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Molecular Characterization of Isolates of *Fusarium* spp. Associated With Wilt in *Capsicum* spp.

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Abstract

Fusarium is a diverse and heterogeneous fungi genus. Its wide genetic variability and similarity in morphological characteristics hinder the identification of species of this genus. Identifying *Fusarium* species is difficult due to the genus. Several molecular methods have been useful for differentiating these species, and the amplification of internal transcribed spacer (ITS) regions of the fungus ribosomal DNA has been successfully used, since ITS are preserved regions of the DNA that assists in distinguishing species. The objective of this work was to collect and characterize isolates of *Fusarium* spp. associated with wilt symptoms in *Capsicum* spp. in the biomes of the state of Mato Grosso, Brazil. Were collected tissue samples of plants with wilt symptoms. The DNAs of the *Fusarium* spp. found were extracted, and subjected to polymerase chain reaction, using the primers ITS1 (5'TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Subsequently, the sequencing was performed. The resulting sequences were, five *Fusarium* species were found *F. solani*, *F. oxysporum*, *F. equiseti*, *F. incarnatum*, *F. chlamydosporum*, predominating *F. solani* and *F. equiseti*.

Keywords: mycobank, genetic variability, pepper, wilt

1. Introduction

Fusarium is a diverse and heterogeneous fungi genus that is important in the food and drug industry, medicine, and agriculture (Luginbuhl, 2010). *Fusarium* species are worldwide spread; thus, this species have greater genetic, and pathogenic variability (Gonçalves, 2015). They are also known for their ability to cause several diseases in diverse hosts (Summerell et al., 2003).

Several pathogens cause wilt in *Capsicum* spp. (Ochoa & Ramirez, 2001; Naik et al., 2008; Singh et al., 2017), however, wilt caused by *Fusarium,* especially *Fusarium solani* and *Fusarium oxysporum*, is the main disease in peppers and chilies, and a serious problem for these crops in the last years (Raghu et al., 2016; Tembhurne et al., 2017).

One of the predominant characteristics of *Fusarium* species is the formation of asexual spores—macroconidia and microconidia (Teixeira, 2015). They also produce resistance structures called chlamydospores (Bedendo, 1995), which assures their survival in plant and soil debris for many years and makes their control and eradication difficult (Raghu et al., 2016; El Kichaoui et al., 2017).

Identifying *Fusarium* species is difficult due to the genus amplitude and the lack of tools for a reliable differentiation of these species (Lievens et al., 2008). Moreover, distinguishing its species, formae speciales, and races is complicated, even for specialists (O'Donnell et al., 1998; Windels, 1991).

However, several molecular methods have been useful for differentiating these species (Kistler et al., 1987). The amplification of internal transcribed spacer (ITS) regions of the fungus ribosomal DNA (rDNA) has been successfully used for this purpose (Hillis & Dixon, 1991; Menezes et al., 2010), since ITS are preserved regions of the DNA that assists in distinguishing species (Chen, 2004). ITS is located between the 18SrDNA and 28SrDNA genes, and this region can be divided into ITS1 (genes 18S to 5.8S) and ITS2 (genes 5.8S to 28S) (Hillis & Dixon, 1991). Amplification of ITS regions is indicated for distinguishing species or varieties, because they are rapidly evolving regions (Fungaro, 2000).

Thus, the objective of this work was to collect and characterize isolates of *Fusarium* spp. associated with wilt symptoms in *Capsicum* spp. in the biomes of the state of Mato Grosso, Brazil, creating a mycobank of high genetic variability.

2. Method

2.1 Study Area

Fusarium species were collected in five cities, representing the three biomes of the state of Mato Grosso, Brazil: Alta Floresta (Amazon), Cáceres (Pantanal), Juína (Cerrado), Mirassol d'Oeste (Amazon), Tangará da Serra (Amazon).

The morphological identification of the isolates from these collections was carried out in the Plant Breeding Laboratory of the Mato Grosso State University, Cáceres campus (PBL-UNEMAT). The molecular identification of the isolates, via sequencing of the ITS1 and ITS2 regions, was carried out by the company ACTGene Análise Moleculares Ltda., in the Biotechnology Center of the Federal University of Rio Grande do Sul, Porto Alegre RS, Brazil.

2.2 Isolate Collection

Producing farms of *Capsicum* spp. were visited and plants presenting the characteristic symptoms caused by *Fusarium*—wilt, yellowing, tipping, and stem base necrosis—were collected, packed, and sent to PBL-UNEMAT for further analysis and identification of the causal agent.

The areas evaluated were delineated using a GPS to obtain the geographical data of each collection point. Subsequently, these data were input into the ArcGis 10.1 software (Esri, Redlands CA, USA) to create a geographic database of the research.

2.3 Pathogen Identification

Stem and root samples of symptomatic plants were collected to isolate the pathogen (Figure 1). These samples were placed in Petri dishes containing PCNB-agar medium (Nash & Snider, 1962) and incubated in BOD with photoperiod of 12 hours, at temperature of 25±2 ºC, for approximately seven days.

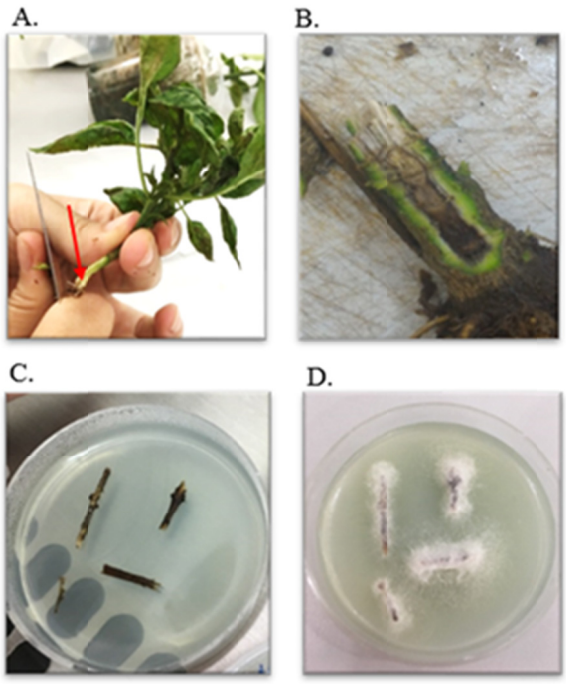

Figure 1. Isolation of symptomatic tissues of Capsicum spp. collected in the biomes of the state of Mato Grosso, Brazil. A) Segmentation of symptomatic root and stem base tissues; arrow showing an initial necrosis in the stem base; B) Stem showing necrosis; C) Fragments of symptomatic tissue in PCNB-agar medium; D) Mycelial growth after the incubation period

The presence of pathogens was confirmed via morphological characterization of the isolates, which was carried out after their growth, in approximately 7 days, using a microscope. The characterization was based on colony coloration (Figure 2); presence, size, and shape of macroconidia and microconidia (Figure 3); presence and arrangement of chlamydospores; and type of phialides where the conidia were formed (Figure 4) (Nelson et al., 1983; Nirenberg, 1990).

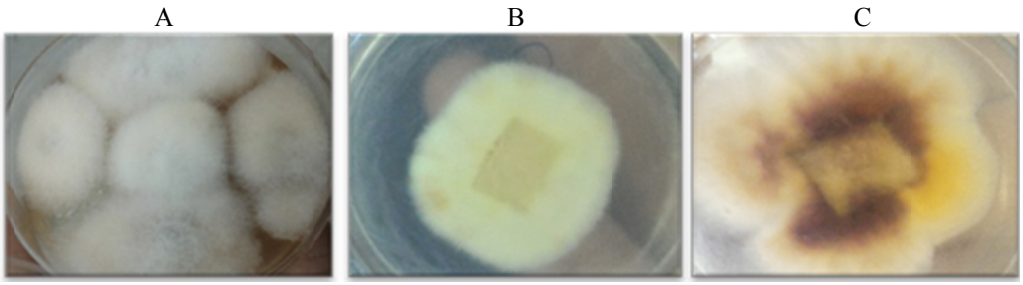

Figure 2. Coloration in PCNB-agar medium of colonies of *Fusarium* spp. collected from *Capsicum* spp. in the biomes of the state of Mato Grosso, Brazil. A) Petri dish with mycelial growth—upper perspective; B) Petri dish with light-colored mycelial growth—lower perspective; C) Petri dish with yellowish-purple mycelial growth—lower perspective

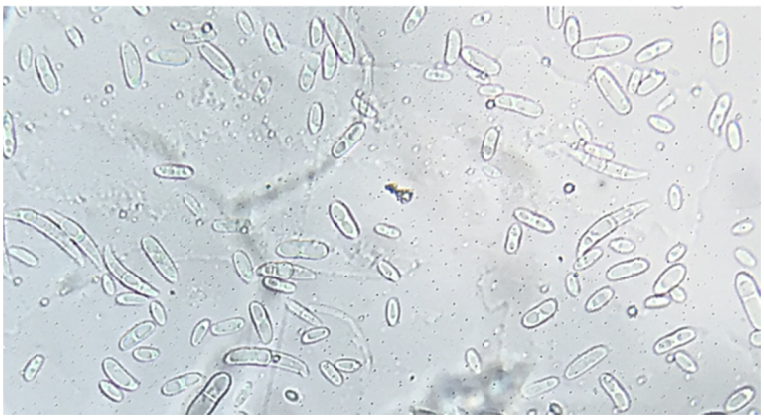

Figure 3. Macroconidia and microconidia of *Fusarium* spp. collected from *Capsicum* spp

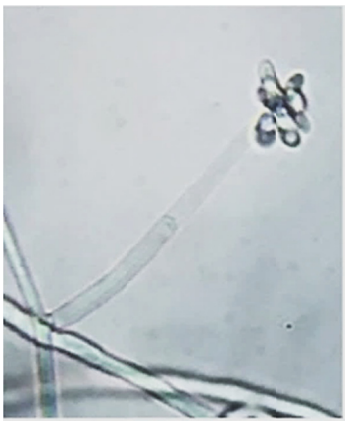

Figure 4. Phialides of *Fusarium* spp. collected from *Capsicum* spp

The isolates confirmed as Fusarium spp. were stored by the filter paper preservation method. Thus, small pieces of sterile filter paper were placed in a potato-dextrose-agar (PDA) culture medium, with mycelium of the isolate to be conserved, for a period of approximately 7 days. Subsequently, these papers were withdrawn and placed in

sterile Petri dishes, which remained for approximately 7 days in BOD to reduce moisture. They were then sealed, stored in a refrigerator, and cataloged in the mycobank of the PBL-UNEMAT.

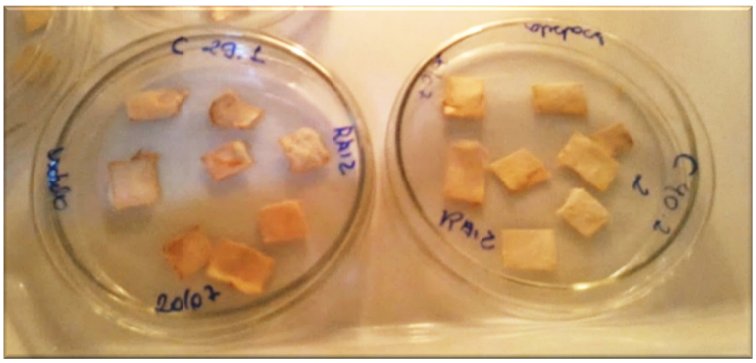

Figure 5. Isolates of Fusarium spp. collected in Capsicum spp. in the biomes of the state of Mato Grosso, Brazil, stored of in the mycobank of the Plant Breeding Laboratory of the Mato Grosso State University, Cáceres campus

2.4 Molecular Characterization

The molecular characterization of the isolates of *Fusarium* spp. begun with DNA extraction from the collected isolates. Thus, the isolates of *Fusarium* spp. were cultured in Petri dishes with PDA medium for approximately 7 days. The mycelium was then withdrawn with spatulas and macerated in liquid nitrogen. The Wizard® Genomic DNA Purification Kit (Promega) and the protocol recommended by its manufacturer were used for the extraction.

The polymerase chain reactions (PCR) were carried out using 2 µL of DNA from each isolate, 2.5 µL of 10× PCR buffer, 2 µL dNTP (2.5 mM), 1.25 µL of each ITS primer, 0.125 µL of Taq polymerase, and ultrapure H_2O qsp to 25 µL. The protocol consisted in denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 secs, 58 °C for 45 secs, and 72 °C for 1 min, and elongation at 72 °C for 10 min in a thermal cycler. PCR products were subjected to electrophoresis in 1.5% agarose gel. Amplicon sizes were determined with the 100pb DNA Ladder marker (Sigma-Aldrich Inc., St. Louis MO, USA).

The samples were then sequenced, and the molecular identification of the isolates was performed by partial sequencing of the ITS regions 1 and 2 of the rDNA, using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for the amplification of the rDNA ITS region (White et al., 1990). The sequences were compared with the GenBank database of the National Center for Biotechnology Information (NCBI) through the BLAST program. A phylogenetic tree with collected isolations and the references *F. solani*-MF401578.1, *F. equiseti*-MF471699.1, *F. oxysporum*-MG356946.1, *F. chlamydosporum*-EU520242.1, *F. incarnatum*-MH045587.1. The phylogenetic tree were obtained using the analysis of Maximum Likelihood, The alignments and analysis were made using the software Mega 5.0 (Tamura et al., 2011).

3. Results

Eighty-nine plants of *Capsicum* spp., collected in the biomes of the state of Mato Grosso, presented characteristic symptoms of those caused by *Fusarium*—wilt, yellowing, tipping, and stem base necrosis. *Fusarium* spp. were found in 74 of these plants.

The geographic data of the collection points were used to develop a thematic map with the regions in which plants with *Fusarium* spp. were found (Figure 6).

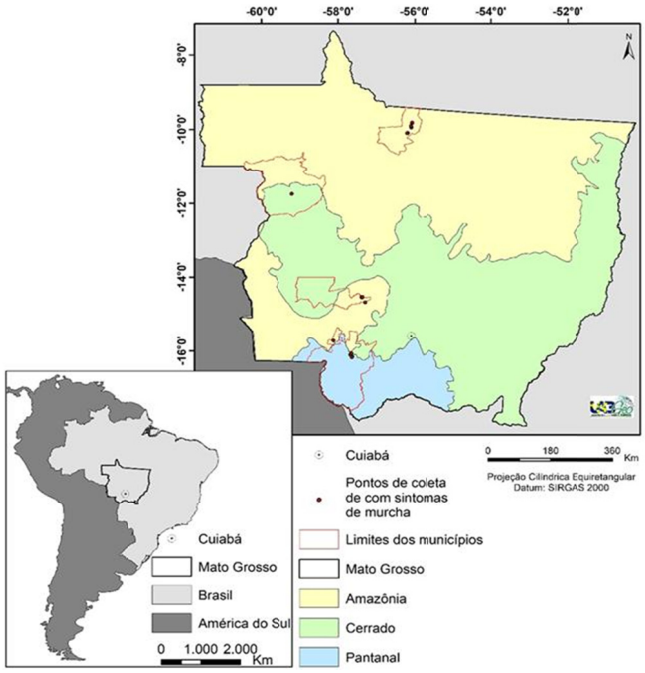

Figure 6. Map of collection points of *Capsicum* spp. in which *Fusarium* spp. were found

Based on the morphological data of the isolates, 26 isolates were selected for sequencing. The species of *Fusarium* spp. identified were *Fusarium solani* (10 isolates), *Fusarium equiseti* (10 isolates), *Fusarium oxysporum* (3 isolates), *Fusarium incarnatum* (2 isolates), and *Fusarium chlamydosporum* (1 isolate) (Table 1).

Table 1. Collected Coordinates points of *Fusarium* ssp. in plants of *Capsicum* spp. in three different biomes of Mato Grosso state

Isolates	Cities	Biomes	CWWrdinates		Species
1	Cáceres	Pantanal	16°9′46.05″ S	57°38′10.38″ W	*Fusarium solani*
2	Cáceres	Pantanal	16°9′43.93″ S	57°38′18.89″ W	*Fusarium solani*
3	Cáceres	Pantanal	16°9′41.83″ S	57°38′18.20″ W	*Fusarium equiseti*
4	Cáceres	Pantanal	16°9′46.47″ S	57°38′1.45″ W	*Fusarium solani*
5	Cáceres	Pantanal	16°10′43.03″ S	57°38′21.96″ W	*Fusarium solani*
6	Cáceres	Pantanal	16°04′34.5″ S	57°39′08.8″ W	*Fusarium equiseti*
22	Cáceres	Pantanal	16°04′34.5″ S	57°39′08.8″ W	*Fusarium solani*
28	Mirassol d'Oeste	Amazônia	15°43′15.45″ S	58°7′18.37″ W	*Fusarium equiseti*
29	Cáceres	Pantanal	16°6′39.92″ S	57°40′48.85″ W	*Fusarium solani*
30	Alta Floresta	Amazônia	10°05′28.7″ S	56°11′35.5″ W	*Fusarium incarnatum*
31	Alta Floresta	Amazônia	10°05′53.5″ S	56°12′34.4″ W	*Fusarium equiseti*
34	Alta Floresta	Amazônia	09°55′56.6″ S	56°04′04.0″ W	*Fusarium equiseti*
35	Alta Floresta	Amazônia	09°49′39.8″ S	056°04′41.3″ W	*Fusarium chlamydosporum*
36	Alta Floresta	Amazônia	09°49′06.8″ S	56°04′41.3″ W	*Fusarium equiseti*
37	Alta Floresta	Amazônia	09°48′39.9″ S	56°04′51.6″ W	*Fusarium oxysporum*
38	Alta Floresta	Amazônia	09°56′37.5″ S	56°05′56.5″ W	*Fusarium oxysporum*
39	Alta Floresta	Amazônia	09°54′29.3″ S	56°06′36.8″ W	*Fusarium equiseti*
40	Alta Floresta	Amazônia	09°54′29.3″ S	56°06′38.5″ W	*Fusarium incarnatum*
41	Tangará da Serra	Amazônia	14°32′58.6″ S	57°22′52.8″ W	*Fusarium equiseti*
42	Tangará da Serra	Amazônia	14°32′17.1″ S	57°23′11.1″ W	*Fusarium oxysporum*
43	Tangará da Serra	Amazônia	14°32′17.7″ S	57°22′41.2″ W	*Fusarium equiseti*
44	Tangará da Serra	Amazônia	14°32′51.7″ S	57°22′19.8″ W	*Fusarium solani*
46	Cáceres	Pantanal	16°04′34.1″ S	57°39′07.6″ W	*Fusarium solani*
48	Juína	Cerrado	11°44′50.13″ S	58°71′50.21″ W	*Fusarium solani*
49	Cáceres	Pantanal	16°04′34.1″ S	57°39′07.6″ W	*Fusarium equiseti*
50	Cáceres	Pantanal	16°04′34.1″ S	57°39′07.6″ W	*Fusarium solani*

The results obtained after sequencing the 26 isolates were used to create a phylogenetic tree of isolates, which showed how these isolates are grouped; they formed four distinct groups (Figure 7).

4. Discussion

The Group I consisted of ten *Fusarium equiseti* isolates found in the Amazon and Pantanal biomes, and two *Fusarium incarnatum* and one *Fusarium chlamydosporum* isolate found only in the Amazon biome. Reports on these isolates are scarce in scientific literature.

Although, Group II consisted of three isolates of *Fusarium oxysporum*, which were found only in the Amazon biome. This species, commonly confused with *F. solani*, is the causal agent of various vascular diseases in wilting plants. It has specific characteristics that is depended on its hosts, thus, it presents more than 100 *formae speciales*, and different races (Leslie & Summerell, 2006). This pathogen was found in plants of *Capsicum annuum* in Mexico (Vásques Lopes et al., 2009), Spain (Martinez et al., 2010), and Pakistan (Sahi & Khalid, 2007).

The Group III consisted of all the isolates characterized as *Fusarium solani*. Based on the geographic data, *F. solani* was found in plants of *Capsicum* spp. in the three biomes of the state of Mato Grosso, Amazônia, Pantanal and Cerrado.

Occurrence of *Fusarium solani* is found in several crops in Brazil, such as *Piper nigrum* (black pepper) (Rocha et al., 2015), and *Passiflora* spp. (Carvalho, 2015, Silva et al., 2014). However, no study on pathogens of the genus *Fusarium* was conducted with plants of *Capsicum* spp. Reports on the occurrence of wilt caused by *F. solani* in *Capsicum* spp. are found many countries. Mejía-Batista et al. (2016) identified *F. solani* as causal agent of wilt in *Capsicum chinense* in Mexico. Cases of wilt in *Capsicum annuum* were also reported in Spain (Martinez et al., 2010), China (Duan et al., 2016) and India (Tembhurne et al., 2017).

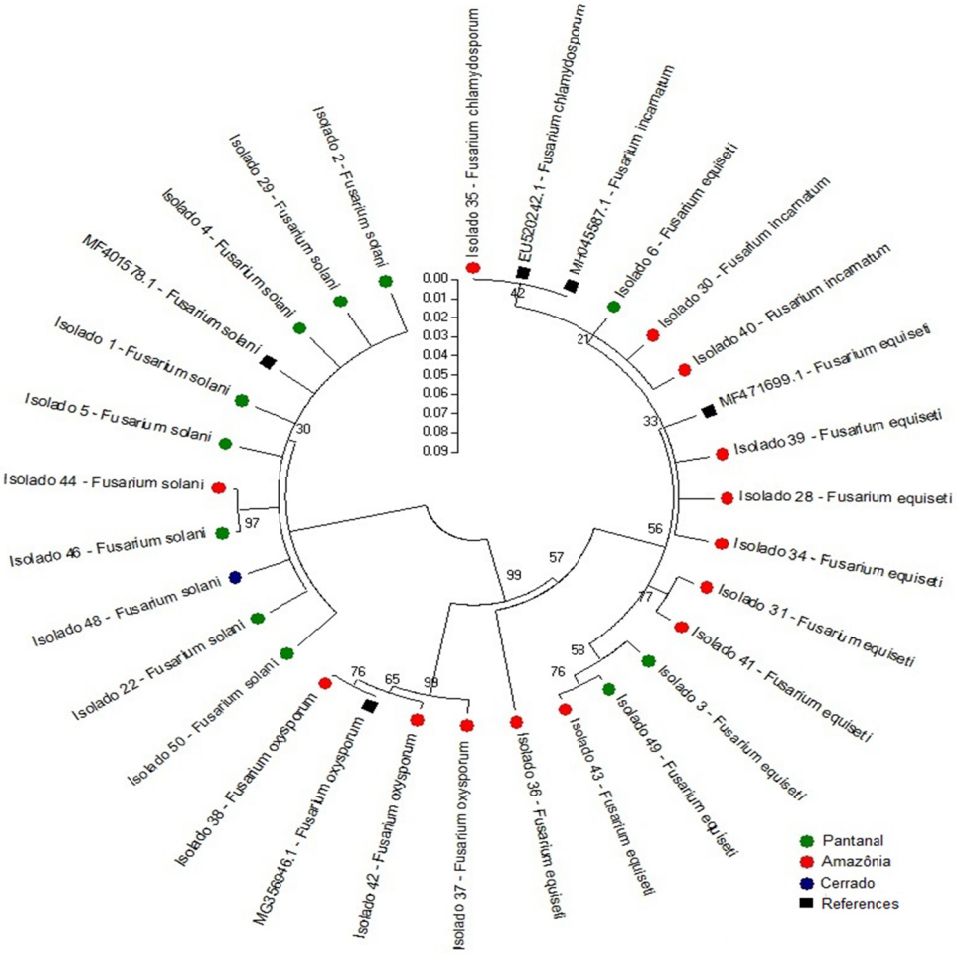

Figure 7. Phylogenetic tree of isolates of *Fusarium* spp. collected in plants of *Capsicum* spp. in the biomes of the state of Mato Grosso, Brazil

Recently the work of Mejía-Batista et al. (2016) found *Fusarium equiseti,* as well as *Fusarium solani,* as the causal agent of wilt in *Capsicum chinense* grown in Mexico.

A first report of occurrence of the pathogen *Fusarium incarnatum* in *C. annuum* plants in Trinidad and Tobago named the disease as pepper rot, since it was found in fruits of this crop and caused necrotic lesions (Ramdial et al., 2016). Plants with *F. incarnatum* presented symptoms of wilting and yellowing.

Fusarium is an important pathogenic fungi genus. It has a conflicting taxonomic history, due to the lack of clear morphological characters separating its species, and its variations and mutations in each host, that end up representing poorly the diversity of *Fusarium* spp. (Geiser et al., 2004). For example, conflicts in nomenclature are found in the literature; *F. incarnatum*, *F. semitectum* and *F. pallidoroseum* are commonly described as the same species (Leslie & Summerell, 2006). Moreover, *Fusarium chlamydosporum*, which is found in very few studies, is treated as *Fusarium sporotrichioides* var. *chlamydosporum*, and *Fusarium fusarioides,* according to the Leslie & Summerell Handbook (2006). This species was found in *C. annuum* seeds in Pakistan (Sharfun-Nahar et al., 2004).

5. Conclusions

Plants of *Capsicum* spp. with *Fusarium* spp. were found in the three biomes of the state of Mato Grosso, Brazil. This is probably the first report on the occurrence of *Fusarium* spp. in *Capsicum* spp. in Brazil. Sequencing ITS regions was efficient in differentiating the *Fusarium* species found. Five species of *Fusarium* were found in plants of *Capsicum* spp.: *F. solani*, *F. oxysporum*, *F. equiseti*, *F. incarnatum* and *F. chlamydosporum*.

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