Geographical distribution, diversity and pathogenicity of *Colletotrichum* associated with soybean anthracnose in Brazil

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**Abstract.** Eighty-five *Colletotrichum* isolates were obtained from soybean croplands in 34 municipalities of Mato Grosso state. Our objectives were characterize the isolates through mycelial growth, cultural and morphological analyses, as well as assessing pathogenicity. To evaluate cultural characteristics, the mycelial growth was daily measured, and the color of colonies was characterized after ten days incubation. The morphology, length, and width of 50 conidia per isolate were assessed. The pathogenicity of twenty-one isolates was evaluated on seed germination rate, incidence, and detection of extracellular enzymes. The cultural and morphology characteristics of *Colletotrichum* isolates were greatly variable. All isolates studied were able to reduce seed germination, produce signs and proteinase activity. These results suggest there are more than one *Colletotrichum* species associated with soybean anthracnose in Mato Grosso State.

**Keywords:** morphological and cultural traits, *Glycine max*.

**Introduction**

Soybean crop (*Glycine Max* (L.) Merr.) is one of the most important agricultural commodities, which 358.65 million metric tons produced in 2018/2019. Brazil and USA were the largest producers of soybeans (~35 million hectares), with 120 and 117 million metric tons produced in USA and Brazil, respectively. Brazil’s 2019/20 soybean production and area are estimated at a record 126.0 million metric tons (mmt) and 36.9 million hectares (mha), respectively (USDA, 2020). The state of Mato Grosso is the largest producer, which is responsible for 28% of the Brazilian production (CONAB, 2019). However, disease is one of the most limiting factors to enhance crop yields, especially in the tropical region. Anthracnose is an important disease, which can cause severe damage under high temperature and moisture. Symptoms are dark, depressed, irregular lesions on pods, leaves, stems and petioles. Anthracnose may induce pods rotting and falling, immature pods opening and premature grain germination, resulting in a reduced pod number, leaf retention and green stem (Hartman et al., 2015).

Disease reduce stands, seed quality and yields by 16-26% or more in the United States, 30-50% in Thailand, and 100% in certain areas of Brazil and India (Hartman et al., 2015). Most recent studies, in the Brazilian Cerrado biome, indicate that for each 1% increment in the disease incidence (in the range of 9 to 17% incidence), a reduction of 90 kg.ha⁻¹ of soybean grain may occur (Dias et al., 2016).

Pathogen-seed association is the most effective cause of an epidemic. *Colletotrichum* is able to establish latent infection without any visible symptom or cause cotyledon lesions, and pre-emergence and post-emergence damping-off at the V1 and V2 development phase. This infection resulting in seed physiological quality losses, as germination, viability and vigor (Begum et al., 2008).

The most common pathogen associated with soybean anthracnose is *Colletotrichum truncatum* (Schw.) Andrus & Moore (syn. *C. dematiurn* (Pers. exFr) Grove var. *truncata* (Schw.) Arx), but another species have been associated to soybean anthracnose. In Brasil, there are reports of *C. claviae* in soybean crop in Mato Grosso (Barbieri et al., 2017) and Tocantins states (Dias et al., 2018). In addition, *C. incanum*, *C. chlorophyli*, *C. gloeosporioides*, *C. cocccodes*, *C. destructivum*, *C. graminicola* were detected in USA (Yang et al., 2014; Yang et al., 2012; Riccioni et al., 1998), Malaysia (Mahmodia et al., 2013), Taiwan (Chen et al., 2006) and Argentina (Ramos et al., 2013).

Traditionally, the species identification of the genus *Colletotrichum* relied on the host infected species, morphological and cultural characteristics, even as size and shape of the conidia (Sutton,
1980). Vinnere (2004) reported that *C. acutatum* has slower growth than *C. gloeosporioides* in culture media, which is a reliable characteristic for identification among them.

Our objective was to identify *Colletotrichum* isolates currently affecting soybean crops in the state of Mato Grosso; compare their morphological and cultural characteristics, including the size and shape of the conidia; and describe their pathogenicity in soybean seeds. Moreover, preserve the isolates in fungal databases at the Federal University of Mato Grosso.

**Methods**

*Colletotrichum* **isolates**

Soybean plants with anthracnose symptoms on pods, stems and petioles were collected from thirty-four municipalities in the Mato Grosso state (Central Brazil) from 2011 to 2014 (Figure 1; table 1).

To obtain fungal isolates, disease pods, stems and petiole were cut into 0.5 x 0.5 cm pieces and surface-disinfested by sequential immersion in sterile water for 3 min, 70% ethanol for 1 min, 1% (w/w) sodium hypochlorite for 2 min, and rinsed three times in sterile distilled water for 2 min and dried on sterile filter paper. Subsequently, the pieces were placed on water agar and incubated at 25 ºC in 12 h light/dark regimes. When a fungus grew, hyphen tips were transferred onto potato dextrose agar (PDA) and incubated at 25 ºC in 12 h light/dark regimes for seven days. Then, pure cultures were obtained by monosporic culture, using the technique of successive dilution.

Eighty-five isolates were preserved in filter paper (Alfenas & Mafia, 2007) and in sterile distilled water (Castellani, 1939) and kept at 4 ºC in the Plant Pathology and Microbiology laboratory, Federal University of Mato Grosso.

**Mycelial growth and cultural characterization**

The mycelial growth of the eighty-five isolates was compared. Mycelial discs (diameter 6 mm) were dissected from previous colony margins and deposited in the center of Petri dishes (9 cm) containing PDA and then incubated at 25 ºC in dark conditions. After two days, two orthogonal diameters of the colonies were measured daily, during 10 days to evaluate the colony size.

Average mycelial growth rate (AMGR) were calculated according to the formula described by Oliveira (1991): AMGR = \( \frac{\Sigma (D-Da)}{N} \). Where, D is current average diameter of colony; Da is the last day average diameter of colony; N is the number of days after inoculation.

After 7 days of incubation, cultural characteristics were identified according to 25 color (7.5YR) range determined by Munsell® Soil Color Charts (1971). In our study, the identified colors were: white (8/0), light gray (7/0), gray (6/0), dark gray (4/0), very dark gray (3/0), pinkish gray (6/2), pinkish gray (7/2), pink (7/4), pinkish white (8/2), and pink (8/4).

The experimental design was a completely randomized with five replicates (Petri dish) per isolate.

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**Figure 1.** Municipalities of Mato Grosso state where soybean plants with anthracnose symptoms were taken
<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Geographic origin</th>
<th>Year</th>
<th>Isolate code</th>
<th>Geographic origin</th>
<th>Year</th>
<th>Isolate code</th>
<th>Geographic origin</th>
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<td>LU-1</td>
<td>Lucas do Rio Verde</td>
<td>2012</td>
<td>RO-1</td>
<td>Rondonópolis</td>
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<td>2014</td>
<td>LU-2</td>
<td>Lucas do Rio Verde</td>
<td>2013</td>
<td>SC-1</td>
<td>Santa Carmen</td>
<td>2012</td>
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<td>SI-3</td>
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<td>2013</td>
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<td>2014</td>
<td>SI-5</td>
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<td>2013</td>
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<td>2013</td>
<td>MA-4</td>
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<td>2014</td>
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<td>MT-1</td>
<td>Matupá</td>
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<td>Querência</td>
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<td>2014</td>
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<td>Querência</td>
<td>2013</td>
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</table>
Morphometric
Conidia were characterized by the sizes and shapes. *Colletotrichum* isolates were grown in PDA Petri dish and incubated at 25 °C, in dark conditions. After 7 days, conidia were observed in semi-permanent slides in lacto-glycerol, and 50 conidia were measured in a light microscope Zeiss, Axio Scope A1 (Gottingen, Germany) equipped with Zeiss Axion Cam Erc 5s. The shapes were classified in three groups: 1) falcate; 2) cylindrical, straight; or 3) fusiform, straight attenuated at each end (Sutton, 1980).

*Colletotrichum* pathogenicity to soybean seed
Twenty-one isolates of *Colletotrichum* (CH-1, CH-3, CJ-1, CN-1, CO-1, IT-3, IT-4, IT-6, LU-2, LU-3, MA-3, NB-1, NB-2, NU-5, PE-4, QU-2, SI-9, SO-11, TN-1 and VR-1) were grown in PDA Petri dish with mannitol solute (Salisbury & Ross, 1991) at water potential of -0.6 MPa and incubated in 25 °C for 10 days.

Soybean seeds (cultivar TMG 132) were surface-disinfested in 1% (w/w) sodium hypochlorite for 1 min and air-dried for 24 h and then, put over the 10 day old colony for 24 h at 25 °C and 12 h photoperiod. After inoculation, 100 seeds per isolate were equidistantly distributed on a Petri dish with wet filter paper. They were incubated at 20 °C with a 12 h photoperiod for 1 week. Un-inoculated seed (control, 20 seeds) were placed directly on PDA with mannitol. Inoculated and un-inoculated seeds were used for germination and incidence (acervuli and mucilage) rates.

The experiment was a completely randomized design, 5 replicates (Petri dish) with 20 seeds each.

Enzyme assays
Specific solid culture media on Petri dishes were used for detection of extracellular enzymes amylase and protease (Pereira, 2009). To determine amylase activity, *Colletotrichum* isolates were grown in solid culture media (6 g NaNO3, 1.5 g KH2PO4, 0.5 g KCl, 0.5 g MgSO4.7H2O, 0.01 g FeSO4, 0.01 g ZnSO4, 10 g starch, 15 g agar and 1 L distilled water, at pH 6.8). The halo formation was measured after the 5-old colony had been in contact with Lugol's iodine solution (1 g iodine, 2 g KI, 300 mL distilled water) for 15 min. The starch is hydrolyzed by amylase when isolates forming a clear halo around the colony, whereas isolates that did not form a halo not produce an extracellular enzyme.

To evaluate protease activity identification, isolates were grown in solid culture media (5 g peptone, 3 g yeast extract, 1g NaCl, 15 g agar, 0.4 g gelatin and 1 L distilled water, at pH 6.0). The gelatin was autoclaved separately and mixed in the media before pouring it onto the Petri dish. Isolates were incubated at 25 °C for 48 h, in the dark.

The halo diameter measurements were done after 10 mL of 2% methyl red been applied on the colony. In addition, the ratio between the halo and the colony diameter (H/C) for each isolate was calculated.

The experiment design was a complete randomized, with 21 isolates and five replicates (Petri dish), totaling 55 experimental units.

Statistical analysis
The data were analyzed using the analysis of variance (ANOVA) of the statistical software SAMS – Agri (Canteri et al., 2001). The treatment means were estimated and comparison was performed using the Scott Knott test (P < 0.05).

Results and discussion
*Colletotrichum* isolates presented high difference in colony color and average mycelial growth rate (Table 2). No correlation was observed between the geographical origin of the isolates and morph-cultural characterization and aggressiveness.

Among 85 *Colletotrichum* isolates, 82% presented gray color colony, range from white to very dark gray, and the remaining isolates presented pinkish gray, pinkish white and pink color colony (Table 2). This pattern was also reported by Hartman et al. (2015). Usually, *Colletotrichum truncatum* produce whitish colonies that eventually turn smoky black and, for that reason, isolates of *C. truncatum* greatly vary the colony characteristics and pathogenicity (Torres-Calzada et al., 2017; Majonjo & Kapooria, 2003).

On the other hand, *C. truncatum* isolates obtained from the symptomatic soybean plants in several states of Brazil presented colonies dark grey or orange-colored colony (Rogerio et al., 2016). Cultural characters of *Colletotrichum truncatum* are dark olive, light olive and yellow brown colonies, and to *C. gloesporioides* are dark olive and light olive colonies from soybean in Taiwan (Chen et al., 2006).

In papaya and apple fruits, a broad variation of *Colletotrichum gloesporioides* and *C. truncatum* isolates was also observed. The authors have organized the isolates into several groups and report no correlation between the species and the colony color in BDA and pepetone-glucose-agar (PGA) (Velho et al., 2015; Andrade et al., 2007).

Mycelial growth rate in vitro differed among isolates, which were classified into twelve groups (Table 2), however the growth rate ranged from 0.16 cm.dia⁻¹ (AB-1) to 1.24 cm.dia⁻¹ (VE-3). Rogerio et al. (2016), evaluating *C. truncatum* isolates, reported growth rate in PDA from 0.60 to 0.89 cm.dia⁻¹. In our study, only 28% of the isolates were identified in this range. 70% were above and 2% below it.
<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Colony color</th>
<th>AMG (cm.day⁻¹)</th>
<th>Shape</th>
<th>Length (min-max)</th>
<th>Width (min-max)</th>
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</thead>
<tbody>
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<td>AB-1</td>
<td>7.5YR 7/0</td>
<td>0.39 d</td>
<td>falcate</td>
<td>20.62 (16.49-22.84)</td>
<td>3.01 (3.53-2.52)</td>
</tr>
<tr>
<td>AF-1</td>
<td>7.5YR 8/0</td>
<td>1.00 k</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>BG-1</td>
<td>7.5YR 6/0</td>
<td>0.70 h</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>BG-2</td>
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<td>0.77 i</td>
<td>*</td>
<td>*</td>
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</tr>
<tr>
<td>BR-1</td>
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<td>0.78 i</td>
<td>cylindrical</td>
<td>14.85 (12.27-18.51)</td>
<td>4.35 (2.97-3.53)</td>
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<td>CH-1</td>
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<td>0.43 e</td>
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<tr>
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<td>0.45 e</td>
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<td>24.45 (18.49-28.69)</td>
<td>4.41 (3.37-5.23)</td>
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<td>1.11 (0.84-1.51)</td>
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<td>LU-1</td>
<td>7.5YR 7/0</td>
<td>0.44 e</td>
<td>falcate</td>
<td>21.34 (14.59-26.48)</td>
<td>3.84 (2.70-5.58)</td>
</tr>
<tr>
<td>LU-2</td>
<td>7.5YR 7/0</td>
<td>0.37 d</td>
<td>falcate</td>
<td>18.03 (13.02-22.42)</td>
<td>3.58 (2.57-4.33)</td>
</tr>
<tr>
<td>LU-3</td>
<td>7.5YR 7/0</td>
<td>0.44 e</td>
<td>falcate</td>
<td>23.84 (20.14-27.94)</td>
<td>3.72 (2.57-4.96)</td>
</tr>
<tr>
<td>LU-4</td>
<td>7.5YR 7/0</td>
<td>0.42 e</td>
<td>falcate</td>
<td>22.80 (18.50-26.44)</td>
<td>3.62 (2.70-4.32)</td>
</tr>
<tr>
<td>LU-5</td>
<td>7.5YR 7/4</td>
<td>0.46 e</td>
<td>falcate</td>
<td>19.36 (13.35-23.56)</td>
<td>3.94 (3.30-4.72)</td>
</tr>
<tr>
<td>LU-6</td>
<td>7.5YR 4/0</td>
<td>0.32 c</td>
<td>falcate</td>
<td>21.73 (19.71-24.29)</td>
<td>3.07 (2.41-3.61)</td>
</tr>
<tr>
<td>MA-2</td>
<td>7.5YR 8/0</td>
<td>0.52 f</td>
<td>falcate</td>
<td>22.37 (17.27-25.83)</td>
<td>3.69 (2.85-4.53)</td>
</tr>
<tr>
<td>MA-3</td>
<td>7.5YR 7/0</td>
<td>0.42 e</td>
<td>falcate</td>
<td>24.26 (20.69-26.97)</td>
<td>4.06 (2.99-5.57)</td>
</tr>
<tr>
<td>MA-4</td>
<td>7.5YR 7/0</td>
<td>0.42 e</td>
<td>falcate</td>
<td>25.08 (21.76-28.38)</td>
<td>3.51 (2.87-4.32)</td>
</tr>
<tr>
<td>MT-1</td>
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<td>0.37 d</td>
<td>falcate</td>
<td>18.89 (14.25-24.20)</td>
<td>3.67 (2.95-4.47)</td>
</tr>
<tr>
<td>NA-1</td>
<td>7.5YR 8/0</td>
<td>0.81 j</td>
<td>cylindrical</td>
<td>15.74 (11.44-18.92)</td>
<td>4.36 (3.55-5.01)</td>
</tr>
<tr>
<td>NB-1</td>
<td>7.5YR 7/0</td>
<td>0.45 e</td>
<td>cylindrical</td>
<td>15.49 (11.65-19.36)</td>
<td>4.66 (3.68-5.66)</td>
</tr>
<tr>
<td>NB-2</td>
<td>7.5YR 6/0</td>
<td>0.30 c</td>
<td>cylindrical</td>
<td>22.75 (19.25-27.11)</td>
<td>3.44 (2.79-4.08)</td>
</tr>
<tr>
<td>NM-1</td>
<td>7.5YR 7/0</td>
<td>0.84 j</td>
<td>cylindrical</td>
<td>15.82 (12.16-19.40)</td>
<td>4.83 (3.64-6.47)</td>
</tr>
<tr>
<td>NO-1</td>
<td>7.5YR 7/0</td>
<td>0.77 i</td>
<td>cylindrical</td>
<td>13.67 (9.04-16.61)</td>
<td>4.26 (3.01-5.46)</td>
</tr>
<tr>
<td>NU-1</td>
<td>7.5YR 3/0</td>
<td>0.85 j</td>
<td>cylindrical</td>
<td>14.47 (11.97-17.55)</td>
<td>4.67 (3.54-5.61)</td>
</tr>
</tbody>
</table>
Comparing mycelial growth rate between two species of *Colletotrichum* Dias et al. (2018) reported slower growth rate in the *C. truncatum* than *C. cliviae*. Authors also observed, after 7 days of incubation at 25°C, *C. cliviae* mycelial growth occupying all Petri dish (9 cm), while *C. truncatum* occupied less than 5 cm. In similar evaluation in the United States, *C. incanum* growth 0.75 cm.day⁻¹, differing from *C. truncatum* that growth 0.58 cm.day⁻¹ (Yang et al., 2014).

The isolates conidia shape in our study were classified in three groups: falcate (58%), cylindrical (31%) and fusiform (11%). All conidia were hyaline, unicellular and aseptate, but with variable size (Table 2). Falcate conidia average size was from 18.9 to 25.5μm in length and 3.0 to 4.2μm in width (except isolate CN-1, CN-1, SI-8 and CL-4). Cylindrical conidia average size varied from 12.8 to 18.4μm in length and from 4.0 to 5.1μm in length (except QU-1, SI-4 and VE-1). Fusiform conidia

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Color</th>
<th>Length</th>
<th>Width</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NU-2</td>
<td>7.5YR 7/0</td>
<td>0.32 c</td>
<td>fusiform</td>
<td>18.94 (15.72-22.56)</td>
</tr>
<tr>
<td>NU-3</td>
<td>7.5YR 7/0</td>
<td>0.40 e</td>
<td>falcate</td>
<td>23.95 (19.11-28.73)</td>
</tr>
<tr>
<td>NU-4</td>
<td>7.5YR 6/0</td>
<td>0.55 f</td>
<td>falcate</td>
<td>24.65 (21.83-28.76)</td>
</tr>
<tr>
<td>NU-5</td>
<td>7.5YR 7/0</td>
<td>0.38 d</td>
<td>falcate</td>
<td>23.23 (18.24-25.30)</td>
</tr>
<tr>
<td>NU-6</td>
<td>7.5YR 7/0</td>
<td>0.54 f</td>
<td>falcate</td>
<td>24.76 (21.78-28.34)</td>
</tr>
<tr>
<td>NX-1</td>
<td>7.5YR 7/0</td>
<td>0.45 e</td>
<td>falcate</td>
<td>20.87 (15.02-23.34)</td>
</tr>
<tr>
<td>PE-1</td>
<td>7.5YR 7/0</td>
<td>0.64 g</td>
<td>falcate</td>
<td>20.37 (17.84-22.93)</td>
</tr>
<tr>
<td>PE-4</td>
<td>7.5YR 3/0</td>
<td>0.61 g</td>
<td>cylindrical</td>
<td>15.31 (12.45-18.92)</td>
</tr>
<tr>
<td>PN-1</td>
<td>7.5YR 4/0</td>
<td>0.37 d</td>
<td>falcate</td>
<td>23.66 (21.56-27.26)</td>
</tr>
<tr>
<td>QU-1</td>
<td>7.5YR 8/4</td>
<td>0.44 e</td>
<td>cylindrical</td>
<td>5.94 (4.18-6.64)</td>
</tr>
<tr>
<td>QU-2</td>
<td>7.5YR 6/2</td>
<td>0.26 b</td>
<td>fusiform</td>
<td>19.75 (15.48-23.10)</td>
</tr>
<tr>
<td>RO-1</td>
<td>7.5YR 7/0</td>
<td>0.63 g</td>
<td>cylindrical</td>
<td>17.17 (13.41-21.20)</td>
</tr>
<tr>
<td>SC-1</td>
<td>7.5YR 7/2</td>
<td>0.43 e</td>
<td>fusiform</td>
<td>21.92 (18.36-25.43)</td>
</tr>
<tr>
<td>SC-2</td>
<td>7.5YR 7/0</td>
<td>0.39 d</td>
<td>falcate</td>
<td>21.41 (16.89-25.71)</td>
</tr>
<tr>
<td>SI-1</td>
<td>7.5YR 7/0</td>
<td>0.86 j</td>
<td>cylindrical</td>
<td>15.66 (13.43-18.10)</td>
</tr>
<tr>
<td>SI-2</td>
<td>7.5YR 7/0</td>
<td>0.39 d</td>
<td>falcate</td>
<td>22.87 (20.33-26.33)</td>
</tr>
<tr>
<td>SI-3</td>
<td>7.5YR 8/0</td>
<td>0.62 g</td>
<td>cylindrical</td>
<td>12.74 (10.10-17.06)</td>
</tr>
<tr>
<td>SI-4</td>
<td>7.5YR 8/4</td>
<td>0.38 d</td>
<td>cylindrical</td>
<td>6.29 (4.35-7.96)</td>
</tr>
<tr>
<td>SI-5</td>
<td>7.5YR 7/0</td>
<td>0.44 e</td>
<td>falcate</td>
<td>23.59 (21.38-27.20)</td>
</tr>
<tr>
<td>SI-7</td>
<td>7.5YR 7/2</td>
<td>0.38 d</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>SI-8</td>
<td>7.5YR 8/0</td>
<td>0.37 d</td>
<td>falcate</td>
<td>15.40 (12.04-17.97)</td>
</tr>
<tr>
<td>SI-9</td>
<td>7.5YR 7/2</td>
<td>0.34 d</td>
<td>fusiform</td>
<td>21.02 (17.23-24.50)</td>
</tr>
<tr>
<td>SO-3</td>
<td>7.5YR 7/0</td>
<td>0.38 d</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>SO-4</td>
<td>7.5YR 7/0</td>
<td>0.37 d</td>
<td>cylindrical</td>
<td>15.23 (11.41-19.52)</td>
</tr>
<tr>
<td>SO-5</td>
<td>7.5YR 7/0</td>
<td>0.32 c</td>
<td>cylindrical</td>
<td>17.47 (13.96-20.14)</td>
</tr>
<tr>
<td>SO-6</td>
<td>7.5YR 7/0</td>
<td>0.64 g</td>
<td>falcate</td>
<td>22.25 (19.36-27.03)</td>
</tr>
<tr>
<td>SO-7</td>
<td>7.5YR 7/0</td>
<td>0.65 g</td>
<td>falcate</td>
<td>21.53 (17.02-24.88)</td>
</tr>
<tr>
<td>SO-9</td>
<td>7.5YR 7/0</td>
<td>0.36 d</td>
<td>cylindrical</td>
<td>15.34 (13.12-18.72)</td>
</tr>
<tr>
<td>SO-10</td>
<td>7.5YR 7/2</td>
<td>0.45 e</td>
<td>falcate</td>
<td>21.94 (17.98-25.53)</td>
</tr>
<tr>
<td>SO-11</td>
<td>7.5YR 4/0</td>
<td>0.67 h</td>
<td>falcate</td>
<td>24.58 (20.65-28.02)</td>
</tr>
<tr>
<td>TB-1</td>
<td>7.5YR 7/0</td>
<td>0.40 d</td>
<td>fusiform</td>
<td>19.67 (12.07-24.79)</td>
</tr>
<tr>
<td>TB-2</td>
<td>7.5YR 4/0</td>
<td>0.31 c</td>
<td>falcate</td>
<td>21.36 (16.26-26.66)</td>
</tr>
<tr>
<td>TN-1</td>
<td>7.5YR 7/0</td>
<td>0.69 h</td>
<td>cylindrical</td>
<td>*</td>
</tr>
<tr>
<td>US-1</td>
<td>7.5YR 4/0</td>
<td>0.43 e</td>
<td>falcate</td>
<td>23.63 (19.07-26.87)</td>
</tr>
<tr>
<td>VE-1</td>
<td>7.5YR 7/0</td>
<td>0.38 d</td>
<td>cylindrical</td>
<td>20.60 (16.42-25.50)</td>
</tr>
<tr>
<td>VE-3</td>
<td>7.5YR 7/0</td>
<td>1.24 l</td>
<td>cylindrical</td>
<td>17.54 (13.67-21.28)</td>
</tr>
<tr>
<td>VE-4</td>
<td>7.5YR 4/0</td>
<td>0.46 e</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>VE-5</td>
<td>7.5YR 6/0</td>
<td>0.82 j</td>
<td>falcate</td>
<td>21.73 (18.64-25.94)</td>
</tr>
<tr>
<td>VR-1</td>
<td>7.5YR 4/0</td>
<td>0.32 c</td>
<td>falcate</td>
<td>22.60 (18.47-26.79)</td>
</tr>
</tbody>
</table>

Means followed by the letter in the column did not differ (P < 0.05) by the Scott-Knott test.

* isolates did not sporulate under environmental conditions.

Geographical distribution, diversity and pathogenicity of *Colletotrichum* associated with soybean anthracnose in Brazil

Moraes et al. 2018
average size was from 18.9 to 21.9µm x 3.3-3.8µm (except CH-3 and CL-6).

Average size measured for falcate conidia is similar to the values reported for C. truncatum, C. chlorophyti and C. incanum isolated from vegetative soybean around the world, except for CJ-1 isolate. The length x width range for C. truncatum conidia had been reported as 14-26 x 2-5µm (Chen et al., 2006; 20.54-25 x 2.95-3.4µm (Rogério et al., 2016) and 18.81-25.5 x 3.56-4.63µm (Jagtap & Sontakke, 2009). On the other hand, C. chlorophyti presented conidia ranged from 15.5-21.3 µm x 2.5-4.3µm (Yang et al., 2012), and C. incanum produce shorter and narrower falcate conidia (16.9-21.9 x 2.3-3.7 µm) (Yang et al., 2014).

AVERAGE conidia length and width in isolates CJ-1, CN-1 and SI-8 were lower than average. According to Sutton (1980), falcate conidia with shorter width have wide host range and, usually is saprophytic. And the largest falcate conidia, as observed in the CL-4 isolate (28, 43 x 3.23µm) was reported on Gramineae.

Colletotrichum cliviae (Barbieri et al., 2017), C. destructivum (Damm et al., 2014) and C. gloeosporioides (Chen et al., 2006) have cylindrical conidia and size (length and width) similar to observed in our study. Except QU-1 and SI-4 isolates presents lower length and width conidia. And VE-1 isolate that had highest size conidia. Colletotrichum sojae is new species that presents cylindrical conidia measuring from 14 to 17 µm in length and from 5 to 6 µm in width (Damm et al., 2019) however the width is greater than found in our work.

Colletotrichum coccodes obtained from the stem soybean presented straight and fusiforme conidia ranged from 15 to 23 µm long and 3 to 4 µm wide (Riccioni et al., 1998) as observed to fusiforme conidia in our study. While CL-6 isolate had the highest width and CH-3 isolate the highest length and width.

The twenty-one of Colletotrichum spp. were pathogenic when inoculated on soybean seeds presenting acervuli and mucilage. However, there was difference in the isolate aggressiveness, which negatively affect seed germination. Seeds germination percentages were classified in five groups (Table 3). The control germination rate was greater than all other isolates. These low germination rate of the control is likely related to the decrease in the seed vigor due to the storage condition.

Table 3. Germination rate and incidence of acervuli and mucilage in soybean seeds (cultivar TMG132) inoculated with Colletotrichum isolates, and halo size

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Germination</th>
<th>Acervuli (%)</th>
<th>Mucilage (%)</th>
<th>Protease (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64.5 a</td>
<td>0 c</td>
<td>0 d</td>
<td>-</td>
</tr>
<tr>
<td>CH-1</td>
<td>0.8 e</td>
<td>100 a</td>
<td>97.5 a</td>
<td>2.1 a</td>
</tr>
<tr>
<td>CH-3</td>
<td>3.3 e</td>
<td>100 a</td>
<td>49.1 b</td>
<td>0 c</td>
</tr>
<tr>
<td>CJ-1</td>
<td>0 e</td>
<td>100 a</td>
<td>97.5 a</td>
<td>3.2 a</td>
</tr>
<tr>
<td>CL-4</td>
<td>36.6 c</td>
<td>100 a</td>
<td>100 a</td>
<td>2.5 a</td>
</tr>
<tr>
<td>CN-1</td>
<td>0 e</td>
<td>100 a</td>
<td>97.5 a</td>
<td>2.7 a</td>
</tr>
<tr>
<td>CO-1</td>
<td>44.1 c</td>
<td>13.3 c</td>
<td>13.3 c</td>
<td>1.5 b</td>
</tr>
<tr>
<td>IT-3</td>
<td>0 e</td>
<td>100 a</td>
<td>95 a</td>
<td>1.8 b</td>
</tr>
<tr>
<td>IT-4</td>
<td>0 e</td>
<td>100 a</td>
<td>86.6 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td>IT-6</td>
<td>0 e</td>
<td>100 a</td>
<td>97.5 a</td>
<td>1.6 b</td>
</tr>
<tr>
<td>LU-2</td>
<td>0 e</td>
<td>100 a</td>
<td>100 a</td>
<td>1.4 b</td>
</tr>
<tr>
<td>LU-3</td>
<td>0 e</td>
<td>100 a</td>
<td>100 a</td>
<td>2.7 a</td>
</tr>
<tr>
<td>MA-3</td>
<td>0 e</td>
<td>99.1 a</td>
<td>90 a</td>
<td>0.6 b</td>
</tr>
<tr>
<td>NB-1</td>
<td>32.5 c</td>
<td>77.5 b</td>
<td>92.5 a</td>
<td>2.0 a</td>
</tr>
<tr>
<td>NB-2</td>
<td>50.8 b</td>
<td>99.1 a</td>
<td>91.6 a</td>
<td>2.1 a</td>
</tr>
<tr>
<td>NU-5</td>
<td>3.3 e</td>
<td>99.1 a</td>
<td>95.8 a</td>
<td>1.7 b</td>
</tr>
<tr>
<td>PE-4</td>
<td>48.3 b</td>
<td>70.8 b</td>
<td>95 a</td>
<td>2.0 a</td>
</tr>
<tr>
<td>QU-2</td>
<td>4.1 e</td>
<td>100 a</td>
<td>100 a</td>
<td>1.3 b</td>
</tr>
<tr>
<td>SI-9</td>
<td>16.6 d</td>
<td>83.3 b</td>
<td>87.5 a</td>
<td>3.2 a</td>
</tr>
<tr>
<td>SO-11</td>
<td>10 d</td>
<td>98.3 a</td>
<td>100 a</td>
<td>2.6 a</td>
</tr>
<tr>
<td>TN-1</td>
<td>23.3 d</td>
<td>91.6 a</td>
<td>99.1 a</td>
<td>1.8 b</td>
</tr>
<tr>
<td>VR-1</td>
<td>3.3 e</td>
<td>100 a</td>
<td>100 a</td>
<td>1.9 a</td>
</tr>
</tbody>
</table>

Means followed by the letter in the column did not differ (P < 0.05) by the Scott-Knott test.
The seeds inoculated with NB-2 and PE-4 isolates presented the highest germination rate, followed by control. These isolates inhibited 21 and 25%, respectively, seed germination considering the control as a reference (100%). After these two, the lesser aggressive isolates were CO-1, NB-1 and CL-4 isolates, which inhibited 32%, 50% and 58%, respectively. The CH-1, CH-3, CJ-1, CN-1, IT-3, IT-4, IT-6, LU-2, LU-3, MA-3, VR-1 isolates greatly affect the germination rates, with values below 23%. Among them, CJ-1, CN-1, IT-3, IT-4, IT-6, LU-2, LU-3 and MA-3 isolates present no seed germination. There was no correlation between the isolate aggressiveness (low germination) and conidial morphology, since the more aggressive isolates present falcate and fusiforme conidia. Cylindrical conidia was identified in moderate or low aggressiveness isolates, although high acervuli and mucilage incidence was observed.

All isolates had acervuli and mucilage incidence on seeds, which was positively related with germination rate (81% of the isolates). In uninoculate seeds, there were no acervuli and/or mucilage incidence from Colletotrichum spp.

Low seed quality was observed in previous studies evaluating soybean-Colletotrichum pathosystem. *C. truncatum* isolates reduced in vitro soybean seed germination and viability by 29.2% and 26.8%, respectively. Under greenhouse conditions, the germination rate was 46.4% less and an increased frequency of pre and post-emergence damping-off was observed compared with the control (Begum et al., 2008). Although, according to Galli et al. (2005), the contact period between soybean seeds and *C. dematium* var. *truncata* influences the germination rate and 40 hours of incubation was sufficient to warrant completely infected seeds.

Extracelluar enzymes amylase was not detected in vitro, however halo formation would indicate degradation by amylase activity. Similar results were found for Colletotrichum spp. (Tozze et al., 2016). According to the authors, amylase production cannot be correlated with halo formation or insufficient amylase for halo formation, because amylase production by filamentous fungi varies according to genus and species.

The proteinase activity was identified in all *Colletotrichum* isolates, except CH-3. The *Colletotrichum* isolates were classified into two groups, according to the halo size. The first group (CH-1, CJ-1, CL-4, CN-1, IT-4, LU-3, NB-1, NB-2, PE-4, VR-1 isolates) present halo size larger than 1.98 cm. The second group was composed by CO-1, IT-3, IT-6, LU-2, NU-5, QU-2, TN-1 and MA-3 isolates. Broad variation of proteolytic activity from *Colletotrichum* spp. (Tozze et al., 2016) and *C. gloeosporioides* (Assis et al., 2010) isolates corroborate with the results of our study. However, *Colletotrichum* spp. from avocado fruits presented protease values up to eight times higher (Tozze et al., 2016) than registered in our study.

The pathogenicity of some fungi is related to the ability to produce enzymes that degrade cell wall (Ramos et al., 2010). Redman & Rodriguez (2002) reported proteinase is an essential enzyme to support pathogenicity and virulence of *C. coccodes* in tomato fruits. The enzyme activity was confirmed by mutants that did not produce the proteinase, which had nonpathogenic endophytic action.

Biochemical markers, such as amilolytic, cellulosolytic, lipolytic and and physiological markers, such as mycelial growth, did not contribute to distinguish isolates into groups by aggressiveness. There were no correlation between in vitro amilolytica and proteolytic enzymatic activities and *Colletotrichum* isolates aggressiveness. The same result was also reported to *Colletotrichum gloeosporioides* (Almeida & Coelho, 2007). However, enzymatic activity was significantly different among five isolates of *C. gloeosporioides* in guava fruits (Wahid, 2010).

**Conclusion**

Our results indicate a broad morphological variation in the *Colletotrichum* isolates in the soybean cropland in Mato Grosso State, Brazil, and the great potential impact on the seed germination rate. It indicates that there are more than one *Colletotrichum* species able to cause anthracnose due to the identified population diversity. Our data indicates that future research is needed to molecular identify the species and, therefore, support the decision-make process in the agricultural practices (e.g., crop rotation, chemical control) and to driven plant breeding programs.

**References**


