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# Sensitivity of *Corynespora cassiicola* from soybean to carbendazim and prothioconazole

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**ABSTRACT**

The incidence of target spot disease on soybean has increased in recent years in Brazil even with intensive use of fungicides, and fungal resistance has been reported in recent studies. The objective of this study was to determine the fungicide sensitivity to carbendazim and prothioconazole in a sample of 24 isolates of *Corynespora cassiicola* from soybean collected from 1996 to 2011 in the states of Paraná, Mato Grosso and São Paulo (Brazil) and Corpus Christi (Paraguay). The 50% effective concentration ($EC_{50}$) values were estimated by the relative mycelium growth reduction on fungicide-amended medium with the doses of 0, 0.5, 1, 10, 100 and 1000 µg of active ingredient/mL. For carbendazim, four highly resistant isolates ($EC_{50}\geq50$ µg/mL) were observed from samples collected from Mato Grosso in 2008 and from Paraná and Mato Grosso in 2011. The $EC_{50}$ values for prothioconazole ranged from 0.47 µg/mL to 26.44 µg/mL (mean: 5.02 µg/ml). The results reinforce the occurrence of *C. cassiicola* resistance to benzimidazole in Paraná and Mato Grosso states.
**Key words:** benzimidazole, fungicide resistance, triazole.

The fungus *Corynespora cassiicola* (Berk. & M. A. Curtis) C.T. Wei infects more than 350 host plants from over 50 tropical and subtropical countries (Farr & Rossman, 2013). On soybean it causes the target spot disease and affects leaves, stems, pods, seeds, hypocotyls, and roots. Severely affected leaves drop prematurely (Sinclair, 1999). Since 1976, when it was first reported in Brazil (Almeida et al., 1976), target spot has occurred mainly in soybean growing regions with moderate temperatures, and in regions at high altitudes in the savannas. In recent years, target spot incidence has increased due to the over utilization of high yielding susceptible cultivars and the disease can now be found in most soybean growing regions.

Fungicides of Methyl Benzimidazole Carbamates (MBC), Quinone outside Inhibitors (QoI) and DeMethylation Inhibitors (DMI) groups are registered to control target spot in Brazil (Agrofit, 2013). Fungicides were first recommended on soybean in Brazil during the 1996/97 season to control powdery mildew caused by *Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takam., and some years later to control late season diseases caused by *Cerspora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner and *Septoria glycines* Hemmi, mainly with sulfur and benzimidazoles (Anonymous, 1997). With the introduction of Asian soybean rust caused by *Phakopsora pachyrhizi* Syd. & P. Syd. in 2001, the use of fungicides was intensified, with an average of two applications during one growing cycle. Because of the reduction of efficiency of the triazoles for rust control, in 2007 it was recommended that only mixtures of triazoles-strobilurins should be used for controlling rust (Godoy, 2012). Even with frequent fungicide application to control these multiple fungal diseases, target spot is still frequently observed in fields of a susceptible cultivar. Due to the low efficiency of triazoles-strobilurins fungicides mixtures to control target spot, it is a common practice to add benzimidazole in the application, or even to start with an application of benzimidazoles at the vegetative stage to increase target spot control.

Recommended and new fungicides were evaluated in trials in different regions in Brazil during the 2011-12 season (Godoy et al., 2012). Carbendazim (benzimidazole) had efficiency of only 16%, averaged across six trials from multiple regions. The mixture of trifloxystrobin + prothioconazole had the highest efficiency among the registered products (32%), but it was not as efficient as the unregistered test mixture of pyraclostrobin + epoxiconazole + fluxapyroxad (66% efficiency).

The lower efficiency of carbendazim can be due to the fungus resistance to this group. Benzimidazole fungicides were introduced for plant disease control in the 1960s and early 1970s. As site-specific inhibitors, benzimidazoles generally carry a high risk of resistance development in pathogens. Since commercialization started, at least 100 species of fungi have developed some degree of resistance to benzimidazoles (FRAC, 2013a) including *C. cassiicola* in tomato (*Solanum lycopersicum* L.) (Date et al., 2004). In Brazil, failures in target spot disease control with benzimidazole were only noticed with the recent increase planting of susceptible soybean cultivars. Avozani (2011) and Teramoto et al. (2012) reported resistance of *C.*

*cassiicola* isolates from soybean in Goiás and Mato Grosso States, respectively. Due to the intensive use of fungicides, resistant populations could have been present in the fields even before 2011.

Field samples of major target pathogens should be tested for levels of sensitivity before the fungicide is used commercially, to provide baseline data for subsequent comparisons (Brent, 2012). According to Hasama & Sato (1996) the 50% effective concentration ($EC_{50}$) values for a baseline population of *C. cassiicola* to benzimidazole is between 0.08 µg/mL and 0.61 µg/mL. Resistance of *C. cassiicola* was also reported for dicarboximides, N-phenylcarbamates, QoI fungicides and boscalid (Hasama & Sato, 1996; Date et al., 2004; Takeuchi et al., 2006; Ishii et al., 2007; Myamoto et al., 2009). Prothioconazole was recommended for soybean in Brazil for the first time in 2010/11. Although some authors considered 1 µg/mL as a cut off for triazoles - *C. cassiicola* (Teramoto et al., 2011), no baseline data has been published for prothioconazole. Prothioconazole is the only DMI-fungicide that belongs to the chemical group triazolinthione (FRAC, 2013b).

The objective of this study was to determine $EC_{50}$ for carbendazim (MBC) and prothioconazole (DMI) for 24 *C. cassiicola* isolates from the Embrapa Soja mycological collection (MES) obtained from multiple regions across Brazil from 1996 to 2011 (Table 1). The isolates were collected from soybean leaves, stem and pods. One isolate was from Paraguay (MES 692) and the others were isolated from different regions in the states of Paraná (10), Mato Grosso (10) and São Paulo (1). Some of the isolates were collected from unknown origins in Brazil (MES 307 and 657). The isolates have been maintained on potato-dextrose-agar (PDA) media (20 g of dextrose, 15 g of agar, and infused of 200 g of potato in 1 L of distilled water) and stored at 5°C until use.

Commercial formulations of carbendazim [50% active ingredient (a.i.) Carbendazim Nortox®; Nortox SA] and prothioconazole (48% a.i., Proline®; Bayer CropScience) were used in all experiments. Carbendazim was chosen for the test as this fungicide was known to be the active degradation component of thiophanate-methyl and benomyl from MBC- fungicide group (Ishii, 2012). Prothioconazole is present in the mixture with the highest field efficiency among the products registered in Brazil (Godoy et al., 2012).

To test the sensitivity of *C. cassiicola* to these selected fungicides, PDA media were amended with 0, 0.5, 1, 10, 100 and 1000 µg of a.i./mL of carbendazim and prothioconazole. Media were poured into 9-mm-diameter Petri plates, using approximately 20 mL per plate, and

**TABLE 1 -** Code of identification of the *Corynespora cassiicola* isolates from Embrapa Soja mycological collection (MES), year and place of isolation, and effective concentrations of carbendazim and prothioconazole that reduce 50% of mycelial growth ($EC_{50}$ - µg/mL)

| Code | Year | Municipality, state, country | Carbendazim | | Prothioconazole |
|---|---|---|---|---|---|
| | | | $EC_{50}$ | Sensitivity | $EC_{50}$ |
| MES 307 | 1996 | Unknown, Brazil | 8.01 | MR | 2.80 |
| MES 310 | 1997 | Sarandi, Paraná, Brazil | 0.77 | S | 3.21 |
| MES 311 | 1997 | Campo Mourão, Paraná, Brazil | 1.03 | MR | 0.47 |
| MES 312 | 1997 | Itiquira, Mato Grosso, Brazil | 0.61 | S | 1.93 |
| MES 313 | 1998 | Nova Mutum, Mato Grosso, Brazil | 1.05 | MR | 1.20 |
| MES 317 | 1999 | Palotina, Paraná, Brazil | 0.57 | S | 0.82 |
| MES 318 | 1999 | Campo Novos dos Parecis, Mato Grosso, Brazil | 0.30 | S | 1.26 |
| MES 322 | 2001 | Nova Ventura de São Roque, Paraná, Brazil | 0.70 | S | 3.31 |
| MES 1027 | 2007 | Chapadão do Sul, Mato Grosso, Brazil | 4.07 | MR | 26.44 |
| MES 629 | 2007 | Campo Verde, Mato Grosso, Brazil | 0.61 | S | 0.59 |
| MES 646 | 2008 | Campos Novos Parecis, Mato Grosso, Brazil | 575 | HR | 0.81 |
| MES 649 | 2008 | Campo Mourão, Paraná, Brazil | < 0.5 | S | 1.15 |
| MES 651 | 2008 | Sorriso, Mato Grosso, Brazil | 0.40 | S | 0.95 |
| MES 657 | 2008 | Unknow, Brazil | 0.83 | S | 2.87 |
| MES 667 | 2008 | Sete Quedas, Mato Grosso, Brazil | 0.86 | S | 1.27 |
| MES 670 | 2008 | Pirassununga, São Paulo, Brazil | 0.76 | S | 10.26 |
| MES 692 | 2008 | Corpus Christi, Canindeyú, Paraguay | 0.50 | S | 1.86 |
| MES 754 | | Sorriso, Mato Grosso, Brazil | 1.21 | MR | 11.82 |
| MES 755 | | Sertanópolis, Paraná, Brazil | 1.55 | MR | 11.64 |
| MES 926 | 2011 | Londrina, Paraná, Brazil | 1.00 | S | 0.97 |
| MES 930 | 2011 | Rolândia, Paraná, Brazil | > 1000 | HR | 11.05 |
| MES 931 | 2011 | Mauá, Paraná, Brazil | 10.19 | MR | 18.09 |
| MES 932 | 2011 | Londrina, Paraná, Brazil | 165 | HR | 4.44 |
| MES 933 | 2011 | Deciolândia, Mato Grosso, Brazil | > 1000 | HR | 1.42 |

HR - Highly Resistant, $EC_{50} \geq 50$ µg/mL; MR - Moderately Resistant, 50 µg/mL$>EC_{50}>$1 µg/mL; S - Sensitive, $EC_{50} \leq 1.0$ µg/mL allowed to solidify for 24 h. Six-millimeter-diameter mycelial plugs taken from the edge of a 10-day-old colony of each isolate were placed mycelium surface down onto PDA plates amended with each concentration of the fungicides. After the plates were incubated at 25±2°C for 10 days in the dark, the radial growth (colony diameter) of each isolate was measured, with the original mycelial plug subtracted from each measurement. The average of the colony diameters measured in two perpendicular directions was used. Four replicates of each fungicide concentration were used per isolate. The experiment was performed once. For each isolate, a linear regression of the percent inhibition (relative to the mycelial growth of the control) versus the $\log_{10}$ transformation was obtained, and the $EC_{50}$ was calculated with the PROC PROBIT function in SAS® Version 9.1.3. (SAS Intitute Inc.). The Pearson's chi-square test was used to check the goodness of fit (p-value>0.1). Cross-resistance was evaluated by the significance of the correlation coefficient ($r$) between log transformed $EC_{50}$ values of carbendazim and prothioconazole.

For carbendazim, based on differences in sensitivity, tested isolates were divided into three phenotypes as follows: Highly Resistant (HR), $EC_{50}\geq 50$ µg/mL; Moderately Resistant (MR), 50 µg/mL>$EC_{50}$>1 µg/mL; Sensitive (S), $EC_{50}\leq 1.0$ µg/mL (Edgington et al., 1971; Ma et al., 2003) (Table 1). For prothioconazole no cut off was assumed and the distribution of $EC_{50}$ among isolates was plotted (Figure 1).

Two different resistance levels (moderate and high) were observed for 11 isolates of *C. cassiicola* based on their sensitivity to carbendazim. For the isolate MES 649, a complete inhibition was observed at concentration of 0.5 µg/mL, whereas isolates MES 930 and MES 933 grew at 1000 µg/mL (Table 1). The calculated $EC_{50}$ ranged from 0.3 µg/mL to 575 µg/mL. The isolates with the highest resistance were collected in 2008 from Mato Grosso (MES 646) and in 2011 from Paraná and Mato Grosso states (MES 930, 932 e 933). From the isolates evaluated, 13 were considered sensitive to carbendazim. The isolate collected in 1996, before the first recommendation of fungicides on soybean, was classified as moderately resistant to carbendazim. Although samples highly resistant to carbendazim were detected in the collection only after 2008, this was not a general trend since sensitive isolates were also detected after 2008 in Mato Grosso (MES651, 667), Paraná (MES 649, 926), São Paulo (MES 670) and Canideyú, Paraguay (MES 692).

For prothioconazole all isolates were completely inhibited at 100 µg/mL. The EC50 ranged from 0.47 µg/

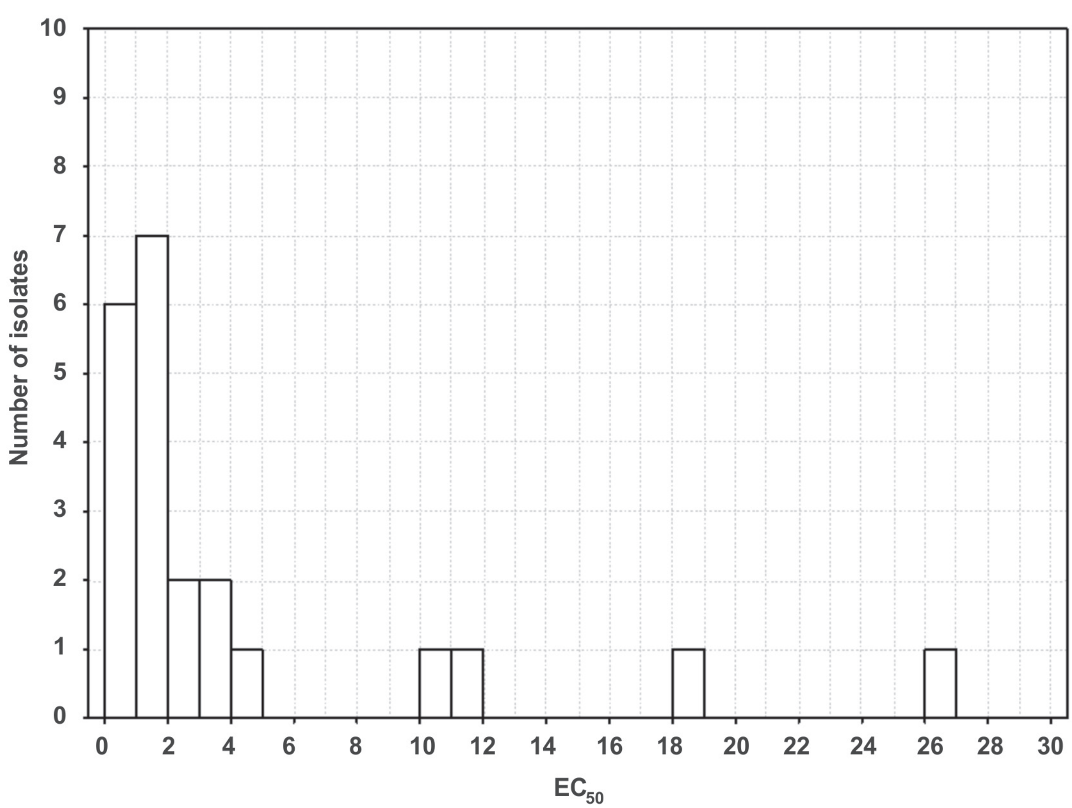


**FIGURE 1** - Distribution of the effective concentration of prothioconazole that reduces 50% of mycelial growth ($EC_{50}$ - µg/mL) for a collection of 24 isolates of *Corynespora cassiicola* from soybean collected from 1996 to 2011 in Paraná, Mato Grosso and São Paulo states in Brazil and Corpus Christi in Paraguay.

mL to 26.44 µg/mL (mean: 5.02 µg/mL). Sixty percent of the isolates presented $EC_{50}$<2 µg/mL (Figure 1). Although four isolates presented EC50>10 after 2007, no trend was observed among years. Prothioconazole is a triazolinthione from DMI group and others DMIs have been intensely used on soybean since *P. pachyrhizi* introduction in 2001. Cross resistance can be present between DMIs fungicides active against the same fungus (Russell, 2004). The ideal situation to establish a baseline should be if the molecule being considered was the only representative on the market for that mode of action (Russell, 2004) but this data is not available for prothioconazole - *C. cassiicola* on soybean. The $EC_{50}$ variation for prothioconazole could be due to the intrinsic characteristics of the isolates or to the selection pressure by the use of DMIs on soybean.

No significant correlation was observed for carbendazim and prothioconazole $EC_{50}$ levels and this was expected since the two fungicides evaluated have different mode of action.

Avozani (2011) studied the sensitivity of five *C. cassiicola* soybean isolates (three from Mato Grosso, one from Minas Gerais and one from Rondônia, Brazil) to carbendazim and observed $EC_{50}$ values of 0.2 mg/L and 0.26 mg/L for Minas Gerais and Rondônia isolates, respectively, and higher than 40 mg/L (maximum evaluated rate) for the Mato Grosso isolates. For the four DMI-fungicides evaluated for mycelium growth inhibition, the $EC_{50}$ values ranged from 0.77 mg/L to 20.32 mg/L. The range of $EC_{50}$ was similar to the one observed for prothioconazole (0.47 µg/mL to 26.44 µg/mL).

Teramoto et al. (2011) evaluated the sensitivity of five cucumber (*Cucumis sativus* L.) *C. cassiicola* isolates from São Paulo and one from Goiás State, Brazil, using mycelium growth inhibition to azoxystrobin, difenoconazole, carbendazim, tebuconazole and thiophanate-methyl and found $EC_{50}$ values higher than 50 µg/mL for azoxystrobin, carbendazin and thiophanate-methyl and below 50 µg/mL for difenoconazole and tebuconazole. Benzimidazoles and QoI resistance has been reported for *C. cassiicola* in cucumber with decrease in its use in Japan (Hasama, 1991; Hasama & Sato, 1996; Date et al., 2004; Takeuchi et al., 2006). The resistance to benzimidazole generally persists after development with some exceptions such as *Mycosphaerella fijiensis* M. Morelet in bananas (*Musa* spp.) (Smith, 1988; Brent, 2012). The use of benzimidazole-mancozeb mixtures is one control strategy that worked for peanut Cercospora leaf spots in the USA (Smith, 1988).

Although this research included a small number of isolates, the results reinforce the occurrence of *C. cassiicola* isolates highly resistant to benzimidazole in regions of Paraná and Mato Grosso States. A more detailed sampling should be conducted to map the distribution of the resistant isolates. The distribution curve of $EC_{50}$ values for prothioconazole can be used to monitor shifts in the distribution peak in future samplings.

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