Final Report Southern Region Small Fruit Consortium 2016

Title: Developing strategies to use polyoxin D for gray mold control of strawberry

Guido Schnabel and Madeline Dowling Department of Plant and Environmental Sciences, Clemson University, Clemson, SC 29634;

This work was published recently: Dowling, M.E., Hu, M.J., Schmitz, L.T., Wilson, J.R., and G. Schnabel 2016. Characterization of *Botrytis cinerea* isolates from strawberry with reduced sensitivity to polyoxin D Zinc Salt. **Plant Dis.** 100:2057-2061.

Abstract

Polyoxin D is a FRAC 19 fungicide that was registered in 2008 for gray mold control of strawberry. In this study we determined the sensitivity to polyoxin D of *B. cinerea* isolates from 41 commercial strawberry farms in South Carolina, North Carolina, Maryland, Virginia, and Ohio and investigated fitness of reduced sensitive isolates. Relative mycelial growth on malt extract agar amended with a discriminatory dose of 5 µg/mL polyoxin D revealed wide range of sensitivity from 0 to 100% growth. Isolates that grew more than 70% at that dose were designated reduced sensitive (RS) and were found in three of the five states. The EC_{50} of three sensitive (S) and three RS isolates ranged from 0.59 to 2.27 and 4.6 to 5.8 µg/µg/ml, respectively. The three RS isolates grew faster on detached tomatoes treated with Ph-D WDG at recommended label dosage than S isolates ($p < 0.008$). RS isolates exhibited fitness penalties

with regard to sporulation ability ($p < 0.0001$) and growth rate ($p < 0.0001$), but a fitness advantage in sclerotia production ($p < 0.0386$). The results indicate that polyoxin D applications must be part of a resistance management approach. To our knowledge this is the first study demonstrating reduced sensitivity to FRAC 19 fungicides in *B. cinerea* isolates from the United States.

Goal and Objectives

As part of a project to provide location-specific resistance management information to eastern US strawberry growers, we determined the sensitivity distribution to polyoxin D for *B. cinerea* isolates. None of the participating growers had begun using this chemistry in their fields in 2015. The goals for this study were to (1) determine mycelial growth inhibition of isolates from the east coast at a discriminatory dose of 5 µg/ml, (2) examine the sensitivity of isolates of both sides of the sensitivity spectrum to the recommended label rate of Ph-D WDG, and (3) determine fitness and pathogenicity of isolates with reduced sensitivity. This study is the first to document reduced sensitivity to polyoxin D in *B. cinerea* isolates collected from strawberry fields in the United States.

Results and Discussion

We investigated sensitivity to polyoxin D in 450 *B. cinerea* isolates from 5 states of the eastern United States and found a wide range of sensitivity. Though most of the isolates collected were sensitive, 18.4% were MS and 6.4% were RS to polyoxin D, despite the absence of pressure from FRAC 19 fungicides. The RS isolates were able to outgrow S isolates on tomato fruit treated with a dose of polyoxin D recommended for field applications, indicating that these isolates may survive field applications at the recommended label rate. However, the reduced sensitivity appears to be associated with a fitness cost, since RS isolates typically had slower growth rates, and were less likely to sporulate than sensitive isolates. However, RS isolates were able to produce more sclerotia than S isolates in our experimental setup. If this ability is confirmed in the field, such isolates may have a survival advantage over S isolates in the absence of polyoxin D fungicide pressure.

Reduced sensitivity to group 19 fungicides, in the absence of prior polyoxin selection pressure was previously found in *B. cinerea* isolates from sweet basil grown in greenhouses in Israel. In that study, similar parameters were evaluated, including relative growth analysis on a discriminatory dose of fungicide, growth rate on unamended media, and EC50 analysis. The main differences between their methods and ours were that they used a modified version of Czapek-Dox media, a discriminatory dose of 1µg/mL for relative growth analysis, and polyoxin A-L fungicide instead of polyoxin D. Polyoxin A-L is a mixture of polyoxins that includes polyoxin D. However, results between the two studies were fairly consistent with 20-35% of the isolates collected prior to polyoxin A-L application showing low-level resistance to polyoxin D. Relative growth on media amended with a discriminatory dose of fungicide showed a bimodal distribution for both studies, and EC50 values of low-level resistant isolates ranged between 4 and 6.5 µg/µg/mL, consistent with the range of 4.6-5.8 µg/µg/mL obtained for our RS isolates. But in contrast to our study, the authors found no fitness penalties in the low-level resistant isolates, though they only examined growth rate *in vitro* and not sporulation or sclerotia production.

There may be several explanations for the observed pre-existence of reduced sensitivity to polyoxin D in B. cinerea isolates. *B. cinerea* populations are inherently so broad in genetic diversity that one study of *B. cinerea* isolates within South Carolina detected genetic differences between all 56 isolates collected, though multiple isolates were collected from sampled greenhouses. A different study from Europe found single plants and even lesions that contained five or more different haplotypes. The cause of this high diversity is currently unknown, but has been attributed to multiple phenomena including sexual recombination, transposons, and heterokaryosis. Because of *B. cinerea's* high diversity, certain specialized isolates may be inherently resistant to a fungicide before it is applied. One European study discovered two genetically distinct groups within *B. cinerea*: one with inherent tolerance to fenhexamid and the other sensitive to fenhexamid. Therefore, the broad diversity of *B. cinerea* may result in the co-existence of isolates with sensitivity and inherent reduced sensitivity to polyoxin D.

Another possibility is that resistance of our isolates to other chemical classes of fungicides predisposed them to reduced sensitivity to polyoxin D. In *Venturia inequalis* isolates, cross-resistance between MBC fungicides and DMI fungicides was detected. The authors proposed that this cross-resistance could be due to fungicide selection for greater genome plasticity potentially by affecting DNA repair systems. This predisposition to resistance across chemical classes could result in reduced sensitivity to fungicides before their widespread use.

The reduced sensitivity we observed also may be related to multi-drug resistance (MDR) which stems from upregulation of drug efflux pumps in the cell membrane. Based on conventional definitions of resistance, RS isolates appear to have quantitative resistance since there are varying levels of sensitivity to polyoxin D. Though multiple mutations conferring increasing levels of resistance are one possible cause of this reduced sensitivity, another possibility is that overexpression of genes such as those involved in drug efflux may cause the RS phenotype. Though the resistance mechanism to polyoxin D is currently unknown, upregulation of ABC transporters after exposure to polyoxins has been documented as well as increased sensitivity to polyoxins when the ABC transporter gene *BMR1* was knocked out. Some work indicates that MDR isolates do not typically have fitness costs associated with them as our isolates did. However, other recent work (unpublished) indicates that though sclerotia production, sclerotia viability, sporulation, growth rate, and oxidative sensitivity were no different between S and MDR1h isolates, osmotic sensitivity is greater and growth rate in colder temperatures is slower in MDR1h isolates. This indicates that MDR may result in fitness cost.

Though further work is necessary to determine the exact cause of reduced sensitivity, it is critical to take resistance management precautions when using polyoxin D to control gray mold on strawberry. Fortunately, polyoxin D has a unique mode of action that has not yet been used on strawberry. This makes it an excellent candidate for tank mixing and rotation with single-site inhibitor fungicides that have risk of resistance development. Its low toxicity and good field efficacy also make it a worthwhile product to use despite the detection of RS isolates.

The results of this study demonstrate that isolates RS to polyoxin D are already present in many locations of the eastern US, but it is unknown whether RS isolates can be controlled effectively by field rates of formulated products containing polyoxin D. Low-level resistant isolates from sweet basil in Israel never developed into highly resistant isolates over a 10-year period, though low-level resistance increased in frequency from 20% to 73% in response to polyoxin A-L application (Mamiev et al. 2013). FRAC 19 fungicides such as polyoxin D should be integrated into resistance management programs to delay the selection of RS isolates.

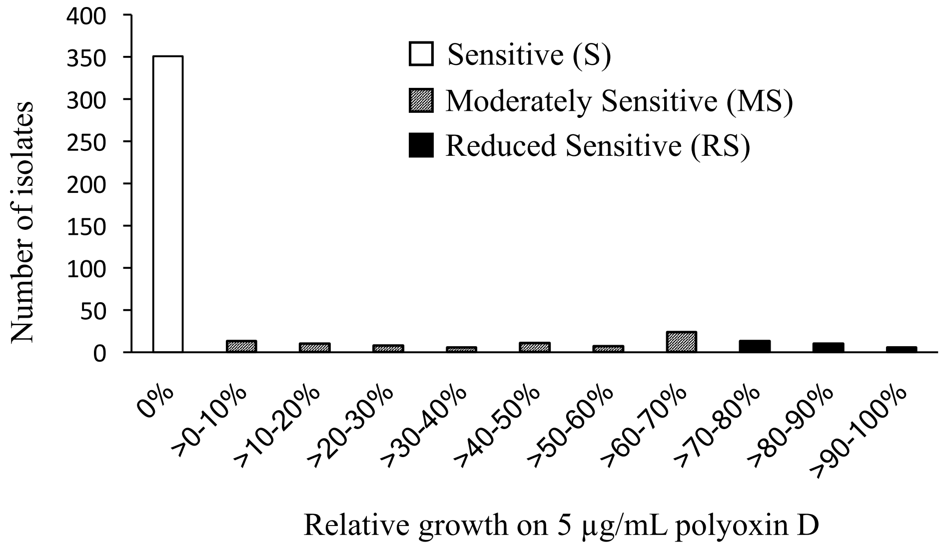

Figure 1. Relative growth of 452 *Botrytis cinerea* isolates from 5 states on PDA amended with polyoxin D.

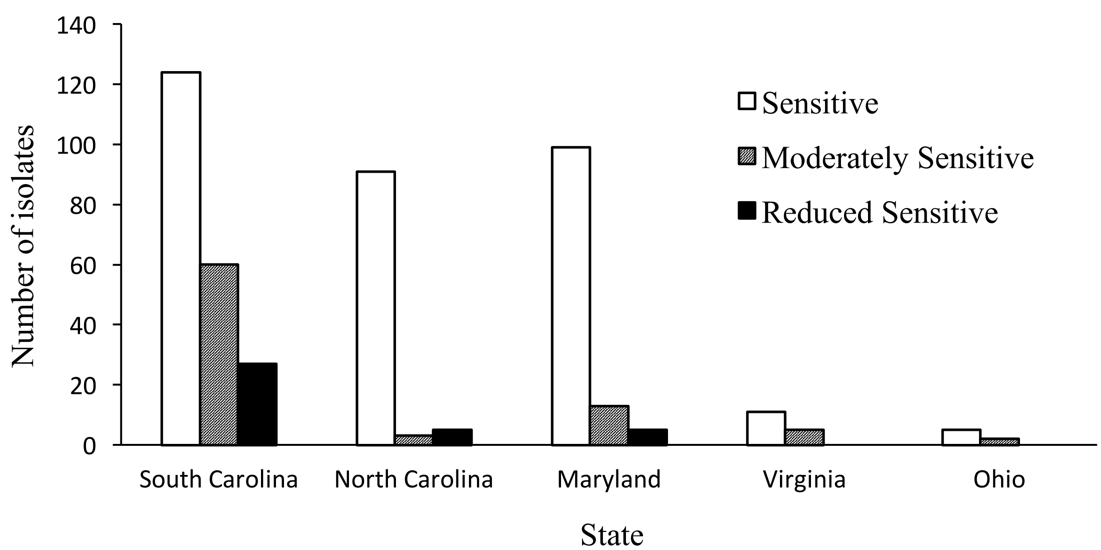

Figure 2. Geographic distribution of *Botrytis cinerea* isolates S, MS, and RS to polyoxin D.

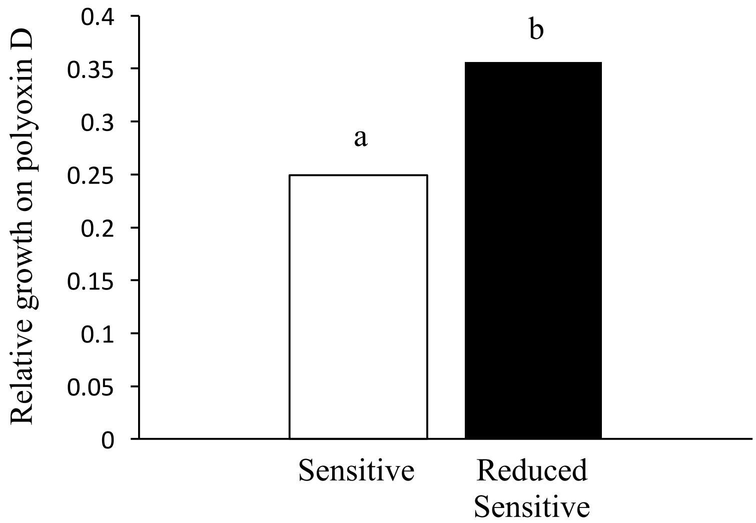

Figure 3. Relative growth of RS and S isolates on detached tomato fruit at a field rate of polyoxin D.