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Evaluation of mycelial sensitivity to fungicides of *Colletotrichum* spp. associated with atemoya in the state of Alagoas

Avaliação da sensibilidade micelial a fungicidas de *Colletotrichum spp*. associadas à atemoia no estado de Alagoas

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A B S T R A C T

Anthracnose, caused by *Colletotrichum spp*., is an important disease of atemoya causing damage both pre and post-harvest. Symptoms of this disease range from foliar anthracnose, flower abortion, branch blight and necrotic fruit lesions. Knowing the action of certain fungicides in the fight against the etiological agent is essential to develop control techniques. The objective of this work was to evaluate the mycelial sensitivity of *Colletotrichum spp*., from atemoya, to the fungicides thiophanate methyl, azoxystrobin and tebuconazole. To determine the sensitivity of *Colletotrichum* species to fungicides, mycelial growth in synthetic potato-dextrose-agar (PDA) supplemented with fungicide was evaluated. The fungicides were dissolved in dimethylsulfoxide (DMSO) and added to the fluxing culture medium (45°C), to reach concentrations of 0.0, 0.1, 0.5, 1.0, 5.0 and 10 µg/ml^{-1} of i.a. The mycelial growth of each colony was measured daily to obtain the mycelial growth index (ICM). EC_{50} was also calculated. After calculating the EC_{50}, the *Colletotrichum* species were classified into three sensitivity categories, where: EC_{50}: < 10 µg/ml^{-1}: high sensitivity (SA); EC_{50}: 10-100 µg/ml^{-1}: moderate sensitivity (MS); EC_{50}: 100-500 µg/ml^{-1}: insensitivity (I). The species *C. theobromicola*, *C. fruticola, C. siamense* and *C. karstii* were highly sensitive to the fungicides tebuconazole and thiophanate methyl. The fungicide azoxystrobin proved to be efficient in the *in vitro* control of the species *C. theobromicola*, however, it proved to be inefficient for the control of the species *C. siamense*. The species *C. fruticola* and *C. karstii* were moderately sensitive to the fungicide azoxystrobin.

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R E S U M O

A antracnose, causada por *Colletotrichum* spp., é uma importante doença da atemoia causando danos tanto na pré como na pós-colheita. Os sintomas desta doença variam de antracnose foliar, abortamento de flores, queima de ramos e lesões necróticas nos frutos. Conhecer a ação de determinados fungicidas no combate ao agente etiológico é essencial para desenvolver técnicas de controle. O trabalho teve como objetivo avaliar a sensibilidade micelial de *Colletotrichum* spp., provenientes de atemoia, aos fungicidas tiofanato metílico, azoxistrobina e tebuconazole. Para determinar a sensibilidade das espécies de *Colletotrichum* aos fungicidas foi avaliado o crescimento micelial em meio de batata-dextrose-ágar (BDA) sintético suplementado com fungicida. Os fungicidas foram dissolvidos em dimetilsulfóxido (DMSO) e adicionados ao meio de cultura fundente (45°C), para alcançar as concentrações de 0.0, 0.1, 0.5, 1.0, 5.0 e 10 µg/ml^{-1} de i.a. O crescimento micelial de cada colônia foi mensurado diariamente para obter o índice de crescimento micelial (ICM). A EC_{50} também foi calculada. Após o cálculo da EC_{50}, as espécies de *Colletotrichum* foram classificadas em três categorias de sensibilidade,

Palavras-Chave:
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onde: EC_{50}: <10 μg/ml^{-1}: alta sensibilidade (AS); EC_{50}: 10-100 μg/ml^{-1}: moderada sensibilidade (MS); EC_{50}: 100-500 μg/ml^{-1}: insensibilidade (I). As espécies *C. theobromicola, C. fruticola, C. siamense* e *C. karstii* foram altamente sensíveis aos fungicidas tebuconazole e tiofanato metílico. O fungicida azoxistrobina se mostrou eficiente no controle *in vitro* da espécie *C. theobromicola*, porém, se mostrou ineficiente para o controle da espécie *C. siamense*. As espécies *C. fruticola* e *C. karstii* foram moderadamente sensíveis ao fungicida azoxistrobina.

Introduction

The family Annonaceae is a group of trees and shrubs, comprising 2440 species distributed in 108 genera (Couvreur et al., 2012; Chatrou et al., 2012; Ferreira et al., 2019). The genus *Annona* is considered the most important of the family Annonaceae, because it constitutes fruit species of economic importance worldwide. The pine (*Annona squamosa* L.), soursop (*Annona muricata* L.), cherimoia (*Annona cherimola* Mill) and atemoya (hybrid *A. squamosa* x *A. cherimola*) are those that stand out for presenting potential for consumption *in natura* and in the processed form, as well as in the production of biocompounds of medicinal, allelopathic or pesticide importance (Lemos, 2014; São José et al., 2014).

Atemoya is an interspecific hybrid, resulting from the crossing between cherimoia and pine fruit. The hybrid brings together interesting characteristics of the two crops producing extremely tasty fruits. Among other advantages, the fruit presents a smaller number of seeds, longer shelf life in the post-harvest, absence of cracks, differentiated flavor and more balanced soluble solids content, attracting interest from some producers due to the flavor and quality similar to cherimoia and superior to pine forest (Ferreira et al., 2006; Donadio, 2010).

Anthracnose, caused by *Colletotrichum spp*., is considered the most important disease of the aerial part, compromising the development of annoaceous in the flowering and fruiting phases, attacking the peduncle and causing the fall of flowers and fruits (Bonaventure, 1999; Tokunaga, 2000; Takahashi, 2008; Firmino et al., 2014; Takahashi, 2009; Lemos, 2014).

In fruits, the initial symptoms of the disease are characterized by small dark spots on the bark. Over time, the lesions increase in size, and can coalesce and reach the entire surface of the fruit, sometimes causing deep cracks. The younger or developing fruits, when they do not fall, become dark and mummified (Kavati, 1992; Junqueira et al., 2003). In new branches, dark, elongated or circular lesions may occur, which impair their growth or new plant in the field or nursery. In leaves, dark lesions with irregular shape may occur that cause deformation and fall. The flowers become dark and usually fall in periods of high humidity, their intense fall occurs (Lemos, 2014).

Chemical control has become an ally in areas of atemoya production because of the efficiency in controlling the etiological agent of the disease when the plant population is susceptible and climatic conditions are ideal for the development of the etiological agent of the disease (Junqueira et al., 2001). Therefore, the objective of this study was to evaluate the *in*

vitro mycelial sensitivity of *Colletotrichum* species to methyl thyophanath, azoxystrobin and tebuconazole fungicides.

Material and methods

Location of the experiment and obtaining the species of *Colletotrichum*

The work was carried out in the Molecular Phytopathology and Plant Virology laboratory of the Campus of Engineering and Agricultural Sciences (CECA) of the Federal University of Alagoas (*Universidade Federal de Alagoas - UFAL*), located at km 85 of BR 101 North (9º27'54.71" S - 35º49'39.27" O), in the county of Rio Largo, 27 km from the city of Maceió, capital of the state of Alagoas.

The isolates used in the experiment, from the phytopathogen collection of the Federal University of Alagoas (*Universidade Federal de Alagoas - UFAL*), were obtained from symptoms of anthracnose in atemoya leaves in commercial planting in the states of Alagoas and Bahia, identified by Bayesian Inference based on multi-locus analysis of the β-tubulin (TUB2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and internal transcribed space region (ITS) genes (Chart 1).

Chart 1. *Colletotrichum* isolates used in the experiments.

Code of the isolated	Species	Collection location
[a]COUFAL0337	*C. siamense*	Bahia
COUFAL0339	*C. theobromicola*	Bahia
COUFAL0335	*C. karstii*	Alagoas
COUFAL0332	*C. fructicola*	Alagoas

Source: Material obtained from COUFAL - Collection of Phytopathogens of the Federal University of Alagoas (*Coleção de Fitopatógenos da Universidade Federal de Alagoas*).

Evaluation of mycelial sensitivity of *Colletotrichum* species to fungicides

To determine the sensitivity of *Colletotrichum* species to fungicides, mycelial growth was evaluated in synthetic potato-dextrose-agar (BDA) medium supplemented with fungicide. The commercial formulations of methyl thyophanath fungicides (Cercobin 700 WP, 700 g kg^{-1} of active ingredient (i.a.) Iharabras, São Paulo, Brazil) (Agrolinkfito, 2020), Azoxistrobin (Amistar 500 WG, 500 g kg^{-1} i.a. Syngenta, São Paulo, SP, Brazil) (Agrolinkfito, 2020) and tebuconazole (Folicur 200EC, 200g l^{-1} i.a., Bayer, São Paulo, Brazil) (Agrolinkfito, 2020) were used in *in vitro* tests. The fungicides were dissolved in dimethylsulfue (DMSO) and added to the founding synthetic culture medium (45°C) to reach concentrations of 0,0, 0,5, 1,0, 5,0 and 10 μg/ml^{-1} of i.a. For all concentrations, including the control, the final concentration of DMSO

in the culture medium was 0,1% (v/v). Mycelium discs (5 mm Ø) of each isolate/species were removed from the margin of colonies with 7 days of growth in BDA medium and transferred to the center of Petri slabs containing synthetic BDA medium supplemented with fungicides. The slabs were incubated at 25 °C in the dark for 7 days.

The experimental design used was completely randomized in a triple factorial arrangement, with five slabs (replications) by combination of species-isolate/fungicides/concentrations. Daily, the mycelial growth of each colony was measured in two perpendicular directions to obtain the Mycelial Growth Index (MCI) that will be determined by the formula MCI=[(C1/N1) +(C2/N2) +...+(Cn/Nn)], with C1, C2, Cn the mycelial growth of the fungus in the first, second and last evaluation; and N1, N2, Nn are the number of days after inoculation. The data were submitted to variance analysis (ANOVA) ($p < 0.05$) by the Tukey Test. EC_{50}, an active ingredient concentration capable of inhibiting 50% of mycelial growth, was also calculated. After the calculation of EC_{50}, *Colletotrichum* species were classified into three sensitivity categories, according to the scale of Edgington et al. (1971), in which: EC_{50}: <10 μg/ml^{-1}: high sensitivity (HS); EC_{50}: 10-100 μg/ml^{-1}: moderate sensitivity (MS); EC_{50}: 100-500 μg/ml^{-1}: insensitivity (I).

Results and discussions

According to the results obtained from EC_{50} (concentration of active ingredient capable of inhibiting 50% of mycelial growth), species *C. theobromicola* was highly sensitive to fungicides azoxystrobin, tebuconazole and methyl thiophadate (Chart 2), values ranged from 7.3211 μg/ml^{-1}, 0.0352 μg/ml^{-1} and 0.8603 μg/ml^{-1}, respectively.

The results of EC_{50} for the fungicides tebuconazole and methyl thyophadate (Chart 2) ranged from 0.8502 μg/ml^{-1} and 0.3269 μg/ml^{-1}, respectively, when the pathogen was *C. fructicola*, being considered highly sensitive. Azoxystrobin was moderately sensitive at the concentrations tested for this species.

For the species *C. siamense*, the values of EC_{50} in the fungicides tebuconazole and methyl thyophanate (Chart 2) ranged from 3.8654 μg/ml^{-1} and 0.3931 μg/ml^{-1}, respectively, and the species was considered highly sensitive. For the fungicide azoxistrobin the EC_{50} value was above 100 μg/ml^{-1}, considering inefficient in the concentrations tested for the species.

For the species *C. karstii*, the values of EC_{50} in tebuconazole and methyl thyophadate (Chart 2) ranged from 0.4364 μg/ml^{-1} and 0.3817 μg/ml^{-1}, respectively, being highly sensitive in the concentrations tested. A similar result was found by Salgado (2021) for the control of *C. karstii*, which causes citrus floral rot, using the fungicide tebuconazole; EC_{50} was 1,110 μg/ml^{-1}, considering the highly sensitive species at the concentrations tested. When the fungicide was

azoxystrobin, it was moderately sensitive, with a value of 25.9826 being between 10-100 µg/ml^{-1}.

Chart 2 - EC_{50} (concentration of active ingredients capable of inhibiting 50% of mycelial growth) of different species of *Colletotrichum* to fungicides.

Fungicides EC_{50} (µg/ml^{-1})*	Species				
	C. theobromicola	*C. fructicola*	*C. siamense*	*C. karstii*	Sensitivity of species**
Azoxistrobina	7.3211	81.2192	503.65	25.9826	AS/MS/I
Tebuconazole	0.0352	0.8502	3.8654	0.4364	AS
Tiofanato metílico	0.8603	0.3269	0.3931	0.3817	AS

Source: own authorship.
*EC_{50} = Concentration that inhibits 50% of mycelial growth;
**Classification according to EC_{50}, where: EC_{50} < 10 µg/ml^{-1}: high sensitivity (HS); EC50 10 – 100 µg/ml^{-1}: moderate sensitivity (MS); EC_{50} 100 – 500 µg/ml^{-1} insensitivity (I).

For the fungicide azoxistrobin, IMC (Minimum Inhibitory Concentration) was less than 10 µg/ml^{-1}, with an inhibitory effect of 52.81% for the species *C. theobromicola*. The fungicide tebuconazole showed an inhibition of mycelial growth of 77.13% for concentrations lower than 1 µg/ml^{-1}, while at 10 µg/ml^{-1} there was an inhibition of 90.92%. Methyl thiophanate showed an inhibition of mycelial growth of 52.09% for concentrations lower than 1 µg/ml^{-1}, at the concentration of 10 µg/ml^{-1} there was an inhibition of 86.21%. The fungicides tebuconazole and methyl thyophanath presented iMC lower than 1 µg/ml^{-1}, but tebuconazole showed the highest percentage of inhibition, being 77.13% and azoxystrobin presented the highest IMC and the lowest percentage of inhibition in the tested concentrations (Chart 3).

Chart 3 - Mean percentage values of inhibition of mycelial growth of *C. theobromicola* and the minimum inhibition concentration (IMC)[1].

Fungicides	**Concentrations of µg/ml^{-1}**					
	0.1	**0.5**	**1**	**5**	**10**	**IMC**
Azoxistrobina	42.47%	43.38%	43.92%	48.09%	52.81%	<10
Tebuconazole	49.73%	77.13%	90.92%	90.92%	90.92%	<1
Tiofanato metílico	2.54%	52.09%	59.53%	78.22%	86.21%	<1

Source: own authorship.
[1]Interval between concentrations, in which values of 90 – 100% inhibition of mycelial growth can be found.

The IMC value for the fungicide tebuconazole was above 1 µg/ml^{-1}, with an inhibiting effect of 76.45% in relation to the species *C. fructicola*. Methyl thiophanate presented an IMC

value lower than 1 μg/ml^{-1}, with an inhibition effect of 94.31% for the concentration of 10 μg/ml^{-1}. In azoxystrobin the IMC value was above 10 μg/ml^{-1}, with an inhibition percentage of 47.89% for the concentration of 10 μg/ml^{-1}. For the species *C. fructicola* the fungicide with the lowest IMC value and highest percentage of inhibition was methyl thiophanate, but azoxystrobin presented the highest IMC value and the lowest percentage of inhibition of mycelial growth (Chart 4).

Chart 4 - Mean percentage values of inhibition of mycelial growth of *C. fructicola* and the minimum inhibition concentration (IMC)[1].

Fungicides	**Concentrations of μg/ml^{-1}**					
	0.1	**0.5**	**1**	**5**	**10**	**IMC**
Azoxistrobina	28.78%	29.35%	34.13%	35.04%	47.89%	>10
Tebuconazole	25.03%	34.81%	49.37%	76.45%	89.53	>1
Tiofanato metílico	13.88%	70.65%	84.07%	94.31%	94.31%	<1

Source: own authorship.
[1]Interval between concentrations, in which values of 90 – 100% inhibition of mycelial growth can be found.

In tebuconazole the inhibiting effect was 52.49% and 59.73% at concentrations of 5 and 10 μg/ml^{-1}, respectively, with an IMC value above 1 μg/ml^{-1}. Methyl thyophalate was more efficient with values lower than 1 μg/ml^{-1}, with inhibition of 71.27% and 94.34%. Azoxistrobin was the fungicide that led the lowest percentage of inhibition of mycelial growth for *C. siamense*, being 32.35% for the concentration of 1 μg/ml^{-1}, where this result is similar to that of Miranda (2018) that evaluating the sensitivity of *C. siamense* associated with *Capsicum*, obtained a inhibition percentage of 22% for the concentration of 1 μg/ml^{-1}, thus showing a low inhibition of mycelial growth. In the present study, the IMC value was greater than 10 μg/ml^{-1} (Chart 5).

Chart 5 - Mean percentage values of inhibition of mycelial growth of *C. siamense* and minimum inhibition concentration (IMC)[1].

Fungicides	**Concentrations of μg/ml^{-1}**					
	0.1	**0.5**	**1**	**5**	**10**	**IMC**
Azoxistrobina	27.15%	33.71%	32.35%	38.46%	39.37%	>10
Tebuconazole	10.9%	32.47%	33.94%	52.49%	59.73%	>1
Tiofanato metílico	12.33%	71.27%	72.06%	94.34%	94.34%	<1

Source: own authorship.
[1]Interval between concentrations, in which values of 90 – 100% inhibition of mycelial growth can be found.

The minimum inhibition concentration for the fungicides tebuconazole and methyl thyophanate tested in the species *C. karstii* presented a value lower than 1 μg/ml^{-1}, with

mycelial inhibition of 55.44% to 91.08% for tebuconazole and 69.82% to 91.08% in methyl thyophanate. The fungicide azoxistrobin presented an IMC value above 10 μg/ml^{-1} and obtained the lowest percentage of inhibition of mycelial growth (Chart 6). Lima (2013) evaluating the sensitivity of the fungicide azoxistrobin in *C. karstii*, observed that among the fungicides tested azoxistrobin provided a lower percentage of inhibition of mycelial growth, being 8.8%, thus confirming the results obtained for *C. karstii*.

Chart 6 - Mean percentage values of inhibition of mycelial growth of *C. karstii* and the minimum inhibition concentration (IMC)[1].

Fungicides	**Concentrations of μg/ml^{-1}**					
	0.1	**0.5**	**1**	**5**	**10**	**IMC**
Azoxistrobina	17.83%	27.81%	28.88%	42.42%	43.49%	>10
Tebuconazole	24.6%	55.44%	65.6%	85.92%	91.08%	<1
Tiofanato metílico	8.2%	69.82%	82.89%	91.08%	91.08%	<1

Source: own authorship.

[1]Interval between concentrations, in which values of 90 – 100% inhibition of mycelial growth can be found.

Oliveira (2018) evaluating the sensitivity of fungicides azoxistrobin, tebuconazole and methyl thiophanate in species of *C. theobromicola*, *C. fructicola*, *C. siamense* and *C. karstii* from *Annona squamosa* and *Annona muricata*, obtained a value of EC_{50}, where all species were highly sensitive to fungicides tebuconazole and methyl thiophanate, but for the fungicide azoxistrobin the species *C. siamense* was insensitive.

A study conducted by Pereira (2018), evaluating the sensitivity to fungicides of *Colletotrichum* isolates from anthracnose symptoms in guava, observed that the species *Colletotrichum gloeosporioides* was highly sensitive to tebuconazole, in *in vitro* condition, thus contributing to the results obtained in the present study, where all species were highly sensitive to the tebuconazole fungicide.

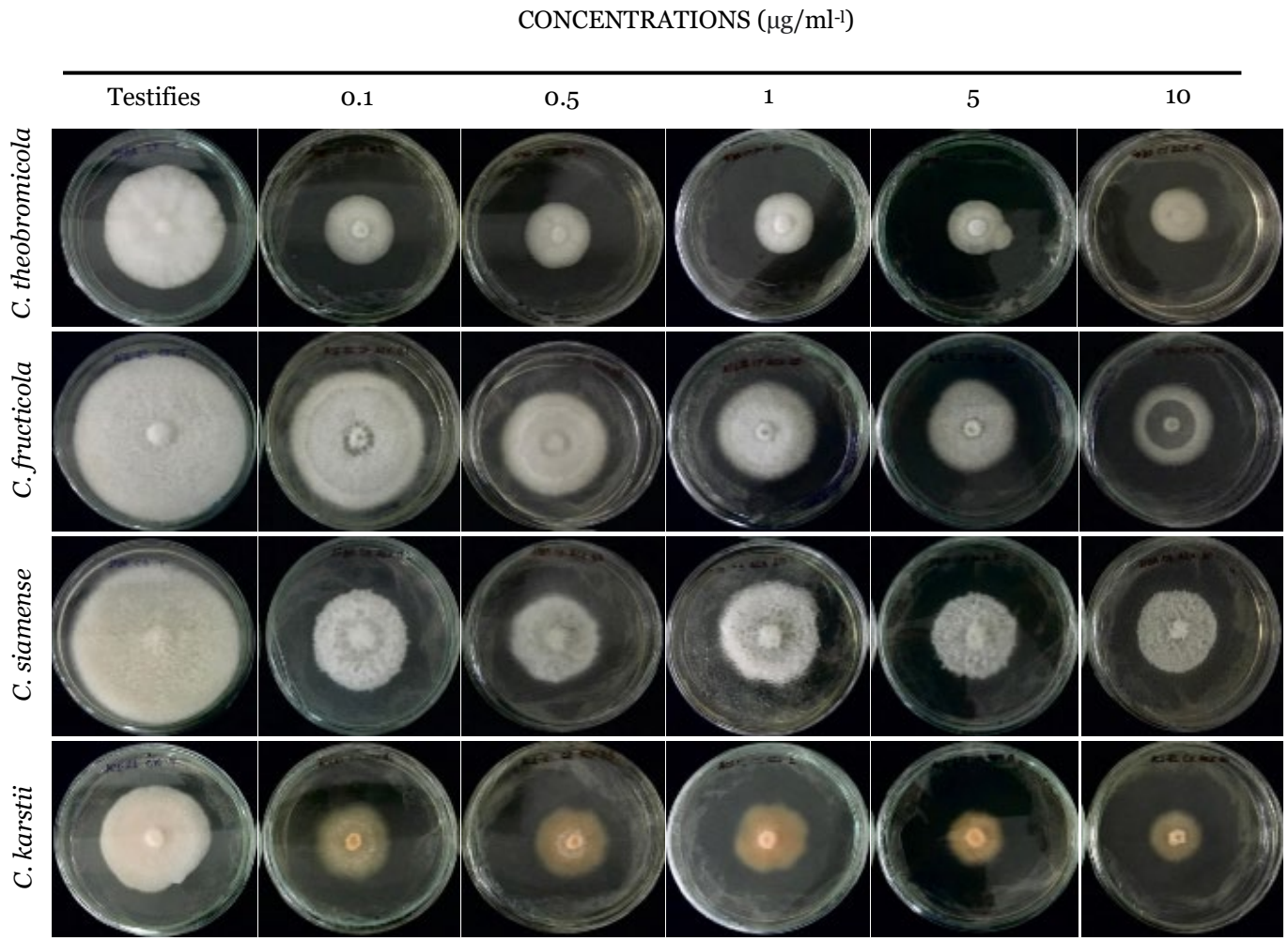
The fungicide methyl tiofanato was efficient in the *in vitro* control of *Colletotrichum* species tested in the present study, where other studies obtained similar results such as Celoto et al. (2011), which evaluating the sensitivity of *C. musae*, obtained from banana crop, reported that this fungicide was efficient in inhibiting mycelial growth and spore germination, having an EC_{50} lower than 1 μg/ml^{-1}, thus being classified with high efficiency.

According to this work, the fungicide azoxistrobin proved inefficient for the control of *C. siamense*, where this resistance may be related to the use of this fungicide in production areas for the control of anthracnose allowing the pathogen to develop resistance to fungicide, but showed a considerable level of control for the species *C. fruticola*, *C. karstii* and *C.*

theobromicola, thus showing that the results were different in relation to the species. Tozze Junior (2007) also obtained a similar difference in relation to fungicide action on species, where in relation to the species *C. acutatum* EC_{50} was below 1 μg/ml^{-1}, however, for *C. gloeosporioides* the EC_{50} was higher than 1000 μg/ml^{-1}, thus showing a resistance between the species of *Colletotrichum* to the fungicide azoxistrobina.

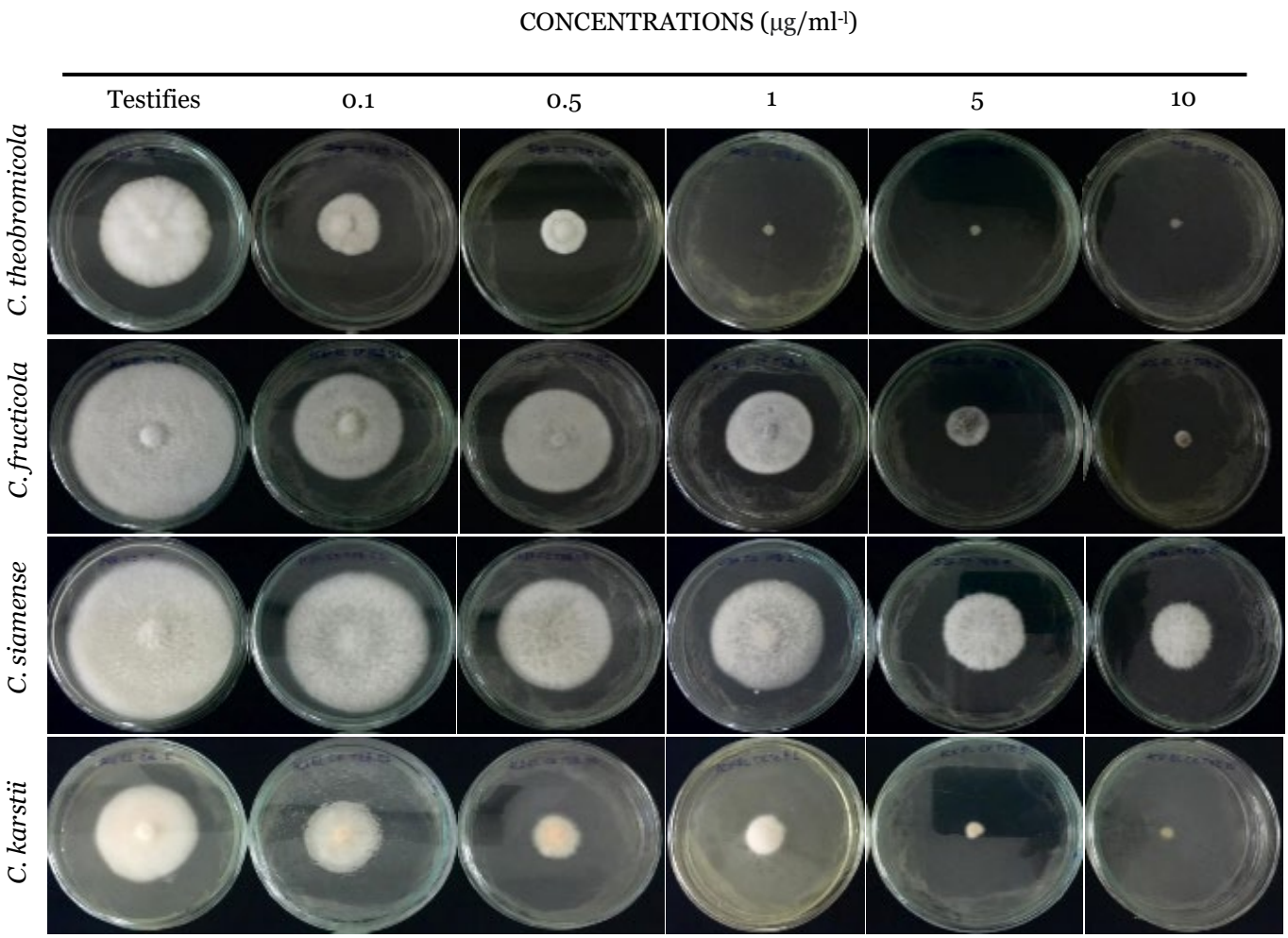
A reduction in mycelial growth of *Colletotrichum* species was observed that were submitted to different concentrations of fungicides azoxystrobin, tebuconazole and methyl thiophanate (Figure 1, 2 and 3).

Figure 1. Effect of the concentrations of fungicide azoxystrobin on the mycelial growth of *Colletotrichum*.



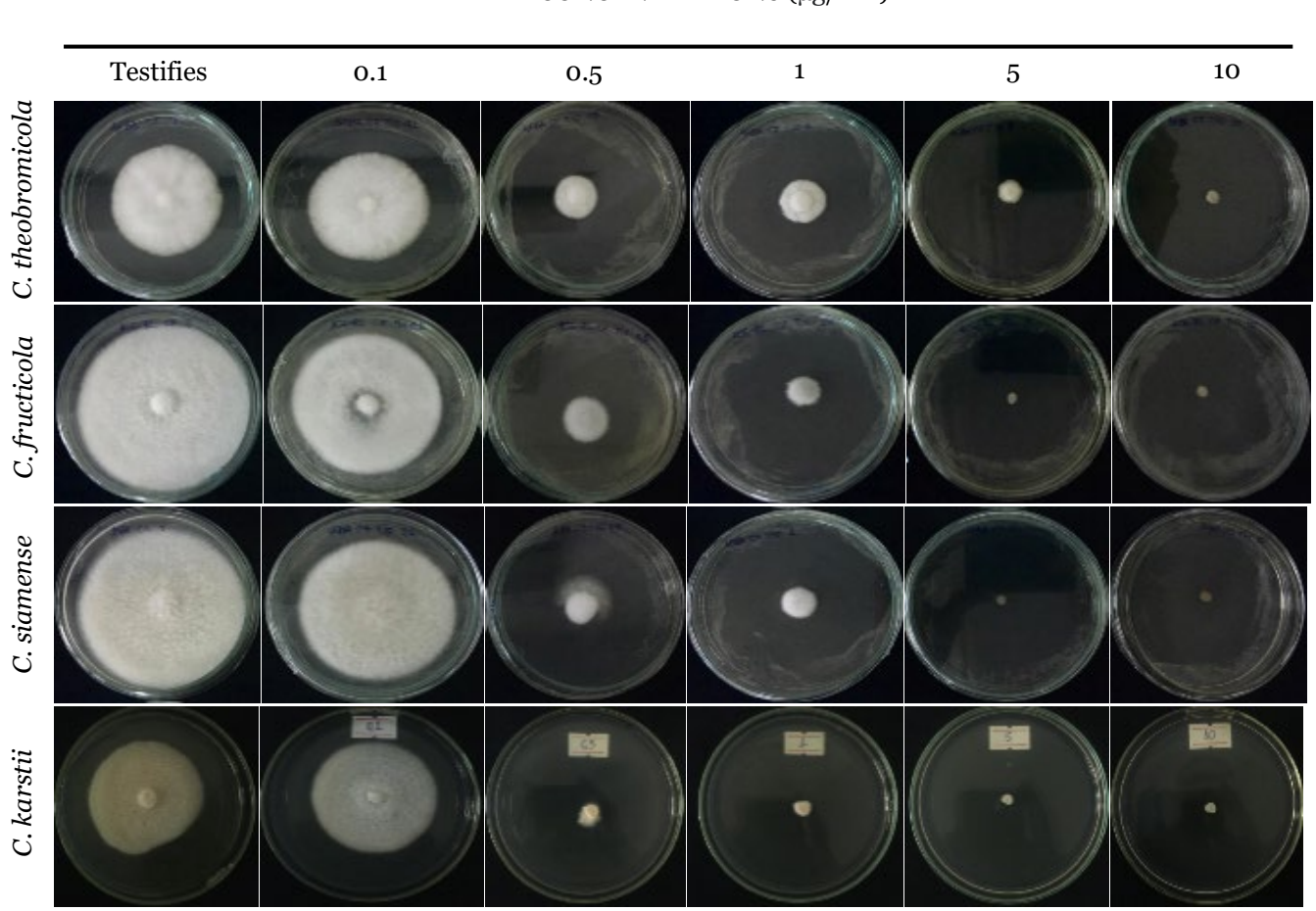
Source: own authorship.

Figure 2. Effect of the fungicide tebuconazole concentrations on the mycelial growth of *Colletotrichum* species.



Source: own authorship.

Figure 3. Effect of concentrations of methyl thiophanate fungicide on mycelial growth of *Colletotrichum*.



Source: own authorship.

The results obtained in this work can be used as a basis to establish strategies to control anthracnose disease, in the culture of atemoya, under field conditions, thus enabling the use of the best fungicides and concentrations, in addition, it avoids the use of fungicides with the same active ingredient.

Conclusions

The species *C. theobromicola*, *C. fructicola*, *C. siamense* and *C. karstii* were highly sensitive to the fungicides tebuconazole and methyl thiophanate.

The fungicide azoxistrobin was efficient in the *in vitro* control of the species *C. theobromicola*, but proved inefficient for the control of the species *C. siamense*.

The species *C. fructicola* and *C. karstii* were moderately sensitive to the fungicide azoxistrobin.

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