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Full Length Article

A Rapid Microtiter Assay to Evaluate Fungicide Sensitivity to *Colletotrichum falcatum* Isolates

Renato de Carvalho Menezes*, Mariana Guimarães Silva, Thayssa Monize Rosa Oliveira, Geisiane Alves Rocha, Vanessa Duarte Dias, Renato Carrer Filho and Marcos Gomes da Cunha
Universidade Federal de Goiás, Goias, Brazil
*For correspondence: renato_cmenezes@hotmail.com
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Abstract

Chemical control of sugarcane red rot, caused by *Colletotrichum falcatum*, forms part of integrated management of the disease. A rapid microtiter bioassay based on the colorimetric changes of resazurin dye was developed to evaluate the sensitivity of *C. falcatum* to the main chemical fungicide groups, including strobilurin, triazole, benzimidazole, isophthalonitrile and dithiocarbamate. There was no significant difference among the isolates in terms of growth inhibition for any of the active ingredients tested ($\alpha = 0.01$). The *C. falcatum* isolates showed almost similar sensitivity to various fungicides. The active ingredients varied in relation to fungitoxicity. Doses that inhibited 50% of *C. falcatum* growth were calculated as a percentage of resazurin reduction due to various fungicides. The colorimetric method used to assess the fungitoxicity of active ingredients to *C. falcatum*, combined with resazurin, proved a fast practical and efficient method. © 2022 Friends Science Publishers

Keywords: Chemical control; Fungitoxicity; Red rot; Resazurin; Sugarcane

Introduction

The fungus *C. falcatum* is the causal agent of sugarcane red rot, one of the most destructive diseases that affects the crop (Khan *et al*. 2011; Bharti *et al*. 2012; Sharma and Tamta 2015). Widely disseminated across all continents, sucrose yield losses of 50–70% have been reported in infected stems (Santiago and Rossetto 2008). Earliest red rot epidemics were atributed to attach of *Diatrea saccaralis*, but the fungus was no longer 100% associated with *Diatrea saccharalis*, more aggressive variants do not need the hole of the insect to penetrate the stalk. With the absence of burned, the pathogen survives in the soil and crop residues after harvest and with each harvest the incidence and severity increases (Viswanathan 2010), it has become a very harmful disease to sugarcane crops in countries like Brazil and India (Viswanathan *et al.* 2020a).

The fungal pathogen exhibits enormous variation under fields conditions and the pathogenics variants emerge regularly in tune with deployment of new host varieties for cultivation making the resistant to susceptible referred as "varietal breakdown" (Viswanathan *et al.* 2020b). So, already many sugarcane varieties were replaced due to their breakdown to a new pathogenic strain, so chemical control may be more one possibility in the management of red rot in sugarcane crop. Given the losses caused by the disease, existence of pathogenic variation in *C. falcatum* and the emergence of new virulent pathotypes were documented over the decades (Viswanathan 2010; Viswanathan *et al.* 2020b).

Among the management strategies used, fungicides are an important supplementary tool in controlling different diseases (Nene and Thapliyal 1993). However, in the case of sugarcane red rot, information on the sensitivity of various *C. falcatum* isolates to different fungicides remains scarce, largely due to the lack of a practical and efficient method to conduct this assessment. Conventional techniques for evaluating the fungitoxicity of active ingredients in a fungicide-enriched growth medium by inhibiting mycelial growth and/or conidial germination are costly, laborious, time-consuming, require a significant amount of laboratory space to accommodate a large number of plates, cannot identify intermediate sensitivity to fungicides and preclude automated data collection (Rampersad and Teelucksingh 2012; Promega Corporation 2019).

An alternative strategy for assessing the fungitoxicity of active ingredients is resazurin, a stable, nontoxic water-soluble dye used as an indicator in oxidation-reduction reactions. Resazurin has been used to measure cell proliferation, as well as the viability and cytotoxicity of different cell types in medical research (Invitrogen Molecular Probes 2021; ThermoFisher Scientific 2021). As such, the present study aimed to (i) adjust a colorimetric method for assessing the sensitivity of *C. falcatum* isolates

to the main fungicide chemical groups strobilurin, triazole, benzimidazole, isophthalonitrile, and dithiocarbamate and (ii) evaluate the fungitoxicity and sensitivity of the different *C. falcatum* isolates of the active ingredients tested.

Materials and Methods

Preparation of the conidial suspension

Eighteen fungal isolates were obtained from monoconidial *C. falcatum* cultures belonging to the microorganism collection of the Plant Pathology Laboratory at the School of Agronomy of the Federal University of Goias, collected in the state of Goias in the 2017 growing season. For the production of conidia, the isolates were cultivated in Petri dishes containing potato dextrose agar (PDA) medium for 20 days at 28–30°C, under constant darkness.

In order to determine the optimal conidia concentration to test the sensitivity to fungicides of 18 *C. falcatum* isolates, suspensions were prepared at initial concentrations of 10^4, 10^5 and 10^6 conidia mL^{-1} for one *C. falcatum* isolate (CFF 12). The conidial suspension (60 μL), 140 μL of Czapek Dox Broth medium + 0.05% agar and 10 μL of 0.02% resazurin were deposited into a clear flat bottom polystyrene microplate. Each conidial suspension concentration was deposited in three repetitions (microplate wells). The negative control used was 60 μL sterile water without conidia, also in three repetitions. The microplate was incubated in a biochemical oxygen demand (BOD) incubator at 28–30°C, under constant darkness. In order to determine the ideal incubation period, the resazurin reduction percentage was evaluated after 48 and 72 h of incubation.

Fungicide sensitivity testing

The following fungicides were tested: Amistar® WG 500 g kg^{-1} of Azoxystrobin, obtained from Syngenta), Tilt® (250 g L^{-1} of propiconazole, Syngenta), Carbendazim (97%, Sigma-Aldrich), Manzate 800 (800 g kg^{-1} of mancozeb, UPL), Bravonil® 720 (720 g L^{-1} of chlorothalonil, Syngenta) and Cercobin 700 WP (700 g kg^{-1} of thiophanate-methyl, Iharabras). Each fungicide was precisely weighed and dissolved in Czapek Dox Broth medium + 0.05% agar (used as a dispersing agent) in serial dilutions. The active ingredient concentrations used were 0; 0.01; 0.1; 0.5; 1; 5; 10 and 50 μg mL^{-1}.

Suspensions with a concentration 10^5 conidia mL^{-1} were prepared for each of the 18 isolates. Each well received 60 μL of conidial suspension, 140 μL of Czapek Dox Broth medium + 0.05% agar, active ingredients at the doses previously mentioned and 10 μL of 0.02% resazurin. For strobilurin, 10 μL of salicylhydroxamic acid (SHAM) was added to the well to suppress the alternative oxidase pathway (Ma *et al.* 2006). Active ingredient doses were tested in three wells for each isolate. The microplates were sealed and incubated in a BOD incubator at 28–30°C under constant darkness. The resazurin reduction percentage was evaluated after 48 h of incubation.

Absorbance of the microplate wells was read in an Epoch microplate spectrophotometer (Biotek, Vermont, United States) after 48 h of incubation, at wavelengths of 570 nm and 600 nm, with the aid of GENE 5 software, version 2.0. The resazurin reduction percentage was estimated using the formula provided by the manufacturer of alamarBlue® reagent (Cox *et al.* 2009).

Data analysis

Data were analyzed in R software version 3.6.2. For each dose of active ingredient (dose y), the growth inhibition percentage (GIP) of the isolate was calculated in relation to dose zero (dose 0) using the estimated resazurin reduction percentage, which indirectly measures initial *C. falcatum* growth. For each active ingredient, the GIP as a function of dose and isolates was submitted to analysis of variance, using the *ExpDes* package of R software. The *nls* and *nlstools* packages were used for nonlinear regression adjustment based on the model proposed on Michaelis–Menten model, where GIP is a function of the active ingredient dose.

Results

Adjustment of spore concentration and incubation time

Preliminary tests to adjust the spore concentration and incubation time were assessed by analysis of variance and Tukey's test ($\alpha = 0.01$). A concentration of 10^5 conidia mL^{-1} provided the greatest resazurin reduction, with no significant difference between incubation times of 48 and 72 h, confirming the stability of the dye after 48 h of incubation.

Sensitivity of *C. falcatum* isolates to fungicides

For each active ingredient, the *C. falcatum* GIP as a function of dose and isolates was submitted to analysis of variance, with no significant difference in GIP between *C. falcatum* isolates ($\alpha = 0.01$) (data not shown). The regression curve that expresses the *C. falcatum* GIP as a function of dose, based on the model proposed by (Michaelis and Menten 1913), was adjusted for each active ingredient. Thus, all the regression curves were significant at 0.1% according to the F-test. The regression equations, coefficients of determination (R^2) and doses that inhibited 50% of growth (EC_{50}) for each active ingredient are described in Table 1.

The effects of the active ingredients varied in relation to fungitoxicity to *C. falcatum*. The sensitivity of *C. falcatum* to the molecules tested can be ranked in descending order based on EC_{50}, as follows: azoxystrobin, propiconazole, carbendazim, mancozeb, chlorothalonil and thiophanate-methyl. Comparison of the fungitoxicity of the active ingredients is shown in Fig. 1 and 2.

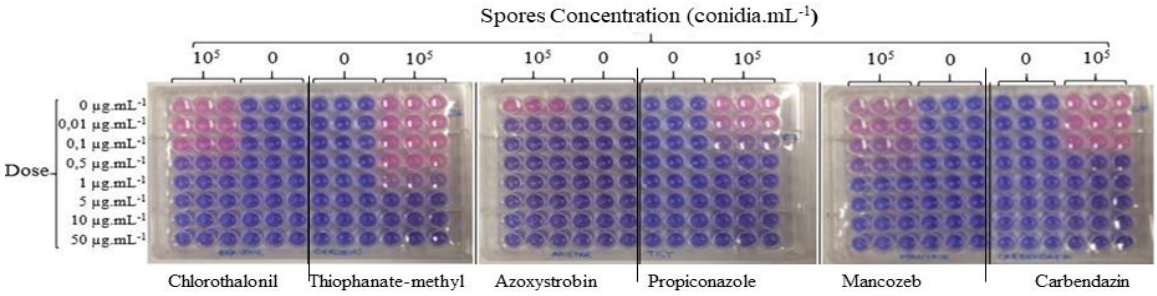

Fig. 1: Visualization of *Colletotrichum falcatum* growth inhibition by resazurin reduction as a function of fungicide doses.

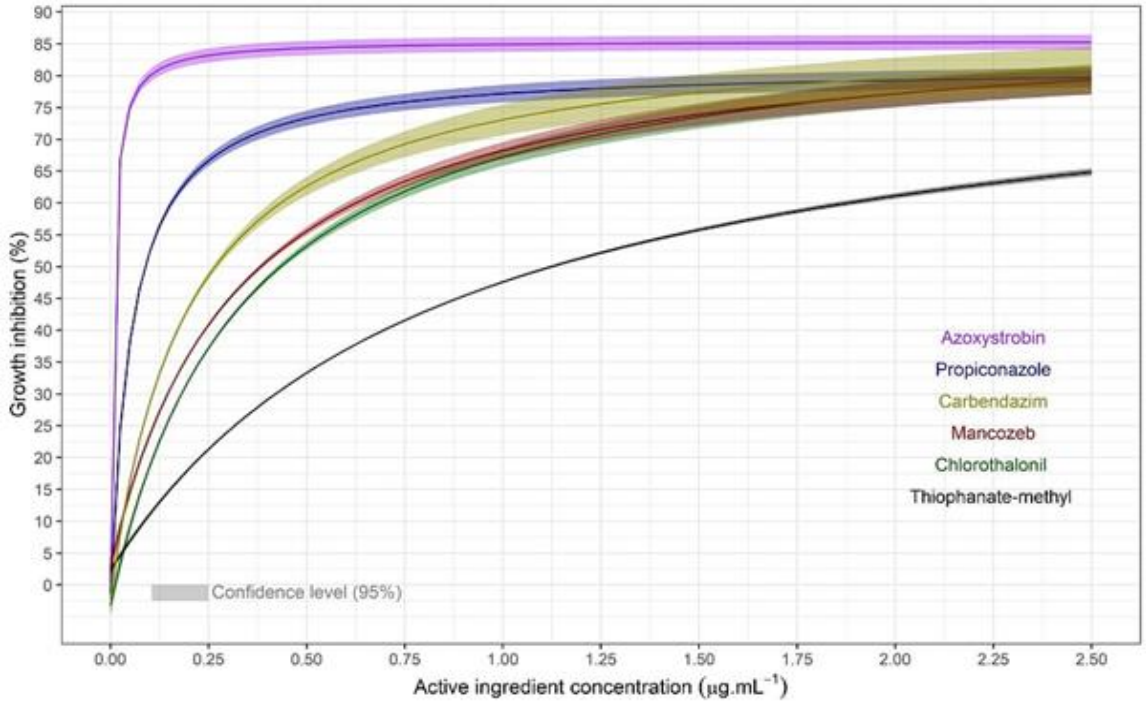

Fig. 2: Comparative regression curves of *Colletotrichum falcatum* growth inhibition as a function of fungicide fungitoxicity. CI: confidence interval

Table 1: Regression equation, coefficients R^2 e EC_{50}

Active Ingredient	Equation	R^2	EC_{50} ($\mu g.mL^{-1}$)
Azoxystrobin	GIP = -0.13 + 85.63. Dose / (0.0069 + Dose)	0.994	0.0097
Propiconazole	GIP = -1.41 + 82.87. Dose / (0.0542 + Dose)	0.989	0.0885
Carbendazim	GIP = -2.26 + 89.78. Dose / (0.1916 + Dose)	0.931	0.2668
Mancozebe	GIP = 2.84 + 86.09. Dose / (0.3161 + Dose)	0.954	0.3829
Chlorothalonil	GIP = -3.24 + 93.56. Dose / (0.3281 + Dose)	0.956	0.4332
Thiofanate methil	GIP = 2.28 + 83.92. Dose / (0.8519 + Dose)	0.987	1.1230

Discussion

With respect to the spore concentration that optimizes resazurin reduction, the present study demonstrated that 10^5 conidia mL^{-1} provided the best results after 48 h of incubation. Resazurin sensitivity to spore density is dependent on the fungal species studied and should be determined empirically for studies involving dye reduction in response to the fungitoxicity of pesticides to phytopathogenic fungi (Cox *et al*. 2009). Larson *et al.* (1997) analyzed the viability of corneal endothelial cells and Pelloux-Prayer *et al.* (1998) the viability of *Botrytis cinereal* conidia, with both studies reporting that densities lower than 10^4 cells mL^{-1} required longer incubation times to reduce resazurin, whereas more than 10^5 cells mL^{-1} produced inconsistent results. Larson *et al.* (1997) also found that high

cell densities and prolonged incubation times cause resazurin to reach an undesirable plateau because the (blue) dye is reduced to resorufin (pink which, in turn, is reduced to hydroresorufin (colorless). The reduction percentage for resorufin is higher than that of hydroresorufin (Rampersad 2011).

In regard to the incubation times tested, resazurin reduction stabilized between 48 and 72 h. This indicates that the dye reached its reduction limit at 48 h, which was the time established for testing to assess *C. falcatum* sensitivity to fungicides. Prolonged incubation times compromise resazurin reduction, as reported by Larson *et al.* (1997).

There was no significant difference in growth inhibition, estimated by the resazurin reduction percentage ($\alpha = 0.01$), between isolates for any of the active ingredients tested. As such, the *C. falcatum* isolates studied showed equal sensitivity to azoxystrobin, propiconazole, carbendazim, mancozeb, chlorothalonil and thiophanate-methyl. Ghazanfar *et al.* (2017) reported that Tilt® (250 $g.L^{-1}$ of propiconazole) was more efficient than mancozebe at inhibiting the mycelial growth of the fungus and Nenhuma fonte bibliográfica especificada. It was found more effective at controlling sugarcane red rot.

In the present study, the active ingredient propiconazole was the second most toxic to the pathogen (Fig. 1 and 2). In research on other chemical molecules, Nikhil and Sahu (2014) observed that carbendazim was more effective than propiconazole at inhibiting mycelial growth, whereas Abbas *et al.* (2016) reported that mancozebe was most effective, followed by propiconazole, and found that benomyl, tebuconazole and mancozeb were more efficient than propiconazole. Although benomyl and tebuconazole were not tested here, carbendazim was only less toxic to *C. falcatum* than azoxystrobin and propiconazole, with mancozeb ranked fourth in terms of fungitoxicity to the molecules tested (Fig. 1 and 2).

Studies that evaluate the sensitivity of *C. falcatum* to different fungicides measure the effect of different products on the mycelial growth of the fungus and/or conidial germination in a culture medium, generally with doses above 5 $\mu g\ mL^{-1}$. However, a number of investigations do not indicate whether the dose refers to active ingredients or commercial products (Bharti *et al.* 2014; Ghazanfar *et al.* 2017). In our study, doses greater than 5 $\mu g\ mL^{-1}$ were too high, which precluded determining the fungitoxicity of azoxystrobin, propiconazole, carbendazim, mancozeb, chlorothalonil, and thiophanate-methyl (Fig. 1). Although the aforementioned studies evaluated the sensitivity of the pathogen to different fungicides, none provided data on the active ingredient dose (EC_{50}) that inhibited 50% of mycelial growth and/or 50% of conidial germination, which precluded comparison with the EC_{50} values found here (Nikhil and Sahu 2014; Bharti *et al.* 2014; Abbas *et al.* 2016).

EC_{50} has been estimated for other species of the genus *Colletotrichum*. Analysis of the conidial germination of *C. gloeosporioides* and *C. capsica* isolates showed EC_{50} values from 0.009 to 0.091 $\mu g\ mL^{-1}$ for both species (Li *et al.* 2005). In our study, the EC_{50} of azoxystrobin for *C. falcatum*, calculated based on the estimated resazurin reduction percentage, was 0.0097 $\mu g\ mL^{-1}$, within the range described above (Table 1). The EC_{50} values for the active ingredients propiconazole and thiophanate-methyl were estimated by the mycelial growth of *C. cereale* isolates and varied from 0.025 to 0.35 $\mu g\ mL^{-1}$ and 0.14 to 2.3 $\mu g\ mL^{-1}$, respectively (Wong and Midland 2007; Wong *et al.* 2008). In the present study, the EC_{50} values of propiconazole and thiophanate-methyl for *C. falcatum* were 0.0885 $\mu g\ mL^{-1}$ and 1.123 $\mu g\ mL^{-1}$, respectively, similar to those reported for *C. cereale*.

The EC_{50} values of carbendazim and mancozeb for *C. acutatum* isolates estimated, respectively, by the inhibition of mycelial growth and conidial germination, were 0.1946 $\mu g\ mL^{-1}$ for carbendazim and between 0.24 and 0.85 $\mu g\ mL^{-1}$ for mancozeb (Cai *et al.* 2008; Gao *et al.* 2017). For *C. falcatum*, our study demonstrated EC_{50} values of 0.2668 $\mu g\ mL^{-1}$ for carbendazim and 0.3829 $\mu g\ mL^{-1}$ for mancozeb, calculated based on the estimated resazurin reduction percentage, similar to the values reported for *C. acutatum*.

The EC_{50} of chlorothalonil was estimated for *C. truncatum* and *C. gloeosporioides* by mycelial growth inhibition, with values from 0.23 to 1.14 $\mu g\ mL^{-1}$ and 0.01 to 0.95 $\mu g\ mL^{-1}$, respectively (Rampersad and Teelucksingh 2012). For *C. falcatum*, our study recorded an EC_{50} of 0.4332 $\mu g\ mL^{-1}$ for chlorothalonil, calculated via the estimated resazurin reduction percentage, which is within the ranges reported for *C. truncatum* and *C. gloeosporioides*.

The aforementioned studies found similar EC_{50} values for the active ingredients in question for species from the genus *Colletotrichum* to those recorded to *C. falcatum*. Some of the studies calculated the EC_{50} for each isolate used and established a range for that dose. However, when isolates exhibit similar sensitivity to fungicides, as observed for the *C. falcatum* isolates tested here, EC_{50} is estimated for a set of fungal isolates. Application of the fungicides showed a reduction in red rot incidence over control but the extent of reduction varied considerably (10.6–39.4% reduction) across the treatments over the 2 years. Overall, among the seven fungicides evaluated, sett treatment with two fungicides (Tebuconazole + Trifloxystrobin and Thiophanate methyl) consistently resulted in > 30% reduction in red rot incidence over control in both years along with significantly higher NMC over control (Joshi 2021).

Resazurin has been used successfully in active ingredient fungitoxicity tests in several agronomic studies. Research has reported a high correlation between the results obtained with the traditional method and the resazurin-based assay (Cox *et al.* 2009; Vega *et al.* 2012). In summary, we found that the resazurin method is a faster, low-cost alternative to mycelial growth assays for *C. falcatum*. This assay may be a more reliable indicator of fungicide resistance because it uses spores instead of mycelia (Hu *et al.* 2007). Furthermore, it can provide sugarcane growers

with needed information about the sensitivity of isolates collected from their orchards in a more cost-effective manner. The technology also adds simplicity to the detection of resistance, especially when quantification is not desired, and results can be taken visually.

Conclusion

The colorimetric method used to assess the fungitoxicity of active ingredients to *C. falcatum*, combined with resazurin, is fast, practical, efficient and viable for application in large sets of isolates. All the *C. falcatum* isolates studied exhibited equal sensitivity to azoxystrobin, propiconazole, carbendazim, mancozeb, chlorothalonil and thiophanate-methyl. The active ingredients displayed different levels of toxicity to *C. falcatum*, ranked in descending order as follows: azoxystrobin, propiconazole, carbendazim, mancozeb, chlorothalonil and thiophanate-methyl.

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Author Contributions

RCM and MGC planned the experiments, RCM, MGS and TMRO executed the tests, GAR, RCM, VDD and RCF made the write up and statistically analyzed the data.

Conflict of Interest

All authors declare no conflict of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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