Molecular and Aggressiveness Characterization of Isolates of *Fusarium solani* and *Fusarium oxysporum* f.sp. *passiflorae* Associated to Passion Fruit Wilting

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Abstract

This work was carried out with the objective of performing a molecular and aggressiveness characterization of *F. solani* and *F. oxysporum* f.sp. *passiflorae* collected in the Pantanal, Cerrado and Amazon biomes. We selected the most aggressive isolates for use in breeding programs aiming resistance to Collar Rot and Fusariosis. For inoculation of the isolates of *F. oxysporum* f.sp. *passiflorae* the washed root method was used. The molecular characterization of the isolates was carried out by partial sequencing of the Transcribed Internal Spacer of the rDNA region. The isolates of *F. solani* formed two distinct groups in relation to aggressiveness. Among all isolates, FSUNEMAT 40 and FSUNEMAT 46 were the most aggressive. The model with K=2 was taken as the best model to explain the genetic structure of the *F. solani* populations, with clear combinations of genes from both gene pools. There were three groups with respect to the aggressiveness of the isolates of *F. oxysporum* f.sp. *passiflorae*, with the isolated FOUNEMAT 22 being the most aggressive. In view of these results, the isolates of *F. solani* collected in *P. edulis* in the state of Mato Grosso presented a high molecular variability independent from the biome of origin, and this was also observed in the tests of aggressiveness. The results indicate the need to consider the molecular variability and the aggressiveness of the pathogens in the evaluation of genotypes of sour passion fruit in programs of selection of resistant cultivars.

Keywords: *Passiflora edulis*, resistance, breeding programs, phytopathogen

1. Introduction

The cultivation of passion fruit, *Passiflora edulis*, has been growing every year in Brazil, occupying a prominent position in tropical fruit growing (MAPA, 2016). The state of Mato Grosso is the largest producer in the Midwest region, totaling 5,275 tons in a cultivated area of 346 hectares. This production is still very low when compared to the state of Bahia, which produces an average of 342 thousand tons/year (IBGE, 2016).

One of the main problems that contributes to the reduction in productivity of the passion fruit crop is the occurrence of diseases, such as collar rot and fusariosis, caused respectively by the fungi *Fusarium solani* Martius and *Fusarium oxysporum* f.sp. *passiflorae* (Fischer & Rezende, 2008). Plants infected by these fungi initially present wilting of the nodes and, with the progress of the disease, there is early defoliation, wilting and death of the plant in a few days. These symptoms are the result of the damage caused by these fungi to the vascular system of plants (Fischer & Rezende, 2016). The presence of *F. solani* and *F. oxysporum* f.sp. *passiflorae* in the soil may even prevent the cultivation of passion fruit for years since these pathogens have a resistance structure (chlamydospore) that guarantees fungus survival (Bueno et al., 2006).

One of the most current alternatives for the control of diseases in passion fruit is the use of resistant cultivars (Silva et al., 2012). However, there are still no passion fruit cultivars resistant to collar rot and fusariosis recorded in the Ministry of Livestock and Supply. In this context, knowledge on the pathogenicity and variability of the pathogen

population is indispensable for breeding programs aiming the selection of a resistant passion flower cultivar or a hybrid in view of the complex pathosystem, where the pathogen involved presents a genetic variability, as reported by previous studies (Carvalho et al., 2015; Dariva et al., 2015; Silva et al., 2017).

In this sense, this work was carried out with the objective of performing a molecular and aggressiveness characterization of *F. solani* and *F. oxysporum* f.sp. *passiflorae* collected in the Pantanal, Cerrado and Amazon biomes. We selected the most aggressive isolates for use in breeding programs aiming resistance to Collar Rot and Fusariosis.

2. Methods

2.1 Characterization of Aggressiveness

Sixteen isolates of *F. solani* and eight isolates of *F. oxysporum* f.sp. *passiflorae* belonging to the collection of the State University of Mato Grosso (UNEMAT) were selected for characterization of aggressiveness in *P. edulis* (Tables 1 and 2). All isolates were collected from plants presenting symptoms of wilting in passion fruit producing areas, including the three biomes of the state of Mato Grosso: Pantanal, Cerrado and Amazon (Carvalho et al., 2015). All isolates are preserved in the UNEMAT fungi collection in filter paper strips stored in a refrigerator at ± 5 °C.

Table 1. Relation of *Fusarium solani* isolates used in the aggression test

Treatment	Nº of Collection	Origin	Biomes
1	FSUNEMAT 4	São José dos Quatro Marcos	Amazon
2	FSUNEMAT 7	São José dos Quatro Marcos	Amazon
3	FSUNEMAT 12	São José dos Quatro Marcos	Amazon
4	FSUNEMAT 17	Curvelândia	Amazon
5	FSUNEMAT 21	Carlinda	Amazon
6	FSUNEMAT 25	Alta Floresta	Amazon
7	FSUNEMAT 28	Carlinda	Amazon
8	FSUNEMAT 31	Nossa Sraª do Livramento	Pantanal
9	FSUNEMAT 33	Santo Antônio do Leverger	Pantanal
10	FSUNEMAT 34	Santo Antônio do Leverger	Pantanal
11	FSUNEMAT 35	Santo Antônio do Leverger	Pantanal
12	FSUNEMAT 38	Santo Antônio do Leverger	Pantanal
13	FSUNEMAT 40	Campo Verde	Cerrado
14	FSUNEMAT 46	Santo Antônio do Leverger	Pantanal
15	FSUNEMAT 47	Campo Verde	Cerrado
16	FSUNEMAT 50	Campo Verde	Cerrado

Table 2. Relation of *Fusarium oxysporum* f.sp. *passiflorae* isolates used in the aggression test

Treatment	Nº of Collection	Origin	Biomes
1	FOUNEMAT 3	Cáceres	Cerrado
2	FOUNEMAT 5	Cáceres	Pantanal
3	FOUNEMAT 6	São José dos Quatro Marcos	Amazon
4	FOUNEMAT 9	Nova Lacerda	Cerrado
5	FOUNEMAT 13	Indiavaí	Amazon
6	FOUNEMAT 22	Carlinda	Amazon
7	FOUNEMAT 41	Campo Verde	Cerrado
8	FOUNEMAT 43	Campo Verede	Cerrado

In order to characterize the aggressiveness, clones (cuttings) were produced from a *P. edulis* plant, accession UFV50, belonging to the GAB (Germplasm Active Bank) of UNEMAT. The cuttings contained three internodes. Then, they were planted in trays of 72 cells containing Plantmax® substrate (Eucatex Mineral Ltda.) and kept in a greenhouse under controlled irrigation and two weekly applications of leaf fertilizer in order to stimulate plant

rooting. The whole period lasted approximately three months until the stakes reached ±20 cm in height. After rooting, for the experiment on *F. solani*, the cuttings were transplanted into pots containing ½ of substrate, ¼ of sand and ¼ of autoclaved soil. For inoculation, filter paper strips of each isolate of *F. solani* and of *F. oxysporum* f.sp. *passiflorae* were placed in Petri dishes containing PDA (potato-dextrose-agar) culture medium, and then incubated in BOD at 25 °C and a photoperiod of 12 hr. for seven days. After this time, a blade of each plate was made to ensure that all the isolates sporulated.

2.1.1 *F. solani*

The procedure of inoculation of the isolates of *F. Solani* was that described by Fischer et al. (2005b), in which a disk of culture medium containing the pathogen (5 mm diameter) was fixed with PVC plastic on a small wound in the plant stem at a height of 2 cm from the soil. The PVC plastic was removed five days after inoculation (DAI), and the same procedure was performed with the controls, except for the application of the fungus.

The experimental design consisted of randomized blocks with 17 treatments (16 isolates + 1 control), with four replications and three plants per plot.

The clones of the inoculated UFV50 genotype were analyzed based on 13 characteristics, as described by Preisigke et al. (2015), which are:

a) lesion expansion, length and width (CE and LW);

b) number of plants in which the lesion reached less than 50% of the circumference (NPL-50%);

c) period of inoculation until the lesion reached more than 50% of the circumference of the damaged plant stem (PIL+50%);

d) period of inoculation until the lesion reached 100% of the circumference of the damaged plant stem (PIL100%);

e) number of plants in which the lesion reached the medulla (NPLRM);

f) normalized area below the expansion of lesion area curve (ABELAC);

g) normalized area below the expansion of lesion width curve (ABELWC);

f) normalized area below the expansion of lesion length curve (ABELLC);

i) period of inoculation until the plant wilts (IPPW);

j) number of dead plants (NDP);

k) survival period (SP); and

l) range of scores (RS).

The scores in each plot were used to calculate the Disease Index (DI) according to McKinney (1923). The evaluations of all treatments began on the 5 days post inoculation (DPI). They were carried out every two days until the 55th DAI or until the death of the plant. At the end of the experiment, the isolates were re-isolated in order to complete the Koch Postulates.

Data evaluated were submitted to analysis of variance, and means were compared by Scott-Knott test at 5% probability.

The genetic distance matrix estimation was performed based on Gower's algorithm (1971). Afterwards, the dendrogram was constructed using the UPGMA grouping method. The adjustment between the distances matrix and the dendrogram was estimated by the Cophenetic Correlation Coefficient (CCC) (Sokal and Rohlf, 1962). To establish a cut-off point in the dendrogram and to define the number of groups, the Mojena (1977) procedure was used based on the relative size of the merging (distances) levels in the dendrogram.

All analyses were performed using the GENES software (Cruz, 2016) with interaction of the "ape" package of the R software (R Development Core Team, 2014).

2.1.2 *F. oxysporum* f.sp. *passiflorae*

For the inoculation of the isolates, the washed root method was used. The seedlings were removed from the trays and the roots were washed in distilled water. Then, the roots were cut using sterile scissors and immersed in 100 mL conidia suspension in 400 mL plastic pots (the root was left in the suspension for 24 hours) (Preisigke et al., 2017). For the control, the procedure was the same, replacing only the suspension of conidia for distilled water. After the 24-hour period, the suspension was withdrawn, and 100 mL of nutrient solution was added as proposed by Clark (1975). This solution was changed every three days.

The experimental design was randomized blocks with 9 treatments (8 isolates + 1 control), with four replications and three plants per plot.

We evaluated the survival period (period in days from inoculation to plant death) and the number of live plants. Evaluations were carried out periodically up to 50 DAI. During this period, symptomatic seedlings had parts of their stem and roots disinfested and transferred to PDA medium to confirm the etiological agent. After data collection, the data were submitted to graphic dispersion using the means of the characteristics. The statistical analysis was performed using the Genes software (Cruz, 2016).

2.2 Molecular Characterization and Population Structure

For the extraction of genomic DNA, the monosporic isolates of *F. solani* and *F. oxysporum* f. sp. *passiflorae* were grown in liquid potato dextrose agar (PDA) medium for two weeks. Subsequently, the mycelium was macerated in liquid nitrogen. DNA extraction was performed using extraction kit (Axygen Biosciences USA) according to the manufacturer.

The molecular characterization of the isolates was carried out by partial sequencing of the internal transcribed spacer (ITS) of the rDNA region. The primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (GGAAGTAAAAGTCGTAACAAGG 5'-3') were used for amplification of the rDNA's ITS region (White et al., 1990). For 25 µl of reaction, approximately 60 ng of total DNA, 2.5 µl of 10x PCR buffer, 2.5 mM of each dNTP, 10 pMol of each primer oligonucleotide, 1 U of Taq polymerase and ultrapure H_2O s.q.f. for 25 ml were used. The amplification program consisted of an initial stage of denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 45 sec and 72 °C for 1 min, and a final step of stretching at 72 °C for 10 min in an Amplitherm thermocycler. The sequencing was performed by the Sanger method using the Big Dye Kit on the Applied Biosystem ABI3100 sequencer (Dunn & Blattner, 1987).

The sequences obtained were edited and aligned using the BioEdit software (Hall, 1999), and then compared to sequences in the GenBank database using the NCBI BLAST software (www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the MEGA software, version 5.0 (Tamura et al., 2011), and the Neighbor-Joining method with a bootstrap of 1,000 replications.

For the structuring of the populations, the Structure v2.3.4 software (Pritchard; Stephens; Donnelly, 2000) was used through clustering based on the Bayesian model. The structure of populations was classified into "K" clusters according to their genetic similarities. The number of clusters (K) tested ranged from 1 to 10, with 5 interactions each. The number of steps of the burnin length was 10,000 and the MCMC (Markov chain Monte Carlo) repetitions was 100,000. To determine the number of genetic groups (most likely K), the criterion proposed by Evanno, Regnaut, and Goudet (2005) was used through the Structure Harvester v0.6.94 software (Earl & Vonholdt, 2012).

3. Results

3.1 Characterization of Aggressiveness F. solani

By analyzing the data contained in the analysis of variance, it was possible to observe a significant difference between the means of the isolates at 1% probability by F test for almost all characteristics analyzed, except for LL, which was significant at 5%.

By applying the Scott Knott test, the variables NPL-50%, IPLR+50%, NPLRM, IPPW and NDP separated the isolates into three groups, and the other variables into only two groups (Table 3). There was a marked variation for the characteristic IPLR+50%: from 13 days (FSUNEMAT 25) to 50 days (FSUNEMAT 21, 28 and 35) among the isolates studied. Regarding the lesion expansion, the characteristic LL showed a mean variation from 14.72 mm (FSUNEMAT 7) to 77.86 mm (FSUNEMAT 25), and for LW from 3.52 mm (FSUNEMAT 50) to 13.54 mm (FSUNEMAT 25).

Table 3. Aggressiveness averages of the 12 characteristics of 16 isolates of *F. solani* inoculated in *P. edulis*

Isolates	RS	SP	LE	LW	NPL-50%	PIL+50%	PIL100%
UNEMAT 4	55.55 b	46.58 a	28,14 b	6.35 b	1.25 c	38.58 a	50.00 a
UNEMAT 7	41.66 b	42.99 a	14,72 b	4.67 b	2.00 b	46.41 a	46.58 a
UNEMAT 12	27.77 b	46.91 a	15,74 b	3.83 b	2.50 a	47.25 a	47.41 a
UNEMAT 17	30.55 b	48.50 a	17,97 b	3.75 b	2.50 a	44.33 a	48.25 a
UNEMAT 21	33.33 b	46.25 a	15,68 b	3.95 b	2.75 a	50.0 a	50.00 a
UNEMAT 25	91.66 a	34.49 b	77,86 a	13.54 a	0.00 c	13.66 c	39.75 b
UNEMAT 28	19.44 b	50.00 a	16,22 b	5.36 b	3.00 a	50.00 a	50.00 a
UNEMAT 31	40.27 b	46.25 a	24,15 b	4.45 b	2.00 a	45.75 a	49.25 a
UNEMAT 33	69.44 a	43.99 a	47,93 a	12.54 a	0.50 c	19.00 c	36.99 b
UNEMAT 34	73.61 a	29.57 b	41,24 a	9.17 a	0.25 c	15.00 c	33.75 b
UNEMAT 35	31.94 b	43.66 a	19,03 b	4.74 b	2.50 a	50.00 a	50.00 a
UNEMAT 38	48.61 b	44.16 a	52,13 a	5.19 b	2.00 b	41.41 a	47.08 a
UNEMAT 40	80.55 a	23.74 c	77,56 a	9.13 a	0.25 c	26.08 b	28.58 b
UNEMAT 46	80.55 a	21.58 c	76,03 a	10.30 a	0.25 c	30.66 b	43.66 a
UNEMAT 47	66.66 a	33.91 b	43,98 a	10.18 a	0.50 c	33.66 b	44.00 a
UNEMAT 50	48.60 b	37.99 a	18,83 b	3.52 b	1.75 b	46.75 a	50.00 a
CONTROL	20.83 b	50.00 a	14,68 b	2.46 b	3.00 a	50.00 a	50.00 a
VC	37.10	17.33	96.38	53.97	40.37	25.97	16.23

Note. Averages followed by the same letter in the columns do not differ from each other by the Scott Knott test at the 5% probability level. Characteristics of LE and LW are expressed in millimeters.

Table 3. Continued

Isolates	Variables					
	NPLRM	ABELAC	ABELWC	ABELLC	IPPW	NDP
UNEMAT 4	1.75 a	114.53 b	32.31 b	4.74 b	46.08 a	0.25 c
UNEMAT 7	1.00 b	19.78 b	17.00 b	1.82 b	42.41 a	0.75 c
UNEMAT 12	0.50 c	24.08 b	18.42 b	2.27 b	46.75 a	0.25 c
UNEMAT 17	0.50 c	46.07 b	24.50 b	2.90 b	48.00 a	0.25 c
UNEMAT 21	0.25 c	29.91 b	18.47 b	2.83 b	46.16 a	0.25 c
UNEMAT 25	3.00 a	610.66 a	87.13 a	11.61 a	32.99 b	1.50 b
UNEMAT 28	0.00 c	26.33 b	19.00 b	2.45 b	50.00 a	0.00 c
UNEMAT 31	1.00 b	69.37 b	31.62 b	3.11 b	45.16 a	0. 50 c
UNEMAT 33	2.50 a	381.61 a	61.16 a	9.73 a	43.24 a	0. 50 c
UNEMAT 34	2.75 a	265.34 b	56.29 a	8.08 a	28.08 b	1.75 b
UNEMAT 35	0.50 c	38.55 b	22.72 b	2.97 b	43.16 a	0. 50 c
UNEMAT 38	1.00 b	147.35 b	57.44 a	3.80 b	43.83 a	0. 50 c
UNEMAT 40	2.75 a	491.40 a	77.26 a	8.31 a	21.83 c	2.50 a
UNEMAT 46	2.75 a	432.67 a	69.93 a	8.35 a	20.08 c	2.25 a
UNEMAT 47	2.50 a	233.23 b	58.77 a	7.25 a	33.24 b	1.25 b
UNEMAT 50	1.25 b	50.51 b	25.77 b	3.08 b	37.33 a	1.00 c
CONTROL	0.00 c	18.92 b	17.88 b	1.81 b	50.00 a	0.00 c
VC	45.42	55.34	68.94	51.24	18.76	74.12

Note. Averages followed by the same letter in the columns do not differ from each other by the Scott Knott test at the 5% probability level. Characteristics of ABELAC, ABELWC and ABELLC are expressed in millimeters.

In this sense, the Gower's algorithm was used for the joint analysis of the 13 variables evaluated in this experiment in order to estimate the divergence of the aggressiveness of isolates of *F. solani*. The isolates FSUNEMAT 21 and FSUNEMAT 40 were the most distant in relation to aggressiveness, presenting a magnitude of 0.62, while the isolates FSUNEMAT 12 and FSUNEMAT 17 were the most similar, with a value of 0.03. It is noteworthy that the

most distant isolates come from collections in different biomes, where one was collected in the Amazon and another in the Cerrado, while the most similar isolates were collected in the Amazon biome.

Based on the relative magnitude of the distances generated by the Gower's algorithm and the UPGMA grouping method, with a 75% cut-off point obtained by the Mojena method, there was the formation of two distinct groups (Figure 1). The dendrogram was representative of the matrix since the Cophenetic Correlation Coefficient (CCC) was 0.91, considered optimal. According to Vaz Patto et al. (2004), a CCC > 0.70 already reflects a good agreement between the genetic distance matrix and the clustering method.
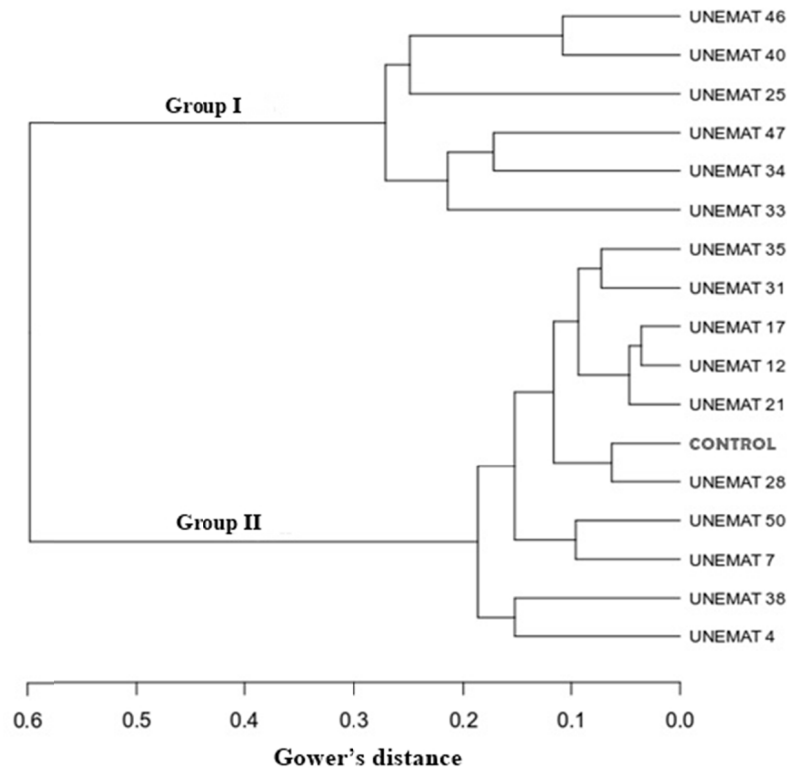
Figure 1. Dendrogram of genetic dissimilarities among 16 isolates of *F. solani* plus the control, obtained by the UPGMA method based on the Gower's algorithm, from 13 characters of aggressiveness

Thus, the isolates were divided into two groups. The Group I consisted of six isolates: FSUNEMAT 33, FSUNEMAT 34, FSUNEMAT 47, FSUNEMAT 25, FSUNEMAT 40 and FSUNEMAT 46. This group was formed by the most aggressive isolates of the UFV 50 cultivar, with a score of 5 in the grading scale (severe symptoms with extensive colonization of the plant collar, necrosis of the cortex and the plant medulla) and a lower SP. It also presented the highest mean values for ABELAC (402.48 mm), for ABELLC (68.42 mm) and ABELWC (8.88 mm). Among all isolates, FSUNEMAT 40 and FSUNEMAT 46 were the most aggressive and, consequently, caused most plant deaths. It is noteworthy that these isolates belong to the Cerrado and Pantanal biomes, respectively.

The Group II consisted of 10 isolates plus the control. The following characteristics were found in common: mild symptoms, necrosis in only part of the plant, *i.e.*, less than 50% of the stem circumference, mean survival period of 45 days, mean ABELAC of 56.64 mm, ABELLC of 26.72 mm and ABELWC of 2.99 mm. The data reflect the low aggressiveness of these isolates to the cultivar UVF 50 of passion fruit. It is worth noting that the control did not show symptoms of the disease. However, due to an injury during the methodological procedure, a mean lesion length of 14.68 mm was observed.

3.1.1 Molecular Characterization and Population Structure—*F. solani*

In order to better understand the structuring of the population, we proceeded with the analysis via Structure. According to the proposed criterion, the model with K = 2 was considered as the best model to explain the genetic structure of the populations of *F. solani* (Figure 2). For this, two populations were formed, with clear combinations of genes from both gene pools. The isolates FSUNEMAT 12, 38, 40, 47 and 21 formed the population 1 and the

population 2 was composed of the other isolates. It is noteworthy that the isolated FSUNEMAT 21 presented introgression of genes from the population 2, and that the isolates FSUNEMAT 50 and FSUNEMAT 7 presented gene combinations of the population 1 in the population 2.
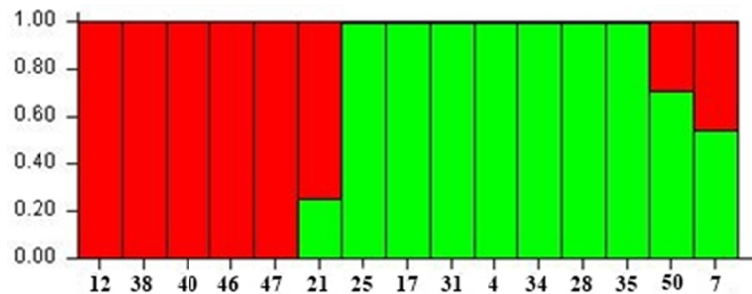

Figure 2. Genetic structure of populations of *Fusarium solani* when evaluated by K = 2 by the Structure Program. Each color indicates a population

The ITS region was amplified by PCR sequenced and compared with DNA sequences deposited in the GenBank, the phylogenetic tree was constructed for this region with 15 isolates. The isolate FSUNEMAT 33 was not used for phylogenetic tree construction because it did not present a significant alignment with sequences in the GenBank database (Figure 3).

The dendrogram generated from the sequencing of the ITS1 and ITS2 regions consisted of three distinct groups. Group I was formed by the highest number of isolates from a total of 10. This group consisted of isolates from the Pantanal and Amazon biomes. The Group II was composed only of two isolates, FSUNEMAT 47 (Cerrado) and FSUNEMAT 46 (Amazonia), while the group III was formed by three isolates belonging to the three biomes analyzed in this study.
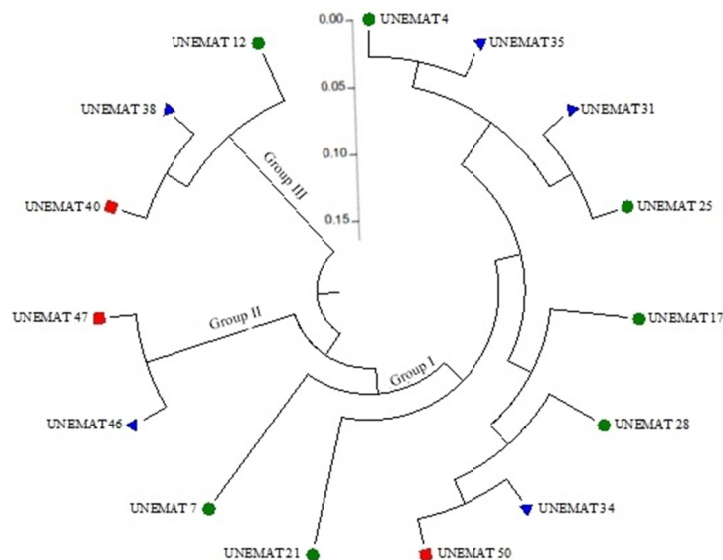

Figure 3. Phylogenetic tree based on the Neighbor-joining method derived from the sequences of the ITS 4 and ITS 5 regions of rDNA from *Fusarium solani* isolates

3.2 Characterization of Aggressiveness—Fusarium oxysporum f.sp. passiflorae

The analyzed variables presented significant differences among the means of the isolates at 1% probability by F test. For the variable survival period, the FOUNEMAT 22 isolate was the most aggressive because the average survival of plants did not exceed 17 days (Table 4). For this isolate, the symptoms in the plant appeared on the 5

DPI, but on average, for the majority of isolates, the symptoms on the cuttings of *P. edulis* appeared on the tenth day after the inoculation. There was a death concentration of *P. edulis* between the 22 and the 25th DPI for most isolates.

Table 4. Mean of the two aggressiveness characteristics of 8 isolates of *F. oxysporum* f.sp. *passiflorae* inoculated in *P. edulis*

Isolates	Variables	
	Survival Period	Number of live plants
UNEMAT 3	44.25 a	1.50 a
UNEMAT 5	39.16 b	1.50 a
UNEMAT 6	40.16 b	1.75 a
UNEMAT 9	37.33 b	1.00 a
UNEMAT 13	38.58 b	1.25 a
UNEMAT 22	17.75 c	0.00 b
UNEMAT 41	43.24 a	1.75 a
UNEMAT 43	33.24 b	0.25 b
CONTROL	48.91 a	2.50 a

Note. Averages followed by the same letter in the columns do not differ by Scott Knott's test at 5% probability level.

According to the graphic dispersion obtained through aggressiveness characteristics, three groups were formed (Figure 6). The first group consisted only of the control, since it presented the highest values for the studied variables. The second group consisted of isolates with a moderate aggressiveness, which had a mean survival period of 39 days and a mean of 1.28 live plants at the end of the experiment. This group comprised 87.5% of the isolates.

The third group was formed by the isolate FOUNEMAT 22, which was the most aggressive. It presented the earliest symptoms in the stakes of *P. edulis*, besides providing the death of all the stakes until the 17th day after inoculation (Figure 4).
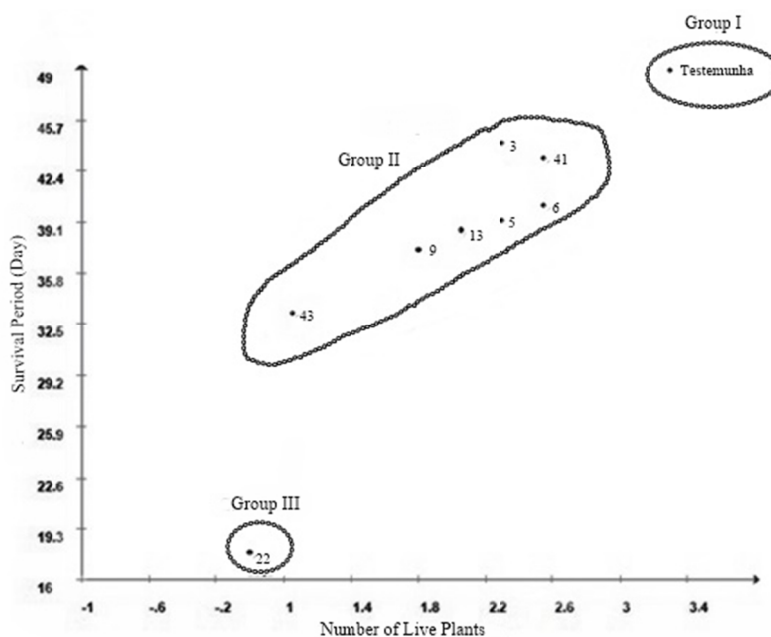

Figure 4. Graphic dispersion of characteristics: Number of Live Plants and Survival Period for eight isolates plus the control of *Fusarium oxysporum* f.sp. *passiflorae*

3.2.1 Molecular Characterization and Population Structure—*F. oxysporum* f.sp. *passiflorae*

The results of the Bayesian analysis performed in the Structure program indicated the optimal value of K = 3. The value 3 was the lowest standard deviation and the log (ln K) was closest to zero indicating that 3 is the most likely value for the group. The groupings can be seen in Figure 5. It is noted that the isolate FOUNEMAT 5 formed a single population, being the most divergent among all other isolates.
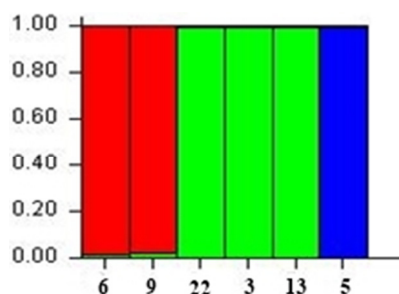

Figure 5. Genetic structure of populations of *Fusarium oxysporum* f.sp. *passiflorae* when evaluated by K = 3

As described in the molecular characterization section of *F. sonali*. by amplification, sequencing and alignment of the ITS1 and ITS2 regions, a genetic dissimilarity matrix and a dendrogram were generated. However, two from eight isolates of *F. oxysporum* f.sp. *passiflorae*, FOUNEMAT 41 and FOUNEMAT 43, showed no significant alignment with any GeneBank sequence and therefore were not used for the preparation of the dendrogram (Figure 6).

The phylogenetic tree based on the Neighbor-Joining method consisted of three groups. The first group consisted of the isolates FOUNEMAT 3, FOUNEMAT 22 and FOUNEMAT 13. This group includes an isolate collected in the Cerrado biome and other isolates collected from the Amazon biome. The second group was formed by the isolates FOUNEMAT 9 and FOUNEMAT 6, collected respectively in the Cerrado and Amazon biomes. Finally, the third group was composed of only one isolate, FOUNEMAT 5, which belongs to the Pantanal biome.
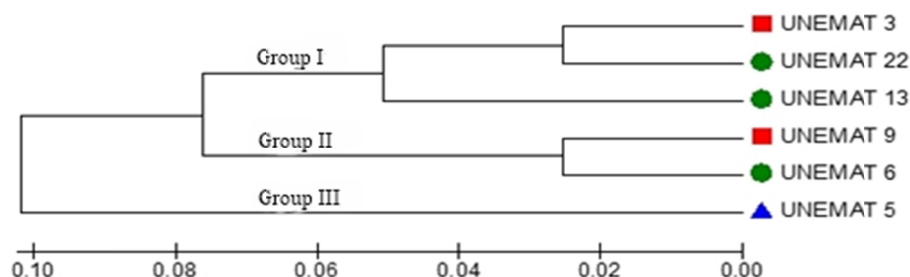

Figure 6. Dendrogram based on the Neighbor-joining method derived from the ITS 4 and ITS 5 region sequences of rDNA from *Fusarium oxysporum* f.sp. *passiflorae* isolates

4. Discussion

4.1 Characterization of Aggressiveness—F. solani

By the results, the groups formed by the UPGMA method, using Gower's distance, promoted a grouping that matched the degree of aggressiveness of the isolates.

Bueno et al. (2010) found similar results upon verifying the behavior of yellow passion fruit, variety Afruvec, in contact with a population of nine isolates of *F. solani* from the states of São Paulo and Rio de Janeiro. In the mentioned study, the fungus population presented a variability in relation to aggressiveness, ranging from isolates that did not cause death of plants, but caused a small lesion in the collar to isolates that caused a high number of plant mortality and large lesions in the collar.

Fischer et al. (2005b) also evaluated the behavior of two yellow passion fruit genotypes against five different isolates of *F. solani*. In that study, the authors found a great variability regarding the aggressiveness by the isolates

(number of dead plants and length of injuries in the collar of the plants), and a variability in the resistance between the materials for each isolate.

By analogy between the biome of origin of the isolates studied here and the grouping formed, there was no relation between the degree of aggressiveness and the origin of the pathogen, that is, the isolates from the three biomes of the state of Mato Grosso (Pantanal, Cerrado and Amazon) were distributed indistinctly in the groups formed.

These data corroborate those of Nakatina et al. (2009) upon studying the variability of five pathogenic isolates of *Xanthomonas axonopodis* pv. *passiflorae* collected at four different locations in the state of São Paulo. In that study, the authors verified the existence of variability in the aggressiveness of the isolates when inoculated in sour passion fruit. However, there was no evident relation between the collection site and the groups' composition. This is different from what was found by Munhoz et al. (2011) by working with isolates of *X. axonopodis* pv. *passiflorae*. The authors noticed a relation between the collection region and the genetic diversity of the isolates.

In addition, Dariva et al. (2015) found a high intraspecific genetic variability for isolates of *F. solani* using the molecular markers ISSR and RAPD, but there was also no clustering of isolates according to the geographic region of origin.

From a technical point of view, the genetic variability observed for the pathogen's aggressiveness (*F. solani*) to *P. edulis* may be related to the genetic variability of the genus *Passiflora* since Brazil is an important center of diversity of this genus, and generally the centers of diversity of the hosts are also centers of diversity of pathogens (Leppik. 1970). It is interesting to emphasize that there is a co-evolution process between pathogen and host, generating variability in both plant and fungal populations, which are usually associated with evolutionary forces, such as mutation, migration and selection (Barbieri & Carvalho, 2001). In addition, another explanation would be the existence of the sexed phase of this fungus, which belongs to the genus *Haematonectria*, where there is genetic recombination and the possibility of crossing between different individuals (Fischer et al., 2005a; Nayaka et al., 2011; Dufresne et al., 2011).

4.1.1 Molecular Characterization and Population Structure—*F. solani*

According to the results, a great genetic diversity was verified among the isolates of *F. Solani* studied. This high variability was also observed by Bahar and Shahab (2012) upon investigating 33 isolates of *F. solani* collected in different regions of Iran and characterized by marked SSR. The DNA analysis showed that the isolates had a high genetic variability and formed 10 distinct groups in the phylogenetic tree.

In studies on passion fruit, Henao-Henao et al. (2018) analyzed populations of *Fusarium* spp. associated with *P. edulis* in five municipalities of Valle del Cauca. Colombia. They analyzed 35 isolates by amplifying the region of the TEF 1 alpha gene, and identified three species of *Fusarium*, among them *F. solani*.

In this same context, using the ITS-5.8S region sequencing and the elongation factor 1α (EF-1α). Bueno et al. (2014) verified an interspecific variability of eight *F. solani* isolates from *P. edulis*. The authors identified that the isolates had host specificity and that the phylogenetic trees of the ITS and EF-1a region showed that the isolates formed a distinct group within the *F. solani* group when compared to other GenBank sequences.

In view of these results, the isolates of *F. solani* collected in *P. edulis* in the state of Mato Grosso presented a high molecular variability independent from the biome of origin, and this was also observed in the tests of aggressiveness.

4.2 Characterization of Aggressiveness—Fusarium oxysporum f.sp. passiflorae

All isolates of *F. oxysporum* f.sp. *passiflorae* were pathogenic. However, the analysis of aggressiveness showed a variation among isolates classified as aggressive and moderately aggressive. Similar results were found by Cunha et al. (2015) working with different isolates of *Fusarium oxysporum* f. sp. *cubense* collected in the north and south of the state of Santa Catarina. Brazil. The authors identified that the isolates from Bananeiras of the Pome subgroup were only classified as aggressive (88.5%) and moderately aggressive (11.5%). None was classified as little aggressive.

The data indicate that there is no relationship between the aggressiveness of the isolates analyzed in this study and their biome of origin. This also occurred with the isolates of *F. solani*. This is probably due to a horizontal transfer of genes between lineages in the species under study.

Baayen et al. (2000) found that *formae speciales* in the complex of *F. oxysporum* might have independent origins. Pathogenicity and virulence evolve through mutations, transposition or dissemination to distant related organisms through parasexuality or horizontal transfer of genes.

Faced with these facts, Hua-Van et al. (2001) reported that the transposition of DNA sequences into the genome might result in genetic, intra- and inter-specific variability. They also point out that, from an evolutionary point of view, there is the possibility of a horizontal transfer of genes between different species, as observed in an experiment on isolates of *F. oxysporum*.

4.2.1 Molecular Characterization and Population Structure—*F. oxysporum* f.sp. *passiflorae*

According to results obtained by molecular analysis, the groups formed demonstrate a high degree of diversity among the isolates of *F. oxysporum* f.sp. *passiflorae*. It should be noted that the isolate FOUNEMAT 5 from the Pantanal biome was the most divergent because it was allocated alone in a group. Note that the clusters formed in the population structure analysis coincide with the groups generated by the phylognetic tree using the Neighbor-Joining method.

Other authors also found a high molecular genetic diversity among isolates of *F. oxysporum* f.sp. *passiflorae*. Silva et al. (2013), using AFLP markers, observed a high variability among 14 isolates of *F. oxysporum* f.sp. *passiflorae* collected in Bahia. Dariva et al. (2014) determined the genetic variability of 13 isolates of *F. oxysporum* f.sp. *passiflorae* pathogenic to passion fruit by using ISSR and RAPD markers. The analyses revealed a population with a high intra-population genetic variability.

Similar results were found by Walker et al. (2016) using the ITS1 and ITS4 markers on *Fusarium acuminatum* and *Fusarium verticillioides*. The authors were able to genetically dissociate these isolates from others of the genus *Fusarium*.

Contrary to what was found in this study in Iran, Nourollahi and Madahjalali (2017) determined the population structure and the genetic diversity of 101 isolates of *Fusarium oxysporum* f. sp. *lentis* by SSR markers. The results showed a high genetic similarity between the isolates probably due to a low gene flow in the collection region.

By making an analogy between the results obtained with the molecular and aggressive tests. a high genetic and pathogenic variability of the isolates can be observed regardless of the biome of origin.

5. Conclusion

The results indicate a need to consider the molecular variability and the aggressiveness of the pathogens *F. solani* and *F. oxysporum* f.sp. *passiflorae* in the evaluation of genotypes of sour passion fruit in programs of selection of resistant cultivars. The genetic structure of pathogen populations may provide insights into the pathogen's evolutionary potential, as well as lead to the development of integrated disease management strategies and breeding programs. The use of the most aggressive pathogens in this study is recommended for the selection of resistant genotypes of *Passiflora* spp. Among all isolates of the *F. solani*, FSUNEMAT 40 and FSUNEMAT 46 were the most aggressive and of the *F. oxysporum* f.sp. *passiflorae* the most aggressive was FOUNEMAT 22.

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Appendix A

NCBI ID information for each fungal isolate used in this research

Nº of Collection	NCBI/GenBank	% Query Cover
Fusarium solani		
FSUNEMAT 4	MG734215.1	96%
FSUNEMAT 7	MH559637.1	99%
FSUNEMAT 12	KM979504.1	99%
FSUNEMAT 17	MH794181.1	98%
FSUNEMAT 21	MG679522.1	86%
FSUNEMAT 25	KM678345.1	93%
FSUNEMAT 28	KM678345.1	93%
FSUNEMAT 31	MF510588.1	99%
FSUNEMAT 33	MF510588.1	99%
FSUNEMAT 34	KU097248.1	94%
FSUNEMAT 35	KU097247.1	94%
FSUNEMAT 38	KR071141.1	94%
FSUNEMAT 40	KU097248.1	94%
FSUNEMAT 46	KR071141.1	94%
FSUNEMAT 47	KU097247.1	94%
FSUNEMAT 50	MH794181.1	99%
Fusarium oxysporum f. sp. *passiflorae*		
FOUNEMAT 3	DQ979012.1	99%
FOUNEMAT 5	MG669176.1	94%
FOUNEMAT 6	MH368097.1	84%
FOUNEMAT 9	MH665483.1	97%
FOUNEMAT 13	MH368097.1	99%
FOUNEMAT 22	MG664775.1	99%
FOUNEMAT 41	MG664774.1	99%
FOUNEMAT 43	MH879859.1	99%