and analyzed through R Studio using R (Desktop 1.4.1106) using the package EZEC and drc (R Core Team, 2018; Ritz et al., 2015; Kamvar, 2014).

Results and Discussion

Two possible threats to the validity of this research project were incorrect fungal identification and contamination on the Petri dish plates from non-targeted fungi. These threats, in conjunction with the primary steps of the project, were key to understanding the resistance levels and stable population collections of the fungi involved. For this project, we used a quantitative experimental design because this research project dealt primarily with numeric levels of potency for FRAC group 3. For fungal identification, polymerase chain reaction (PCR) was used to amplify DNA of our samples and determine if they had the same number of base pairs as known samples of *Cercospora* spp. and *Corynespora* spp. The second concern for validation came from potential contamination of the Petri dish plates on which the fungi were grown and fungicide was applied. In

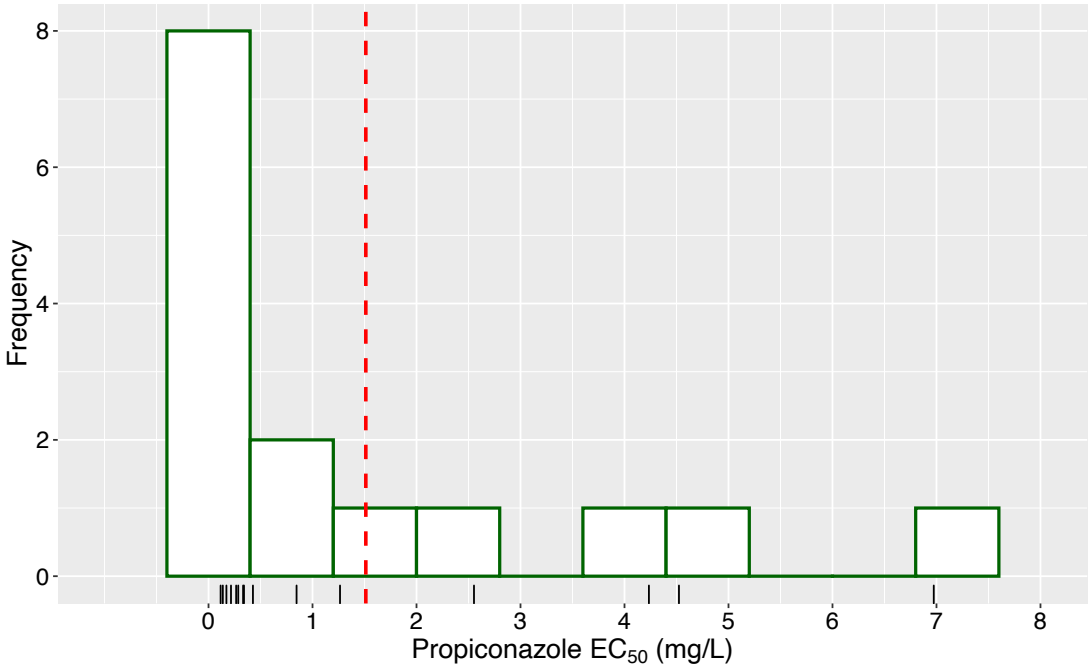

Fig. 1. Effective Concentration of propiconazole that inhibits at least 50% of the fungal growth for 15 isolates of *Cercospora* spp. and *Corynespora cassiicola* in Arkansas Soybeans. The dashed red line represents the mean of all EC_{50} values for the entire sample population.

the laboratory, thousands of other fungi spores are present and could land on Petri dishes being prepped, causing unwanted growth. The threats mentioned were factors viewed in a study by Xavier et al. (2013) on baseline sensitivities to *Corynespora* spp.

A total of 15 isolates were used in this study. Three isolates were *C. flagellaris*, 6 were *C. cassiicola*, and 6 were *C. sojina*. For DMI, all isolates were completely inhibited at 50 mg/L. The greatest number of isolates inhibited by the fungicide had EC_{50} values ranging from 1 to 10 mg/L (Fig. 1).

Effective Concentration values for 50% fungal inhibition were inconsistent across the three species. However, greater EC_{50} values were associated with *Corynespora cassiicola* compared with EC_{50} values obtained for *Cercospora sojina* and *Cercospora flagellaris*. In addition to greater EC_{50} values, there were some abnormal distributions of isolates regarding growth over time. This abnormality was observed as plates with low concentrations (0.01 and 1 mg/L) of fungicide having a larger percentage of growth than plates with other concentrations of fungicide. For all 6 isolates of the *C. cassiicola*, isolate 1601 was the only one to show this abnormal growth between 0 and 1 mg/L (Fig. 2); this phenomenon is known as the hormetic effect since some isolates could respond with an increased growth under sublethal concentrations of fungicides (Pradham et al., 2017).

The EC_{50} values for the Frogeye Leaf Spot were less than *C. cassiicola* as the value was close to 1.00 mg/L but no greater than 10.00 mg/L. This relationship shows the higher levels of sensitivities of *C. sojina* isolates compared to *C. cassiicola*. Isolates for this pathogen represented even distribution for all treatments (Fig. 3).

Effective Concentration value for 50% fungal inhibition was the least for the Cercospora Leaf Blight isolates. No inhibition was reported at greater than 1 mg/L, and all isolate growth was distributed across the ranges tested without abnormality (hormetic effect) in percent growth. Isolates used for this fungal genus were *C. flagellaris,* and it is associated with the lineage of Cercospora Leaf Blight (Albu et al., 2016). Cercospora Leaf Blight is associated with Purple Seed Stain, and *C. kikuchii* and *flagellaris* have been linked in lineage to several other *Cercospora* diseases in soybean- growing regions (Albu et al., 2016; Price et al., 2015). Cercospora Leaf Blight isolates represented a sigmoidal distribution for all treatments. There was a steady decrease in the percent growth for the increasing concentrations (mg/L) of the fungicide, which also represents the current sensitivity levels of these isolates to propiconazole, and EC_{50} values were overall below 1 mg/L in comparison to *C. cassiicola,* which was greater than 1 mg/L. Growth decline was steeper than *C. sojina* and *C. cassiicola* (Fig. 4).

Conclusions

Of the three fungal species tested, no cross-resistance was reported as multiple FRAC 3 fungicides were not test-

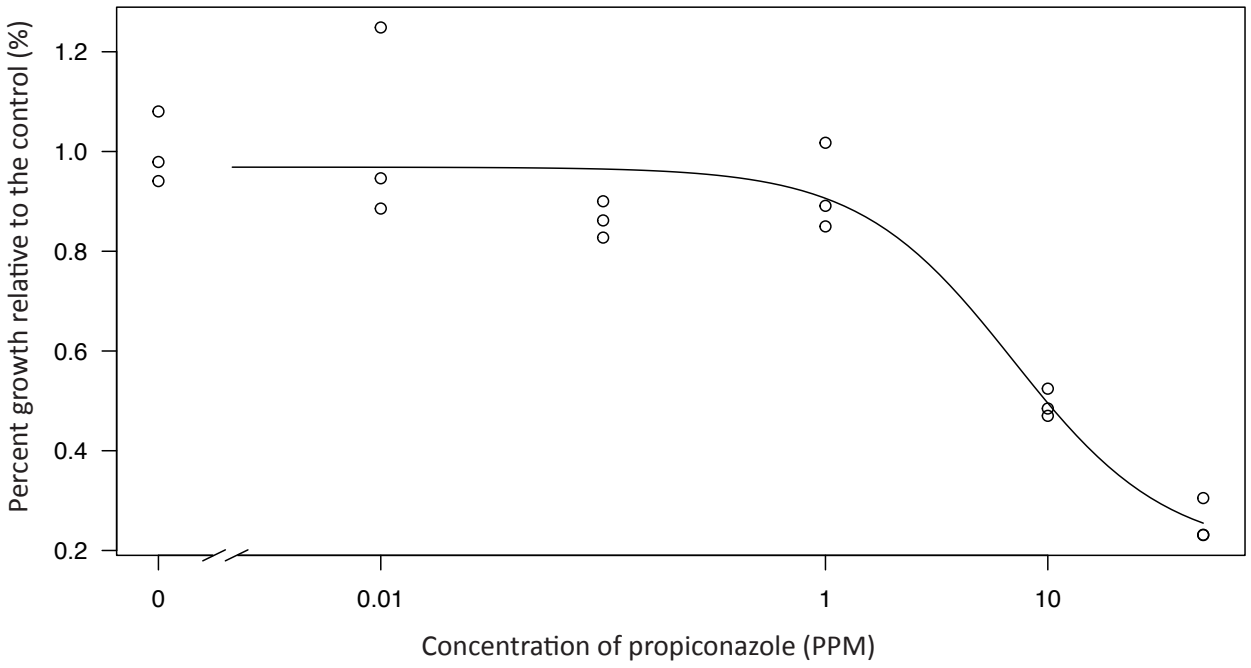

Fig. 2. Distribution of EC_{50} values for *C. cassiicola* isolates 1601. With percent growth on the *y*-axis, the EC_{50} value for isolate 1601 fell in the range of 10.00 mg/L (ppm).

ed. However, varying sensitivity levels to the fungicide for all species may prove a future concern to growers as similar chemical control practices may continue to be in place. *Corynespora cassiicola* had the greatest EC_{50} values indicating a sensitivity lower than the latter two species. This may also point to the growing tolerance of this pathogen to DMI fungicides, and that resistance may be reached earlier in *Corynespora* spp. In addition, there were differences in sensitivity values between isolates of the same fungal species. This difference in sensitivity values may be attributed to local adaptation under different fungicide exposures under different management programs. In comparing the two *Cercospora* species, *C. sojina* had greater EC_{50} values and is at a larger risk of developing resistance than *C. flagellaris*. An integrated pest management regime in conjunction with the integrated FRAC chemistries is the current recommendation of this research project to aid in decreased disease resistance.

Acknowledgments

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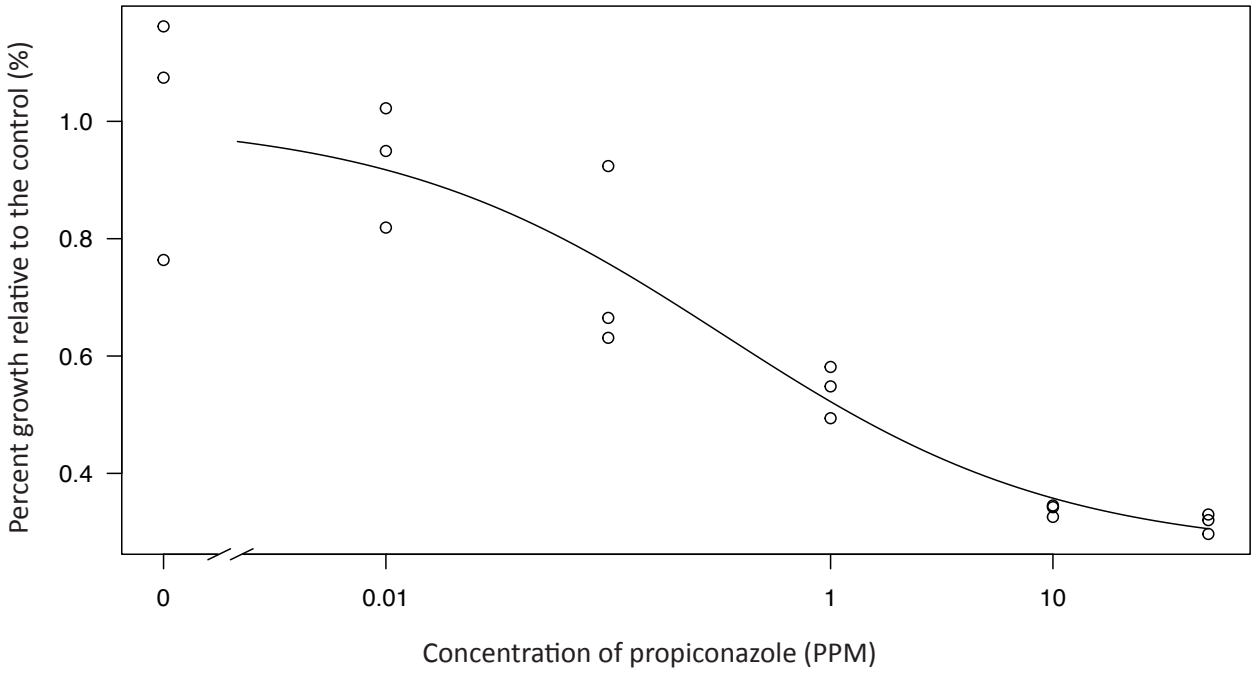

Fig. 3. Distribution of EC_{50} values for *C. sojina* isolate hSB-421.

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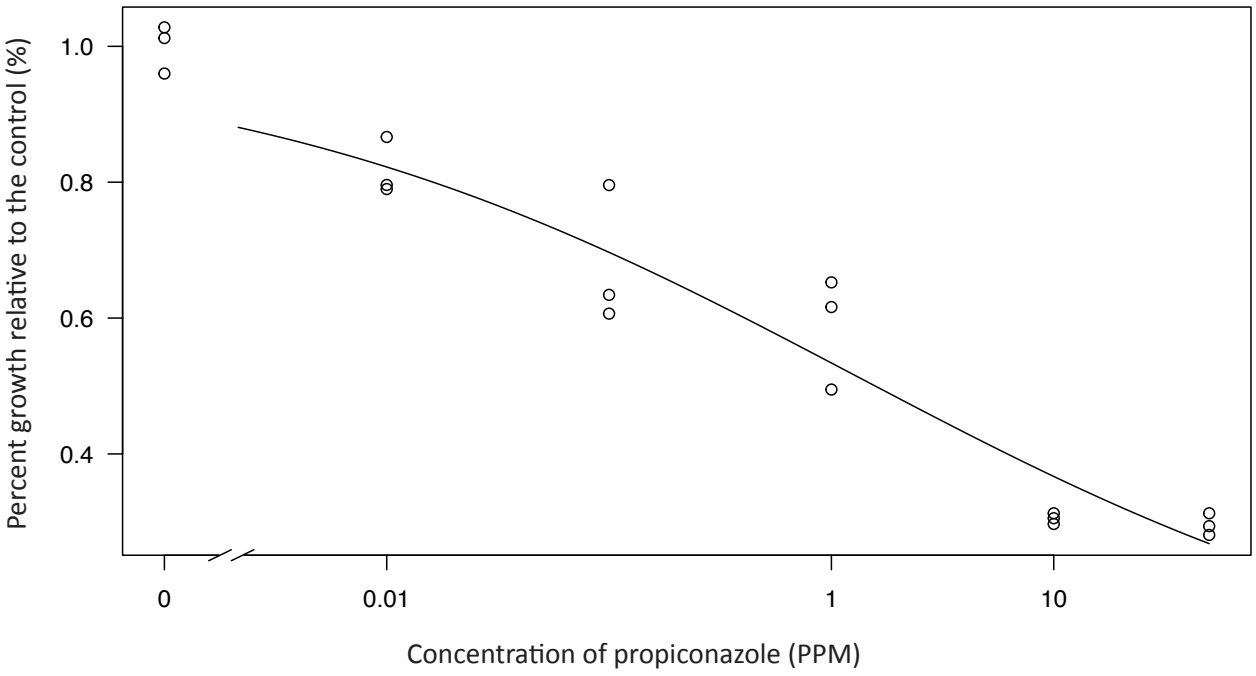

Fig. 4. Distribution of EC_{50} values for *Cercospora flagellaris* isolate 1901.