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Morphological and molecular characterization of *Curvularia lunata* pathogenic to Andropogon grass

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ABSTRACT: The fungal genus *Curvularia* is associated with a number of diseases in plants, commonly producing foliar spots in forage grasses. The objective of this study was to characterize the morphological and molecular diversity of the isolates of *Curvularia* sp. associated with Andropogon seeds, and to assess both their capacity to transmit disease and the pathogenicity of this fungus to crop. Ten isolates of *Curvularia* sp. were sourced from Andropogon seeds from agricultural producing regions in the Brazilian states Tocantins and Pará. Morphological characterization was achieved by observing fungus colonies and conidia and molecular characterization by DNA extraction and amplification with sequence-specific primers. The disease transmission was evaluated from seed sowing, in which after 40 days typical symptoms of *Curvularia* sp. were observed. Pathogenicity was evaluated by inoculating conidial suspension into the leaves of healthy plants, and after ten days, inspecting for pathogenic symptoms. Based on morphological and molecular features, the pathogen associated with Andropogon seeds was identified as *Curvularia lunata*, which, as such, is transmitted through the Andropogon plants via its seeds and is pathogenic to this species of forage grass.

Key words: *Andropogon* L., *Curvularia lunata*, diagnosis, phylogeny, pathogen.

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The genus *Curvularia* consists of more than 40 species distinguished by differences that are very often evident in the conidia morphology, number of septa and the colony morphology (Zhang et al. 2004; Chung and Tsukiboshi 2005). The species of this fungal genus consists of saprophytes, endophytes and pathogens (Sanchez-Marquez et al. 2008). Most of these species are pathogenic and give rise to losses in agricultural production. In vegetables, there are several examples of diseases caused by this genus responsible in the main part for necrotic spots in the leaves of several plant families (Dasgupta et al. 2005), especially Poaceae (Toledo et al. 1990; Yago et al. 2011; Silva et al. 2014; Santos et al. 2014; Kusai et al. 2016).

In the Poaceae family, the Andropogoneae group has 85 genera and 960 species, of which the most representative genus is *Andropogon*, which belongs to the Panicoideae subfamily. This genus is characterized by its wide distribution in the tropical and subtropical regions of the Planet (Clayton and Renvoize 1986). In Brazil, Andropogoneae comprise 10% of all species that make up the family and are found in all regions (Hartley 1958). This species is for the most part perennial, often spawns multiple shoots, and is especially noted for its feathery inflorescences (Zanin and Longhi-Wagner 2006).

The state of Tocantins has an area of approximately 7.5 million hectares recognized for its stretches of pasture, and is notable for the presence of the genus *Andropogon* (Seagro 2015). Furthermore, this forage grass has a substantial presence in areas that have undergone some kind of transformation, along roadsides and in clearings (Zanin and Longhi-Wagner 2006), and takes on the role of an invasive plant thanks to its ability to easily disseminate seeds.

It is commonly known that, despite the vast areas under cultivation, there is little information available relating to diseases which affect forage species of the *Andropogon* genus. Thus, it is against this background that the chosen objective of this study was to characterize the morphological and molecular diversity of *Curvularia* sp. isolates associated with Andropogon seeds, and to evaluate the transmissibility capability and pathogenicity of *Curvularia* sp. in Andropogon plants.

For the assay, a total of ten monosporic isolates collected from Andropogon seeds were characterized. Each isolate corresponds to one region, i.e., Araguacema (ARA), Araguaína (ARG), Brejinho de Nazaré (BJN), Caseara (CAS), Gurupi (GUR), Lagoa da Confusão (LGC), Marianópolis (MAR), Palmas (PAL) and Sucupira (SUC), cities located in the state of Tocantins and Santa Maria das Barreiras (SMB) in the State of Pará. All cities are important agricultural producers.

Morphological identification was carried out drawing on micro and macromorphological observations, based on characteristics described by Ellis (1971) and Sivanesan (1987) for the *Curvularia* genus. The isolates were grown in Petri dishes in a potato-dextrose-agar medium (BDA). Subsequently, the characteristics of the colonies and the conidia were observed. The analyzed colonies morphological characteristics were as follows: mycelial aspect (cottony or thin), border shape (regular or irregular), colony coloration (black, moss green or grey), colony reverse side (with or without dark pigmentation) and absence or presence of conidia in BDA after ten days of incubation in a chamber at a temperature of 25 °C ± 2 °C and a 12-hour photoperiod. The conidia were collected with a platinum loop and transferred to slides, where 50 conidia were randomly counted per sample, using an optical microscope (400X) coupled to a camera, which was designed and measured by the TSView7 software program. From the data obtained by conidia measuring, a Scott-Knott test ($p < 0.05$) was performed to generate an isolates clustering according to the conidia characteristics, using the SISVAR software program (Ferreira 2014).

DNA extraction was carried out by scraping the mycelium and reproductive structures formed in monocultures of the *Curvularia* sp. previously identified by morphological analysis and cultivated in BDA for ten days in a chamber at a temperature of 25 °C ± 2 °C and a 12-hour photoperiod. With the help of a sterile scalpel, the mycelium was transferred to 2 mL tubes, and the biomass obtained in this process was macerated in liquid nitrogen with a glass rod. DNA extraction was carried out according to the CTAB-based protocol (Murray and Thompson 1980).

To amplify the DNA from the isolates, primers P1 (forward: 5'- ATGGACGAGAACAACAGGATAACGA-3') and P2 (reverse: 5'CTACCAGCATTTAAGTTTACTCCAG-3') based on the nucleotide sequence of the *Clg2p* Ras protein gene described by Hou et al. (2013), specific to *C. lunata* were used. The total volume of the amplification reactions was 50 µL, comprising 25 µL of Taq DNA Polymerase 2x Master Mix RED (Ampliqon III PCR Enzymes & Reagents), 5 µL of each 10 µM primer oligonucleotide, 10 µL of ultrapure water and 5 µL DNA of the samples. PCR reactions were run on a thermal cycler (TC-5000) programmed for an initial cycle of 5 min at 95 °C (initial denaturation), followed by 35 cycles of 30 s at 95 °C (denaturation), 40 s at 55 °C (annealing), 30 s →

at 72 °C (extension) and a final cycle of 5 min at 72 °C (final extension). The PCR product was subjected to electrophoresis in 0.8% agarose gel (TAE 1X) stained with Neotaq Brilliant Green Plus DNA Stain (Neobio). DNA concentration was measured at 230 and 280 nm using a spectrophotometer (Biodrop – μLite).

The products used to amplify the *C. lunata* samples were purified using a Purelink® purification kit (Invitrogen) following the manufacturer's recommendations and were subjected to direct sequencing using the P1 forward primer. The DNA concentration after purification ranged from 88.06 to 192.8 ng·μL^{-1}. The products were sent for sequencing by the Sanger method to the DNA Sequencing Laboratory of the Catholic University of Brasilia, Brazil. After the sequences were obtained, they were alignment using the ClustalW in MEGA (version 7.0) software program, then, similar sequences were identified using the GenBank BLASTn tool. After alignment, these sequences were processed using the MEGA software program (version 7.0) (Tamura et al. 2011) to elaborate a phylogenetic tree of the isolates, using the Maximum Likelihood method and the Kimura 2 evolution model with 2000 Bootstrap replicates.

After molecular characterization and confirmation of the *C. lunata* specie, the capacity to transmit the fungus via seedlings was evaluated, as well as the capacity of this pathogen to infect Andropogon plants.

For the transmissibility test a total of 200 seeds from each sample was used without fungicides. Pots, with a capacity of 5 L, were sown with a mixture of sand, soil and commercial substrate (Bioplant – Type Biofibra) autoclaved in a ratio of 1:1:1. The pots were placed at a distance of 1 m between each other as to avoid contamination between plots. The material was kept in a greenhouse, the humidity of the pots being maintained at field capacity. Forty days after sowing, the plants with symptoms of Curvularia spots were assessed. In order to fulfill the Koch's Postulates, fragments of leaves that presented symptoms were isolated in BDA medium.

The pathogenicity of *C. lunata* isolates from Andropogon seeds was evaluated by inoculating the fungus into healthy plants. After 25 days of sowing, a conidial suspension containing 1×10^6 conidia m·L^{-1} was sprayed onto the plants leaves. Adjacent plants were sprinkled with water and kept under the same condition. After inoculation, the plants remained in a dark humid chamber for 36 h, and were then placed in a greenhouse, where after ten days pathogenicity was evaluated. When symptoms were observed in the inoculated tissue, the fungus was reisolated and grown in BDA medium (Alfenas and Mafia 2007).

The results found in the morphological characterization assay, in relation to the fungal mycelium, showed little variation between cotonous and thin, only the LGC isolate had a thin aspect. This variation of LGC isolate is common in fungal isolation. The difference in mycelium color or texture can often occur with the fungus removed in the same Petri dish, being, therefore, a characteristic that alone should not be considered in the species identification. The shape of the colony borders also presented variations between regular and irregular throughout its growth. The BJN, PAL and SMB isolates presented an irregular shape. There was a prevalence of coloration of greyish colonies in all the isolates obtained, dark pigmentation was observed on the reverse side of the colony. After ten days of incubation, the presence of dark brown conidia was observed. According to Sun et al. (2003), only the morphological analysis of the *Curvularia* genus does not support a safe identification, requiring analysis based on another marker, such as DNA region sequencing.

Regarding of qualitative characteristics from *Curvularia* sp. isolates, the conidia were mostly similar. All isolates presented smooth and curved conidia, varying only in the presence or absence of hilum. The presence or absence of hilum was dependent on the conidia's age, with older conidia having prominent hilum whereas younger conidia do not. The LGC, PAL, SUC and SMB isolates demonstrated variations in the range of 60% – 90% for the presence of hilum in the conidia. As for the number of septa per conidia, there was, in fact, a significant effect on the isolates analyzed (CV 16.51%), and MAR and PAL had a higher number of septa in relation to the other isolates, with means of 3.30 and 3.40 septa per conidia, respectively. The SMB isolate presented the lowest number of septa per conidia (2.20). The remaining isolates presented mean values varying between 2.80 and 2.90 septa per conidia. The *Curvularia* sp. isolates showed variations in both length and width of the conidia, with a mean of 30.62 μm in length (CV 14.01%) and 13.65 μm in width (CV 13.69%).

The morphological characteristics observed during the microscopic analysis suggested that the fungus genus isolated from Andropogon seeds is *Curvularia lunata*, similar to that described in the relevant literature (Ellis 1971; Sivanesan 1987). Other authors have also obtained similar results when examining species infecting other plants, for example, rice, Silva et al. (2014) and Kusai et al. (2016), characterized *C. lunata* colonies in BDA with cottony appearance and →

blackish-gray coloration. Also for these authors, the conidia presented three transverse septa, with a more voluminous median cell, pronounced curvature and dark hilum at one end, having average dimensions of 20.62 µm × 8.58 µm.

Studies demonstrate morphological variation in conidia when they are produced at different substrates (Ellis 1971; Sivanesan 1987). Thus, morphological variation of conidia of the same species is seen in relation to the number of septa and conidia size. In the present study, the random collection of conidia in different positions in the Petri dish may also have influenced these variations, due to the conidia being more aged when located in the center of the plate than on the edge. However, morphological analysis alone is insufficient to guarantee accurate identification, and therefore additional analysis, based on molecular methods, are required.

PCR amplification using the P1 and P2 primers resulted in fragments of approximately 870 base pairs for all *C. lunata* samples analyzed (Figure 1). No amplification was observed when an isolate from another fungal species was used (negative control – *Fusarium verticillioides*). These results suggest that all isolates detected in Andropogon seeds belong to the *C. lunata* species. This information corroborates the data obtained by Hou et al. (2013) who, using the same primers, found fragments between 827 and 870 bp for the region of the *Clg2p* Ras protein gene from *C. lunata* isolated from maize leaves.

The *C. lunata Clg2p* Ras protein gene region was sequenced and deposited in the Genbank (KY273134) for better characterization of the isolate, providing a subsidy to complement identification with morphological analysis. The nucleotide sequence of ten *C. lunata* isolates obtained from Andropogon seeds shared 89% identity with the *C. lunata* (teleomorphic: *Cochliobolus lunata*) (HQ655805) isolate from China, available from the GenBank. As expected, phylogenetic analysis demonstrated that all *C. lunata* DNA sequences obtained from Andropogon seeds were framed in the same branch as the reference isolate described by Hou et al. (2013) with bootstrap of 99% (Figure 2). The tree also demonstrates the phylogenetic relationships of *Clg2p* protein of *C. lunata* with other Ras proteins from related fungi such as *Bipolaris victoriae* (XM014700014), *B. oryzae* (XM007686909), *Pyrenophora tritici-repentis* (XM001933533) and *Paraphaeosphaeria sporulosa* (XM018176978).

Special primers using the Ras protein target gene for the best detection of *C. lunata* isolates was successfully developed by Hou et al. (2013) and is reproduced in this study. The Ras *Clg2p* protein gene has been used as a molecular marker to identify a number of fungi because *exons* are conserved and *introns* vary in almost all fungal species, thus demonstrating application potential in detection studies of different fungal species.

Detailed knowledge of a disease causal agent is essential to ensuring that the result of epidemiological studies will be the development of efficient management strategies. Specifically, efforts search to identify the species commonly associated with the disease, as well as to understand the phylogenetic relationship between the pathogen and the dynamics of its populations (Kohn 2004; Jeger and Pautasso 2008). Using a combination of morphological data with molecular information it was possible to reliably identify all *C. lunata* isolates obtained from Andropogon seeds.

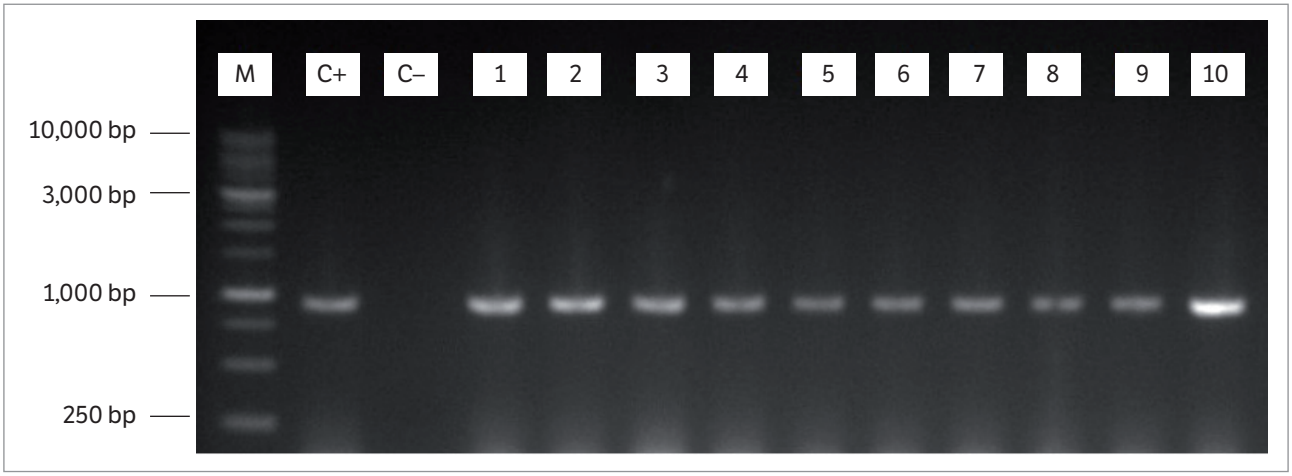

Figure 1. Electrophoresis in agarose gel of amplification products with specific primers for *Curvularia lunata*. M: Molecular marker of 1Kb; C+: Positive control; C–: Negative control (*Fusarium verticillioides*); 1: Araguacema; 2: Araguaína; 3: Brejinho de Nazaré; 4: Caseara; 5: Gurupi; 6: Lagoa da Confusão; 7: Marianópolis; 8: Palmas; 9: Sucupira; 10: Santa Maria das Barreiras.

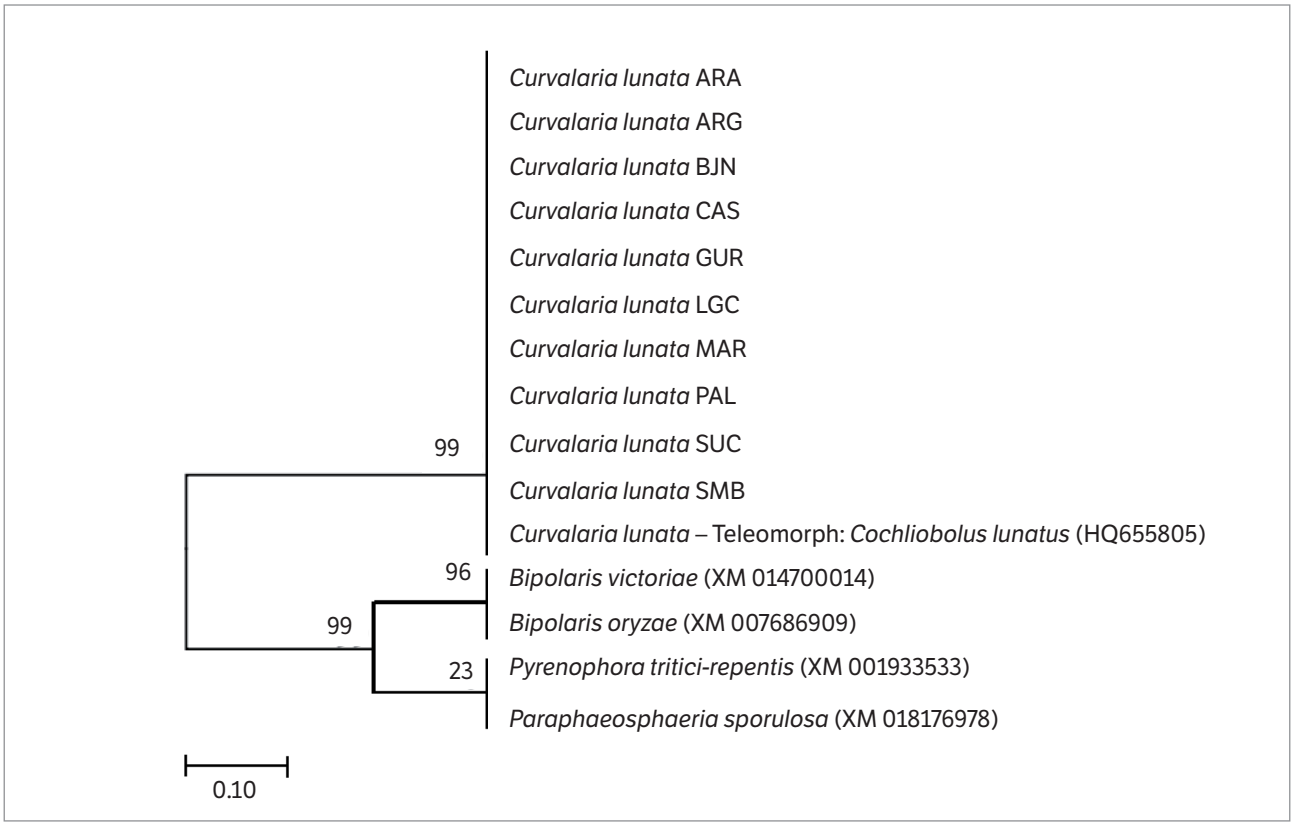

Figure 2. Phylogenetic tree of representative sequences of the amplified Ras *Clg2p* protein gene from *Curvularia lunata* DNA isolated from Andropogon grass, derived by the Maximum Likelihood Method using the Kimura 2-parameter evolution model. The nodes numbers indicate a bootstrap value of 2000 replicates. Nucleotide sequences of *Bipolaris victoriae* (XM014700014), *Bipolaris oryzae* (XM007686909), *Pyrenophora tritici-repentis* (XM001933533) and *Paraphaeosphaeria sporulosa* (XM018176978) were used as outgroup.

The transmission of *C. lunata* via seeds was evidenced in Andropogon plants 40 days after sowing. Plants from Araguacema, Araguaína, Brejinho de Nazaré, Caseara, Gurupi, Marianópolis, Palmas and Sucupira regions showed symptoms of Curvularia spot disease, with small elliptic necrotic spots, slightly oval with a reddish brown to light brown center. These same symptoms were evidenced in the pathogenicity test, after inoculation of *C. lunata*, in Andropogon plants in all seed samples from the regions analyzed.

It is commonly understood that seed infection does not ensure the pathogen transmission, because factors linked to the environment and the host can bring influence to bear which must be taken into account (Machado 1994). Lasca et al. (2004) and Silva et al. (2014) presented evidence regarding the transmission of *Curvularia* sp. from seeds of other species of grass, *Brachiaria*, rice, sorghum and millet. In the African continent, Toledo et al. (1990) reported species of the genus *Curvularia* causing leaf spot in *Andropogon gayanus* and *Andropogon tectorum*.

Based on morphological and molecular markers, the fungus identified with high incidence associated with Andropogon seeds collected in different agricultural producing regions was *Curvularia lunata*. This fungus is transmitted to Andropogon plants via seeds, is pathogenic to this species of forage grass, and causes foliar necrotic spots.

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