SENSITIVITY OF *Colletotrichum gossypii* var. *cephalosporioides* STRAINS TO CARBENDAZIM, TEBUCONAZOLE, AND AZOXYSTROBIN

Nelson D. Suassuna (Embrapa Algodão / suassuna@cnpa.embrapa.br), Joel A. Queiroz (Embrapa Algodão), Aldenise B. de Oliveira (Embrapa Algodão), Luiz A. Maffia (UFV), Eduardo S. G. Mizubuti (UFV).

ABSTRACT - “Ramulose”, caused by *Colletotrichum gossypii* var. *cephalosporioides* (Cgc), is a cotton disease that reduces fiber yield and increases production costs in Brazil. Due to the lack of resistant cotton varieties with suitable agronomic traits, fungicide application either associated or not with cultural methods, is an effective control measure commonly adopted by growers. Frequent sprays of protectant and systemic fungicides are routinely used, but reduced efficacy of chemicals has been noticed. Thus, sensitivity of Cgc isolates to carbendazim, tebuconazole, and azoxystrobin was assessed. Baseline population sensitivity was established with isolates collected where no fungicide was used. For *in vitro* assays, the ED$_{50}$ values of carbendazim (n=88 isolates) ranged from 0.037 to 1.474 µg a.i./mL, a 40-fold sensitivity factor. No resistance to carbendazim (ED$_{50}$ > 10 µg a.i./mL) was detected. Similarly, ED$_{50}$ values for tebuconazole (n=91) and azoxystrobin (n =82) were low and ranged from 0.020 to 0.191 and from 0.019 to 0.068 µg a.i./mL, respectively.

Key words: fungicide, chemical control, and cotton ramulose

SENSIBILIDADE DE ISOLADOS DE *Colletotrichum gossypii* var. *cephalosporioides* A CARBENDAZIM, TEBUCONAZOLE E AZOXYSTROBIN

RESUMO - A ramulose do algodoeiro, causada por *Colletotrichum gossypii* var. *cephalosporioides* (Cgc), é uma importante doença que reduz a produtividade e onera os custos de produção. Devido a falta de resistência genética nas cultivares de algodoeiro em uso, aplicações de fungicidas são rotineiramente empregadas no manejo da doença. O propósito do trabalho foi gerar informações acerca da sensibilidade *in vitro* de isolados do patógeno quanto aos fungicidas carbendazim, tebuconazole e azoxystrobin. Isolados oriundos de áreas onde não há relatos do uso de fungicidas compuseram a população *baseline*. Os valores de ED$_{50}$ (dose necessária para inibir o crescimento micelial em 50%) para carbendazim (n=88 isolados) variaram de 0,037 a 1,474 µg i.a./mL. Não foi detectada resistência a carbendazim (ED$_{50}$ > 10 µg i.a./mL). De maneira análoga, os valores estimados de ED$_{50}$ para os fungicidas tebuconazole (n=91) e azoxystrobin (n=82) foram baixos e variaram de 0,020 a 0,191 e de 0,019 a 0,068 µg i.a./mL, respectivamente.

Key words: Fungicida, controle químico e ramulose
INTRODUCTION

In the savannah region of the west-central Brazil the hot and wet weather that prevails during the cotton growing season (November to July) is highly favorable to several foliar diseases. “Ramulose” (“escobilla” in Spanish or “witches’ broom”, in English), firstly reported in Brazil in 1937, is an important fungal disease during the growing season and is apparently confined to South America. Ramulose is caused by a distinct physiological variety of C. gossypii, named C. gossypii var. cephalosporioides (Cgc), although no authoritative description of the morphology of this pathogen has been reported. Cotton plant can be infected at all developmental stages, and disease symptoms comprise shortening of internodes and excessive development of branches and leaves, causing witches’ broom type of symptom (MIRANDA e SUASSUNA, 2004, THAXTON e EL-ZIK, 2001).

Currently, management of ramulose is based on crop rotation to reduce initial inoculum, use of cultivars with some level of resistance, and fungicide sprays (Miranda e Suassuna, 2004). Nevertheless, these practices are not always integrated, and application of fungicides is the only control measure adopted. Among the fungicides, site-specific compounds are commonly used either solely or combined. It is well known that frequent sprays of the same active ingredients favor selection of fungicide resistant isolates. Although there are no reports of insensitive populations of Cgc in Brazil, growers frequently complain about the lack or reduced efficacy of fungicides after spraying crops for ramulose and areolate mildew, caused by Ramularia areola.

Because of their specific, single-site, mode of action, the systemic and translaminar fungicides are generally more at-risk for resistance development than contact fungicides (MCGRATH, 2001). Among the systemic fungicides, the benzimidazoles, triazoles, and strobilurins (quinone outside inhibitors - QoI) have been commonly used to manage ramulose in Brazil. Benzimidazole binds to ß-tubulin and prevents the formation of microtubules, consequently preventing cell division. Tebuconazole is a demethylation inhibiting fungicide (DMI) that interferes with ergosterol biosynthesis (GISI et al., 2000). The QoI’s interfere with respiration at the electron transport chain in mitochondrial respiration at the Q0 site of the cytochrome bc1 complex (complex III, Q-cytochrome C reductase) (BRENT e HOLLOMON, 1998). Fungicides are usually applied when the first lesions appear on young leaves before the apical meristem is damaged. Where crop rotation is performed, ramulose is not noticed or sometimes disease symptoms appear at the end of the season (“late ramulose”). However, where cotton is continuously cultivated (e.g. in areas without crop rotation), fungicides are sprayed as early as 30 days after plant emergency. Aerial applications are routinely employed and sometimes exceed two sprays per season, particularly in rainy years.

Given the frequent usage of these fungicides in cotton crops and the high genetic variability of Cgc (LIMA e CHAVES, 1992), the reduced efficacy of fungicides in controlling ramulose could be associated with insensitive pathogen populations. Thus, was tested the hypothesis of occurrence of insensitive isolates of Cgc to the most commonly used systemic fungicides in the major Brazilian cotton growing areas. The approach was to sample isolates from several fields where systemic fungicides were used; estimate the effective dosage to inhibit fungal growth by 50% (ED50); and compare these ED50 values to ED50 values estimated with isolates from fields with no fungicide application – baseline population. We also assessed the effect of systemic fungicides on ramulose progress in the field.
Commercial formulations of azoxystrobin, tebuconazole, and carbendazim were used in all experiments. Isolates were sampled from cotton growing areas in counties across the west-central region of Brazil (Fig. 1). Isolates from areas with no reports of fungicide usage in cotton plants (Campina Grande, Paraíba State, and Viçosa, Minas Gerais State) were considered to form the baseline population whereas the other isolates were considered non-baseline. In each county, one to four areas (georeferenced with a portable global positioning system device and distant at least 100m from each other) were sampled, depending on disease occurrence and intensity. In each area, one to six diseased leaves from plants with typical ramulose symptoms were sampled. From each sample, to indirectly isolate the pathogen, leaf fragments with ramulose lesions were surface sterilized for 60s with sodium hypochlorite (NaClO 1.5 %), rinsed once in sterilized distilled water (SDW), immersed in alcohol (70%) for 30s, and washed again in SDW. The fragments, each with a single lesion, were placed on sterile filter paper to remove water excess and transferred to Petri dishes (9-cm-diameter) containing potato dextrose agar medium (PDA) with lactic acid (1%). One isolate was obtained directly from a conidium at the top of setae in an old lesion. The Petri dishes were sealed and maintained at 25°C, 12 h day length. After 3 to 5 days, hyphal tips were cut from each colony and transferred to other PDA plates. To confirm each colony as of Cgc, microscopical observations of conidia slides were performed. Five 7-mm diameter mycelial disks were transferred to vials (6 cm) with silica gel for long term preservation. For each experiment, an isolate was subcultured through five transfers, at the most. Three (for carbendazim and azoxystrobin tests) or two (for tebuconazole tests) isolates of C. gloeosporioides from mango, apple, or pepper were included to compare fungicide sensitivity. In the tebuconazole assays three spontaneous carbendazim resistant strains found on PDA amended with 1000 g carbendazim/mL were also included, to test for cross-resistance to tebuconazole.

Sensitivity of 82, 91, or 88 Cgc isolates to azoxystrobin, tebuconazole, or carbendazim, respectively, was estimated based on colony growth inhibition. Azoxystrobin and tebuconazole were dissolved in dimethyl sulfoxide (DMSO) and carbendazim on 100% ethanol, to obtain stock solutions at 1.000 µg a.i. of either azoxystrobin or tebuconazole/mL, or 10.000 µg a.i. carbendazim/mL. The stock solutions were added to PDA medium after sterilization to produce final concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 µg a.i. of azoxystrobin or tebuconazole/mL, or 0, 0.01, 0.1, 1, 10, and 100 µg a.i. of carbendazim/mL.

For each isolate, a 9-mm diameter mycelial plug cut from the edge of a colony grown on PDA was placed in the center of dishes with PDA, amended with each fungicide concentration. The plates were kept at 25°C, in the dark. After 6 days, colony diameter was measured in two perpendicular directions, and the diameter of the mycelial plug was subtracted to calculate the mean diameter of the colony (MD).
For each concentration/fungicide, inhibition of colony growth (ICG) of an isolate $i$ was calculated by $\text{ICG}_i = (\text{MD}_c - \text{MD}_i / \text{MD}_c) \times 100$, in which $\text{MD}_c$ = colony mean diameter for the control (no fungicide amended), and $\text{MD}_i$ = colony mean diameter for isolate $i$. For each replicate of each isolate-fungicide concentration combination, values of ICG were linearly regressed on the logarithm ($\log_{10}$) of fungicide concentration to estimate the dose that inhibited mycelial growth by 50% ($\text{ED}_{50}$ values). The whole range of $\text{ED}_{50}$ values for each fungicide was divided at constant intervals (0.01, 0.04, and 0.005 μg/mL, for tebuconazole, carbendazim, and azoxystrobin, respectively) for each population (baseline and nonbaseline). The frequency of isolates in each $\text{ED}_{50}$ interval for each fungicide was determined. For each fungicide, comparisons were conducted with the NPAR1WAY procedure by using the Kolmogorov-Smirnov test to determine significant differences in distribution of $\text{ED}_{50}$ values between baseline and nonbaseline. The experiment was conducted once. All statistical analyses were performed using SAS version 8.0 (SAS Institute, Cary, NC).

Figure 1. Baseline (boxes) and non baseline (circles) populations of *Colletotrichum gossypii* var. *cephalosporioides* sampled in cotton growing areas of Brazil.

**RESULTS AND DISCUSSION**

The $\text{ED}_{50}$ values for tebuconazole ranged from 0.020 to 0.191 μg/mL and no insensitive isolate was detected. Baseline and nonbaseline isolates were equally sensitive to tebuconazole, although the range of sensitivity of baseline isolates was narrower (from 0.041 to 0.095 μg/mL) than of nonbaseline isolates (0.020 to 0.191). There was no difference ($p=0.270$) between the distribution of $\text{ED}_{50}$ values of the baseline and nonbaseline populations. The $\text{ED}_{50}$ of the two *C. gloeosporioides* and three *C. gossypii* var. *cephalosporioides* isolates considered spontaneous mutants resistant to carbendazim also fell in the same range of sensitivity of the nonbaseline population.
The ED$_{50}$ values for carbendazim ranged from 0.037 to 1.470 $\mu$g/mL among the non baseline isolates, while ED$_{50}$ values for baseline isolates ranged from 0.270 to 1.248 $\mu$g/mL. Frequency distributions of ED$_{50}$ values of baseline population differed (p=0.001) from ED$_{50}$ values of nonbaseline population. A sensitivity factor, the highest ED$_{50}$ value/the lowest ED$_{50}$ value, was approximately 40-fold, indicating great variation between samples. Nevertheless, the maximum ED$_{50}$ value was less than 2 $\mu$g/mL, which is considered sensitive. ED$_{50}$ values for the three C. gloeosporioides isolates from mango, pepper, and apple were 0.259, 0.698, and 2.702 $\mu$g/mL, respectively.

The range of ED$_{50}$ values for azoxystrobin varied from 0.019 to 0.068 $\mu$g/mL. The sensitivity factor was 3.5 fold. The ED$_{50}$ values for the baseline population ranged from 0.029 to 0.063 $\mu$g/mL. There was no difference between sampled population and baseline (p=0.576). ED$_{50}$ values of the three C. gloeosporioides isolates were 0.539, 0.519, and 0.046 $\mu$g/mL. For one isolate of C. gloeosporioides (ED$_{50}$ = 0.539) there was a 27 fold sensitivity factor in relation to the smallest ED$_{50}$ value of C. gossypii var. cephalosporioides.

There was no evidence of insensitivity of Brazilian isolates of C. gossypii var. cephalosporioides to any of the tested fungicides. The ED$_{50}$ range for tebuconazole estimated in the present study is higher than values previously reported for other ascomycetes such as Monilinia fructicola in which 0.001 < ED$_{50}$ < 0.063 (YOSHIMURA et al., 2004) and Sclerotinia homoeocarpa, in which 0.002 < ED$_{50}$ < 0.094 (Hsiang et al., 1997). The values estimated for tebuconazole are in the range reported for sensitive populations of Mycosphaerella graminicola, in which 0.01 < ED$_{50}$ < 0.90 (GISI et al., 1997). The ED$_{50}$ sensitivity factor (relation between the most and the least sensitive isolates in a population) for Cgc varied by a factor of 10. This variation is relatively low compared with cyproconazole data from M. graminicola in Europe for which ED$_{50}$ varied from 7 to 40 fold (GISI et al., 2000). As the action mode of DMI s involves several pathways (GISI et al., 2000) and, as for many plant pathogens, sensitivity seems to be rather stable, and it is expected a long time of reliable disease control if anti-resistance strategies are deployed, including the use of mixtures and alternation of products from different classes.

It was not found differences between ED$_{50}$ of isolates of the baseline and nonbaseline populations and there is no evidence of cross resistance between tebuconazole and carbendazim. Knowledge of whether or not a fungicide can control strains of the target pathogen that are known to be resistant to other fungicides is a key component of resistance risk assessment. In this study, spontaneous carbendazim resistant strains, selected on high concentration media amended with the fungicide, were not able to grow in medium amended with tebuconazole. In severe outbreaks of either ramulose or areolate mildew, several fungicide applications are routinely employed. Despite tebuconazole widespread usage, there is no evidence of field resistance to this fungicide, as well as there is no apparent risk of cross-resistance between tebuconazole and carbendazim.

Selection of insensitive individuals to benzimidazole fungicides is well-known for many groups of plant pathogenic fungi (LAMONDIA e DOUGLAS, 1997). Benzimidazole resistance has developed independently in a number of fungal species and it has always been associated with point mutations in the $\beta$-tubulin gene (YARDEN e KATAN, 1993). The cutoff ED$_{50}$ values for sensitivity may differ depending on the pathogen. Botrytis cinerea strains were considered resistant if ED$_{50}$ >250 $\mu$g/mL for benomyl or > 1,000 $\mu$g/mL for thiophanate-methyl (LAMONDIA e DOUGLAS, 1997). For Monilinia fructicola isolates with ED$_{50}$ values below 2 $\mu$g/mL were considered sensitive to thiophanate-methyl, isolates with values between 2 and 30 $\mu$g/mL were considered low resistance, and above 30 $\mu$g/mL high resistance (YOSHIMURA et al., 2004). Isolates of Helminthosporium solani were grouped on class
of resistant to benomyl if able to grow on media amended with 35 µg/mL of fungicide and resistant to thiophanate-methyl if able to grow on media amended with 400 µg/mL of fungicide (CUNHA e RIZZO, 2003). Regarding carbendazim as a typical benzimidazole and adopting the last criterion to classify isolates as resistant to benomyl, all strains used in this study can be considered sensitive. One reason for non emergence of carbendazim resistant strains is the frequent use of a fungicide mixture (carbendazim and fentin hydroxide) and a gradual substitution of these molecules for novel ones.

Taken together the data reflects an optimist scenario for both fungicides tested. Alternation of fungicide and use of mixtures (e.g. two different action modes) may have contributed to these results. The frequent use of protectant fungicides like fentin hydroxide, commonly adopted to lower production costs, or copper compounds, used to control bacterial blight, must have played an important role in controlling putative resistant strains at low frequency in population. The inefficiency of wind dispersal of Cgc compared with other pathogens, may also have contributed negatively to the establishment of resistant strains. Constant Cgc population monitoring is necessary in order to detect any shift in a population, mainly in areas where fungicide usage is more frequent.

Cotton is the most profitable crop in Central Brazil, and the same cultivars are commonly grown year after year in the same areas. This can contributed to initial inoculum build up in crop debris. Moreover, as discussed above, in very wet conditions it may be impossible to spray fungicides at all, considering large areas. Tank-mix involving growth regulators, insecticides, foliar nutrients, and even selective herbicides, frequently used to minimize application costs, can contribute to reduce fungicide effect by incompatibility. Thus, this mix should be avoided. Good crop management practices, especially crop rotation, and sanitation procedures to reduce seed contamination, associated with fungicide reducing risk practices can contribute to ramulose management and extend life span of fungicides.

**CONCLUSION**

This was the first survey of fungicide resistance to a cotton pathogen in Brazil and the results do not support any lack of efficacy due to fungicide resistance.

**REFERENCES**


