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Morphological and phylogenetic characterization of *Fusarium* Link.

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Abstract

Identification of *Fusarium* isolates in a sample at the species level is an important and difficult task because many *Fusarium* species have similar morphological characteristics. The phylogenetic relations of species have been applied in *Fusarium* systematic and may solve taxonomic difficulties. The aim of the present study is to characterize pathogeny of Fusarium isolates through morphological analysis (concept of morphological species) associated with symptoms in hosts together with phylogeny analysis (concept of phylogenetic species) using internal transcribed spacer region (ITS) of ribosomal DNA for species identification. For the morphological characterization, *Fusarium* isolates were grown in PDA culture medium. Then, they were classified based on colony color and the microconidial, macroconidial and chlamydospore structures. The isolates were characterized molecularly by amplifying and sequencing the ITS region of the ribosomal DNA. The sequences generated were compared with those placed in the Genbank and Maximum Likelihood phylogenetic trees were constructed. Out of 14 isolates characterized morphologically and molecularly, five isolates were grouped in the *Gibberella fujikuroi* species complex in the Liseola section, seven presented characteristics of species from the *Elegans* section within the *F. oxysporum* species complex and two isolates presented characteristics of the section *Gibbosum* species complex in the *F. incarnatum-equiseti* species complex. Thus, seven isolates (section Elegans) belonged to the species *F. oxysporum*, two isolates (section Gibbosum) to the species *F. equiseti*. However, the ITS region of the ribosomal DNA did not provide sufficient evidence to define the species of G. fujikuroi species.

Keywords: Phylogeny, Morphology, *Fusarium oxysporum*, *F. equiseti*, *Gibberella fujikuroi*.
Abbreviations: ITS_Internal Transcribed Spacer. GFSC_*Gibberella fujikuroi* species complex, FGSC, FOSC, *Fusarium oxysporum* species complex, FIESC, *Fusarium incarnatum-equiseti* species complex, FSSC, *Fusarium solani* species complex ICN_ International Code of Nomenclature for algae, fungi, and plants

Introduction

The *Fusarium* species are commonly associated to many diseases of economically important crops and to mycotoxin producers (Summerell, 2019). The types of diseases induced by this species are varied, as well as their severity, which can include root or stem rot, canker, wilt, fruit or seed rot and leaf diseases. However, other habitats and hosts, including natural ecosystems and diversified agroecosystems, clinical and veterinary samples (O'Donnell et al. 2016; Papizadeh et al., 2018) and insects (Aoki et al., 2019; Santos et al., 2019) have also been shown to be important sources for the investigation of *Fusarium* species.

Therefore, identification of *Fusarium* isolates in sample of diseased plants, animal, insects generally at species level, is an important and difficult task in many plant diagnosis laboratories (Aoki et al., 2014; Stepień et al., 2013). Given these circumstances, it is critically important that *Fusarium* has a stable taxonomy with well-defined generic and species concepts that allows practitioners diagnosing diseases, identifying these fungi, and developing management strategies to make decisions about the identity of the species (Summerell, 2019).

The difficulty of studying these species is mainly due to their similar morphology and existence of different biological groups that include endophytes, saprophytes, and plant pathogens, and also human pathogens (Imazaki and Kadota, 2015; Moussa et al., 2017; Nayaka et al., 2009; Zarrin et al., 2016), which does not allow the precise resolution for adequate identification of new species (Choi et al., 2017).

As an alternative, the difficulties of morphological (morphological species concept) identification of the *Fusarium*, the molecular tools (phylogenetical species concept) have been used for precise identification of species (O'Donnell et al., 2018). Therefore, the phylogenetic studies have been utilized in *Fusarium* systematic and can be used to solve taxonomic difficulties by providing reliable pathogen identification (O'Donnell et al., 2015)

Fusarium includes 300 phylogenetically different species that were discovered via molecular phylogeny. However, most of the species have not yet been described formally (O'donnell et al., 2018).

Within the large genus of *Fusarium*, it has become customary to cluster closely related sibling species or lineages from little- to no-morphological differences in so-called species complexes (Van Diepeningen and Hoog, 2016). The term species complex is not well defined but usually implies a grouping of species with shared morphological characteristics and phylogenetic markers, and some level of cryptic speciation the ICN (Al-Hatmi et al., 2019). There are 23 species complexes that provide a mechanism to introduce researchers to the taxonomy of *Fusarium* and allow them to better comprehend the diversity of species within the genus (Summerell, 2019). The generic and species concepts in *Fusarium* have endured significant changes. Currently, *Fusarium* has been distributed within a species complex (Sandoval-Denis et al., 2018).

In addition to these results, recent studies have shown that most of the plant pathogens of this genus are grouped in species complexes such as the *Fusarium fujikuroi* species complex (FFSC), *Fusarium oxysporum* species complex (FOSC), *Fusarium incarnatum-equiseti* species complex (FIESC), *Fusarium solani* species complex (FSSC) (Leyva-Madrigal et al., 2015;Epstein et al. 2017; Maryani et al., 2019; Papizadeh et al., 2018)

Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successfully identifying a large variety of fungi, with the difference in barcodes more clearly defined between intra and interspecific variations in different variations including the genus *Fusarium.* The recognition of ITS as the official DNA barcode marker for fungi represents a noteworthy advance, which has greatly benefited the research community (Zarrin et al., 2016; Schoch et al., 2012; Nadarajah et al., 2015).

Thus, the aim of the present study was to characterize pathogenic *Fusarium* isolates from Brazil through morphological analysis (concept of morphological species) associated with symptoms in hosts such as vascular wilting, rotting (fruits, stems, and leaves), and insect colonization together with phylogenetic analysis (concept of phylogenetic species) of the internal transcribed spacer region (ITS) of ribosomal DNA for species identification

Results

Morphological characterization of the Fusarium isolate

The morphological characterization showed a range of variability in species that were observed pathogenic to plants and insects.

The macromorphological aspect of the coloring of the isolate colonies was separated into five groups. The first group had completely pink coloring. This predominant pink color was observed in the following isolates (MGSS 15, MGSS 42, MGSS 118, MGSS 141, MGSS 149, MGSS 182, MGSS 183), the second group presented pink colored colonies with pale borders (MGSS 54, MGSS 68, MGSS 157), the third group was formed by purple colored colonies (MGSS 09, MGSS 14), the fourth group was formed by a colony a salmon colored colony (MGSS 61) and the fifth group was formed by a burnt yellow colored colony (MGSS 138) (Fig. 1 and Table 4).

The micromorphological evaluation separated the isolates into six groups. Group 1 (Macroconidia 2- 5 septa, presence of Microconidia, Short Monophialides, presence of Chlamydospore): MGSS 14, MGSS 15, MGSS 42, MGSS 182, MGSS 183; Group 2 (Macroconidia 2- 5 septa, presence of Microconidia, Short Monophialides, absence of Chlamydospore): MGSS 09, MGSS 118; Group 3 (Macroconidia 2-5 septa, presence of Microconidia, Monophialides and polyphialides, absence of Chlamydospore): MGSS 54, MGSS 61, MGSS 68; Group 4 (absence of Macroconidia, presence of Microconidia, Monophialides and polyphialides, absence of Chlamydospore): MGSS 141, MGSS 157; Group 5 (Macroconidia 3-7 septa, absence of Microconidia, Monophialides, presence of Chlamydospore): MGSS 138; Group 6 ((Macroconidia 3-7 septa, absence of Microconidia, presence of Mesoconidia, Monophialides, presence of Chlamydospore): MGSS 149 (Table 2).

Thus, according to the morphological characteristics and associated to the types of symptoms caused by the fungus plant pathogens, the following strains were classified in *Fusarium oxysporum* (section *Elegans*): MGSS 09, MGSS 14, MGSS 42, MGSS 118, MGSS 182 and MGSS 183 that were associated to vascular darkening and wilting in the host species (Fig. 2).

The isolates MGSS 09, MGSS 14, MGSS 15, MGSS 42, MGSS 118, MGSS 182, MGSS183 presented characteristics of the species *F. oxysporum* with aseptate microconidia formed in false heads in short monophialides and chlamydospores formed individually, in pairs, grouped in short chains, straight or slightly curved macroconidia that presented 3-5 septa.

The colony coloring of the isolates of the *F. oxysporum* species also presented different shades. For example, the isolates MGSS 14 and MGSS 183 showed different colors.

The isolates MGSS 138 and MGSS 149, from fruits of the *Carica papaya* and leaves of *Lactuta sativa* were classified as *F. equiseti* in the *Gibbosum* section (Fig. 3).

The isolates MGSS 54, MGSS 61, MGSS 68, MGSS 141, MGSS 157 presented macro and micromorphological characteristics similar to the species belonging to the *Gibberella fujikuroi* species complex. The pigmentation of the colony coloring varies from pink to salmon, thin, straight macroconidia with 3 to 5 septa or septa absence, oval, elliptic shaped microconidia with no or one septum, presence of conidia in false heads or in chains and absence of chlamydospores.

The strains MGSS 68 and MGSS 54 that were associated to exudation symptoms in *Ananas comosus* shoots and fruits was classified as *F. guttiforme* (*Liseola* section). The isolate MGSS 15 was also associated to gum exudation, but some micromorphological characteristics placed it in the *Elegans* section, such as chlamydospore production (Fig. 4).

The isolates MGSS 141 and MGSS 157 presented similar morphological characteristics and came from *Hibiscus sabdariffa* stem rot (Fig. 5).

Table 3 shows a summary of the association of the morphological characterization and symptomological aspects observed in hosts. Out of the 14 isolates, five isolates were grouped morphologically in the *Gibberella fujikuroi* species complex in the *Liseola* section, seven presented characteristics of the species of the *Elegans* section in the *Fusarium oxysporum* species complex (FOSC) and two isolates presented characteristics of the *Gibbosum* section in the *F. incarnatum-equiseti* species complex (FIESC).

Molecular characterization of the Fusarium spp. isolates

The phylogenetic tree was constructed with 43 nucleotide sequences and 14 isolate sequences identified morphologically as belonging to the genus *Fusarium.*

The phylogenetic analysis showed the formation of two groups. The first group consisted of the *Liseola* and *Elegans* section and the second group of species in the *Gibbosum* section.

The group with the species from the *Liseola* and *Elegans* section presented a branch supported by 89% of bootstrap. In the *Liseola* section, the evolutionary history of the ITS gene for the isolates MGSS 157, MGSS 141, MGSS 61, MGSS 54 and MGSS 68 showed that they grouped with lines of the *G. fujikuroi* species complex (GFSC). *G. fujikuroi* is a fairly diverse species complex of approximately 50 lines where many species are unknown (O'Donnell et al., 2013). In the *Elegans* section, seven isolates (MGSS 118, MGSS 09, MGSS 15, MGSS 183, MGSS 182, MGSS 14, MGSS 42) clustered with the *F. oxysporum* lines. The group of the Gibbosum section presented two well-defined clades, the first clade was highly significant with 99% bootstrap grouped the isolates MGSS 138 and MGSS 149 with the *F. equiseti* lines (Fig. 6).

The 14 *Fusarium* isolates were identified morphologically and the species determination confirmed by PCR primers specific for ITS gene sequencing. The nucleotide sequences were subsequently placed in the NCBI/Genbank database (Table 1). The information obtained in this research based on the ITS gene is adequate to understand the evolutionary relationships of the *Fusarium* populations where the isolates were separated into three species complexes as follows: *G. fujikuroi species complex* (GFSC), *F. oxysporum* species complex (FOSC) and *F. incarnatum-equiseti* species complex (FIESC).

Discussion

The survival resistance structure observed in the isolates MGSS 15, MGSS 138, MGSS 149 (individual chlamydospores), MGSS 42 (individual and in pairs) MGSS 182 (clustered or in short chains) and MGSS 183 may remain resting in substrates or the soil for many years when conditions are unfavorable, so that the pathogen is able to persist in the environment until there are favorable conditions when it becomes active, returning to cause the disease in the susceptible hosts (Fischer and Rezende, 2016).

The morphological characteristics of isolates of the genus *Fusarium* have been investigated in several culture media as such as de agar clove - CLA (Ramdial et al., 2017), synthetic nutrient agar - SNA (Figueroa-rivera et al., 2010; Moussa et al., 2017) and Potato-Dextrose-Agar - BDA (Akbar et al., 2018). The use of the previously mentioned referred culture media is indicated because they are standard media for genus identification and according to Leslie and Summerell (2006).

The colonies showed variation in the pigmentation of the different isolates cultured on PDA culture medium that was expected, because they are different species of the genus. Thus, the micromorphology observation also showed variation for presence or absence, shape and macroconidia and microconidia septa number.

The characteristics presented in the isolates MGSS 09, MGSS 14, MGSS 15, MGSS 42, MGSS 118, MGSS 182, MGSS183 are fundamental for correct identification of the species *F. oxysporum*. The variation in macromorphological coloring characteristics within the species presented in the the isolates MGSS 14 and MGSS 183 was also reported by Teixeira et al. (2017).

The isolates MGSS 138 and MGSS 149 presented macroconidia with 5-7 septa and coloring that ranged from burnt yellow (MGSS 138) to pink (MGSS 149) in PDA culture medium. One isolate presented septate mesoconidia (MGSS 149). Both isolates formed individual chlamydospores but no microconidia (absence) that are typical characteristics of the species reported by the authors Bonde et al. (2014) and Ramdial et al., (2017).

F. esquiseti is easily confused morphologically with the species *F. compactum* (brown pigmented isolates), *F. semitectum*, and *F. scirpi*. *F. compactum* and *F. equiseti* which are distinguished based on their growth rate in PDA (Leslie and Summerell, 2006). Regarding the hosts, this species has been associated to diseases in different crops such as rice (Nadarajah et al., 2015), fruit and vegetables (Bonde et al., 2014), wheat (Castellá and Cabañes, 2014).

Regarding the *Gibberella fujikuroi* species complex, there are a few morphological traits to differentiate species within complex the species as stated by Hsuan et al. (2011) who observed that isolates classified morphologically as *F. subglutinans* at first were later called classified as *F. sacharri* based on sequences of the gene TEF- 1α (elongation factor 1-α).

The occurrence of *Fusarium* disease in *H. sabdariffa* has been reported in countries on the African continent, in Nigeria by Amusa et al. (2005) and Agbenin and Ogunlana (2006), in Egypt by Hassan et al. (2014). With that, it can be said that the isolates MGSS 141 and MGSS 157 which had similar morphological characteristics are originated from this disease.

The isolate MGSS 61 pathogenic to black fly in citrus *Aleurocanthus woglumi*, presented characteristics of the *G. fujikuroi* species complex in the *Liseola* section and classified as *F. proliferatum* and differentiated from *F. verticillioides* by the presence of mono and polyphialides. Other authors identified species of this complex that were associated to insects such as the species *F. verticillioides* (Pelizza et al., 2011) and *F. fujikuroi* (Sharma et al., 2018).

Some studies have been carried out to define the species level of the genus *Fusarium* using the ITS region of the rDNA and this region was shown adequate for reliable identification of the fungus isolates of this genus. Zarrin et al. (2016), Yuan et al. (2013) and Nadarajah et al. (2015) also differentiated the *Fusarium* species with medical and agricultural importance using the gene of the ITS region.

Table 1. *Fusarium* sequences used for phylogenetic analysis of the rDNA gene ITS.

Species	Strain Number	Host/ Substrate	Code in Genbank[1]
Fusarium circinatum	6DTJF01	Root of aquatic plant	KT184799
Fusarium dimerum	CBS 116527	-	EU926284
Fusarium equiseti	MGSS 149	*Lactuca sativa*- Leaf	MF449200
F. equiseti	C12	-	AY147368
F. equiseti	D	Soil	KT634075
F. equiseti	FE9	*Oryza sativa*	HQ995668
F. equiseti	MGSS 138	*Carica papaya* -Fruit	MF449219
F. guttiforme	NRRL 22945	-	U34562
Fusarium hostae	DAOMC235655	-	KR909426
Fusarium longipes	DEB13	Soil	KF918597
F. longipes	BCCM/IHEM 18093	Soil	KJ125587
Fusarium oxysporum	-	Soil	EF017214
F. oxysporum	KP10	*Pynus sylvestris*	DQ093759
F. oxysporum	PARC682c	*Prunus avium*	KT455376
F. oxysporum	MGSS 42	*Solanum lycopersicon*- Vascular System	MF449220
F. oxysporum	MGSS 14	*Heliconia* sp.- Vascular System	MF449210
F. oxysporum	MGSS 182	*S. lycopersicon*- Vascular System	MF449207
F. oxysporum	MGSS 183	*S. lycopersicon*- Vascular System	MF449199
F. oxysporum	MGSS 15	*Ananas comosus* – plant base	MF449197
F. oxysporum	MGSS 09	*Musa* sp.- Vascular System	MF449193
F. oxysporum	MGSS 118	*Coriandrum sativum* – Sistema vascular	MF449190
F. oxysporum f. sp. *Cubense*	115HT	-	EF155534
*F. oxysporum*f. sp. *lycopersici*	FO29	*S.lycopersicum*	KF914468
Fusarium guttiforme	MGSS 68	*A.comosus*- fruit	MF449192
F. guttiforme	MGSS 54	*A.comosus* - plant base	MF449201
F. proliferatum	MGSS 61	*Aleurocanthus woglumi*	MF449213
Fusarium sp. *(Gibberella fujikuroi complex)*	MGSS 141	*Hibiscus sabdariffa - stalk*	MF449217
Fusarium sp. *(Gibberella fujikuroi complex)*	MGSS 157	*H.sabdariffa - stalk*	MF449221
F. proliferatum	NRRL 31071	*Triticum aestivum*	AF291061
F. proliferatum	UTcp8	*Vigna unguiculata*	KT376487
F proliferatum	D2	*Zea mays*	EU151485
Fusarium phyllophilum	PEN6	*Vitis* sp.	KR909206
Fusarium pseudocircinatum	ATCC MYA-4835	-	JQ070125
F. sterilihyposum	MRC7602	-	AF430130
F. sterilihyposum	MRC7602	-	AF430130
F. sterilihyposum	MRC7602	-	AF430130
F. subglutinans	-	*Zea mays*	X94167
Fusarium sp.	MC-23-F NRRL125184	*Monarda citriodora*	KU527804
F. lateritium	-	*Rosa* sp.	AY904057
F. verticilloides	-	*Zea mays*	X94166
F. verticilloides	JJTKCL	*Saccharum officinarum*	KJ544799
Fusarium sacchari	NRRL 43543	-	EF453121
F. sacchari	ATCC 201263	-	KR909411
Lecanicillium lecanii	CBS122175	*Hylurgops palliatus*	DQ449654
Microdochium nivale	MTCC 6580	-	JN642711

[1]The 14 sequences in bold were obtained from the present study and registered in the Genbank.

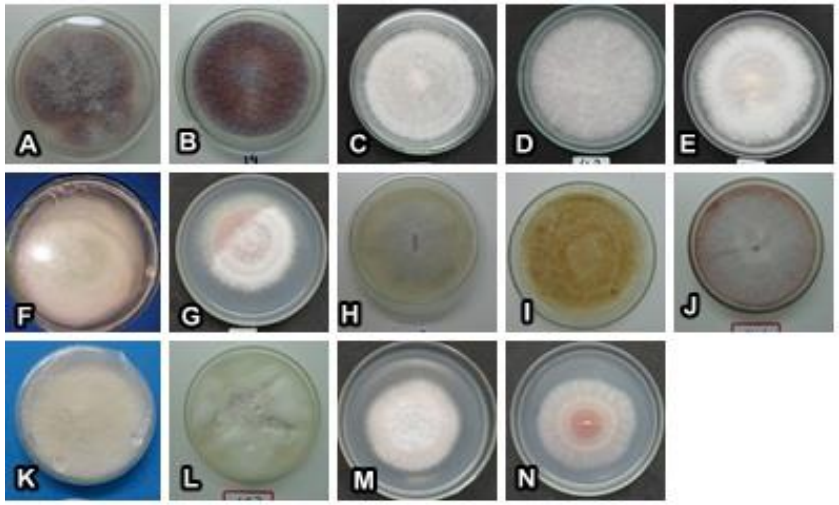

Fig 1. Macromorphological characterization of the isolates studied. Purple coloring (A-MGSS 09, B- MGSS 14,), pink coloring (C- MGSS 15, D- MGSS 42, H- MGSS 118, J- MGSS 141, K- MGSS 149, M-MGSS 182, N-MGSS 183), salmon coloring (F- MGSS 61), pink coloring with pale borders (E- MGSS 54, G- MGSS 68, L- MGSS 157) burnt yellow coloring (I- MGSS 138).

Table 2. *Fusarium* spp. macro and micromorphological characteristics.

MGSS	MORPHOLOGICAL ASPECTS								
	MACROSCOPIC	MICROSCOPIC							
		MICROCONIDIA		PHIALIDES	CHLAMYDOSPORES	MACROCONIDIA		MESOCONIDIA	
	COLOR	SHAPE	NUMBER OF SEPTA			SHAPE	NUMBER OF SEPTA	SHAPE	NUMBER OF SEPTA
09	Purple coloring	Oval	0	Short monophialides	-	Allantoid	3-5	-	-
14	Purple coloring	Oval	0-1	Short monophialides	-	Allantoid	3-5	-	-
15	Pink coloring	Oval	1	Short monophialides	Individual	Allantoid	3-5	-	-
42	Pink coloring	Oval	0-1	Short monophialides	Individual and in pairs	Allantoid	3-5	-	-
54	Pink coloring with pale borders	Oval and obovoid	0-1	Monophialides and poly phialides	-	Allantoid	3-5	-	-
61	Salmon coloring	Oval	1	Monophialides and poly phialides	-	Allantoid	3-5	-	-
-68	Pink coloring with pale borders	Oval	0-2	Monophialides and poly phialides	-	Allantoid	2-5	-	-
118	Pink coloring	Oval	0-1	Short monophialides	-	Allantoid	3-5	-	-
138	Burnt yellow coloring	-	-	Monophialides	Individual	Allantoid	5-7	-	-
141	Pink coloring	Oval	1	Monophialides and poly phialides	-	Absent	-	-	-
149	Pink coloring	-	-	Monophialides	Individual	Allantoid	5-7	Elliptic	1-2
157	Pink coloring with pale borders	Oval	0	Monophialides and poly phialides	-	Absent	-	-	-
182	Pink coloring	Oval	1	Short monophialides	clustered or in short chains	Allantoid	3-5	-	-
183	Pink coloring	Oval	1	Short monophialides	Individual	Allantoid	3-5	-	-

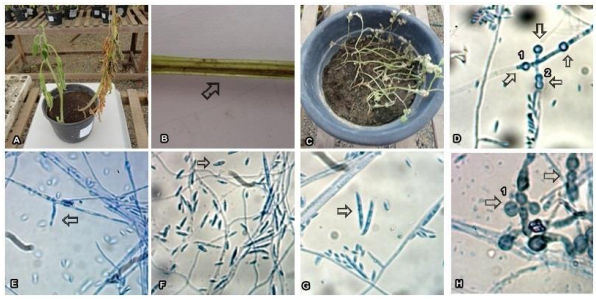

Fig 2. Morphological characteristics and typical symptoms of the species *F. oxysporum* observed in isolates obtained in the present study. A – fusarium wilt in tomato (MGSS 183), B- Vascular darkening (MGSS 182), C- fusarium wilt in *Coriandrum sativum* (MGSS 118), D- Chlamydospores 1(individual) and 2 (in pairs) (MGSS 42), short E- Monophialide curta (MGSS 09), F- Microconidia (MGSS 14), G- Macroconidia 3-5 septa (MGSS 42), H- Chlamydospores 1 (clustered) (MGSS 182).

Table 3. Association between morphological characterization of the isolates and symptomological aspects caused in the respective hosts.

MGSS	Symptomatological Aspects		Morphological Classification	
	Pathogen Isolation Place	Symptoms	Section[1]	Species Complex
09	Vascular system	Wilt	*Elegans*	FOSC
14	Vascular system	Wilt	*Elegans*	FOSC
15	Plant base	Exudation	*Elegans*	FOSC
42	Vascular system	Wilt	*Elegans*	FOSC
54	Plant base	Exudation of gum.	*Liseola*	GFSC
61	Insect	Colonization of nymphs	*Liseola*	GFSC
68	Fruit	Exudation of gum	*Liseola*	GFSC
118	Vascular system	Wilt	*Elegans*	FOSC
141	Stalk	Stem rot	*Liseola*	GFSC
138	Fruit	Fruit rot	*Gibbosum*	FIESC
149	Leaf	Leaf Rot	*Gibbosum*	FIESC
157	Stalk	Stem rot	*Liseola*	GFSC
182	Vascular system	Wilt	Elegans	FOSC
183	Vascular system	Wilt	Elegans	FOSC

[1] Wollenweber and Reinking Sections

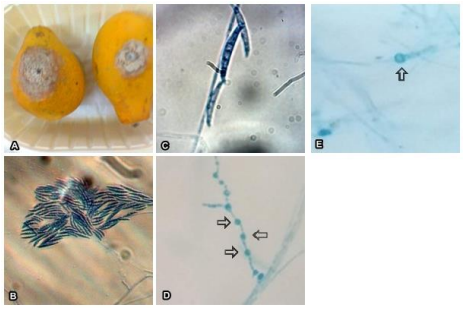

Fig 3. Morphological characteristics and symptoms associated to isolates belonging to the *Gibbosum* section A – rot in *Carica papaya* fruit *(*MGSS 138), B – Macroconidi abundance (MGSS 149), C – Macroconidia with dorsoventral curve (MGSS 138), D- Arrows indicate chlamydospores (MGSS 138) and E – Arrows indicate individual chlamydospore (MGSS 149).

Table 4. Isolates used in the morphological and molecular characterization

Code	Host	Year of Registration in the MGSS Fungi Collection[1]
MGSS 09	*Musa* sp.	2011
MGSS 14	*Heliconia* sp.	2011
MGSS 15	*Ananas comosus*	2011
MGSS 42	*Solanum lycopersicon*	2012
MGSS 54	*A. comosus*	2012
MGSS 61	*Aleurocanthus woglumi*	2012
MGSS 68	*A. comosus*	2012
MGSS 118	*Coriandrum sativum*	2014
MGSS 138	*Carica papaya*	2014
MGSS 141	*Hibiscus sabdariffa*	2014
MGSS 149	*Lactuca sativa*	2014
MGSS 157	*H. sabdariffa*	2014
MGSS 182	*S. lycopersicon*	2014
MGSS 183	*S. lycopersicon*	2014

[1]MGSS – Micoteca Prof. Gilson Soares da Silva.

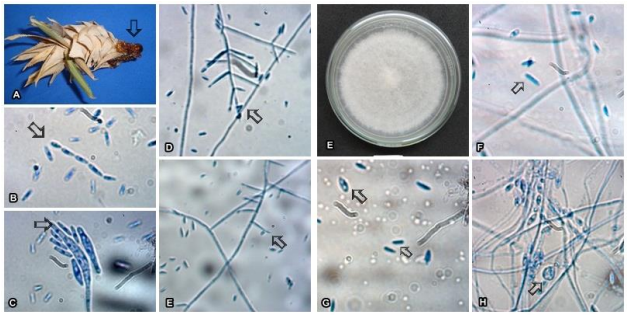

Fig 4. Morphological characteristics and typical symptoms of *F. guttiforme* associated to *A. comosus.* A. Plant base with gum exudation (MGSS 54), B- Microconidia chain (MGSS 68), C- Macroconidia (MGSS 68), D- Polyphialides (MGSS 54), E- Monophialide (MGSS 54). Isolate MGSS 15 (E-G) common characteristics between *F. guttiforme* and *F. oxysporum* (H) characteristic that excludes it from *F. guttiforme.* E- Colony, F-G- Microconida and H- Chlamydospores.

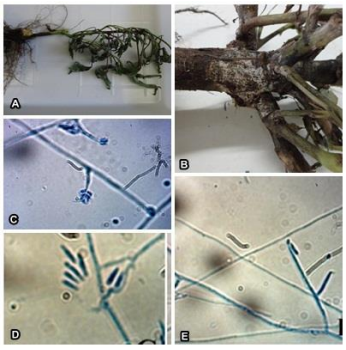

Fig 5. Characteristics of the isolates belonging to the *Liseola* section. A and B- Rot in *H. sabdariffa* roots*.* C- Short monophialide curta and false head formation (MGSS 141), D- Microconidia (MGSS 157) , E -Polyphialide (MGSS 157).

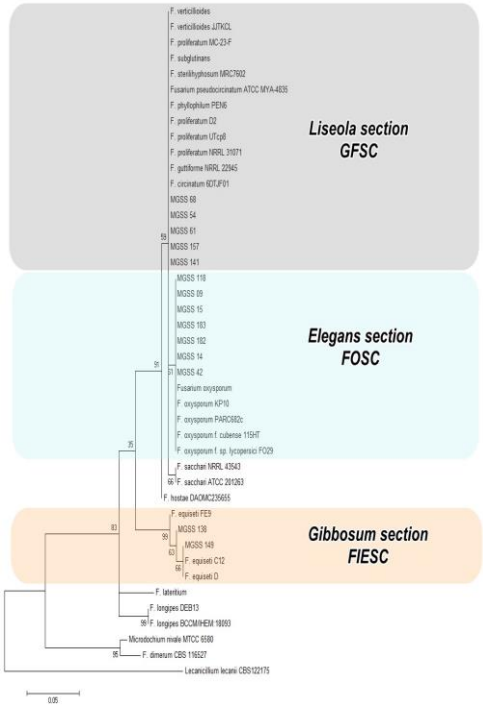

Fig 6. Phylogenetic tree by the Maximum Likelihood method (ML) based on the Jukes-Cantor model derivates of the gene ITS for the genus *Fusarium*. The consensus bootstrap tree inferred from 1000 replications. *Lecanicillium lecanii* Q449654 was used as outgroup.

Research on *F. oxysporum* phylogeny indicates that there is a larger complexity in the evolutionary history of this species regarding its *formae speciales*. Epstein et al. (2017) observed that out of 12 isolates of *F. oxysporum* f. sp. *apii* race 2 are more directly related to *F. oxysporum* f. sp. *lycopersici* race 2 and *F. oxysporum* f. sp. *melonis* clustered in clade 3 and this performance can be explained due to the polyphylectic nature. Nirmaladevi et al. 2016 also concluded the polyphyletic nature

of *F. oxysporum* f. sp. *lycopersici* based on sequences from the ITS region. These results confirmed our findings, where all the different *formae speciales* formed a single group in the phylogenetic analysis.

The nature or symptoms of the disease give important clues regarding the probable species responsible for the disease in these hosts and often limit the range of species that should be distinguished (Leslie and Summerell, 2006). In the present study, the fungi isolated from plants with symptoms of vascular darkening and severe wilt were classified both morphologically and phylogenetically as belonging to *F. oxysporum* Schlechtendal. The isolates obtained from the following pathogen systems: *S. lycopersicon*/vascular darkening (MGSS 42, MGSS 182, MGSS 183); *Heliconia* sp./ vascular darkening (MGSS 14); Musa sp./ vascular darkening (MGSS 09); *C. sativum*/ vascular darkening (MGSS 118); *A. comosus*/gummosis (MGSS 15).

Of the pathogen systems mentioned above, the morphological and molecular classification of the isolate MGSS 15 that was obtained from *A. comosus* was surprising because in Brazil fusariosis in *A. comosus* has been reported by several authors (Carnielli-Queiroz, 2019; Ventura and Góes, 2016) with *F. guttiforme* as causal agent. However, this isolate was identified as *F. oxysporum* according to the morphological characteristics and the phylogeny of the ITS gene. The results showed that more than one species is the causal agent of fusariosis in this host; Vásquez-Jiménez and Mata-Granados (2014); Stepień et al. (2013) also reported the occurrence of *F. oxysporum* in *A. comosus* crops in Costa Rica, Vietnam and Ecuador causing different forms of rot in this host.

The *G. fujikuroi* species complex (GFSC) encompasses monophylectic taxon in a set of *Fusarium* species with similar morphological and overlaid characteristics. This complicates their differentiation at species level. A large part of this species is associated with devastating diseases in several economically important plants (Kvas et al., 2009) and pathogenic to insects (Pelizza et al., 2011; Sharma et al, 2018).

The isolates that were morphologically classified within the (GFSC) species complex were also grouped in the same complex by characterization of the ITS region of the ribosomal DNA, indicating that this region of the DNA is very useful to identify species complex of the genus, a result also reported by Al-Hatmi et al. (2016).

Although the ITS region combines the highest power of resolution to discriminate closely related species and successful sequencing in a wide variety of fungi (Schoch et al., 2012), the phylogeny of species within this complex (GFSC) is difficult to define using only the gene of the ITS region (Al-Hatmi et al., 2016; O'Donnell et al., 2015; Schoch et al., 2012). Our results confirm that the phylogenetic evolution for information at species level through the ribosomal DNA ITS region is not enough for the sequences belonging to the *G. fujikuroi species complex* (GFSC) and the use of multiple genes is more suggested by authors (Kvas et al., 2009) and (Leyva-Madrigal et al., 2015).

Materials and Methods

Experimental location and obtaining Fusarium isolates

The experiments were carried out in the Plant Pathology Laboratory of the Agricultural Biotechnology Nucleus Genetic Laboratory- Labwick University Campus Paulo VI, São Luís – MA, Brazil and the Genetics and Molecular Biology Laboratory at the State University of Maranhão – UEMA, Caxias Center Campus of Higher Studies – CESC, Caxias –MA, Brazil.

The isolates were obtained from the "Prof.° Gilson Soares da Silva" Library at the State University of Maranhão – UEMA, University Campus Paulo VI, São Luís – MA, Brazil, and conserved in the continuous replication method (Table 4).

Morphological characterization of the Fusarium spp. isolates

The isolates were placed to grow on monospore cultures and to classify the colonies for color, macroconidia, microconidia and chlamydospores. Microcultures were also made to observe the fungi structures under an optical microscope for later photographic registration by AxioCam model ERc 5s (ZEISS®). The keys were the Genus *Fusarium* (Booth, 1971), the *Fusarium* Laboratory Manual (Leslie and Summerell, 2006) and *Fusarium* species. An Illustrated manual for identification (Nelson et al., 1983) were used to identify the isolates. The isolates were grouped in species sections and complexes associated to the sites of isolation and symptoms in the hosts.

Molecular characterization characterization of the Fusarium isolate

DNA extraction and amplification of the rDNA ITS region

The genomic DNA was extracted using the KIT HiPuraTM (Himedia®). The samples were prepared for extraction by culturing the isolate in solid medium and later scraping. After obtaining the samples, about 100 mg fungus tissue were squashed in the presence of liquid nitrogen to form a fine powder and then DNA was extracted according the extraction protocol supplied together with the kit.

An aliquot was removed from the samples of the extracted DNA to verify the DNA quality and concentration by comparative analysis in 0.9% agarose gel containing ECG and ethidium bromide at 0.01% (V/V). After this process the samples were stored at -80°C until use.

The ITS1-5.8S-ITS2 regions of the ribosomal DNA (rDNA) were amplified following methodology described by White et al. (1990) using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5-'TCCTCCGCTTATTGATATGC-3') in a thermocycler programmed for the following conditions: 94ºC for 12 minutes (denaturation and enzymatic activation), 35 cycles – 94ºC for 30 seconds (denaturation), 55ºC for 30 seconds (aneling) and 72ºC for 30 seconds (extension), 72ºC for 10 minutes (final extension). For the PCR reaction were used 20 ng DNA molde, 10 mM each primer, 1.5 mM and $MgCl_2$, 0.08 mM of each dNTP, 02 U/µL Taq DNA polymerase, 50 mM Tris-HCl (pH 8.4) and 20 mM KCl in 50 µL final volume. An aliquot of the amplification reaction product (10 µL) was submitted to electrophoresis in 1.5 % (p/v) agarose gel in TBE 1X buffer containing ethidium bromide at a concentration of 0.01 % (v/v), at 90 V for one hour. The Scada 100 bp DNA Ladder (Sinapse inc.) was used as molecular weight marker. After electrophoresis, the gel was removed from the cube and visualized under UV light and then photographed.

The products of the PCR reaction were purified using commercial kits of purification by column (Purelink® – Thermo Fisher Scientific, Inc.), and were sent for sequencing in the Genetics and Molecular Biology Laboratory of the State

University of Maranhão (Campus CESC, Caxias – MA, Brazil).
The consensus sequences generated were compared with those deposited in the NCBI databank (National Centre for Biotechnology Information website -http://www.ncbi.nlm.nih.gov), using the BLAST search tool to confirm the genus of each one of the isolates.

Phylogenetic analyses of the genus Fusarium isolates

The phylogenetic analyses were carried out using the MEGA 6 program (Tamura et al., 2013). The sequences were aligned using the CLUSTALW implemented by the MEGA 6 and adjusted to the Jukes-Cantor model. The Maximum Likelihood phylogenetic trees and the significance of the groupings of the phylogenetic trees were estimated by 1000 bootstrap replications and comparison to the other sequences deposited in the GenBank (Bioproject PRJNA730933) (http://www.ncbi.nlm.nih.gov/genbank/), and /or type-species present (Table 1).

Conclusion

All the isolates classified morphologically as *Fusarium oxysporum* also presented phylogenetic relationships with strains belonging to the same species. Two of the three isolates obtained from *A. comosus* with gummosis symptoms. The isolates MGSS54 and MGSS 68 were classified morphologically as *F. guttiforme* and molecularly belonged to (GFSC) and one isolate MGSS 15 was classified as *F. oxysporum* (FOSC), confirming that two species are responsible for the diseases in this host. The isolates classified morphologically within the *Gibberella fujikuroi* species complex (GFSC) were presented evolutionary by the ITS rDNA gene within clades with species belonging to the same species complex. However, there was no species definition indicating an analysis with multiple loci for better elucidation of the species. Two isolates MGSS 138 and MGSS 149 classified morphologically in the *Gibbosum* section in the *F. incarnatum-equiseti* species complex (FIESC) presented evolutionary history through the rDNA ITS gene grouping in the *F. equiseti* species.

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