In vitro Test to Evaluate the Efficacy of Flutriafol (Rhyme) for Selected Fungal pathogens Using Spiral Plate Gradient Dilutions

Molly Arreguin, Karina Elfar, Akif Eskalen
Dept of Plant Pathology, UC Davis, CA 95616

Fungal Isolates, Culturing, and Inoculum preparation

Fungal isolates used in this study were obtained from the isolate collections of the Eskalen and Trouillas Labs at the Department of Pathology, University of California, Davis. Isolates were maintained on Potato dextrose agar amended with 0.01% lactic acid (APDA) and kept at 22-25C until use. To produce the mycelium inoculum, 5mm mycelium plugs were taken from 7-day-old cultures of *Botryosphaeria dothidea, Diplodia seriata, Diaporthe ampelina, Neofusicoccum parvum, and Neofusicoccum mediterraneum* and evenly distributed on a 100mmx15mm APDA petri dish containing sterilized cellophane strips. All cellophane strips were cut to 50mmx4mm before sterilization. Plates were incubated for 4-5 days at 25ºC. For conidial production, *Calosphaeria pulchella* and *Ceratocystis destructans* were grown at 25ºC for 14 days on the same media. Conidia were harvested by flooding the plates with 5 mL of sterilized water and gently scraping the surface with a sterile scalpel. The liquid was then passed through a sterilized cheesecloth to separate the conidia from the mycelium. Conidia of *Cytospora sorbicola* were harvested from the pycnidia of one-month-old cultures using a sterilized needle and then dispensed into 1mL of sterile water. All conidia suspensions were brought to a final concentration of 1 x 10^6.

Preparation of Petri Dishes for Spiral Plating

The spiral plate assays were conducted on 150mmx15mm Petri dishes containing 50mL of PDA at the height of 5mm. Any plates that had an uneven surface, bubbles, or precipitation were discarded. All plates were prepared 24 hours in advance.

An Eddyjet-2 ® Spiral Plater was used to dispense the fungicide. Flutriafol (Rhyme) was diluted to the desired concentrations using sterilized distilled water. Two concentrations were used in this test (5000ppm and 1000ppm) due to the pathogens differing sensitivities to fungicides. Approximately 54.3µL of the fungicide was dispensed onto the plates in a concentration gradient starting at 12mm from the center and moving towards the edge. A 24mm cork-borer was used to remove the centerpiece of agar from the plates due to diffusion being highly influenced by a zero-concentration area (Forster et al. 2004).

Application of Fungicide and Fungal Inoculum

Conidial suspensions were applied by pipetting a 10µL aliquot of spores to the far and center edge of the agar. The tip of a sterile glass rod was then used to spread the suspension radially across the fungicide gradient. For the mycelium assays, the cellophane strips were placed radially mycelium-side down. Adjacent from each isolate placed was a duplicate of itself. Controls consisted of PDA plates without fungicide, to which the mycelium strips and conidia suspensions were applied as described above. The plates were left at 25ºC for 2-4 days before measurements were taken. The experiment was conducted with ten replicates and repeated at least once.

Calculation of EC50 Values
An R-package developed by Gabriel Andres Torres et al. (2016) was used to determine the EC50 for each pathogen. The following formula was used to calculate the values.

Ecal(rad1=a, rad2=b, EC=c, mx=x, pp=y, AH=z

In this equation, a is the total inhibitory concentration (TIC), b is the maximum inhibitory concentration (MIC), c is the observed effective concentration, x is the molecular weight of the test compound, y is the parts per million concentration of the test compound, and z is the height of the agar (Fig 1).

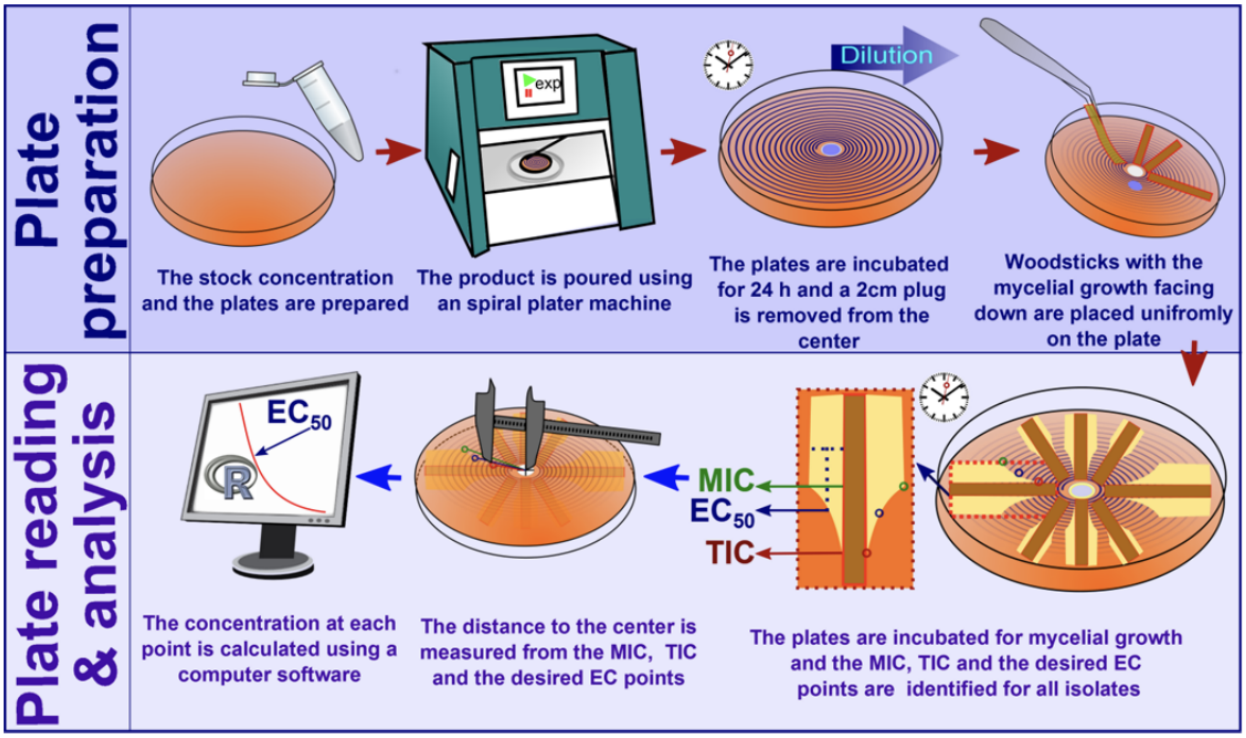


Fig 1. Schematic procedure of calculating chemical concentration using spiral plate technique (Torres et al 2016)

Analysis of EC50 values at Different Flutriafol (Rhyme) Concentrations
A student t-test was run to evaluate whether there was a significant difference between the EC50 values derived from 1000ppm and 5000ppm Flutriafol (Rhyme) tests.

Results
Neofusicoccum parvum showed similar EC50 values for both 1000ppm and 5000ppm for Rhyme, while other isolates showed a significant difference between the two tests ($P<0.05$) (Table 1). Due to the difference in susceptibility to Flutriafol (Rhyme), the final EC50 was

chosen based on whether the isolate showed growth at the centermost edge of the plate. All isolates except for *C. destructans* displayed growth to the center-most edge, indicating that they are less susceptible to Flutriafol (Rhyme). Because of this, EC50 values obtained from 5000ppm tests were used for all isolates except for *C. destructans* (Table 2, Fig 2).

Table 1. T-test Analyses on Comparing Spiral Plating Tests Flutriafol (Rhyme)

	Paired t-test	
	P	**95% confidence interval for the mean difference**
C. pulchella	<0.05	-0.504 to -0.0261
C. destructans	<0.05	0.0153 to 0.225
C. sorbicola	<0.001	-3.199 to -1.546
N. mediterraneum	<0.001	0.694 to 1.871
N. parvum	0.372	-0.162 to 0.412
B. dothidea	<0.001	0.968 to 2.223
D. seriata	<0.05	0.163 to 2.706
D. ampelina	<0.01	-2.379 to -0.576

Table2 . EC50 Values Determined with Flutriafol (Rhyme)

Fungi	**Active Ingredient (Flutriafol) EC50 (µg/mL)**	**Product (Rhyme) EC50 (µg/mL)**	**Standard Deviation**	**Standard Error of The Mean**
C. pulchella	1.147176	5.05363877	0.270003	0.085382
N. parvum	1.30762	5.76044053	0.325342	0.102882
C. sorbicola	4.054113	17.8595286	1.159298	0.366602
N. mediterraneum	0.502056	2.21170044	0.303918	0.096107
D. seriata	0.783205	3.45024229	0.301175	0.09524
B. dothidea	1.120017	4.93399559	0.513314	0.162324
D. ampelina	4.513923	19.8851233	1.053412	0.333118

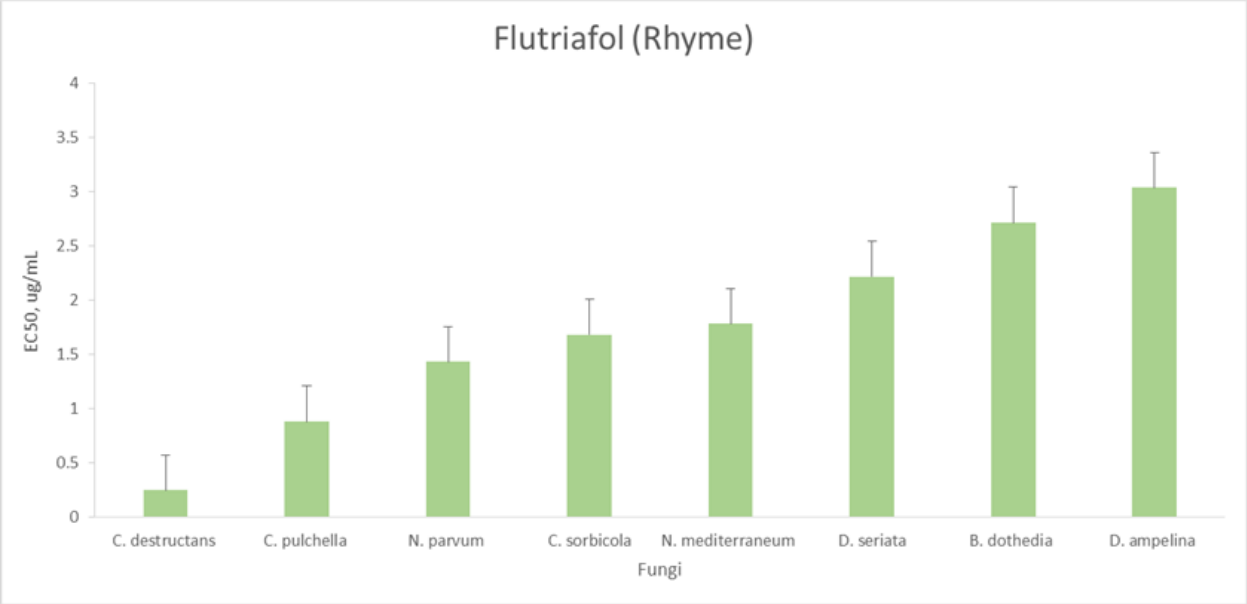
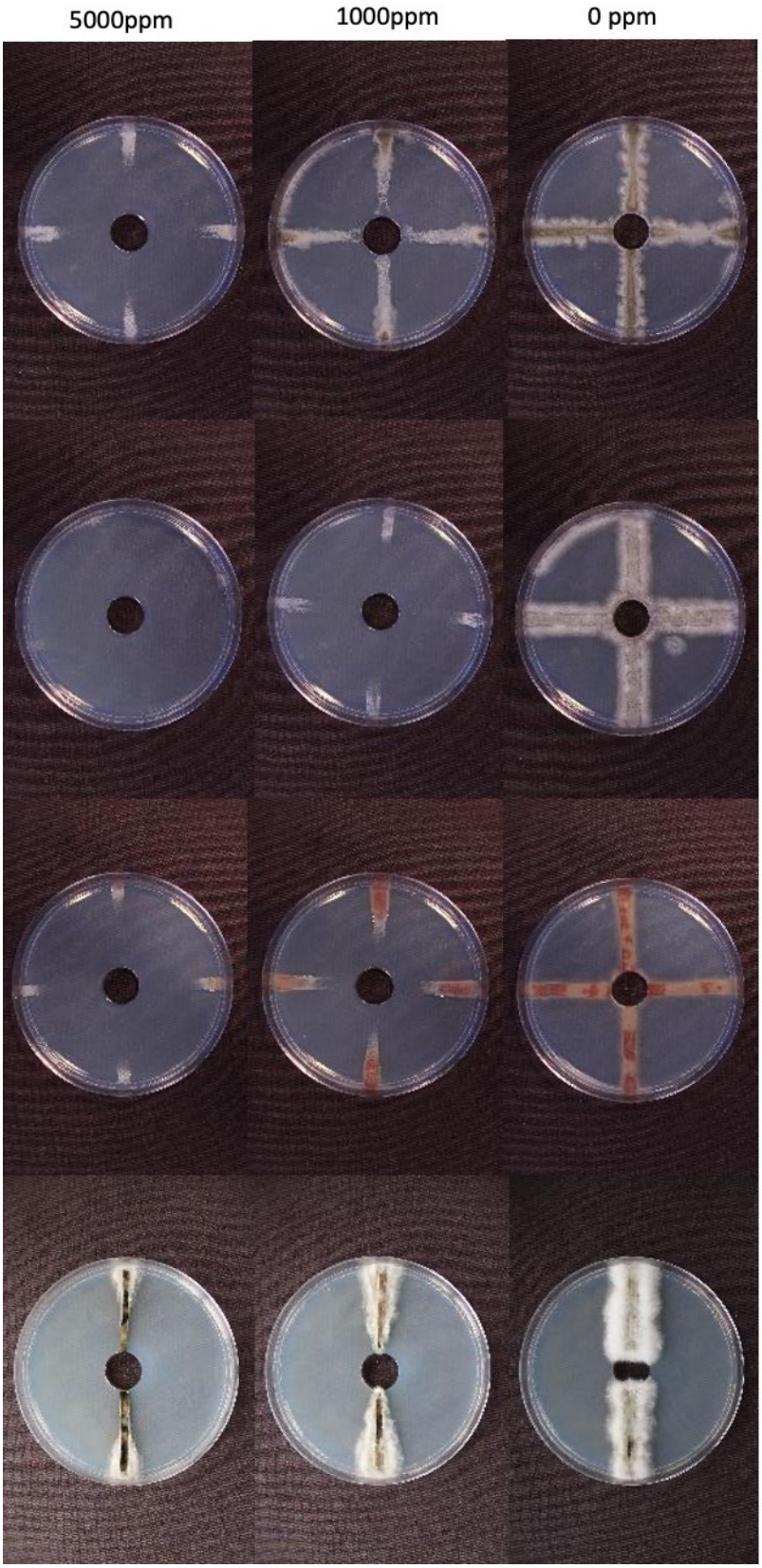


Fig 2. Most Representative EC_{50} Values from Flutriafol (Rhyme)

Literature cited

Förster, H., Kanetis, L., and Adaskaveg, J. E. 2004. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. Phytopathology 94:163-170.

Torres-Londoño, G.A., Hausbeck, M. and Hao, J. 2016. ECX: An R Package for Studying Sensitivity of Antimicrobial Substances Using Spiral Plating Technology. Plant Health Progress. 17. 188-194


5000ppm
1000ppm
0 ppm
Cytospora sorbicola
Ceratocystis destructans
Calosphaeria pulchella
Neofusicoccum Parvum


5000ppm
1000ppm
0 ppm
Neofusicoccum mediterraneum
Botryosphaeria dothidea
Diplodia seriata
Diaporthe ampelina