Research Note

SENSITIVITY OF *COLLETOTRICHUM ALATAE* TO A QUINONE OUTSIDE INHIBITOR (QOI) FUNGICIDE[1,2]

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Yam (*Dioscorea alatae* L.) is a staple crop of economic importance in tropical and sub-tropical regions worldwide. In Puerto Rico, yam occupies first place in economic importance among root and tuber crops, contributing $4.3 million in 2018-19 and producing 2,059 t (DAPR, 2022). Local yam production has been affected by diverse pathogens, causing significant economic loss. Anthracnose, a fungal disease caused by *Colletotrichum* spp., is one of the most destructive diseases of yam (*D. alata* cv. Florido) in Puerto Rico. Chemical control using Quinone outside Inhibitor (QoI) fungicides has been essential in managing fungal diseases in several food crops in the USA (Neves et al., 2022; Ding et al., 2019; Forcelini et al., 2017; Forcelini et al., 2016). In Puerto Rico, one of the most used QoI fungicides to manage anthracnose has azoxystrobin as the active ingredient. Azoxystrobin is a broad-spectrum active ingredient with systemic and curative properties recommended for controlling many important plant pathogenic fungi (Pasche et al., 2004). Azoxystrobin is a member of Group 11 (QoI), classified as high risk for resistance development due to its site-specific mode of action (FRAC CODE, 2022). Azoxystrobin inhibits mitochondrial respiration, blocking the electron transport chain between cytochrome b and cytochrome c by binding the cytochrome bc1 enzyme complex to the mitochondrial quinol oxidation site. Therefore, it affects the synthesis of adenosine triphosphate (ATP), interfering directly with cells' energy production. Different resistance mechanisms have been reported since the commercial release of QoI-fungicides in 1996 (Fernández-Ortuño et al., 2008). Most resistance cases described are target-site mutations involving the amino acid substitution from glycine to alanine at position 143 (G143A) in the cytochrome b gene (Cox et al., 2014). Moderate resistance levels have been linked to a mutation at F129L (change from phenylalanine to leucine at position 129) and G137R (change from glycine to arginine at position 137). The second resistance mechanism uses an alternative oxidase (AOX) in the electron transport chain located on the mitochondrial membrane for fungal respiration (Rebollar-Alviter et al., 2007). An AOX inhibitor, salicylhydroxamic acid (SHAM), is commonly included in QoI-sensitivity in vitro assays to suppress alternative respiration (Liang et al., 2015; Duan et al., 2012).

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Recently, anthracnose control failures have been observed in the use of QoI fungicides on commercial yam farms throughout the island (Fuentes-Aponte, 2015). The objective of this research was to determine the sensitivity of *C. alatae* to QoI-fungicide (azoxystrobin) at four concentrations. *Colletotrichum alatae,* isolate F52253A, used in the assays was collected from a commercial yam farm in San Sebastián, Puerto Rico. This isolate was previously characterized based on morphological, pathogenic and molecular analysis of various genetic regions, otherwise amplifying and sequencing three genetic regions: ITS, TUB2, and GADPH (Fuentes-Aponte et al., 2021). Fungal monosporic culture was obtained following a previously published method (Giacomin et al., 2021) and preserved in a non-metabolizing state on PDA vials at 4° C. The sensitivity of *C. alatae* was evaluated using two methods, fungicide-amended plates and conidial germination assays (Bosques-Martínez and Feliciano-Rivera, 2017; Forcelini et al., 2016).

Technical-grade azoxystrobin (Syngenta Crop Protection[5]) was used for fungicide sensitivity evaluations. Azoxystrobin was diluted in sterile deionized water to prepare a stock solution of 1000 µg/ml and added to Potato Dextrose Agar (PDA, Difco Lab., Detroit, MI) plates at 0.1, 1.0, 5.0, and 10 µg/ml. Azoxystrobin treatments were evaluated with and without the AOX inhibitor salicylhydroxamic acid (SHAM - 99% a.i; Sigma-Aldrich) at 100 µg/ml (Liang et al., 2019; MacKenzie et al., 2020; Forcelini et al., 2016; Bosques-Martínez and Feliciano-Rivera, 2017). Treatments included fungicide-unamended media and fungicide-amended media with and without SHAM. Mycelial plugs (3 mm) from an actively growing seven-day-old single conidia colony of *C. alatae* were transferred upside down to the centers of the plates and incubated for 10 days at 25° C. Radial mycelium growth was measured at two-day intervals. Treatments were replicated three times, and the assay twice. For the conidial germination assay, conidia were harvested by flooding plates with 10 ml of sterile deionized water, gently scrapping off the surface, and filtering through two sterile cheesecloth layers (Chaky et al., 2001). Conidia concentration was adjusted with a hemacytometer to 10^6 conidia/ml (Wong et al., 2007). The assay was conducted as follows: 100 µl of conidial suspension was added to 400 µl of potato dextrose broth (PDB, Difco Lab., Detroit, MI), then the amount required for each test material plus sterile deionized water to achieve the desired concentration of 1 ml in the final volume. The mixture was vortexed for 5 to 10 seconds. Three 10 µl drops from each treatment were placed on plastic coverslips inside Petri dishes that served as humid chambers. Petri dishes were incubated at 25° C, and conidia germination was assessed after 8 h of incubation. A total of 50 conidia per drop were counted from microscope fields arbitrarily located within four quadrants (Kim et al., 2003). Conidia were recorded as geminated if at least one germ tube was observed with or without appressorium (Chaky et al., 2001). SHAM at 100 µg/ml and non-treated conidia were used as controls. The experiment was performed four times with three replicates per treatment. All results were analyzed using InfoStat Statistical Software Version 2020 (Di Rienzo et al., 2020). Analysis of variance (ANOVA) was performed at the significance level of $P<0.05$. Fisher's least significant test (LSD, $P=0.05$) was conducted to compare treatments.

In vitro evaluations combined with molecular detection of mutations on cytochrome b are the most commonly used methods to describe the resistance development of fungal pathogens to QoI fungicides (Avenot et al., 2020; Giorgio et al., 2020; Saito and Xiao, 2018; Liang et al., 2019; Liang et al., 2015; Duan et al., 2012). SHAM, added to the artificial medium to inhibit AOX, is a well-established practice in the in vitro sensitivity assay

[5] Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

TABLE 1.—*Fungicide sensitivity assays against* Colletotrichum alatae *expressed as a percent of inhibition.*

Treatments (µg/ml)	Amended media (%)[1]	Conidial germination (%)
S[2] - 100	0 D	17.6 B
Az[3] - 0.1	0 D	3.4 A
Az -1.0	6.0 C	16.3 B
Az- 5.0	37.3 B	75.3 D
Az - 10.0	40.3 B	92.8 E
Az - 0.1 + S - 100	0 D	14.1 B
Az - 1.0 + S - 100	38.8 B	35.3 C
Az - 5.0 + S - 100	40.3 B	94.1 E
Az - 10.0 + S - 100	47.8 A	98.0 E

[1]Percent of inhibition to the unamended control. [2]S = SHAM or salicylhydroxamic acid. [3]Az = Azoxystrobin. All values represent the percentage of growth reduction compared to the nontreated control. Means within a column followed by the same letter are not significantly different according to Fisher's least significant difference (P = 0.05).

of fungal pathogens to QoI fungicides (Duan et al., 2012; Malandrakis et al., 2006; Markoglou et al., 2006). However, previous studies showed that SHAM significantly inhibited the mycelium growth of *Botrytis cinerea* and *Sclerotium sclerotiorum* and affected the inherent toxicity of QoI fungicides (Liang et al., 2019; Liang et al., 2015). Furthermore, previous research showed that SHAM increased the control efficacy of the QoI fungicide against fungal plant pathogens (Liang et al., 2019; Liang et al., 2015). In our study, during fungicide-amended media assays, SHAM (S) alone did not reduce *C. alatae* mycelial growth (Table 1; Figure 1F). However, SHAM mixed with azoxystrobin significantly increased the control efficacy at concentrations equal to or higher than 10 μg/ml (Table 1). Radial growth of *C. alatae* decreased in all azoxystrobin-based treatments at a concentration equal to or higher than 1.0 μg/ml alone or in a mixture with SHAM (Table 1).

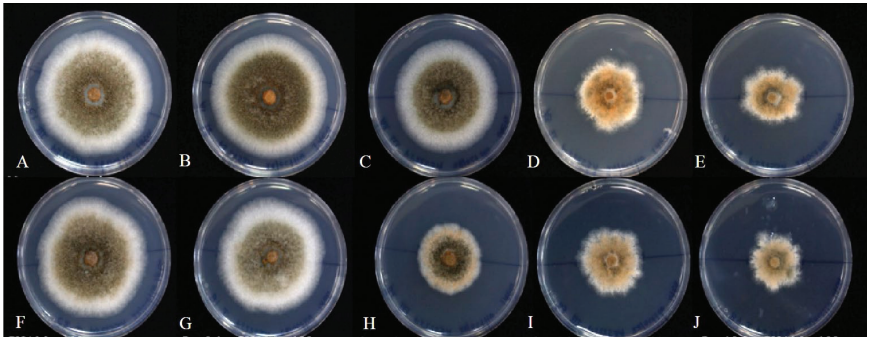

FIGURE 1. Fungicide-amended plate assays after 10 days of incubation. A. Unamended control (PDA); B. Az 0.1 µg/ml; C. Az 1.0 µg/ml; D. Az 5.0 µg/ml; E. Az 10.0 µg/ml; F. SHAM 100 µg/ml; G. Az 0.1µg/ml + SHAM 100 µg/ml; H. Az 1.0 µg/ml + SHAM 100 µg/ml; I. Az 5.0 µg/ml + SHAM; 100 µg/ml and J. Az 10.0 µg/ml + SHAM 100 µg/ml. Az = Azoxystrobin and SHAM = salicylhydroxamic acid.

On the amended media assays, none of the treatments showed values equal to or higher than 50% inhibition, indicating the loss in sensitivity of *C. alatae* to azoxystrobin and possible buildup of resistance. Mycelial growth inhibition higher than 50% is a common parameter used to indicate significant inhibition and sensitivity to the fungicide (Giorgio et al., 2020).

In the conidial germination assays, SHAM had significant inhibition ($P<0.05$) of conidial germination (Table 1). *Colletotrichum alatae* treated with azoxystrobin alone or mixed with SHAM showed a sensitivity response in a dose-dependent manner (Table 1). SHAM's increased the control efficacy of the QoI fungicide in all doses evaluated. Overall, conidia were more sensitive to azoxystrobin than mycelium, with inhibition values higher than 75% at concentrations of 5 and 10 µg/ml. Considering previous reports on SHAM's toxicity on pathogenic fungi and the increase in antifungal activity in the azoxystrobin combinations observed in our trials, we based our conclusions only on the azoxystrobin-based treatments. Thus, we hypothesized that the success in managing *C. alatae* might be affected by the structure and location of the pathogen (conidia on the surface or mycelium inside the plant) present during fungicide applications. Farmers could expect better results based on conidial germination assays as a preventive (conidia on surface) rather than a curative (mycelium colonization) application.

The current study provides information on the potential buildup of resistance in *C. alatae* exposed to a QoI fungicide, but broader studies increasing the *C. alatae* bank of isolates are necessary to support our findings. We consider that various factors are involved in the development of QoI resistance in populations of *C. alatae* in Puerto Rico. First, the lack of low winter temperatures and winter dormancy allow *Colletrotrichum* species to stay active year-round as saprophytes (Wong et al., 2007; Weir et al., 2012) and second, farmers are highly dependent on the QoI fungicides due to the limited management methods available on the island. As recommended by FRAC, growers should not rely solely on one fungicide; they should restrict the number of applications per season and use a fungicide as a protectant rather than as a curative application (Brent and Hollomon, 2007).

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