# *IN VITRO* EFFECT OF FUNGICIDES ON *CORYNESPORA CASSIICOLA* ISOLATES FROM COTTON AND SOYBEAN IN ALABAMA

**M.N. Rondon**
**B.R. Lawaju**
**K.S. Lawrence**
**Auburn University**
**Auburn, AL**

## Abstract

*Corynespora cassiicola*, the causal agent of target spot disease, is a fungal pathogen with increasing importance in cotton and soybean producing countries. Disease management has been a concern in other countries due to its increasing occurrence. Severe disease symptoms and significant yield losses can occur when the pathogen is not properly controlled. Fungicides have been a crucial tool in disease management; however, reported cases of C. cassiicola resistance to Methyl Benzimidazole Carbamates (MBC, FRAC code 1), Succinate-dehydrogenase inhibitors (SDHI, FRAC code 7) and Quinone outside Inhibitors (QoI, FRAC code 11) have been published from tomato, cucumber and soybean isolates. Specific genes known as cassiicolin-encoding genes (*Cas2*, *Cas6*, and *Cas2+6*) were found in *C. cassiicola* isolates from cotton and soybean in Alabama, reflecting an overall genetic differentiation of the isolates. Therefore, the objective of this project was to explore the effectiveness and to determine the sensitivity ($EC_{50}$) of fungicides over different isolates of cotton and soybean based on the cassiicolin-encoding genes from Alabama. Cotton and soybean leaf samples were collected from different locations of Alabama and 12 isolates were obtained. All isolates were submitted to DNA extraction and polymerase chain reaction (PCR) with specific primers covering *Cas* sequences for gene detection. Sensitivity ($EC_{50}$) of *C. cassiicola* isolates to Headline (pyraclostrobin) and Priaxor (pyraclostrobin + fluxapyroxad) was determined using a mycelial growth assay and $EC_{50}$ values for each isolate were estimated by logarithmic regression analysis (P d 0.05). Genetic diversity based on cassicolin-encoding genes was found among the 12 isolates with a different profile of the genes. $EC_{50}$ for the fungicide Headline (pyraclostrobin) ranged between 17.71 – 66.01 mg/L for *C. cassiicola* isolates from cotton and ranged between 50.03 – 94.50 mg/L for *C. cassiicola* isolates from soybean. For the fungicide Priaxor (pyraclostrobin + fluxapyroxad), $EC_{50}$ ranged between 0.57 – 1.03 mg/L for *C. cassiicola* isolates from cotton and ranged between 1.45 – 11.80 mg/L for *C. cassiicola* isolates from soybean. The results suggested that soybean isolates have been exposed to these fungicides more often than cotton isolates. High $EC_{50}$ values found for the fungicide Headline (active ingredient pyraclostrobin) indicate a loss of sensitivity of *C. cassiicola* isolates to the active ingredient. A potential loss of sensitivity of *C. cassiicola* isolates to fluxapyroxad was observed when Priaxor was the fungicide tested. Our results will be useful to monitor resistance to fungicides of *C. cassiicola* and help with fungicide resistance management.

## Introduction

*Corynespora cassiicola* (Berk. & Curt.) C.T. Wei is a widespread plant pathogenic fungus that causes target-shaped necrotic spots on plant leaves and on stems, roots, flowers, and fruits. As a cosmopolite pathogen, *C. cassiicola* has been recorded in over 70 countries on more than 700 plant species including fruits, vegetables, grains, perennial crops, forestry and various ornamental plants (Farr & Rossman, 2017). Symptoms on the cotyledonary leaves appear as small circular spots. The foliar phase of the disease is characterized as small circular spots, varying between 2 mm and 10 mm. Well-developed lesions are necrotic and show typical “target spot” symptoms, with some depression at the center of the lesion. In severe cases of infection, the lesions coalesce and the leaves show severe necrosis followed by complete premature senescence and death of the leaf (Galbieri et al., 2014).

Onerirosan et al. (1975) reported a toxin produced by highly pathogenic *C. cassiicola* isolates were found affecting susceptible cultivars of tomato and it was not effective against resistant cultivars of tomato. It was the first report on toxin production by *C. cassiicola* isolates. Déon et al. (2012) demonstrated that there was some difference between aggressive and moderate aggressive isolates in their levels of a putative effector protein, cassiicolin. Following these studies, Déon et al. (2014) found a variation in the cassicolin gene for several isolates of *C. cassiicola* that could be related to the host range. The cassicolin gene was found expressing in the early phase of infection and six cassicolin isoforms (*Cas1*, *Cas2*, *Cas3/4*, *Cas5* and *Cas6*) could be identified by PCR-detection revealing a genetic diversity. Some of the isolates from cotton were detected with two Cas genes (*Cas2+6*) and others with no detectable Cas gene (Cas0).

Disease management has been a concern in other countries due to increasing occurrence of target spot (Godoy et al., 2015). Severe disease symptoms and significant yield losses can occur when the pathogen is not properly controlled (Hagan and Sikora, 2012; Koenning et al., 2006). Fungicides have been a crucial tool in disease management; however, there are reported cases of *C. cassiicola* isolates from tomato, cucumber, and soybean resistant to specific fungicides. Methyl Benzimidazole Carbamates (MBC, FRAC code 1) (Date et al., 2004; Xavier et al., 2013; Avozani et al., 2014; Teramoto et al., 2017), Succinate-dehydrogenase inhibitors (SDHI, FRAC code 7) (Miyamoto et al., 2009, 2010) and Quinone outside Inhibitors (QoI, FRAC code 11) are fungicides included in those reported cases of resistance.

Due to the intensive use of fungicides to control several diseases, resistant populations of *C. cassiicola* might be present in the United States. For this reason, we explored the effectiveness and determined the sensitivity ($EC_{50}$) of fungicides over different isolates of cotton and soybean from Alabama based on the cassiicolin-encoding genes.

## Materials and Methods

### Isolates

During 2017/18, isolations of *C. cassiicola* were carried out using direct isolation in which small amounts of mycelia and conidia from symptomatic lesions on leaves of cotton and soybean were directly transferred onto acidified potato dextrose agar (APDA). APDA plates were sealed and incubated at room temperature (RT) for mycelial growth. Pure colonies were transferred to potato dextrose agar (PDA; DIFCO Laboratories) plates to establish obtain a new collection of *C. cassiicola* isolates from Alabama. The identification of *C. cassiicola* was through morphological characters (Ellis, 1971) and *C. cassiicola* isolates were stored at 4˚C on PDA slants until further use.

### DNA extraction

Mycelial plugs of each isolate were placed over a cellophane membrane onto a fresh PDA plate (Cassago et al., 2002). After 10 days of incubation at RT, DNA were extracted from each isolate using a ZR Fungal/Bacterial MiniPrepTM kit from Zymo Research (California, USA). Extracted DNA concentrations were evaluated using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and stored at -20˚C prior to use.

### Detection of the cassiicolin-encoding genes

Primers covering available Cas sequences were used for polymerase chain reaction (PCR) amplification (Déon et al., 2014). CasF18 (CCCAAGATACATGTTTTGAATGT) and CasR27 (CCACACAAAGCAAGATACAGAATGAGC) for Cas1 as a target gene; CasF17 (GGATTTGCCTGAGATCCTA) and CasR24 (CAAACAATGCTAACCAAACAAAC) for Cas2; CasF20 (GTCGGCTAACTTGGGAAAAACTCT) and CasR28 (GCAGGAAGCAAAACACAGAACAAG) for Cas3, Cas4; CasF19 (CGGGGAGGTATCAGGTGTGAGATA) and CasR26 (CAGAACAAGCCAAAAGAGAACTAC) for Cas5; CasF16 (GCTTGATTTGCCTGTGAGATACT) and CasR25 (AAAACGATGCTAAACAAAAGGA) for Cas6. PCR was performed on 20 ng/µL of *C. cassiicola* genomic DNA. A 50-µl reaction mix was prepared for each isolate containing 2 µl of each purified DNA sample, 1 µl of forward primers, 1 µl of reverse primers, 21 µl of deionized water, and 25 µl of JumpStart *Taq* ReadyMix (Sigma-Aldrich, St. Louis, MO, USA). Reactions (50 µl) were pipetted into 8-tube strips, and PCR was conducted. DNA amplification was carried out in a MultiGene DNA thermal cycler (Labnet International; Edison, NJ) with a program consisting of initial denaturation for 5 min at 94˚C; followed by 35 cycles of 1 min at 94˚C, 1 min at 55˚C, and 2 min at 72˚C; and a final cycle of 5 min at 72˚C. Amplification products were visualized under UV light on 1.0% agarose gels stained with GelRed® Nucleic Acid Gel Stain (Biotium Inc., Fremont, CA, USA) to detect the presence of the cassicolin-encoding genes. A 1 kb DNA ladder (Biolabs, California, USA) was used as the marker and water, without DNA extract, was used as the control.

### Mycelial growth assay

Sensitivity of *C. cassiicola* to two fungicides, Headline®, a QoI fungicide group (pyraclostrobin) and Priaxor®, a QoI + SDHI fungicide group (pyraclostrobin + fluxapyroxad), was evaluated based on mycelial growth. Twelve *C. cassiicola* isolates were grown on potato dextrose agar (PDA) amended with six fungicide concentrations (0.01, 0.1, 0.5, 1, 10 and 100 mg/L of active ingredient) as well as a control, without the addition of fungicide. Fungicide stock suspensions were prepared by dissolving the commercial fungicide in sterile deionized water (SDW) prior to use. PDA media amended with each fungicide and fungicide concentration were poured into plastic Petri dishes. The day

after PDA Petri dishes preparation, mycelial plugs (5 mm in diameter) of each isolate from a 10-day-old *C. cassiicola* culture were placed surface down on the center of each Petri dish. All Petri dishes were incubated at 28±2˚C and 12 hours of photoperiod. When one colony in the control treatment (without fungicide) reached the edge of the plate, the mycelial growth (colony diameter) was measured in two perpendicular directions. The diameter of the mycelial plugs for each plate was subtracted before calculating the average of the colony and transformed into growth percentage. The sensitivity of each fungicide ($EC_{50}$) was categorized according to Teramoto et al. (2017). For QoI fungicide group $EC_{50}$ values were classified as follows: S = sensitive (< 0.16 mg/L); MS = moderate sensitive (0.16 – 1.0 mg/L); NS = non-sensitive (1 – 25 mg/L); HNS = highly non-sensitive (> 25 mg/L). For the fungicide mixture (QoI + SDHI fungicide) the categories were based on the response to the SDHI group, which $EC_{50}$ values were classified as follows: S = sensitive (< 1.0 mg/L); MS = moderate sensitive (1 – 10 mg/L); NS = non-sensitive (10 – 25 mg/L); HNS = highly non-sensitive (> 25 mg/L).

**Data analysis**

The experiment was set in a completely randomized design with four replicates per concentration of the fungicide. A Petri dish was used as an experimental unit and the experiment was performed once. $EC_{50}$ values (estimated fungicide concentration that inhibited 50% of the mycelial growth) for each isolate were estimated by logarithmic regression analysis using SAS 9.4 PROC REG procedure (*P d 0.05*).

## Results and Discussion

PCR products were obtained from the genomic DNA of the *C. cassiicola* isolates from cotton (HSV01, FHP22, BRW03, EVS01, HSV12 and FHP01) and soybean (LIM02, PBU06, LIM14, PBU07, LIM13 and PBU04). The primers covering the Cas sequences were able to amplify fragments around 750 base pairs (bp) on agarose gels. No amplification product was obtained from the negative control, where water was used instead of DNA. Between the five possible cassicolin-encoding genes, two of them (*Cas2* and *Cas6*) were amplified in our *C. cassiicola* isolates. The absence of amplified fragments by PCR was observed for three isolates (FHP01, HSV12, and LIM13), which was classified as Cas0 (Déon et al., 2014). A genetic diversity based on cassicolin-encoding genes was found for the isolates sampled in Alabama from cotton and soybean infected leaves (Figure 1).

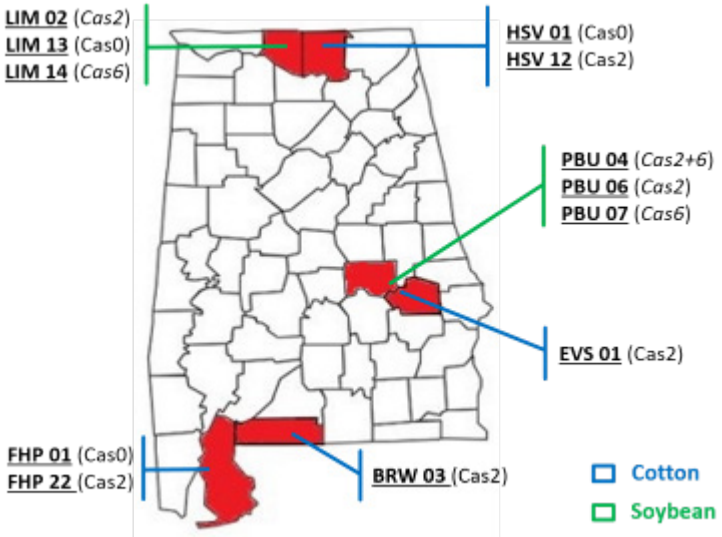


Figure 1. Distribution map of the cassicolin-encoding genes found *in Corynespora cassiicola* isolates sampled from cotton (blue colored) and soybean (green colored) in Alabama, U.S.

The concentration of the fungicides ranged between 0.01 – 100 mg/L, and the control treatment without the use of fungicides (Figure 2). $EC_{50}$ for the fungicide Headline (pyraclostrobin) ranged between 17.71 – 66.01 mg/L for *C. cassiicola* isolates from cotton and ranged between 50.03 – 94.50 mg/L for *C. cassiicola* isolates from soybean (Table 1). The mean of the $EC_{50}$ for the fungicide Headline for cotton isolates was 41.89 mg/L, while for soybean isolates was 73.65 mg/L. Most of the isolates were classified as highly non-sensitive (HNS) to Headline, except for

Table 1. Isolates of *Corynespora cassiicola* and their respective origin and cassiicolin-encoding genes, regression equation, coefficient of determination ($R^2$), significance (*P-value*), effective concentration of the fungicides Headline and Priaxor ($EC_{50}$) and sensitivity (S) to the fungicide.

| Isolate | Origin | Gene | Headline (pyraclostrobin) | | | | | Priaxor (pyraclostrobin + fluxapyroxad) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Equation [z] | $R^2$ | *P-value* | $EC_{50}$ (mg/L) | S [y] | Equation | $R^2$ | *P-value* | $EC_{50}$ (mg/L) | S [x] |
| BRW03 | Cotton | *Cas2* | $y = 66.712\ e^{-0.015x}$ | 0.79 | <.0001 | 19.29 | NS | $y = 88.191\ e^{-0.929x}$ | 0.94 | <.0001 | 0.61 | S |
| EVS01 | Cotton | *Cas2* | $y = 79.090\ e^{-0.026x}$ | 0.21 | 0.0015 | 17.71 | NS | $y = 90.028\ e^{-0.882x}$ | 0.94 | <.0001 | 0.67 | S |
| FHP01 | Cotton | Cas0 | $y = 74.306\ e^{-0.008x}$ | 0.69 | <.0001 | 49.96 | HNS | $y = 66.092\ e^{-0.270x}$ | 0.75 | <.0001 | 1.03 | MS |
| FHP22 | Cotton | *Cas2* | $y = 85.683\ e^{-0.008x}$ | 0.86 | <.0001 | 66.01 | HNS | $y = 85.541\ e^{-0.949x}$ | 0.92 | <.0001 | 0.57 | S |
| HSV01 | Cotton | *Cas2* | $y = 77.683\ e^{-0.008x}$ | 0.75 | <.0001 | 55.84 | HNS | $y = 66.841\ e^{-0.419x}$ | 0.91 | <.0001 | 0.73 | S |
| HSV12 | Cotton | Cas0 | $y = 74.939\ e^{-0.010x}$ | 0.82 | 0.0002 | 42.55 | HNS | $y = 66.815\ e^{-0.312x}$ | 0.81 | <.0001 | 0.93 | S |
| LIM02 | Soybean | *Cas2* | $y = 77.461\ e^{-0.009x}$ | 0.79 | 0.0059 | 50.03 | HNS | $y = 71.705\ e^{-0.161x}$ | 0.78 | <.0001 | 2.24 | MS |
| LIM13 | Soybean | Cas0 | $y = 79.702\ e^{-0.009x}$ | 0.87 | <.0001 | 51.81 | HNS | $y = 74.638\ e^{-0.181x}$ | 0.83 | <.0001 | 2.22 | MS |
| LIM14 | Soybean | *Cas6* | $y = 94.979\ e^{-0.007x}$ | 0.92 | <.0001 | 94.50 | HNS | $y = 86.393\ e^{-0.378x}$ | 0.99 | <.0001 | 1.45 | MS |
| PBU04 | Soybean | *Cas2+6* | $y = 94.812\ e^{-0.008x}$ | 0.94 | 0.0003 | 83.21 | HNS | $y = 57.100\ e^{-0.021x}$ | 0.54 | <.0001 | 6.27 | MS |
| PBU06 | Soybean | *Cas2* | $y = 94.919\ e^{-0.007x}$ | 0.90 | <.0001 | 90.28 | HNS | $y = 64.857\ e^{-0.032x}$ | 0.66 | <.0001 | 8.13 | MS |
| PBU07 | Soybean | *Cas6* | $y = 89.570\ e^{-0.008x}$ | 0.92 | <.0001 | 72.06 | HNS | $y = 65.013\ e^{-0.022x}$ | 0.75 | <.0001 | 11.80 | NS |

[z] y represents the percentage of mycelial growth inhibition; x represents the fungicide concentration.

[y] Sensitivity (S) of *Corynespora cassiicola* to QoI fungicide according to Teramoto et al. (2017): S = sensitive (< 0.16 mg/L); MS = moderate sensitive (0.16 – 1.0 mg/L); NS = non-sensitive (1 – 25 mg/L); HNS = highly non-sensitive (> 25 mg/L).

[x] Sensitivity (S) of *Corynespora cassiicola* to SDHI fungicide according to Teramoto et al. (2017): S = sensitive (< 1.0 mg/L); MS = moderate sensitive (1 – 10 mg/L); NS = non-sensitive (10 – 25 mg/L); HNS = highly non-sensitive (> 25 mg/L).

two isolates, BRW03 and EVS01 from cotton that were non-sensitive (NS). *Corynespora cassiicola* isolates from soybean were less sensitive to Headline compared to our isolates from cotton. Among 34 *C. cassiicola* isolates sampled from soybean in Brazil, Teramoto et al. (2017) reported $EC_{50}$ less than 0.16 mg/L for 10 isolates, considered as sensitive (S) to pyraclostrobin. Only one isolate was considered as HNS with an $EC_{50}$ equal to 36.55 mg/L. The high $EC_{50}$ values found in our isolates suggest loss of sensitivity to Headline (pyraclostrobin) in Alabama, U.S.

$EC_{50}$ for the fungicide Priaxor (pyraclostrobin + fluxapyroxad) ranged between 0.57 – 1.03 mg/L for *C. cassiicola* isolates from cotton and ranged between 1.45 – 11.80 mg/L for *C. cassiicola* isolates from soybean (Table 1). The mean of the $EC_{50}$ for the fungicide Priaxor for cotton isolates was 0.76 mg/L, while for soybean isolates was 5.35 mg/L. Teramoto et al. (2017) reported the sensitivity of soybean isolates to fluxapyroxad fungicide (SDHI group) with EC < 1 mg/L for the majority (85%) of the isolates. Sensitivities of *C. cassiicola* isolates to Priaxor followed the same pattern as sensitivity to Headline, with soybean isolates more sensitive to the fungicides. *Corynespora cassiicola* isolates from cotton were mostly classified as sensitive (S), and just one isolate (FHP01) classified as moderate sensitive (MS). On the other hand, one *C. cassiicola* isolate from soybean was classified as NS, while the rest of the isolates as MS. In this case, high $EC_{50}$ values, such as 11.80 mg/L, indicate a possible case of resistance to SDHI that is present in isolates assessed in Brazil with $EC_{50}$ higher than 91.43 mg/L (Teramoto et al., 2017).


Figure 2. *Corynespora cassiicola* mycelial growth of one replicate for 12 isolates tested with Headline and Priaxor. At the left, control treatment (0 mg/L) and at the right, the higher concentration (100 mg/L) of the fungicides.

## Summary

Our results indicate an important loss of sensitivity of *C. cassiicola* isolates to pyraclostrobin, a QoI fungicide. A potential loss of sensitivity of *C. cassiicola* isolates to fluxapyroxad was observed when pyraclostrobin + fluxapyroxad were tested. Soybean isolates have been more exposed due to the intensive use of these fungicides at the same season or even the long period (years) that these fungicides have been used on soybean fields. Over the years, cotton isolates tend to increase their $EC_{50}$ if the intensive use of the same active ingredients continues. Our findings may help to monitor *C. cassiicola* sensitivity to fungicides, which is important to manage fungicide resistance.

## Acknowledgements

We acknowledge and thank Dr. Patricia A. Donald, Affiliate Professor with the Department of Entomology and Plant Pathology at Auburn University, for reviewing the manuscript.

## References

Avozani, A., Reis, E.M, Tonin, R.B. 2014. Sensitivity loss by *Corynespora cassiicola*, isolated from soybean, to the fungicide carbendazim. Summa Phytopathologica, 40 (2), 273-276. http://www.scielo.br/pdf/sp/v40n3/a10v40n3.pdf

Cassago, A., Panepucci, R., Baiao, A., Henrique-Silva, F. 2002. Cellophane based mini-prep method for DNA extraction from the filamentous fungus *Trichoderma reesei*. BMC Microbiology, 2:14. https://doi.org/10.1186/1471-2180-2-14

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. Annals of the Phytopathological Society of Japan, 70, 7-9.

Déon, M., Bourre, Y., Gimenez, S., Berger, A., Bieysse, D., De Lamotte, F., et al. 2012. Characterization of a

cassiicolin-encoding gene from *Corynespora cassiicola*, pathogen of rubber tree (*Hevea brasiliensis*). Plant Science, 185, 227±37. https://doi.org/10.1016/j.plantsci.2011.10.017

Déon, M., Fumanal, B., Gimenez, S., Bieysse, D., Oliveira, R.R., Shuib, S.S., et al. 2014. Diversity of the cassicolin gene in *Corynespora cassiicola* and relation with the pathogenicity in *Hevea brasiliensis*. Fungal Biology, 118 (1), 32-47. https://doi.org/10.1016/j.funbio.2013.10.011

Ellis, M.B. 1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute: Kew, Surrey, UK.

Farr, D.F., & Rossman, A.Y. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved October 1, 2017, from https://nt.ars-grin.gov/fungaldatabases/

Galbieri, R., Araújo, D.C.E.B., Kobayasti, L., Girotto, L., Matos, J.N., Marangoni, M., Almeida, W.P., Mehta, Y.R. 2014. Corynespora leaf blight of cotton in Brazil and its management. American Journal of Plant Sciences, 5, 3805-3811. http://dx.doi.org/10.4236/ajps.2014.526398

Godoy, C.V. 2015. Target spot. In: Hartman GL, Rupe JC, Sikora EF, Domier LL, Davis JA, Steffey KL, eds. *Compendium of Soybean Diseases and Pests*. St Paul, MN, USA: APS Press, 62

Hagan, A.K., Sikora, E.J. 2012. Leaf spot management in Alabama cotton, control of potash- incited leaf spot diseases and Corynespora leaf spot. Plant Pathology Series. Extension Plant Pathology, 961 S.

Koenning, S.R., Creswell, T.C., Dunphy, E.J., Sikora, E.J., Mueller, J.D. 2006. Increased occurrence of target spot of soybean caused by *Corynespora cassiicola* in the southeastern United States. *Plant Disease,* 90, 974.

Miyamoto, T., Ishii, H., Seko, T., Kobori, S., Tomita, Y. 2009. Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki prefecture, Japan. *Plant Pathology* 58, 1144-1151.

Miyamoto, T., Ishii, H., Tomita, Y. 2010. Occurrence of boscalid resistance in cucumber powdery mildew in Japan and molecular characterization of the iron-sulfur protein of succinate dehydrogenase of the causal fungus. Journal of General Plant Pathology 76, 261-267.

Onesirosan, P.T., Mabuni, C.T., Durbin, R.D., Morin, R.B., Righ, D.H., Arny, D.C. 1975. Toxin production of *Corynespora cassiicola*. Physiological Plant Pathology, 5, 289-295.

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 Phytopathologica, v.43, n.4, p.281-289. http://dx.doi.org/10.1590/0100-5405/2195

Xavier, S.A., Canteri, M.G., Barros, D.C.M., Godoy, C.V. 2013. Sensitivity of *Corynespora cassiicola* from soybean to carbendazim and prothioconazole. Tropical Plant Pathology, *38*, 431–435. http://dx.doi.org/10.1590/S1982-56762013005000020