5-2021

Baseline Sensitivity to DMI Fungicides in Cercospora Spp. and Corynespora Spp. in Arkansas Soybeans

Evan Buckner

BASELINE SENSITIVITY TO DMI FUNGICIDES IN *CERCOSPORA* SPP. AND *CORYNESPORA* SPP. IN ARKANSAS SOYBEANS

By

Evan Buckner

Honors Thesis

Submitted to the Bumpers Honors College in partial fulfillment of the Bumpers Honors Program

Department of Crop, Soil, and Environmental Sciences

University of Arkansas

April 2021

Supervised by Drs J. A. Rojas and M. Egan

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Abstract

Cercospora spp. and *Corynespora* spp. are two common foliar fungal pathogens in Arkansas amongst 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*). Both diseases affect foliage, and when lesions collapse, leaves may fall prematurely resulting in yield loss. In the specific case of CLB, this is a disease on the rise since 2000, and also causes seed infection reducing seed quality. Target spot is a disease caused by *Corynespora cassiicola*, and is of less damaging for farmers in larger soybean producing countries like the US, however disease incidence has been increasing overtime. However, Target Spot has still caused significant harm to plants when left unchecked. Over time, many fungicides used to combat these diseases have become ineffective as the pathogens have developed a resistance to them. The mode of action of the fungicides in question are Quinone Outside Inhibitors (QoIs – FRAC 11) and Triazoles (DMIs – FRAC 3). The primary goals of this research project were to establish a collection of *Cercospora* spp. and *Corynespora* spp., establish a method for testing fungicide resistance determining EC50 (Effective concentration at 50% growth) using fungicide levels 0.01, 0.1, 1, 5, 10, and 50 mg/L, and establish a baseline of resistance against the two chemistries to determine resistance in the field.

Baseline Sensitivity to DMI fungicides in *Cercospora* spp. and *Corynesopra* spp. in Arkansas soybeans

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, 2019; Arkansas Farm Bureau, 2019). Twoof the primary diseases in soybean in Arkansas is Cercospora Leaf Blight (*Cercospora kikuchii*) and Frogeye Leaf Spot (*Cercospora sojina*). 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 causal agent of Cercospora Leaf Blight is also the cause of Purple Seed Stain in soybean. The initial symptoms of this disease cause a faint purple color on the upper surface of the leaf. Throughout the disease stage 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, necrosis of the leaf sets in followed by defoliation around the upper canopy. The reduced number of leaves from the plant hinders the production of photosynthesis ultimately killing the soybean crop. (Faske et al., 2014; Crop Protection Network, FLS 2021

Corynespora cassiicola is a fungal pathogen first described by Berkeley and Curtis in 1868. This pathogen is common in the tropic regions and in greenhouses existing as saprophytes, and endophytes and infects over 500 species of plants. (Schlub et al., 2007; MacKenzie et al., 2018). Though symptoms of the pathogen can be present on the stems and leaves, symptoms

primarily seen on the leaves of plants and was reported by some Arkansas farmers to have caused at least a 15 to 20 bushel/acre loss (Faske et al., 2014; Berkeley & 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, allowed for the fungus to resurface in many parts of the country (Molina et al., 2019).

Problem Statement

Cercospora spp. 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 were with fungicides with Fungicide Resistance Action Committee (FRAC) groups 11 Quinone Outside Inhibitors (QoI), 1 Benzimidizole (MBC), and 3 Demethylation Inhibitors (DMI). Fungicides in groups 1, 3, and 11 have varying levels of resistance to the fungi labeled. In Arkansas, growers using the FRAC group 11 have reported fungicide resistance to the *Cercospora* spp. However, with continuous use of the fungicides in group 3, there is a threat of future fungicide resistance to the *Cercospora* spp. and *Cercospora cassiicola* (Faske T et al., 2014; A. Rojas, personal communication, January 2020)).

Purpose of the Study

Demethylation Inhibitors (DMIs), as part of an integrated fungicide management program, need to be evaluated to determine the efficacy at varying rates of application to prepare a baseline sensitivity (A. Rojas, personal communication, January 2020). The purpose of this

study was to evaluate the effectiveness of the DMI fungicide Tilt (Propiconazole) fungicides to control the pathogens *Cercospora. spp.* (*C. kikuchii* and *C. sojina*) and *Corynespora cassiicola* in soybean (*Glycine max*).

Research Objectives

The primary objective of this research was to determine fungicide resistance levels to *Cercospora.* spp. (*C. kikuchii* and *C. sojina*) and *Corynespora cassiicola.* To accomplish this objective, the first step was to establish a collection of *Cercospora* spp. (Frog eye leaf spot and Cercospora Leaf Blight) and *Corynespora cassiicola* (Target spot) in collaboration with a Master's student in the Rojas lab, Amber Lancaster. The second objective of this research was to establish a protocol for testing EC50s using DMI fungicides (Demethylation Inhibitor). The third objective was to be undertaken only if resistance was present. This objective was to identify any potential mutations present on resistant isolates based on fungicide treatment levels.

Literature Review

A growing number of research efforts on soybean foliage pathogens, *Cercospora* spp. and *Corynespora cassiicola,* show these fungi are becoming resistant to certain fungicides. These fungicides include Quinone Outside Inhibitors (QoI) and thiophanate methyl (MBC) fungicides. Other fungicides, such as Demethylation Inhibitors (DMIs) are being used in the field, and no tolerance has been reported, however, monitoring is important for delaying resistance.

Arkansas soybean pathogens account for a significant economic impact to farmers each year. On average, a 10% reduction in yield occurs throughout the growing season followed by

some diseases causing varied losses yearly (Faske et al., 2014). Three of these diseases common to the state are Target Spot (*Corynespora cassiicola*), Frogeye Leaf Spot (*Cercospora sojina*), and Cercospora Leaf Blight (*Cercospora kikuchii*) (Faske et al., 2014)

Target spot, like Frogeye Leaf Spot, is a foliar fungal pathogen reported in all United States (U.S.) soybean producing states (Allen et al., 2017). Though Target Spot does not cause as high a yield loss in Arkansas, losses have been reported to be as high as 32%. Target Spot causes lesions that are reddish-brown, non-uniformly shaped and surrounded by a chlorotic halo. Some infected areas on the leaves may resemble large diagnosable sections (Faske et al., 2014). A similar symptomatic fungal pathogen, Frogeye Leaf Spot, is an even more significant problem to soybean growers.

Frogeye Leaf Spot is caused by the pathogen *Cercospora sojina*. Symptoms include round to angular shape and may occur in multiple locations throughout the leaf surface. Some older more established lesions may appear as translucent and have white centers with visible stromata. The more severely infected a plant is the larger and more misshaped the spots may be. *Cercospora sojina* may also appear on the stems and pods of some of the plant, though less likely than the leaves. Environments conducive to *C. sojina* include warm and wet weather with temperatures of 81° to 85° Fahrenheit. In addition to Frogeye Leaf Spot, Cercospora Leaf Blight is important in terms of yield losses.

Cercospora Leaf Blight is caused by *Cercospora kikuchii*. This pathogen survives after seasons by staying in soybean debris. Infection of plants by the pathogen is encouraged when temperatures reach 75° to 80° in conjunction with moist field conditions. Symptoms of this pathogen include a yellow and or a bronze or purple tint. Darker colors that show on leaves are due to cercosporin, a toxin caused by the pathogen. Infection occurs around the pod filling stages

and symptoms show on upper most leaves in canopy (Crop Protection Network FLS, 2021;Cochran & Thiessen, n.d.)). In controlling these pathogens, FRAC 3 is used in addition to FRAC groups 1 and 11. The triazole group of fungicides (FRAC 3) are deemed effective for the strobilurin-resistant strains of *Cercospora sojina* (Faske et al., 2014;; Allen et al, 2017).

As far back as 2012 Louisiana soybean producers reported losses from Frogeye Leaf Spot even with the use of QoI fungicides (Price et al., 2015). The QoI Fungicides work by interrupting the function of mitochondrial respiration through blocking the electron transport at the quinol-oxidizing site of a specific cytochrome. This later affects the germination and viability of fungal spores and hyphal growth (Price et al., 2015; Bartlett et al., 2002). The next class of fungicides to target *Cercospora* spp. are the DMI fungicides (FRAC 2013;Price et al., 2015; Bartlett et al., 2002;). The demethylation group is in the triazoles group of fungicides. The mode of action is aimed at ergosterol, a sterol on the cellular membrane of many plant pathogenic fungi. Resistance to this fungicide ranges from highly sensitive (fungus) to mainly resistant fungicide. The ranges in resistance is associated with qualitative resistance and can vary on the application rates ((FRAC 2013; Price et al., 2015; Bartlett et al., 2002;Cochran & Thiessen, n.d.).

Methodology

Two possible threats to the validity of this research project are 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, are key to understanding the resistance levels and stable population collections of the fungi involved.

Research Design

For this project we used a quantitative experimental design because this research project dealt primarily with numeric levels of potency of two fungicide groups. When dealing with fungal identification we need to specify which of the fungi from the plant samples we collected now were in fact *Cercospora* spp. or *Corynespora* spp. (A. Rojas personal communication, January 2020). To do this we use Polymerase Chain Reaction (PCR) to amplify DNA of our samples and if they have the same number of base pairs as known samples of these fungi. The second concern for validation comes from contamination of the Petri dish plates with which the fungi are grown on and fungicide is applied. In the laboratory, thousands of other fungi spores are present and can land on petri dished being prepped causing unwanted growth. The threats mentioned were factors viewed in a study by Xavier (2013) on baseline sensitivities to *Corynespora* spp. (Xavier et al., 2013).

Population and Sampling

Foliage samples of 10–15 leaves were collected during the fall semester 2019 of *Cercospora kickuchii* and *C. cassiicola.* . Once the leaves were collected various methods of fungal isolation were conducted. First, moist chambers were set up using moist towel paper place at the bottom of a container and leaves were placed on top in sealed container and incubated at room temperature for 48 hours, tracking spore development. Spores were picked using a needle and transferred to PDA with antibiotics. Isolates from purple seed stain were recovered directly from seed after sterilizing the seeds in 70% ethanol and dry them in a sterile laminar flow hood. Seeds were plated on PDA media plus antibiotics danitol, ampicillin, streptomycin, and rifampicin.

Rigor

Fungal Identification

Fungal tissue was collected from the foliage samples previously collected and stored. Fungal tissue was then grown on PDA or potato dextrose agar to obtain enough fungal growth to identify. PCR was then conducted with the primersEF1-986R and EF1-728F that are used to match with *Cercospora* and *Corynespora* species. PCR will ensure that the fungal identification matches *Cercospora* and *Corynespora* as identified.

Petri Dish Contamination

Rigor was also assured by the steps taken to eliminate any risk of contamination to the petri dishes prior to inoculation. Ethanol (70%) was used to wipe down all biological fume hoods used. All media were properly autoclaved to manufacturer's instructions prior to plating. Fungicides were prepared in the fume hoods and stored in the laboratory refrigerator to ensure no photodegradation occurred. Additionally, all petri plates were stored in closed containers to prevent excess spores from entering.

Data Collection

Plant tissue samples were collected from four Arkansas locations, Kibler, Marianna, Newport, and Rohwer. Sample collection was limited to the time of the planting season in which the fungi heavily infect and reproduce on the soybean plants. Therefore, sampling was conducted during the fall of 2019. The sampling consisted of four major steps.

The initial step was to collect foliage/leaf samples from the locations and place them in sealed Ziploc bags for each separate location and for each pathogen. After this, in a secure

biological fume hood take, leaves were taken from Ziploc bags and specimens with abundant fungal growth were used. The second step was to take a metal needle and lightly scrape the surface of the infected leaf area and poke into potato dextrose agar (PDA). 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. Next the scraper was rubbed on the PDA. If he same metal scraper was used for multiple extractions then close attention was made to disinfect it of unwanted fungi by placing the needle in an open flame followed by rinsing with ethanol (70%). The third step was to confirm the DNA of the fungal growth with PCR.

Our focus on the instrumentation used for the fungal ID revolved around polymerase chain reaction (PCR). For this, a thermocycler is used to break down the DNA before it goes into a gel. This gel is placed in a chamber in which voltmeters run currents throughout the solution so that the DNA can run from the positive volts to the negative volts. Because DNA is attracted to the negative voltage that will allow us to see which samples, when matched with known ones, have matched base pairs. The fourth step was to set up the fungicide trials.

We had three replications each for the treatment levels of 0, 0.01. 0.1, 1, 10, and 50 mg/L (parts per million). Each of these levels were used for the DMI fungicide for each of the 3 isolates, *C. kikuchii, C. sojina,* and *C. cassiicola*. Each fungal sample was placed on the media and allowed to grow over a period of 1 week, in which at the end of the time frame the growth was 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).

Discussion and Conclusion

A total of 15 isolates were used in this study. Three isolates were *C. flagellaris* (associated with positive presence of *kikuchii*), 6 with *C. cassiicola*, and 6 with *C. sojina***.** For propiconazole, all isolates were completely inhibited at 50 mg/L. The EC_{50} ranged from 1 to 10 mg/L for all isolates with the highest number of isolates falling sensitive to the fungicide (See fig. 1).

Baseline Sensitivity Amongst Isolates

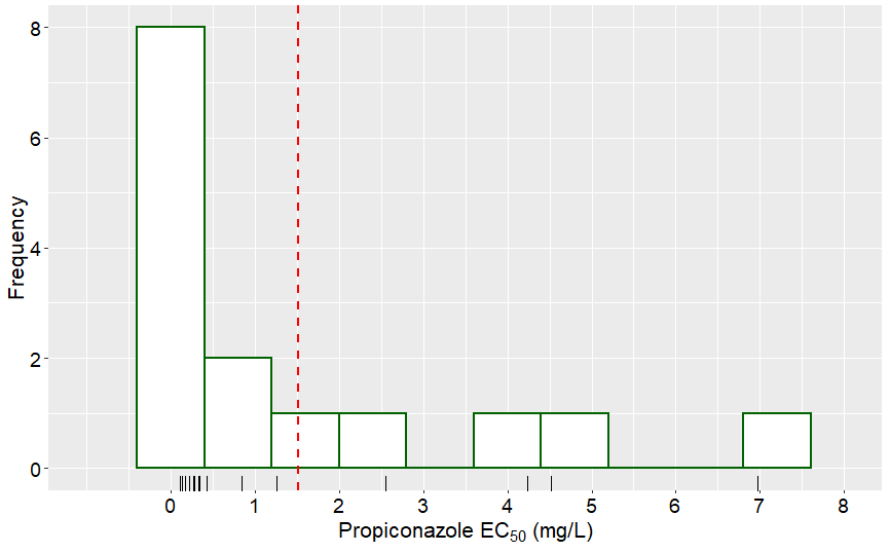

Figure 1. Effective Concentration of propiconazole that inhibits at least 50% of the fungal growth for 15 isolates of *Cercospora* spp. and *Corynespora cassiicola* in Arkansas Soybeans.

Corynespora cassiicola

Effective Concentration values for 50 % fungal inhibition were inconsistent across all three isolates. However, highest EC_{50} values were associated with *Corynespora cassiicola*. In addition to higher EC_{50} values there were some abnormal distributions of isolates regarding growth overtime. This abnormality was seen as plates for higher ppm having larger percent growth than plates with lower ppm. For all 6 isolates of the *C. cassiicola*, isolate 1601 was the only to show this abnormal growth in between 0 and 1 mg/L (ppm).

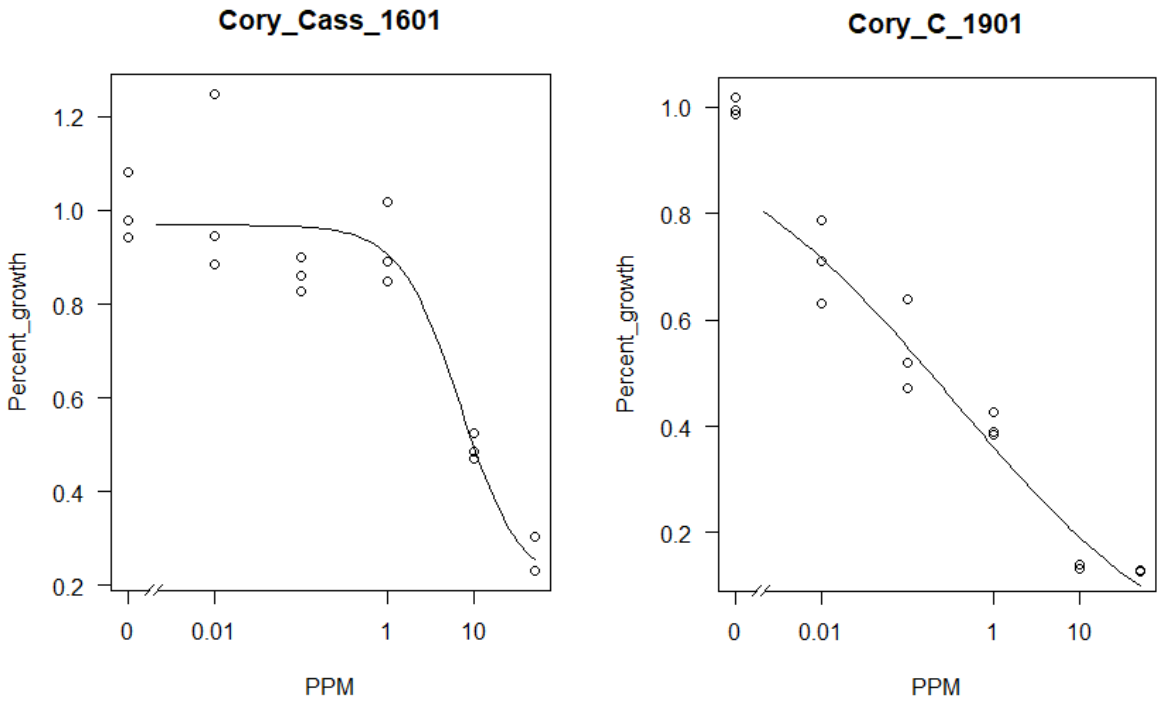

Figure 2. Distribution of EC_{50} values for *C. cassiicola* isolates 1601 and 1901. With percent growth on y-axis, the EC_{50} value for isolate fell in the range of 10.00 mg/L (ppm) while the EC_{50} value for 1901 fell in the range of 1.00 mg/L (ppm). Range in EC_{50} between the two isolates show a difference in levels of sensitivity to the propiconazole between the two isolates. Remaining isolate EC_{50} values for this fungus are included at the end of the is document and was left out of the above section to ensure clarity.

Cercospora sojina

The EC_{50} values for the Frogeye Leaf Spot were low compared to that of *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 to *C. cassiicola.* Isolates for this pathogen represented even distribution for all treatments (see figure 3).

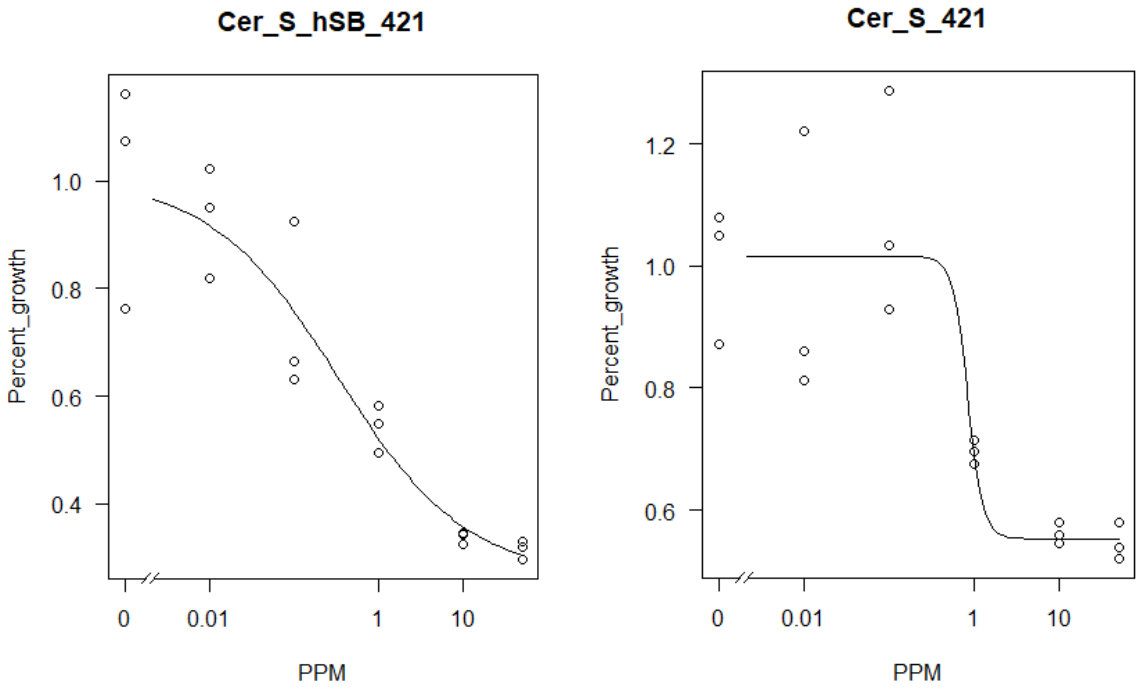

Figure 3. Distribution of EC_{50} values for *C. sojina* isolates hSB-421 and S-421.

In addition, there are differences in sensitivity values between isolates of the same fungal species. This difference in sensitivity values may be attributed to the varying application rates in locations of isolates or local adaptation under different fungicide exposures.

Cercospora kikuchii

Effective Concentration values for 50 % fungal inhibition was the lowest for the Cercospora Leaf Blight isolates. No inhibition was reported at greater than 1 mg/L (ppm) and all isolate growth were distributed across the ranges tested without abnormality (hermetic 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., 2014). Cercospora Leaf Blight is associated with Purple Seed Stain and *C. kikuchii* has been linked in lineage to several other *Cercospora* diseases

in soybean growing regions (Albu et al., 2014). 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 compare to the differences seen across all three fungal species (see figure 4).

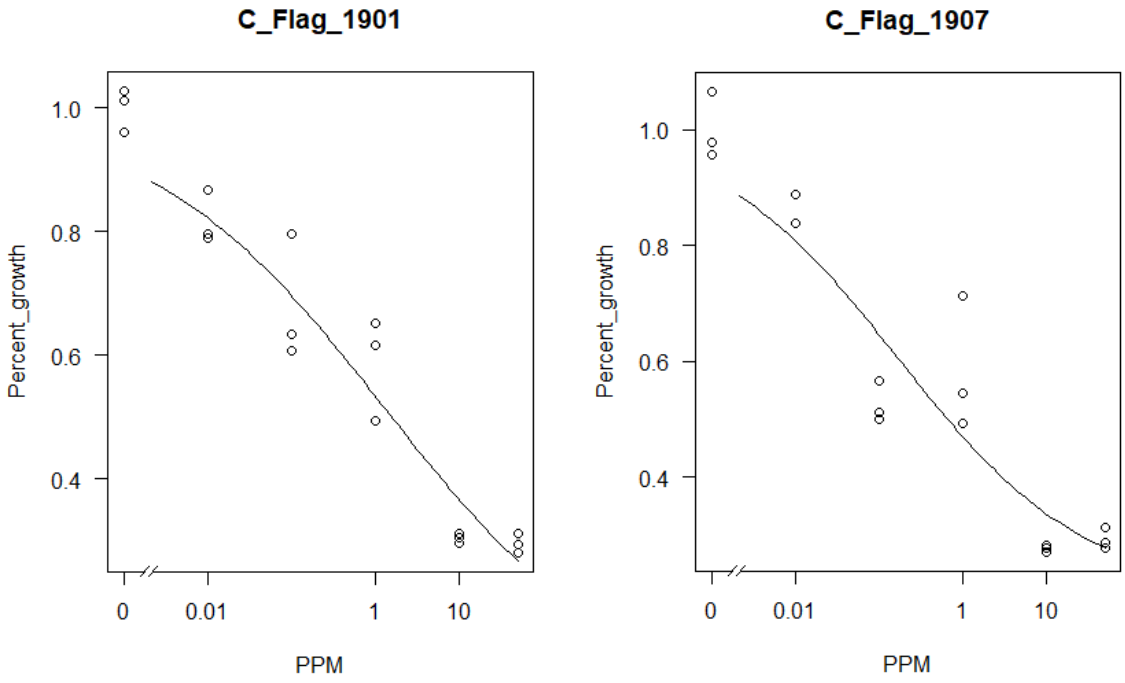

Figure 4. Distribution of EC_{50} values for *Cercosopora flagellaris*

Of the three fungal species tested, no cross resistance was reported as multiple FRAC 3 fungicides were not tested. 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* showed highest 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 earlierin *Corynespora spp*. In comparing the two *Cercospora* isolates, *C. sojina* had higher sensitivities and is at a higher risk of developing resistance than *C. kikuchii.* The DNA isolations and sequencing of the target gene to determine r potential mutations of the isolates is an ongoing procedure that is the future work of this research project. Determining the resistance mutations on the isolates may make easier the recommendations to growers. In addition, effective integration of FRAC chemistries for fungal control is recommend against resistance build up. An integrated pest management regime in conjunction with the integrated FRAC chemistries is the current recommendation of this research project to aid in decreased resistance.

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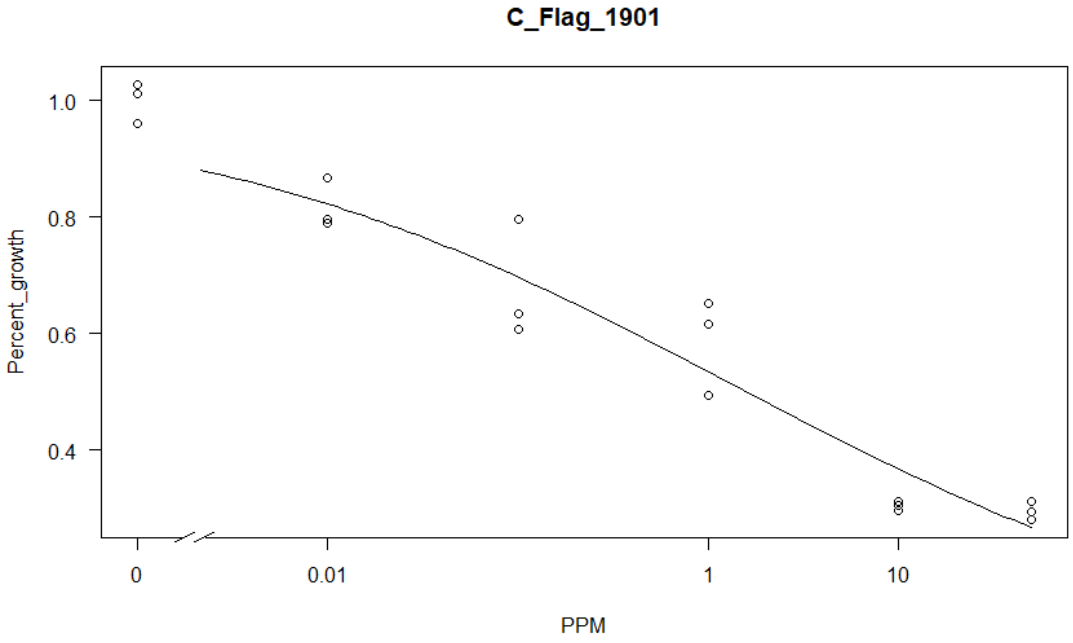
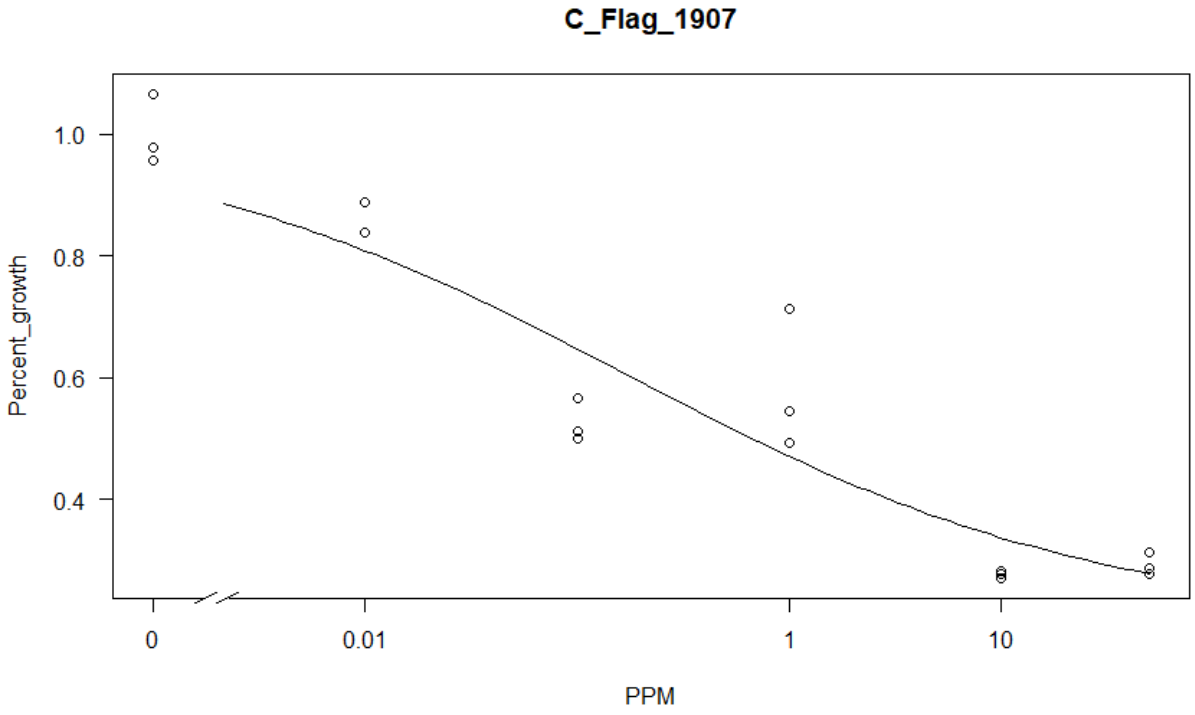
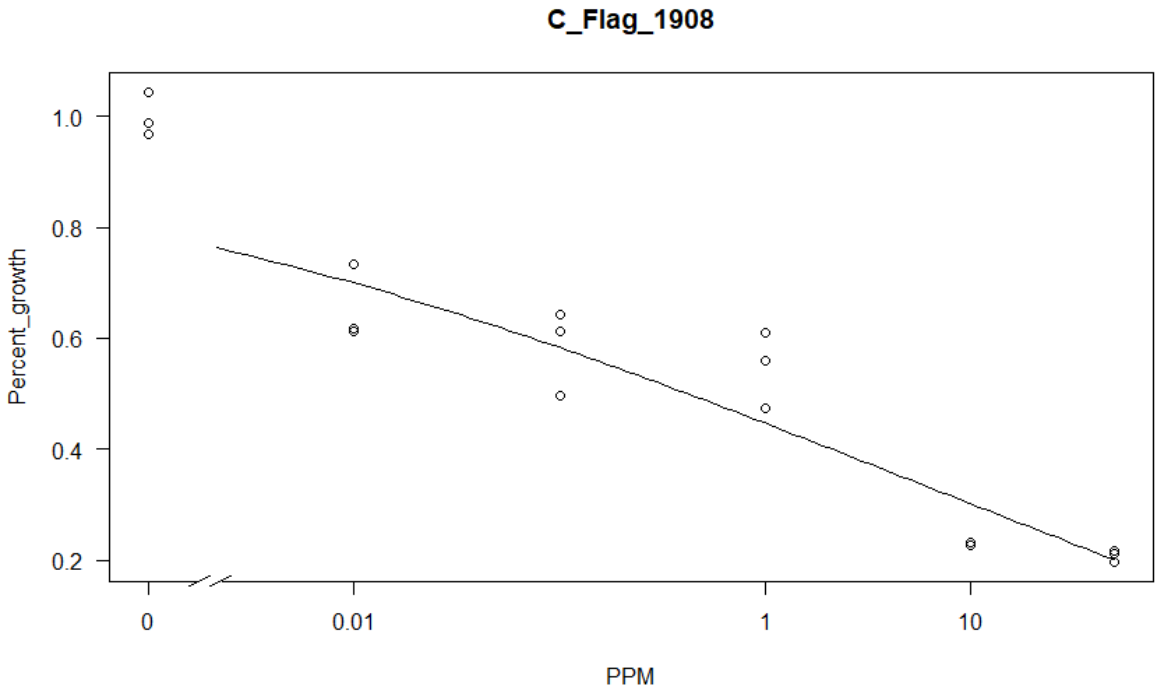
EC_{50} values for total 15 isolates of *C. flagellaris*, *C. sojina*, and *C. cassiicola*



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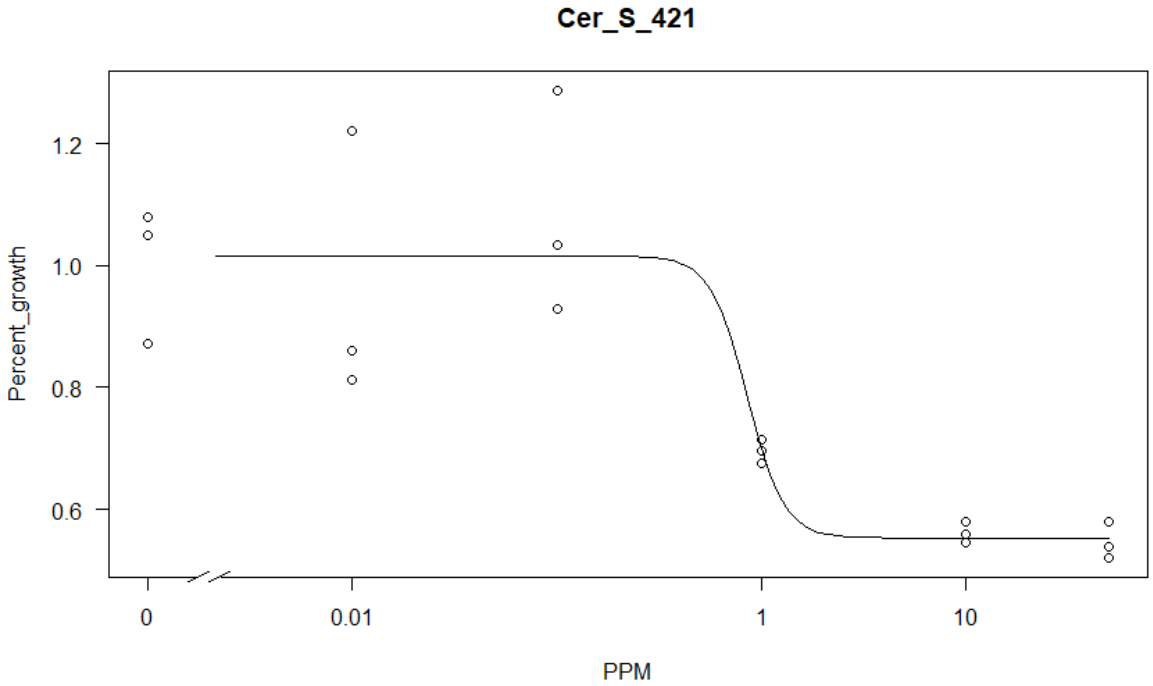
1
10
PPM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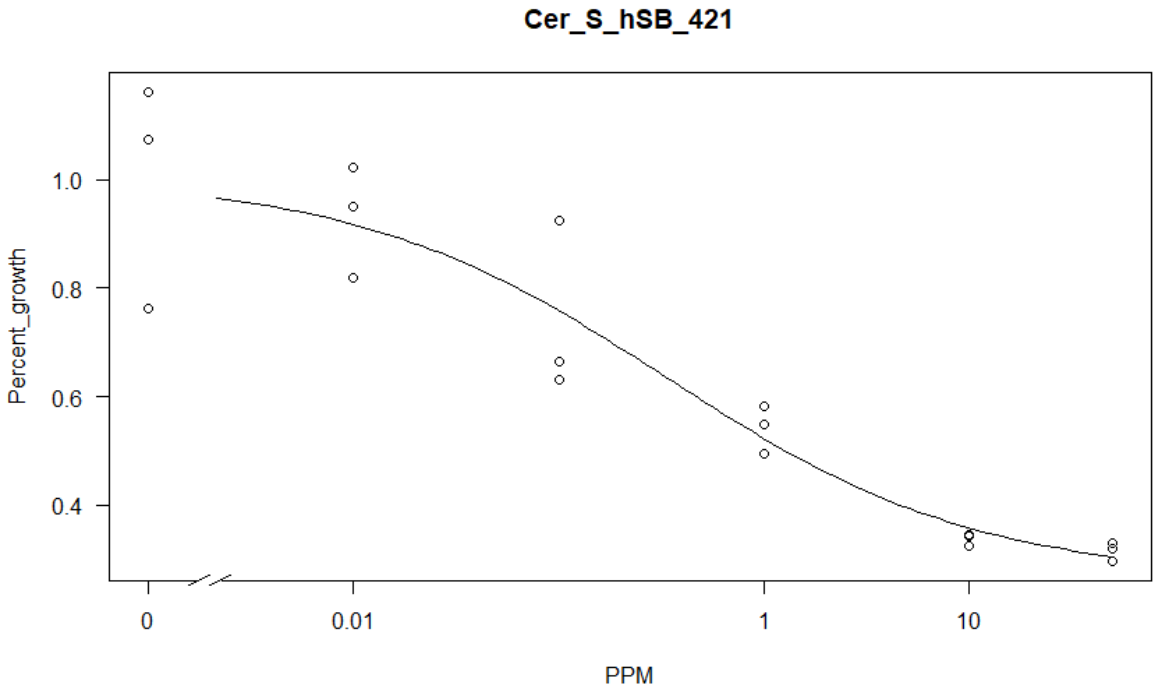
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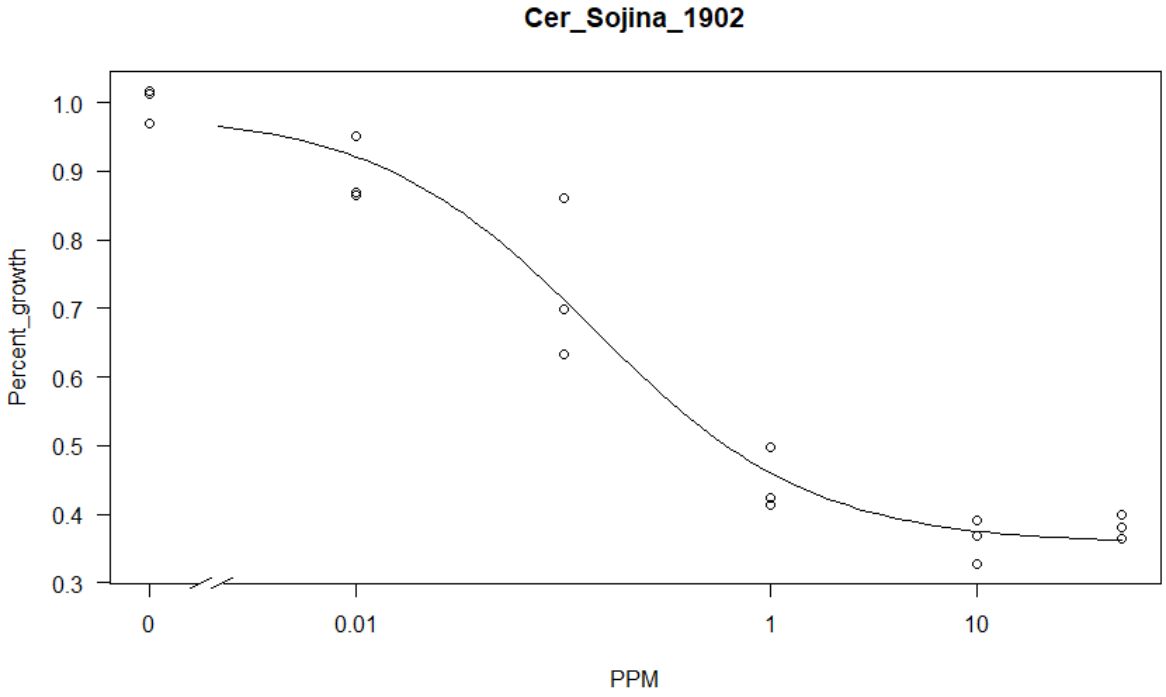
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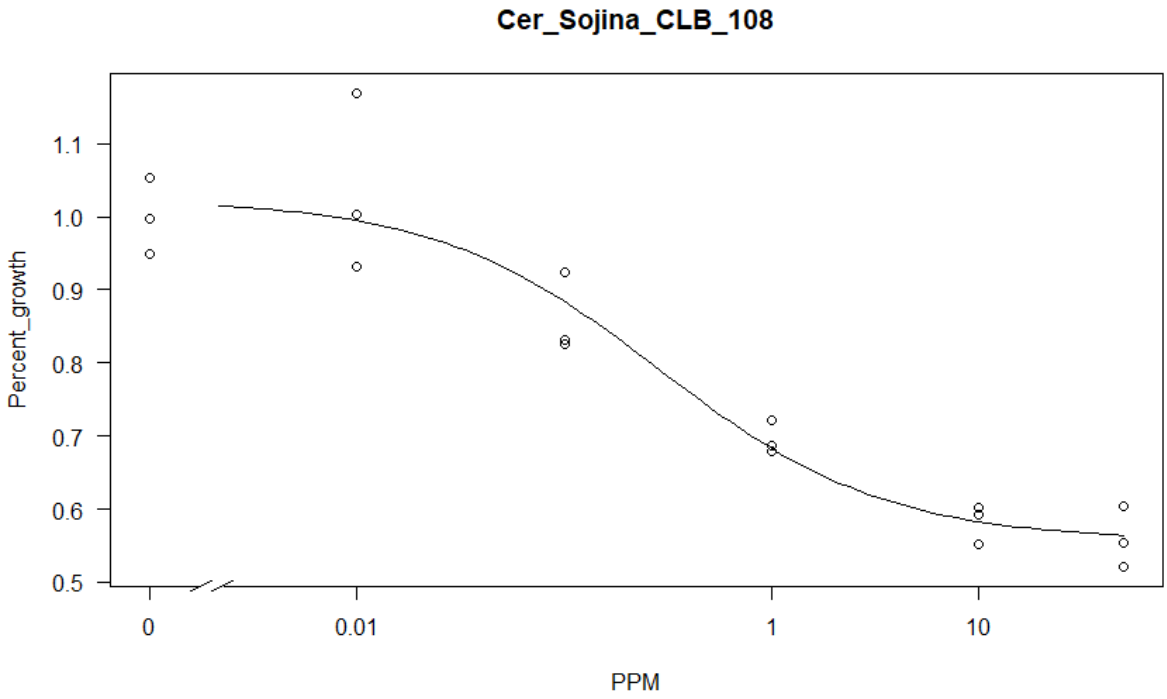
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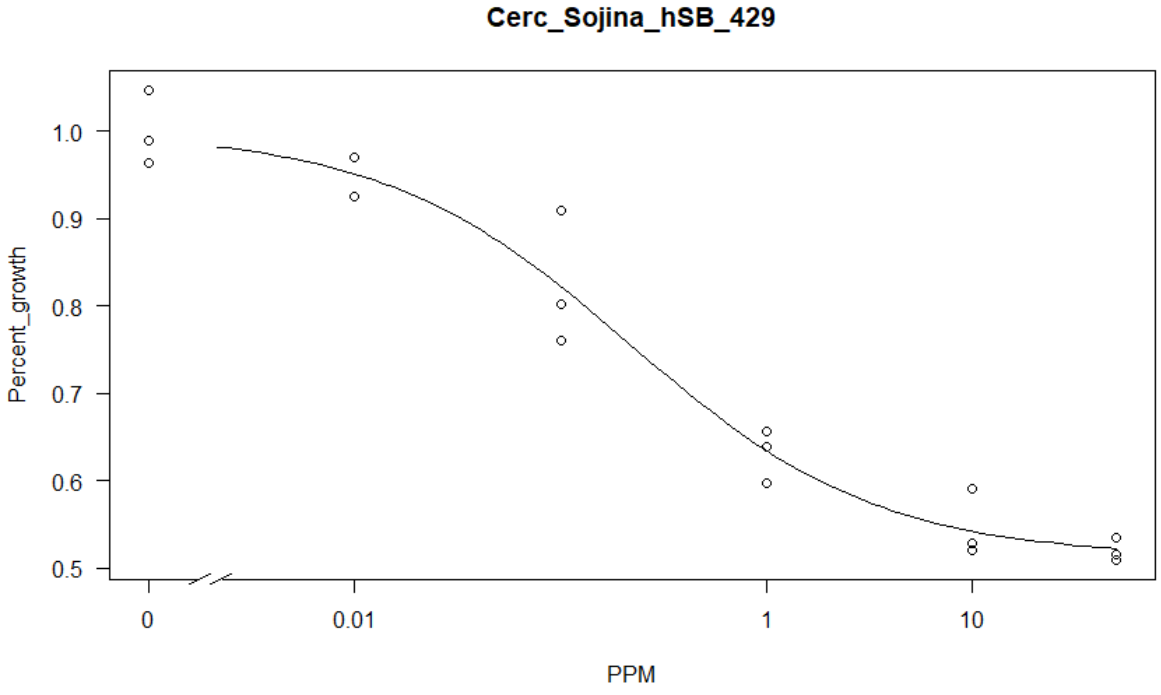
PPM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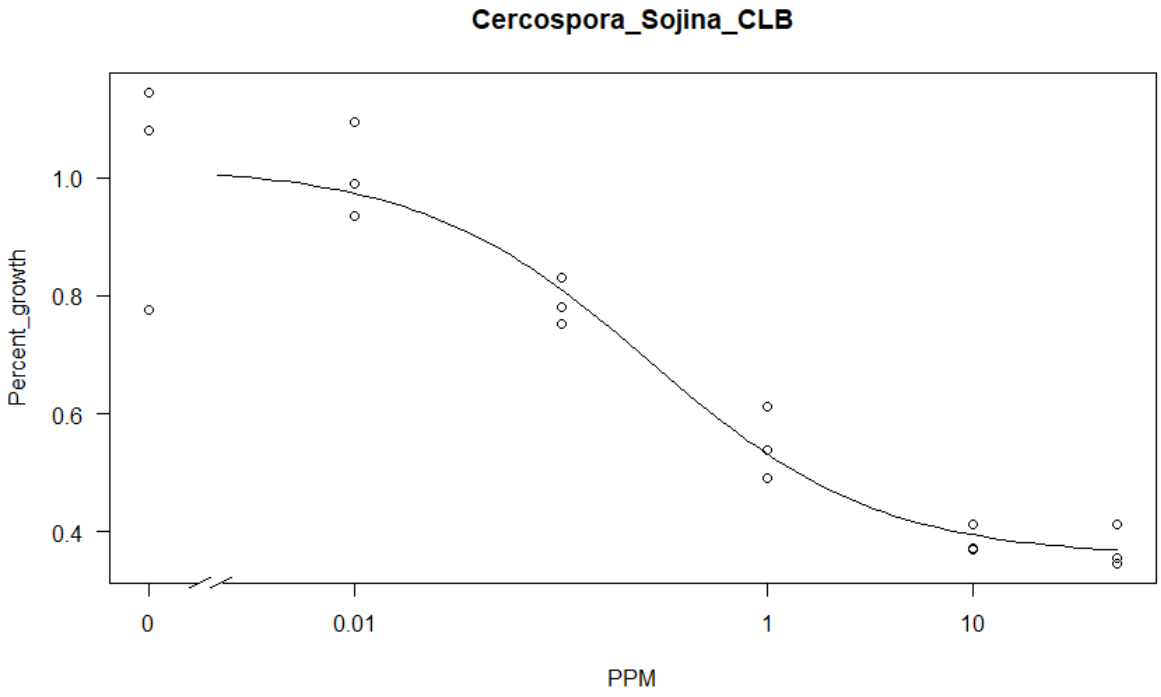
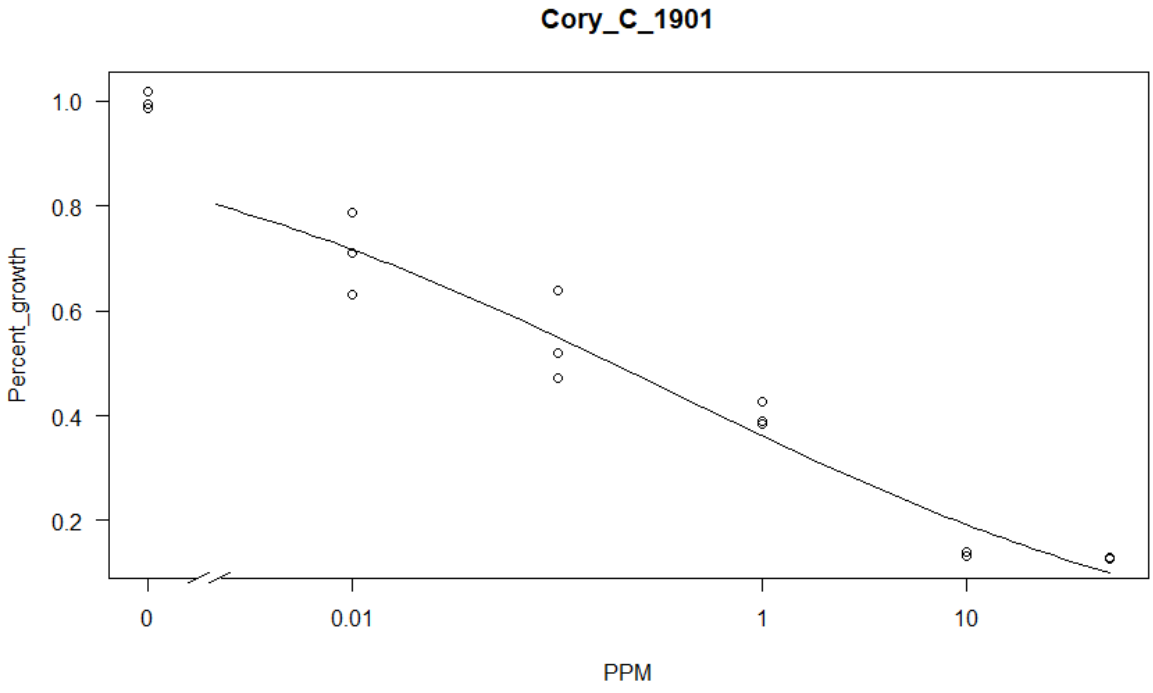
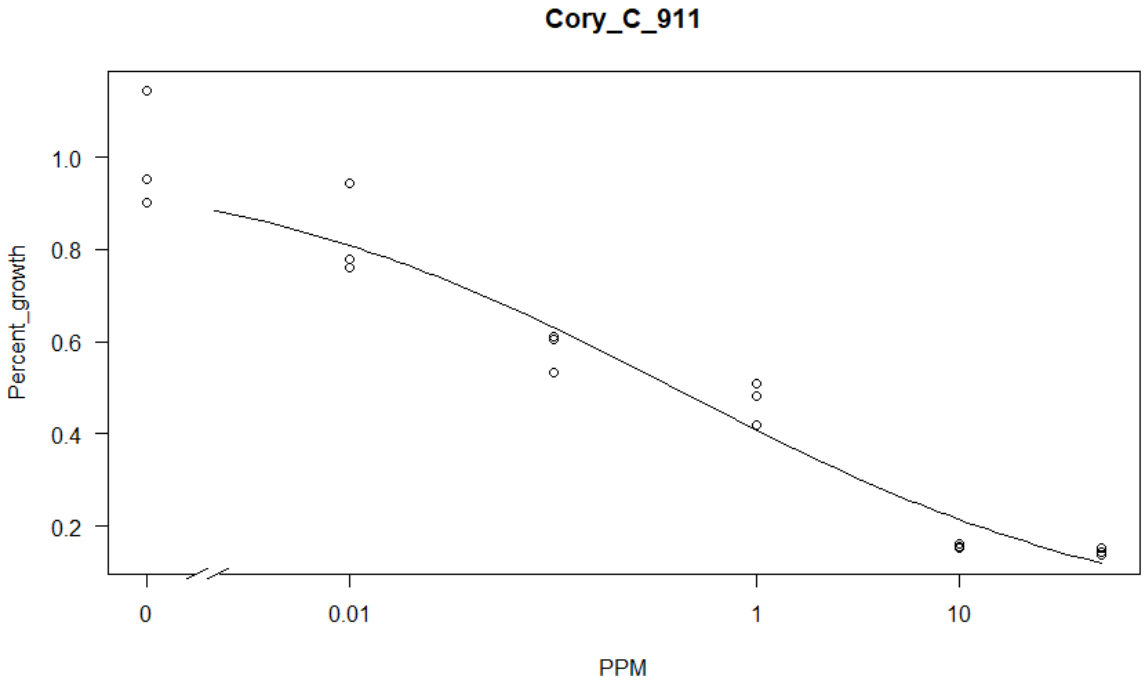
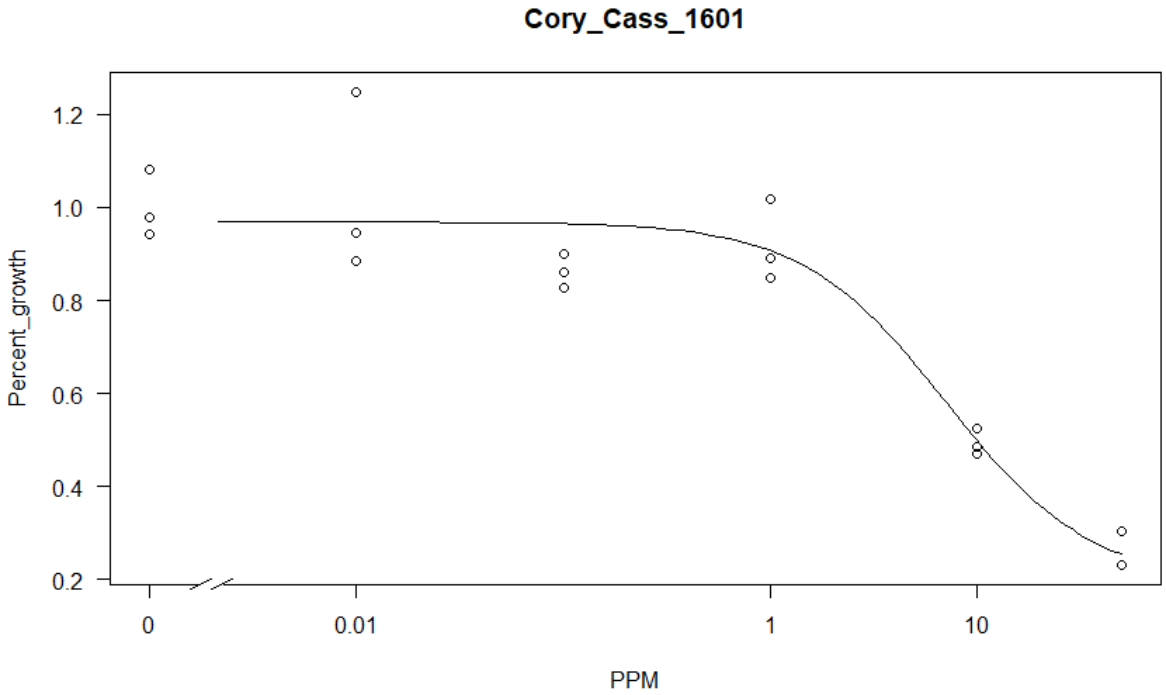
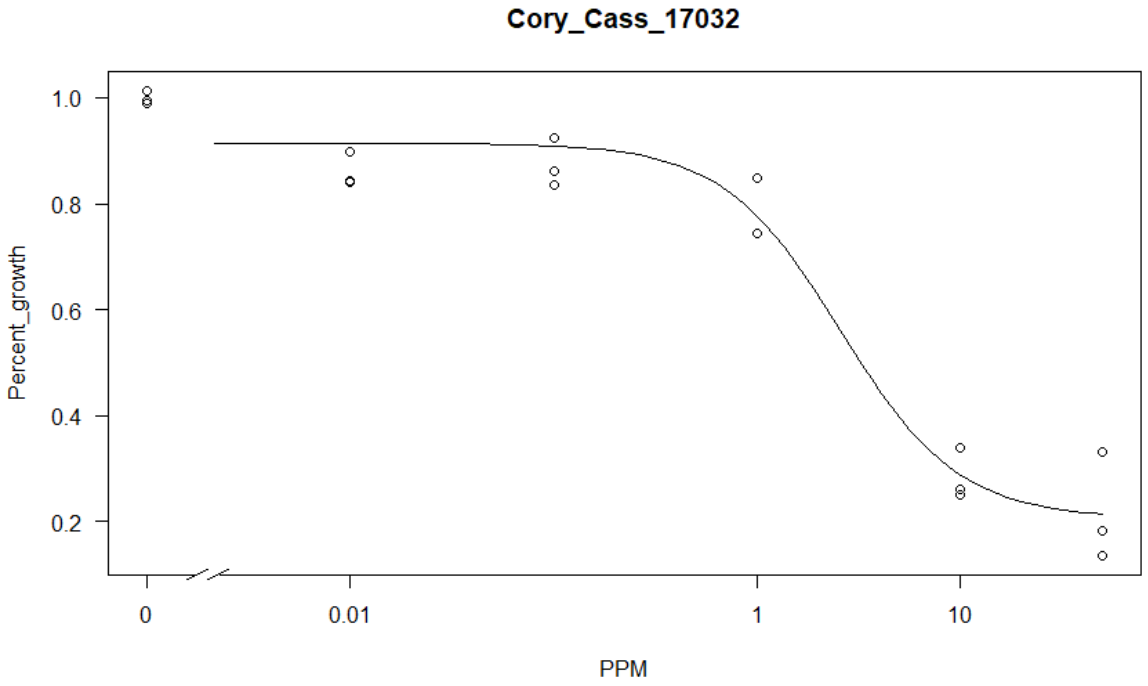
10
PPM

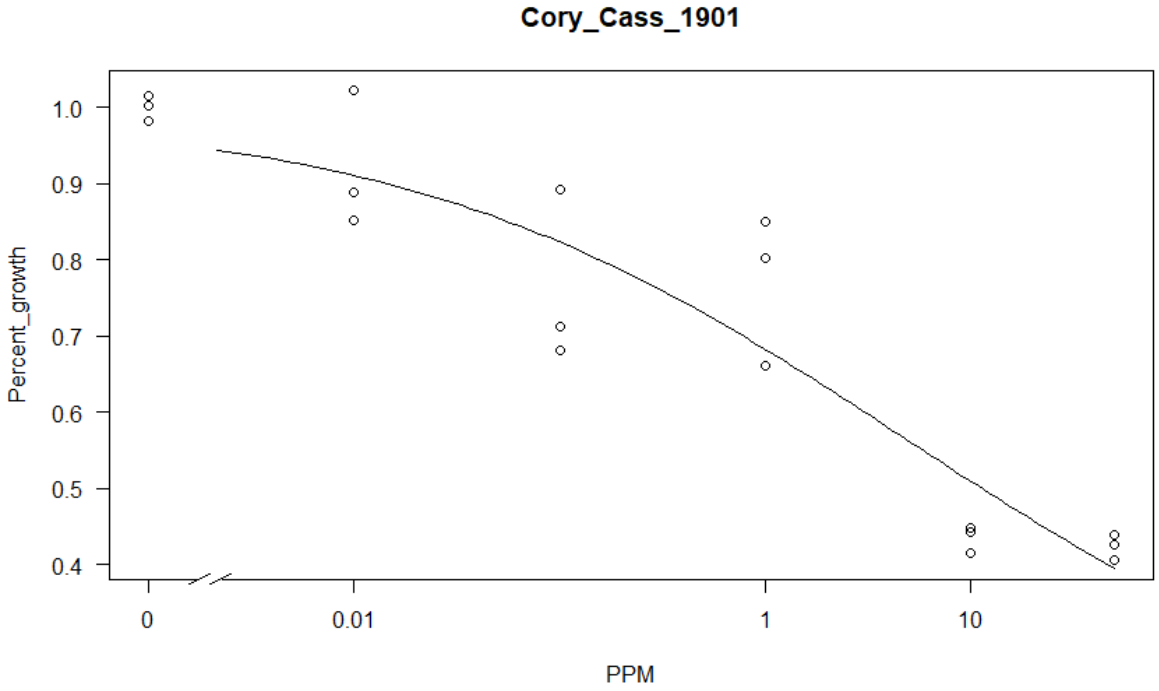

Cercospora_Sojina_CLB
Percent_growth
1.0
0.8
0.6
0.4
0
0.01
1
10
PPM

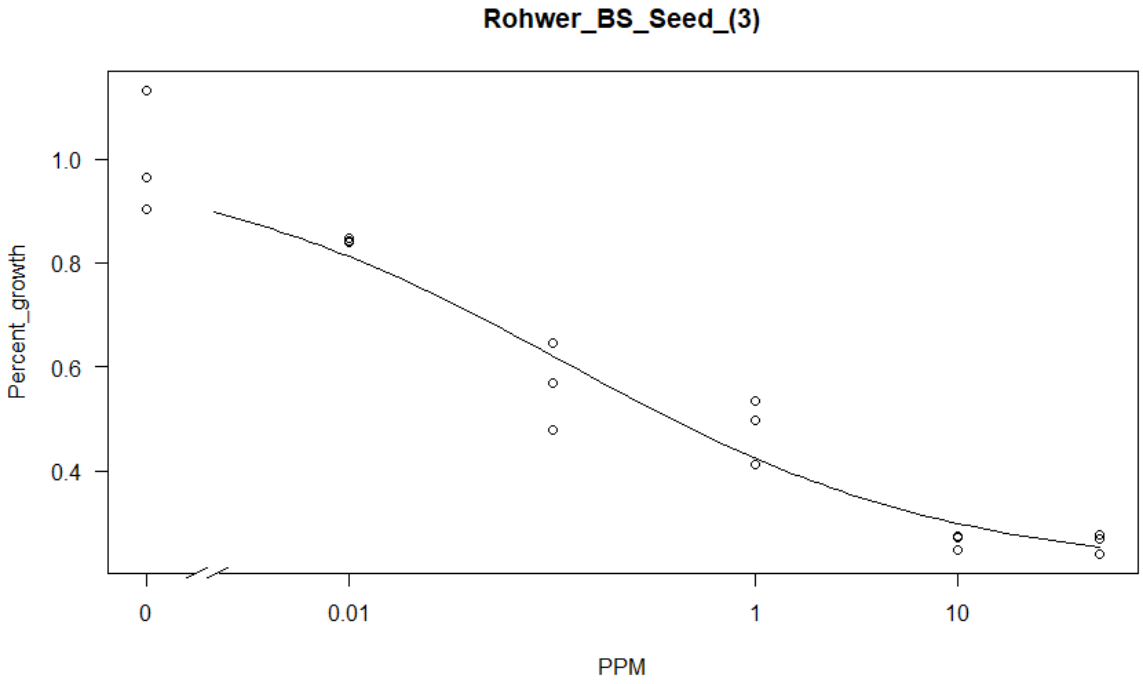

Cory_C_1901
Percent_growth
1.0
0.8
0.6
0.4
0.2
0
0.01
1
10
PPM

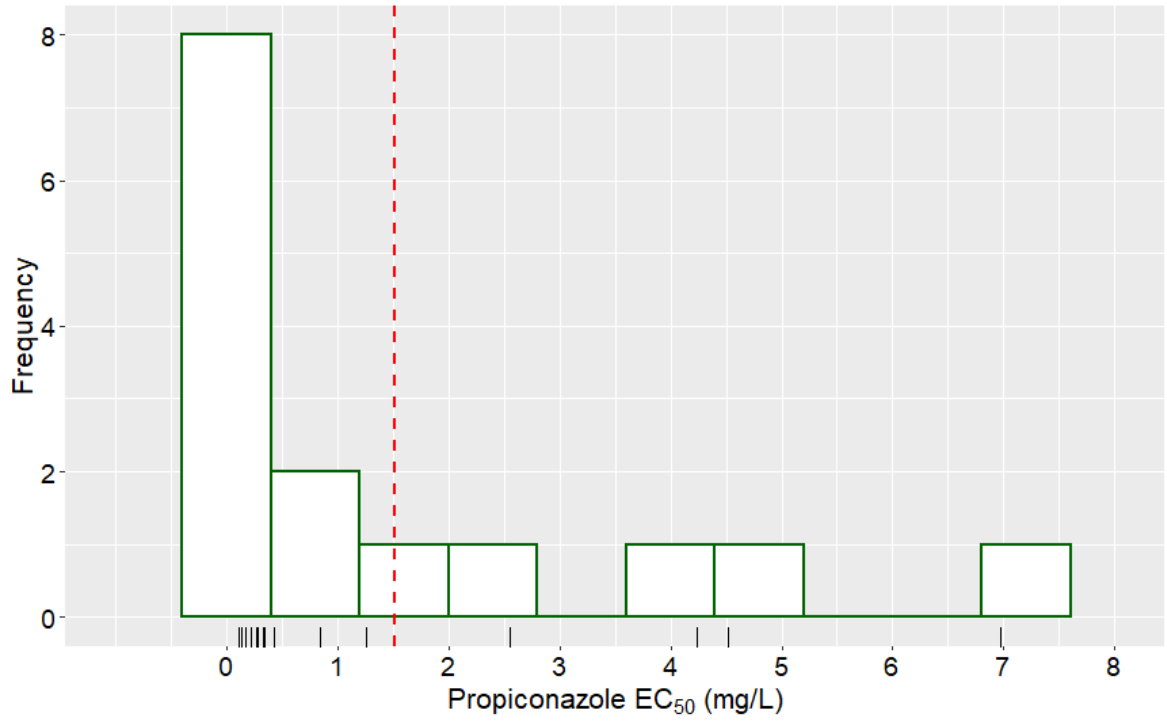

Cory_C_911
Percent_growth
1.0
0.8
0.6
0.4
0.2
0
0.01
1
10
PPM


Cory_Cass_1601
Percent_growth
1.2
1.0
0.8
0.6
0.4
0.2
0
0.01
1
10
PPM


Cory_Cass_17032
Percent_growth
1.0
0.8
0.6
0.4
0.2
0
0.01
1
10
PPM


Cory_Cass_1901
Percent_growth
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0
0.01
1
10
PPM


Rohwer_BS_Seed_(3)
Percent_growth
1.0
0.8
0.6
0.4
0
0.01
1
10
PPM


Frequency
8
6
4
2
0
0
1
2
3
4
5
6
7
8
Propiconazole EC50 (mg/L)