Crop Protection 144 (2021) 105554

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Microtiter method to monitor *Corynespora cassiicola* and sensitivity of the pathogen to carbendazim, prothioconazole and pyraclostrobin

Sheila Ariana Xavier [a,*], Flávia Elis de Mello [a], Helen Prudente da Silva [a], Marcelo Giovanetti Canteri [a], Lucimara Junko Koga [b], Ivani de Oliveira Negrão Lopes [c], Claudia Vieira Godoy [c]

[a] Departamento de Agronomia, Universidade Estadual de Londrina, Londrina, PR, 86051-970, Brazil
[b] Corteva Agriscience, Barueri, SP, 06454-080, Brazil
[c] Embrapa Soja, Londrina, PR, 86001-970, Brazil

ARTICLE INFO

Keywords:
Target spot
Benzimidazole
Triazolinthione
Methoxy-carbamates
Sensitivity to fungicide
EC50

ABSTRACT

The fungus *Corynespora cassiicola* causes target spot on soybeans in Brazil and one of the recommended controls for this disease involves the use of fungicides. Here, we evaluated the sensitivity of *C. cassiicola* isolates to fungicides, comparing the effective concentrations required to inhibit 50% of fungal growth (EC50) using a colorimetric microtiter method, with the EC50 determined by mycelial growth inhibition in a fungicide-amended medium obtained by Xavier et al. (2013), for 16 isolates for carbendazim and prothioconazole. The correlation between EC50 values for both methods were 0.90 and 0.88 for carbendazim and prothioconazole, respectively. After this, the sensitivities of 134 *C. cassiicola* isolates collected in soybean fields in Paraná (PR) and Mato Grosso (MT) in the 2012/13 and 2013/14 crop seasons to carbendazim, prothioconazole, and pyraclostrobin fungicides were evaluated using microtiter method. The spores of the isolates were diluted in Yeast Bacto Acetate culture medium and added to fungicide solutions at concentrations of zero; 0.0032; 0.016; 0.08; 0.4; 2; 10; and 50 μg mL^{-1}. A microplate reader with a 540 nm wavelength was used to estimate the EC50 values. Isolates showed growth inhibition at concentrations higher than 50 μg mL^{-1}, showing a high frequency of resistant isolates, in total, 67% of the isolates from PR and 88% from MT (carbendazim); 99% from PR and 82% from MT (pyraclostrobin); and 11% from PR and 9% from MT (prothioconazole). The results showed that the lower control efficiency for target spot in soybeans in PR and MT to MBC and QoI fungicides could be associated with the presence of resistant isolates.

1. Introduction

Target spot caused by the fungus *Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei was first reported on soybeans in Brazil in 1976 in the Paraná and São Paulo states (Almeida et al., 1976). In 1989, the disease was reported in the Mato Grosso, Mato Grosso do Sul, and Rio Grande do Sul states (Yorinori, 1988). In recent years, the target spot severity has increased and the disease can now be found in most soybean-growing regions in Brazil (Godoy et al., 2016). The disease affects the leaves, stems, pods, seeds, hypocotyls, and roots. Leaf lesions are round to irregular and reddish-brown, frequently surrounded by yellowish halos. Severely affected leaves drop prematurely. The recommended management strategies include the use of resistant cultivars; seed treatments; crop rotation or succession cropping using corn or other grass species; and chemical control with fungicides (Godoy et al., 2016).

Despite the contribution of site-specific fungicides to disease control, their intensive use may result in the selection of fungi isolates that are less sensitive or resistant (Brent, 2011). In Brazil, fungicide resistance has been detected in soybean crops after the failure of control in the field. Among the most-used fungicide modes of action for soybeans in Brazil are methyl benzimidazole carbamates (MBC), demethylation inhibitors (DMI), quinone outside inhibitors (QoI), and succinate dehydrogenase inhibitor (SDHI) compounds (Godoy et al., 2016).

Results from 2012 to 2016 showed a lower efficiency of the fungicide carbendazim in target spot control relative to other fungicides (Molina et al., 2019). The lower efficacy has been associated with the presence of isolates of the fungi that are less sensitive to MBC fungicides. Resistance of *C. cassiicola* isolated from soybean leaves to MBC fungicides has been

* Corresponding author.
E-mail address: sa_xavier@yahoo.com.br (S.A. Xavier).

https://doi.org/10.1016/j.cropro.2021.105554
Received 29 July 2019; Received in revised form 28 June 2020; Accepted 18 January 2021
Available online 2 February 2021

reported in recent years in Brazil in different growing regions including the Paraná, Mato Grosso, and Goiás states (Avozani, 2011; Xavier et al., 2013; Teramoto et al., 2017).

Due to the high risk of pathogen populations developing resistance to site–specific fungicides, rapid detection in commercial fields is essential for disease management (FRAC, 2018). A commonly used method to measure fungicide sensitivity in a nonobligate pathogen population is to determine the dosage at which the colony diameter in fungicide-amended medium is reduced by half (EC50) (Dekker, 1987; Georgopoulos, 1982). However, the mycelial inhibition growth method on medium requires a large amount of medium and space and is time-consuming, and only linear growth is measured, while the density of the colony is not considered (Dekker, 1987). Another accurate technique is to measure the dry weight inhibition in liquid medium, but this method requires even more space, time, and material (Georgopoulos, 1982). An automated quantitative assay using microtiter plates has been widely recommended by Fungicide resistance action committee (FRAC) for monitoring resistance to several pathogens (Stammler and Speakman, 2006; Stammler et al., 2007; Rampersad, 2011; Vega et al., 2012).

The microtiter method consists of growing a fungus in a microplate well and measuring its growth spectrophotometrically (Broekaert et al., 1990; Ludwig and Boller, 1990). This method allows automatic measurement of the fungal biomass increase in the presence of a fungicide, and a large number of isolates can be assessed simultaneously, overcoming the disadvantages of the mycelial inhibition growth assay. The assay is accurate, easy and reliable, and these features make the method very suitable for the rapid measurement of fungicide resistance in a fungal population (Raposo et al., 2015).

Raposo et al. (2015) studied the use of the microtiter assay to determine the sensitivity of *Botrytis cinerea* to fungicides and reported a positive correlation to the EC50 determined using mycelial growth. The volume in the well should be between 75 μL and 200 μL, and the initial spore concentration should be between 10 and 100 spores per microliter.

This study aimed to evaluate the efficiency of the colorimetric microtiter method in measuring *C. cassiicola* sensitivity to prothioconazole (DMI) and carbendazim (MBC), comparing the results with previous studies (Xavier et al., 2013). In the sequence, it aimed to determine the sensitivity of isolates collected in the Paraná (PR) and Mato Grosso (MT) states in Brazil during the growing seasons of 2012/13 and 2013/14 to the fungicides carbendazim (MBC), prothioconazole (DMI), and pyraclostrobin (QoI).

2. Material and methods

The efficiency of the colorimetric microtiter method in measuring the sensitivity of *C. cassiicola* to prothioconazole and carbendazim fungicides was compared to the inhibition of radial mycelial growth (MGI) in a fungicide-amended medium method using 16 *C. cassiicola* monosporic isolates selected from 24 previously used by Xavier et al. (2013). Pyraclostrobin was not evaluated by Xavier et al. (2013). The isolates were obtained from infected soybean leaflets and stem in different regions of Brazil and old isolates from Embrapa Soybean Mycological Collection (ESMC). All isolates were grown in potato-dextrose-agar (PDA - 20 g of dextrose, 15 g of agar, and an infusion of 200 g of potato in 1 L of distilled water) medium and stored at 5 °C.

To monitor the sensitivity of *C. cassiicola* to fungicides, isolates were obtained from infected soybean plants in the PR (101) and MT (33) Brazilian states during the crop seasons of 2012/13 and 2013/14 (Table 1). The isolations were performed on the lesions of leaflets with symptoms typical of target spot. Small fragments of infected tissue were disinfected using a 70% alcohol solution for 60 s, 0.1% sodium hypochlorite for 60 s, and distilled water for 60 s. The fragments were transferred to Petri dishes with a 9-cm diameter containing PDA culture medium.

A total of 8 mL of Yeast Bacto Acetate (YBA) culture medium (10 g yeast extract, 10 g bacto peptone, 20 g sodium acetate: 82.03 placed in 500 mL of distilled water and autoclaved) was added to the fungal colony after 10 days, then rubbed using a sterile glass slide, aiming to collect *C. cassiicola* spores. The spore suspension obtained was filtered on sterile gauze, and the concentration was adjusted to 10^5 spores mL^{-1} with the aid of a hemocytometer. Streptomycin (0.03 g L^{-1}) was added to the YBA medium to reduce bacterial contamination.

Table 1
Identification code for the *Corynespora cassiicola* isolates from the Embrapa Soybean Mycological Collection (ESMC).

Isolate	Year	City	State	Number of isolates
1163	2013	Londrina	PR	5
1196	2013	Boa Esperança	PR	5
1197	2013	Campo Mourão	PR	5
1198	2013	Janiopolis	PR	5
1205	2013	Arapongas	PR	5
1412	2014	Tamarana	PR	5
1415	2014	Mauá da Serra	PR	5
1424	2014	Cascavel	PR	1
1434	2014	Boa Esperança	PR	5
1443	2014	Cruz Maltina	PR	5
1449	2014	Paiquerê	PR	5
1469	2014	Arapuã	PR	5
1472	2014	Londrina	PR	5
1713	2013	Janiopolis	PR	5
2313	2013	Boa Esperança	PR	5
2713	2013	São José dos Pinhais	PR	5
6413	2013	Arapongas	PR	5
6613	2013	Mauá da Serra	PR	5
6913	2013	Grandes Rios	PR	5
8613	2013	Tamarana	PR	5
9513	2013	Cruzmaltina	PR	5
6313	2013	Jaciara	MT	5
5113	2013	Primavera do Leste	MT	1
5713	2013	Sorriso	MT	1
1460	2014	Aral Moreira	MT	1
1478	2014	Sorriso	MT	4
1473	2014	Nova Mutum	MT	5
1474	2014	Sorriso	MT	5
1479	2014	Nova Mutum	MT	5
1480	2014	Sorriso	MT	5
1485	2014	Rio Verde	MT	1

Commercial formulations of the fungicides carbendazim [50% active ingredient (a.i.), Carbendazim Nortox®; Nortox SA], prothioconazole (48% a.i, Proline®, Bayer CropScience), and pyraclostrobin (25% a.i, Comet®, BASF) were used in the tests.

The fungicides were tested at concentrations of zero; 0.0032; 0.016; 0.08; 0.4; 2; 10 and 50 μg mL^{-1}. The fungicides were previously prepared in stock solutions diluted with water to the concentration of 300 μg mL^{-1}, followed by the use of serial dilutions until the final concentrations were reached.

The sensitivity of each isolate to the fungicides was estimated using a colorimetric microtiter test. A 50 μL of the 10^5-spore mL^{-1} suspension was added to each well of the microtiter plate (96-well sterile polystyrene microplates, Kasvi), followed by the addition of 50 μL of the different concentrations of the fungicide products, with a final volume of 100 μL. The concentrations were distributed into lines (8 concentrations) and the isolates into columns, with 4 replicates per isolate (4 columns). Each microplate contained a white control (50 μL of YBA + 50 μL of the different concentrations of the fungicide products). The microplates were capped, wrapped and placed on a shaker table for 1 h to allow mixing of the products with the spore suspension. The microplates were conditioned in a growth chamber at 25 °C with a photoperiod of 12 h for seven days. The experiments were repeated.

The absorbance was measured on a microplate reader (ASYS, Eugendorf, Austria) at a wavelength of 540 nm. The final absorbance value was calculated by subtracting the absorbance of the control from the absorbance of the isolates.

To evaluate the efficiency of the colorimetric microtiter method

compared to mycelial growth inhibition, the EC50 values were related to the values obtained by Xavier et al. (2013). For correlation, when it was not possible to estimate the EC50 because the values occurred below and above the highest doses evaluated (0.0032 μg mL^{-1} and 50 μg mL^{-1}, respectively) the values were set as the limits.

For monitoring study, the EC50 values for each isolate were estimated by a linear regression probit model, as measured by the relative percentage of the control absorbance to the absorbance of the blank reading and the logarithm base 10 of the concentrations (doses) of the products. The existence of interactions between the experiments was verified, and in the absence of interactions, the data were pooled together. The probit model parameters and EC50 estimates were obtained using the proc probit function of the SAS® System Version 9.1.3 (SAS/STAT, 2003). Pearson's chi-squared coefficient was used to verify the quality of the fit ($p > 0.01$).

3. Results

3.1. Efficiency of the colorimetric microtiter method in measuring the sensitivity of C. cassiicola to fungicides

The EC50 values obtained by the colorimetric microtiter method were compared to MGI (Xavier et al., 2013). The correlations (r) of the fungicides carbendazim and prothioconazole were 0.90 and 0.88, respectively. For pyraclostrobin correlation was not obtained, because this fungicide was not tested in MGI by Xavier et al. (2013).

For carbendazim, the estimated EC50 ranged from 0.0045 μg mL^{-1} to 6.5 μg mL^{-1}, and five isolates grew above the maximum dose (Table 2). Based on the sensitivity differences, the tested isolates were divided in highly resistant (HR), EC50 ≥ 10 μg mL^{-1}; moderately resistant (MR), 1 μg mL^{-1} < EC50 < 10 μg mL^{-1}; and sensitive (S), EC50 ≤ 1.0 μg mL^{-1}. The EC50 values of the isolates ESMC 646, ESMC 930, ESMC 931, ESMC 932, and ESMC 933 occurred above 50 μg mL^{-1}, and these isolates were classified as highly resistant. The mean values of the EC50 of carbendazim for the isolates collected in 1997 (ESMC 310, ESMC 311, and ESMC 312), before the recommendation of fungicide in the field, were 0.0165 μg mL^{-1}, and these isolates were classified as sensitive (Table 2).

For prothioconazole, none of the isolates grew above 50 μg mL^{-1}. The estimated EC50 ranged from 0.00617 μg mL^{-1} to 19.4 μg mL^{-1}, by the colorimetric microtiter method. The EC50 values for the ESMC 311, ESMC 312, ESMC 318, ESMC 322, and ESMC 926 isolates occurred below the lowest dose tested (0.0032 μg mL^{-1}) (Table 2).

3.2. Sensitivity of C. cassiicola to the fungicides carbendazim, prothioconazole and pyraclostrobin

Resistance levels (moderate and high) were found for *C. cassiicola* isolates from the Paraná (PR) and Mato Grosso (MT) states, based on the isolate sensitivities to the fungicides carbendazim and pyraclostrobin.

For the fungicide carbendazim, the estimated EC50 ranged from 0.0045 μg mL^{-1} to 14.27 μg mL^{-1} for the PR state isolates and from 0.69 μg mL^{-1} to 2.66 μg mL^{-1} for the MT state isolates. The average of the EC50 of the isolates from 1997 using the colorimetric microtiter method was used as the sensitivity limit (0.0165 μg mL^{-1}). Therefore, 22% of the isolates collected in the state of PR had EC50 below 0.0165 μg mL^{-1}, 8% had EC50 between 0.017 μg mL^{-1} and 1 μg mL^{-1}, and 67% above 1 μg mL^{-1} or the highest dose tested (50 μg mL^{-1}) (Fig. 1A). Of the isolates

Table 2
Identification code for *Corynespora cassiicola* isolates from the Embrapa Soybean Mycological Collection (ESMC), including the year, city, state and country and the effective concentration required to reduce 50% (EC50 μg ml^{-1}) of the target spot severity for carbendazim and prothioconazole fungicides.

			carbendazim		prothioconazole	
Isolate	Year	City, State, Country	Colorimetric microtiter method (EC50)	MGI (EC50)[a]	Colorimetric microtiter method (EC50)	MGI (EC50)[a]
ESMC 310	1997	Sarandi, Paraná, BR	0.0160 S	0.767 S	0.0118	3.214
ESMC 311	1997	Campo Mourão, Paraná, BR	0.0045 S	1.034 MR	<0.0032	0.4724
ESMC 312	1997	Itiquira, Mato Grosso, BR	0.0290 S	0.613 S	<0.0032	1.933
ESMC 313	1998	Nova Mutum, Mato Grosso, BR	0.0203 S	1.051 MR	0.020	1.199
ESMC 317	1999	Palotina, Paraná, Brasil	0.0053 S	0.570 S	0.0061	0.824
ESMC 318	1999	Campo Novos dos Parecis, Mato Grosso, BR	0.0072 S	0.302 S	<0.0032	1.264
ESMC 322	2001	Nova Ventura de São Roque, PR, BR	0.2958 S	0.700 S	<0.0032	3.306
ESMC 629	2007	Campo Verde, Mato Grosso, BR	0.0222 S	0.608 S	0.134	0.588
ESMC 646	2008	Campos Novos dos Parecis, MT, BR	>50 HR	575.06 HR	0.21698	0.811
ESMC 649	2008	Campo Mourão, Paraná, BR	6.505 MR	<0.5 S	0.368	1.149
ESMC 692	2008	Corpus Christi, Canindeyú, PY	0.1444 S	0.395 S	0.277	1.858
ESMC 926	2011	Londrina, Paraná, BR	0.0195 S	0.999 S	<0.0032	0.971
ESMC 930	2011	Rolândia, Paraná, BR	>50 HR	>1000 HR	19.40	11.049
ESMC 931	2011	Mauá da Serra, Paraná, BR	>50 HR	10.19 MR	14.37	18.092
ESMC 932	2011	Londrina, Paraná, BR	>50 HR	165.10 HR	6.28	4.437
ESMC 933	2011	Deciolândia, Mato Grosso, BR	>50 HR	>1000 HR	2.25	1.423

[a] Data extracted from Xavier et al. (2013). Inhibition from the radial mycelial growth method (MGI); HR – Highly resistant, EC50 ≥ 10 μg mL^{-1}; MR – Moderately resistant, EC50 1 μg mL^{-1} < EC50 < 10 μg mL^{-1}; S - Sensitive, EC50 ≤ 1 μg mL^{-1}. BR – Brazil, PY – Paraguay.

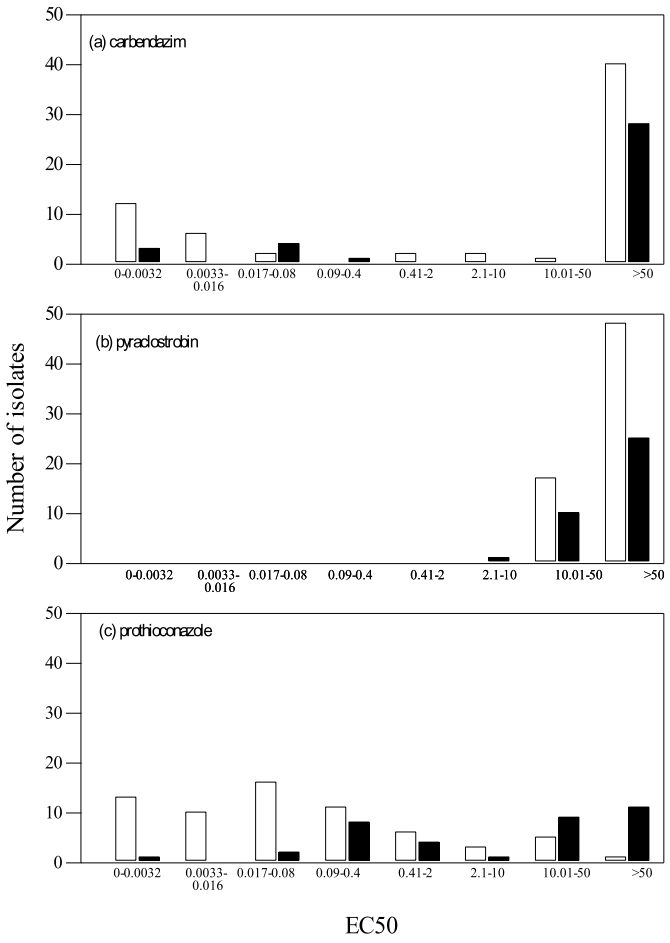

Fig. 1. Distributions of the effective concentrations of carbendazim (a), pyraclostrobin (b) and prothioconazole (c) that reduce 50% of the mycelial growth (EC50 - μg/mL) in a collection of 101 isolates of Corynespora cassiicola from soybeans collected from 2013 to 2014 in the Paraná state, Brazil.

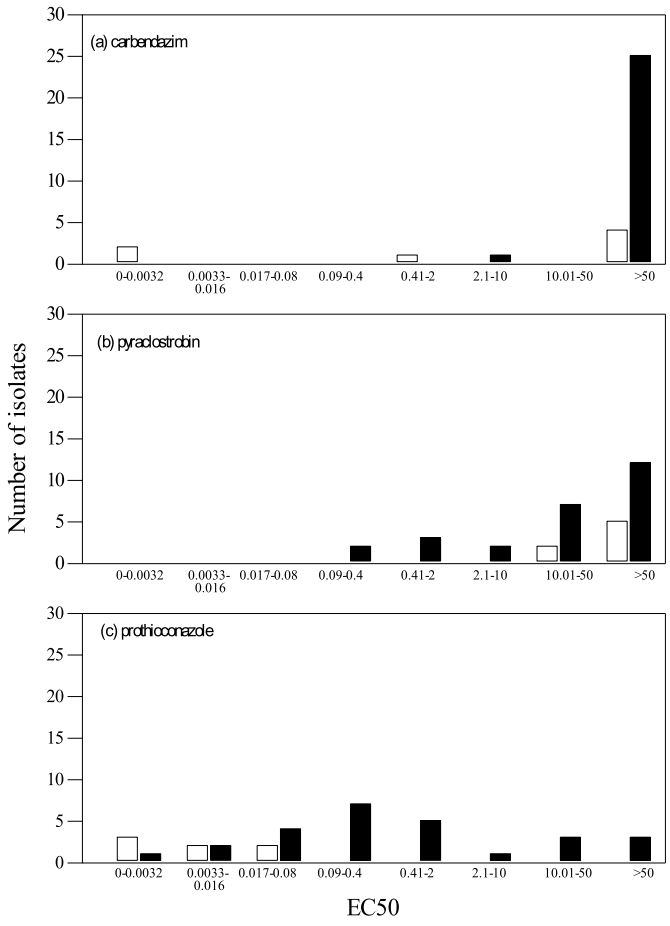

Fig. 2. Distributions of the effective concentrations of carbendazim (a), pyraclostrobin (b) and prothioconazole (c) that reduce 50% of the mycelial growth (EC50 - μg/mL) in a collection of 33 isolates of Corynespora cassiicola from soybeans collected from 2013 to 2014 in the Mato Grosso state, Brazil.

evaluated in the MT state, 88% had EC50 value above the highest dose tested (50 μg mL^{-1}), 2% below 0.0165 μg mL^{-1}, and 2% between 0.017 μg mL^{-1} and 2.66 μg mL^{-1} (Fig. 2A).

The EC50 estimated for pyraclostrobin ranged from 4.17 μg mL^{-1} to 45.65 μg mL^{-1} for isolates collected in the PR state and from 0.29 μg mL^{-1} to 20.43 μg mL^{-1} for the isolates collected in the MT state. In PR, 72% of the isolates had EC50 above the highest dose tested (50 μg mL^{-1}) and 27% had EC50 above 10 μg mL^{-1}, showing a highly resistant pattern (Fig. 1B). For the isolates collected in the MT state, 82% had EC50 above 10 μg mL^{-1} or above the highest dose tested (50 μg mL^{-1}), 6% had $1 <$ EC50 < 10, and 12% had EC50 below 1 μg mL^{-1} (Fig. 2B).

For the prothioconazole fungicide 75% of the isolates collected in the PR state (Fig. 1C) and 76% of the isolates collected in the MT state (Fig. 2C) had EC50 values < 1 μg mL^{-1}. The EC50 for prothioconazole ranged from 0.0033 μg mL^{-1} to 45.11 μg mL^{-1} for the isolates collected in the Paraná state and from 0.0044 μg mL^{-1} to 17.70 μg mL^{-1} for the isolates collected in the MT state.

For all active ingredients, the isolates collected in the same municipality showed a similar sensitivity pattern.

4. Discussion

4.1. Efficiency of the colorimetric microtiter method in measuring the sensitivity of C. cassiicola to fungicides

The EC50 values obtained by the colorimetric microtiter method were lower than the values determined by the MGI method presented by Xavier et al. (2013) being on average 36 times lower for carbendazim and 288 times lower for prothioconazole. According to Raposo et al. (2015), this was probably due to the use of the liquid nutrient medium, which affects the dose-response relationship between the fungus and each fungicide concentration by increasing the contact surface between the fungus and the spore. Moreover, in the method used in this study, the fungal spores are completely immersed in the solution containing the fungicide, which is unlike the MGI method, in which a part of the colony of the fungus is on the fungicide solid culture medium.

In both methodologies, the isolates with the highest resistances to carbendazim fungicide were collected in 2008 in the state of MT (CMES 646) and in 2011 in the states of PR and MT (ESMC 930, 931, 932 and 933). The ESMC 649 isolate showed moderate resistance to carbendazim in the colorimetric microtiter test and was classified as sensitive by the MGI method (Xavier et al., 2013). This variation may have occurred because of the variability of *C. cassiicola* and because the assays were not performed using the isolates at the same time. Fungi with asexual reproduction, such as *C. cassiicola*, do not undergo regular recombination and genetic variation, resulting in mainly spontaneous mutations (Taylor et al., 1999), which may occur during the process of fungus multiplication in the laboratory. Another hypothesis explaining this difference involves the culture medium used in each methodology. In the colorimetric microtiter test, YBA liquid medium was used, while in the MGI method, the isolates were grown in PDA culture medium. According to Griffin (1993), the culture medium can produce distinct behaviors in isolates, since the isolates can metabolize nutrients at different speeds, causing the synthesis of other molecules or products

that may influence fungus growth.

The isolates with the highest values of EC50 (CMES 930, 931, 932, 933) for prothioconazole determined by the colorimetric microtiter method were the same isolates determined to have the highest values by the MGI method. Prothioconazole was used in soybeans for the first time in Brazil in 2010, and the highest values observed for populations in 2011 may indicate a trend of sensitivity change in the fungus compared to the populations of previous years. Resistance to DMI fungicides occurs gradually and is described as a sensitivity change (Brent, 2011). Although cross-resistance may occur within the group, and though other DMIs have been intensively used in soybean cultivation since 2001, prothioconazole has a much lower resistance factor than do other DMIs (Brent and Hollomon, 2007).

The colorimetric microtiter method has been recommended by FRAC to test the sensitivities of *Botrytis cinerea*, *Fusarium graminearum*, *Microdochium nivale*, *Phytophthora infestans*, *Plasmopara viticola*, *Oculimacula* spp., *Pyrenophora teres*, *Pyrenophora tritici-repentis*, *Rhynchosporium secalis* and *Mycosphaerella graminicola* to different groups of fungicides (FRAC, 1991). This method has been used as a simple, accurate, and fast method capable of processing a large number of samples in a short period of time (Rampersad, 2011; Raposo et al., 2015; Stammler and Speakman, 2006). Microbial contamination in microplate cells can occur and can be detected by an abnormal increase in absorbance. For this reason, replications in microplate cells are recommended.

Due to the correlation obtained between the two methods, (0.90 and 0.88 for carbendazim and prothioconazole, respectively), it is concluded that the colorimetric microtiter method could be used to determine the sensitivity of *C. cassiicola* isolates to fungicides.

4.2. Sensitivity of C. cassiicola to the fungicides carbendazim, prothioconazole and pyraclostrobin

In the present study, the sensitivity of *C. cassicola* to fungicides was monitored in populations of the fungus from the states of Paraná and Mato Grosso, as these are the main soybean producing states in Brazil, with intense fungicide application, with an average of two to three applications during a growth cycle (Kleffmann, 2012).

The resistance of *C. cassiicola* isolated from soybean leaflets toward MBC fungicides has been reported in recent years in Brazil in different producing regions (Avozani, 2011; Teramoto et al., 2017; Xavier et al., 2013). Despite the low efficiency, MBCs represented 6% of the recommendations for the control of diseases in soybeans in 2012 in Brazil, corresponding to 10.6 thousand tons of commercialized fungicides (Kleffmann, 2012).

Avozani (2011) studied the sensitivity of five isolates of *C. cassiicola* from soybeans (three from Mato Grosso, one from Minas Gerais and one from Rondônia, Brazil) toward carbendazim. The author observed the EC50 values of 0.2 μg mL $^{-1}$ and 0.26 μg mL^{-1} for isolates collected in the states of Minas Gerais and Rondônia, respectively, and a value higher than 40 μg mL^{-1} (the highest dose evaluated) for the Mato Grosso isolate, highlighting the occurrence of isolates resistant to MBC fungicides in the Mato Grosso state.

In a study of the sensitivity of 24 isolates of *C. cassiicola*, conducted by Xavier et al. (2013), the authors observed isolates highly resistant to carbendazim (EC50 $\geq$ 50 μg mL^{-1}) collected in the state of Mato Grosso in 2008 and of Paraná and Mato Grosso in 2011. The data obtained in this research using isolates of *C. cassiicola* collected in the years of 2013 and 2014 reinforce the presence of high frequency of isolates resistant to carbendazim in Paraná (67%) and Mato Grosso (87%). The high frequency of resistant *C. cassiicola* isolates reflects in the low efficiency of the fungicide to control target spot in the meta-analysis data from trials conducted in the years 2012–2016 (Molina et al., 2019).

Date et al. (2004) tested the sensitivity of 193 isolates of *C. cassiicola* isolated from cucumbers using the minimum inhibitory concentration method and concluded that 29 isolates were highly resistant to thiophanate methyl, which belongs to the MBCs group, and that one isolate was highly resistant to azoxystrobin, which belongs to the QoI group. Detection of the reduction of the sensitivity of *C. cassiicola* to MBCs was also found in a tomato crop in Okayama, Japan.

In this research, *C. cassiicola* populations isolated from soybeans had a high frequency of isolates considered resistant to the fungicide pyraclostrobin (QoI) (99% of isolates from Paraná state and 82% from the Mato Grosso state). Three point mutations mediated field resistance to QoI. Mutations with amino acid substitutions from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R). The G143A has the strongest effect on the resistance and is the most widely spread in number and frequency among the mutations reported in pathogens species (Gisi et al., 2002; Sierotzki, 2015). Resistance levels in G143A mutation are high and have a significant impact on disease control (Sierotzki, 2015). In *C. cassiicola* isolated from soybean, the presence of G143A mutation was detected in a significant number of samples from Brazil in 2015 and 2016 (FRAC, 2018).

Teramoto et al. (2017) evaluated the sensitivity of six isolates of *C. cassiicola* isolated from cucumbers (five from São Paulo and one from Goiás, Brazil) using the MGI methodology and found values of EC50 $>$ 50 μg mL^{-1} for azoxystrobin (QoI), carbendazim and thiophanate (MBC) and EC50 values $<$ 50 μg mL^{-1} for difenoconazole and tebuconazole (DMI).

Prothioconazole was the fungicide with more EC50 values below 1 μg mL^{-1} in this study (75% in Paraná, and 76% in Mato Grosso). The results from fungicides trials from 2012 to 2016 showed an intermediate efficiency of the fungicide prothioconazole + trifloxystrobin (66.5% of control) in target spot control relative to other fungicides. Values of EC50 $>$ 10 μg mL^{-1} were observed less frequently in Paraná and Mato Grosso compared to other fungicides.

In the present study, isolates were classified as resistant using colorimetric microtiter method. Monitoring the fungi resistance to fungicides should be constant to evaluated changes in pattern of the populations due to the introduction of new fungicides and changes in the production systems.

5. Conclusions

The colorimetric microtiter methodology proved to be adequate to determine the sensitivity of *C. cassiicola* to the fungicides carbendazim and prothioconazole. The results showed a high frequency of isolates resistant to carbendazim and azoxystrobin in Parana and Mato Grosso in 2012/13 and 2013/14.

CRediT authorship contribution statement

Sheila Ariana Xavier: Conceptualization, Methodology, Validation, Writing - review & editing. **Flávia Elis de Mello:** Methodology, Validation. **Helen Prudente da Silva:** Methodology, Validation. **Marcelo Giovanetti Canteri:** Conceptualization, Writing. **Lucimara Junko Koga:** Methodology. **Ivani de Oliveira Negrão Lopes:** Software, Formal analysis. **Claudia Vieira Godoy:** Term, Conceptualization, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The first author thanks the National Council for Scientific and Technological Development – CNPq for providing a research fellowship.

References

Almeida, A.M.R., Machado, C.C., Ferreira, L.P., Lehman, O.S., Antonio, H., 1976. Ocorrência de *Corynespora cassiicola* (Berk. & Curt.) Weino Estado de São Paulo. Fitopatol. Bras. 1, 111–112.

Avozani, A., 2011. Sensibilidade de Corynespora cassiicola, isolados da soja, a fungicidas in vitro. Passo Fundo, Brasil: Faculdade de Agronomia e medicina Veterinária. MSc Thesis. http://tede.upf.br/jspui/handle/tede/510.

Brent, K.J., 2011. Historical perspective of fungicide resistance. In: Thind, S.T. (Ed.), Fungicide Resistance in Crop Protection, vol. 3. Punjab Agricultural University, India, p. 18.

Brent, K.J., Hollomon, D.J., 2007. Fungicide Resistance: the Assessment of Risk. FRAC, Global Crop Protection Federation, Brussels, pp. 1–53. Monograph No.2.

Broekaert, W.F., Terrras, F.R.G., Cammue, B.P.A., Vanderleyden, J., 1990. An automated quantitative assay for fungal growth inhibition. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 69, 55–60.

Date, H., Kataoka, E., Tanina, K., Sasaki, S., Inoue, K., Nasu, H., Kasuyama, S., 2004. Sensitivity of *Corynespora cassicola*, causal agent of Corynespora target spot of tomato (*Lycopersicon esculentum*), to thiophanate-methyl and diethofencarb. Ann. Phytopathol. Soc. Jpn. 70, 7–9.

Dekker, J., 1987. How to detect and measure fungicide resistance. In: Kranz, J., Rotem, J. (Eds.), Experimental Techniques in Plant Disease Epidemiology. Springer-Verlag, Berlim.

FRAC, 2018. Online publication. Fungicide resistance action committee. https://www.frac.info/docs/default-source/working-groups/qol-fungicides/qoi-meeting-minutes/minutes-of-the-2018-qoi-wg-meeting-and-recommendations-for-2019-telco-update-june-2019.pdf?sfvrsn=10f3489a_2.

Fungicide resistance action commitee, (FRAC), 1991. FRAC methods for monitoring fungicide resistance. EPPO Bull. 21, 291–354.

Georgopoulos, S.G., 1982. Detection and measurement of fungicide resistance. In: Dekker, J., Georgopoulos, S.G. (Eds.), Fungicide Resistance in Crop Protection. Wageningen Centre for Agricultural Publishing and Documentation, pp. 24–31.

Gisi, U., Sierotzki, H., Cook, A., Mccaffery, A., 2002. Mechanisms Influencing the Evolution of Resistance to Qo Inibitor Fungicides, vol. 58. Pest Management Science, West Sussex, pp. 859–867.

Godoy, C.V., Almeida, A.M.R., Costamilan, L.M., Meyer, M.C., Dias, W.P., Seixas, C.D.S., Soares, R.M., Henning, A.A., Yorinori, J.T., Ferreira, L.P., Silva, J.F.V., 2016. Doenças Da soja. In: Amorim, L., Rezende, J.A.M., Bergamin Filho, A., Camargo, L.E. A., Org (Eds.), Manual de Fitopatologia: v. 2. Doenças das plantas cultivadas. 5. São Paulo, Ceres, pp. 657–675, 2016.

Griffin, D.H., 1993. Fungal Physiology. John Wiley, New York, p. 458.

Kleffmann, G., 2012. Crop protection products - Foliar fungicides - AMIS soybean, 11, 12.

Ludwig, A., Boller, T., 1990. A method for the study of fungal growth inhibition by plant proteins. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 9, 61–66.

Molina, J.P.E., Paul, P.A., Amorim, L., Silva, L.H.C.P., Silva, L.H.C.P., Siqueri, F.V., Borges, E.P., Campos, H.D., Nunes Junior, J., Meyer, M.C., Martins, M.C., Balardin, R.S., Carlin, V.J., Grigolli, J.F.J., Belufi, L.M.R., Godoy, C.V., 2019. Meta-analysis of fungicied efficacy on soybean target spot and cost–benefit assessment. Plant Pathol. 68, 94–106.

Rampersad, S.N., 2011. A rapid colorimetric microtiter bioassay to evaluate fungicide sensitivity among *Verticillium dahliae* isolates. Plant Dis. 95, 248–255.

Raposo, R., Colgan, R., Delcan, J., Melgarejo, P., 2015. Aplication of na automated quantitative method to determine fungicide resistance in *Botrytis cinerea*. Plant Dis. 97, 294–296.

SAS/STAT, 2003. Version 9.1.3 of the SAS System for Windows, Copyright (C) 2002-2003. SAS Institute Inc., Cary, NC, USA.

Sierotzki, H., 2015. Respiration inhibitors: complex III. In: Ishii, H., Hollomon, D. (Eds.), Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. Springer, Japan, pp. 119–143.

Stammler, G., Benzinger, G., Speakman, J., 2007. A rapid and reliable method for monitoring the sensitivity of *Sclerotinia sclerotiorum* to boscalid. J. Phytopathol. 155, 746–748.

Stammler, G., Speakman, J., 2006. Microtiter method to test the sensitivity of *Botrytis cinerea* to boscalid. J. Phytopathol. 154, 508–510.

Taylor, J.W., Jacobson, D.J., Fisher, M.C., 1999. The evolution of asexual fungi: reproduction, speciation and classification. Annu. Rev. Phytopathol. 37, 197–246.

Teramoto, A., Meyer, M.C., Suassuna, N.D., Cunha, M.G., 2017. In vitro sensitivity of *Corynespora cassiicola* isolated from soybean to fungicides and field chemical control of target spot. Summa Phytopathol. 43, 281–289.

Vega, B., Liberti, D., Harmon, P.F., Dewdney, M.M., 2012. A rapid resazur in based microtiter assay to evaluate Qoi sensitivity for *Alternaria alternata* isolates and their molecular characterization. Plant Dis. 96, 1262–1270.

Xavier, S.A., Canteri, M.G., Barros, D.C.M., Godoy, C.V., 2013. Sensitivity of *Corynespora cassiicola* from soybean to carbendazim and protioconazol. Trop. Plant Pathol. 38, 431–435.

Yorinori, J.T., 1988. Levantamento e avaliação da situação de doenças da soja na safra 1987/88. In: Centro Nacional de Pesquisa de Soja (Londrina, PR). Resultados de pesquisa de soja 1988/89. EMBRAPACNPSO-Documentos, vol. 43, pp. 158–159.