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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Fusarium* spp. PATHOGENIC TO *Ilex Paraguariensis*

MEZZOMO, R.; ROLIM, J. M.; POLETTTO, T.; ROSENTHAL, V. C.; SAVIAN, L. G.; REINIGER, L. R. S.; MUNIZ, M. F. B. Morphological and molecular characterization of *Fusarium* spp. pathogenic to *Ilex Paraguariensis*. **CERNE**, v. 24, n. 3, p. 209-218, 2018.

HIGHLIGHTS

Due to the low initial costs and ease of employment of agrosilvopastoral systems, the cultivation of yerba-mate constitutes an alternative for small properties in the RS to the diversification of production.

The isolates of *Fusarium* spp. were submitted to the UPGMA analysis and grouped by the similarity between the characters averages.

Through the UPGMA clustering test, the thirty-nine isolates of *Fusarium* spp. were allocated to seven large groups.

The analysis of the TEF I- α gene region was enough to discriminate species of *Fusarium* spp.

The reflex symptoms resulted from the interaction of *Fusarium* spp. isolates with yerba-mate roots.

ABSTRACT

The cultivation of yerba-mate (*Ilex paraguariensis*) has been intensified in the State of Rio Grande do Sul, as result of the valuation of the price paid to the producer. However, studies related to *Fusarium* pathosystem vs. yerba-mate are still insufficient, so that many producers have faced significant losses in herbal production due to the occurrence of the roots rotting. Considering the above, the objectives of this study were: a) morphologically characterize the *Fusarium* spp. isolates collected in sick plants of yerba-mate; b) select efficient morphological characters on separating the *Fusarium* spp. isolates in similarity groups; c) identify the *Fusarium* spp. isolates to species level through the sequencing of the genomic region of elongation factor I- α (TEF-I α); and d) testing the pathogenicity of the *Fusarium* spp. isolates selected based on the morphological characterization. For such, collections for the pathogen isolates were made in five cities and the collection points were georeferenced. Thirty-nine isolates were morphologically characterized by variables as mycelial growth, sporulation, macroconidia length and width, microconid format, colonies pigmentation and formation of chlamydospore, and tested for pathogenicity by substrate inoculation. The variables used in the morphological characterization were efficient in discriminating the isolates in seven groups, especially length of macronidia associated to sporulation. Seven pathogenic isolates of *Fusarium* spp. were identified, also through molecular sequencing of the TEF-I α gene region. The sequencing of the TEF-I α region consolidated the identification of pathogenic isolates as *F. solani* and *F. oxysporum*.

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INTRODUCTION

The yerba-mate is a native species of the Mixed Ombrophilous Forest, with wide distribution and of great environmental and socioeconomic importance especially in southern Brazil, northeastern Argentina and a great part of Paraguay and Uruguay (Fowler et al., 2007). In an area corresponding to 94,945 hectares, 602,899 tons of green yerba-mate were produced in Brazil in the year of 2015, resulting in an average productivity of 7,126 kg·ha⁻¹. The State of Rio Grande do Sul (RS) concentrates the largest part of the marketed and exported yerba-mate production, with 292,386 tons, which corresponds to 48.5% of the total produced. The State of Paraná follows with 217,851 tons or 36.1% of the production (Brazilian Institute of Geography and Statistics, 2015).

However, these yerba-mate plantations are subject to pathogen attacks such as the *Fusarium* fungi, known to cause diseases such as the roots rotting, which lead to a reduction in productivity and, consequently, to plant death.

Among the main symptoms of root rotting are the ascending yellowing of leaves, the growth stagnation and excessive drop in older leaves and in many cases, the death of the plant. As result of the pathogen attack, the roots darken and rot, compromising the development of the plantations. In the attacked plantations, the production losses may be greater than 30% (Poletto et al., 2006).

To ensure that control and prevention strategies can be deployed there is the necessity for studies that seek to identify the fungus causing the disease correctly.

Morphological characterization is among the main mechanisms employed in the identification of phytopathogenic fungi. It is used widely, and various analyses such as colonies pigmentation, texture, shape, marginal form and mycelial growth rate, process of formation, spore size and shape are involved (Burgess et al., 1995). Although, due to their great similarity, the differentiation of fungi through morphological characters is imprecise. Nevertheless, by directly accessing the DNA, the use of molecular techniques become more efficient and accurate for the discrimination of fungal species (Steenkamp et al., 2000).

The objectives of this study were: a) morphologically characterize the *Fusarium* spp. isolates collected in sick plants of yerba-mate; b) select efficient morphological characters on separating the *Fusarium* spp. isolates in similarity groups; c) identify the *Fusarium* spp. isolates to species level through the sequencing of the genomic region of the elongation factor 1- α (TEF-

1 α); and d) test the pathogenicity of the *Fusarium* spp. isolates based on the morphological characterization.

MATERIAL AND METHODS

Obtaining and purification of isolates

The samples collections were performed in yerba-mate plantations in the cities of Anta Gorda, Arvorezinha, Putinga, Ilópolis, and Machadinho of the State of Rio Grande do Sul (RS), from January through March 2015. All the collection (Table 1) points were georeferenced using a Global Positioning System (GPS) equipment.

The plant material collected in the field (roots of adult plants and seedlings with tipping symptoms) was duly identified and placed in thermal boxes containing ice and transported for further analysis in the Elocy Minussi Phytopathology Laboratory of the Department of Plant Protection of the Federal University of Santa Maria (UFSM). Then, the material was placed in a humid chamber. After that, the material was observed in stereoscopic and optical microscopes to check for the presence of pathogen structures (mycelium and spores). If positive, with the aid of a histological needle, structures of the pathogen were transferred to Petri dishes containing potato-dextrose-agar (PDA) culture medium, as recommended by Alfenas and Mafia (2007). After seven days of growth, the isolates grown on PDA were purified according to the technique of monosporic culture, described by Fernandez (1993).

Morphophysiological characterization of *Fusarium* spp. isolates

The isolates obtained followed the methodology described by Michereff et al. (2003), where each isolate was evaluated according to the parameters displayed below. The mycelial growth rate (M.G.R.) was determined from the transfer of PDA medium discs with a 6 mm diameter, obtained from colonies with seven days of growth, for other plates with PDA. For this, five replications were used, each one constituted by one plate. The mycelial growth of each isolate was evaluated after seven days of incubation by measuring the mean diameter of the colony into two diametrically opposite directions. The sporulation was assessed at 10 days of incubation by adding 20 mL of sterile distilled water in four Petri plates, after the scraping of the colonies and sieving in a double layer of gauze for the subsequent estimation of the conidial concentration (conidia·mL⁻¹), with the aid of a Neubauer chamber. The coloration and

TABLE I Areas of collection of *Fusarium* spp. isolates from *Ilex paraguariensis* plantations in RS..

Area code	Collection date	Region	Coordinates (GMS)
AT	January 2015	Anta Gorda	-28°55'48.5" S, -52°10'40.3" W
AV	January 2015	Arvorezinha	-28°52'13.5" S, -52°10'52.3" W
I1	January 2015	Ilópolis	-28°55'42.5" S, -52°08'32.6" W
I2	January 2015	Ilópolis	-28°55'42.7" S, -52°08'33.6" W
I3	January 2015	Ilópolis	-28°56'53.5" S, -52°08'15.1" W
I4	January 2015	Ilópolis	-28°57'03.6" S, -52°04'56.0" W
I5	January 2015	Ilópolis	-28°54'17.6" S, -52°07'31.6" W
I6	January 2015	Ilópolis	-28°53'58.1" S, -52°04'48.0" W
I7	January 2015	Ilópolis	-28°55'55.4" S, -52°11'00.3" W
I8	January 2015	Ilópolis	-28°55'48.6" S, -52°10'40.1" W
PF	January 2015	Putinga	-28°00'34.1" S, -52°06'00.2" W
M1	March 2015	Machadinho	-27°33'41.9" S, -51°39'51.4" W
M2	March 2015	Machadinho	-27°32'23.1" S, -51°39'40.1" W
M3	March 2015	Machadinho	-27°32'46.6" S, -51°40'47.7" W
M4	March 2015	Machadinho	-27°32'45.9" S, -51°40'48.1" W
M5	March 2015	Machadinho	-27°32'16.1" S, -51°42'32.4" W
M6	March 2015	Machadinho	-27°33'36.6" S, -51°41'34.7" W
M7	March 2015	Machadinho	-27°33'25.0" S, -51°40'04.2" W

pigmentation of the colonies and the aerial mycelium were observed with the aid of the Munsell Soil Color Chart (2009). Then, with the purpose of verifying the macroconidia form, microconidia formation and chlamydospore presence or absence, the isolates were minced for clovers-leaf agar - CLA culture medium (Nelson, 1983). For each isolate, 30 conidia were measured. It was also counted the number of septa of the macroconidia. The width and length were measured in an optical microscope, with a coupled micrometer, at 40x magnitude (Leslie; Summerell, 2006).

Molecular characterization of *Fusarium* spp. isolates

From the morphological characterization, the *Fusarium* spp. isolates were submitted to a pathogenicity pilot test in tobacco seeds (*Nicotiana tabacum*) according to the methodology described by Rego et al. (2012). The

percentage of non-germinated seeds and symptomatic seedlings were evaluated. Based on the three analyzes (morphophysiological characterization, multivariate analysis of grouping and pathogenicity pilot test) seven *Fusarium* spp. isolates with the greatest potential pathogenicity revealed by the pilot test (I1AR1, R6AR2, I8AR1, M3AR2, M4, M5AR2, M7C1) were selected for the molecular characterization and pathogenicity test in yerba-mate seedlings. The seven *Fusarium* spp. isolates were duly identified, with their respective codes, forwarded to DNA extraction, as CTAB method described by Doyle and Doyle (1991). The samples of genomic DNA extracted were submitted to Polymerase Chain Reaction (PCR) for the amplification of the region of elongation factor -I- alpha (TEF-1 α), rDNA with the oligonucleotide primers for sequencing with the pair of EF1-T (ATGGGTAAGGARGACAAGAC) and EF1-1567R (ACHGTRCCRATACCACCRATCTT) primers (REHNER; BUCKLEY, 2005).

The reaction was carried out with 30 ng of DNA, 10x buffer, 2.5 μ M of each dNTP, 20 nM of MgCl₂, 25 μ moles of each of the oligonucleotide primers, 5 units of the Taq polymerase enzyme and ultrapure water to complete the reaction volume. The reactions were carried out in the MJ Research, Inc. thermocycler. PTC - 100MT, under the following thermal conditions: 94°C for 2 min, 30 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 35 s and 72°C for 10 min. At the end of the reaction, the pCR products were kept at 4°C. A negative control without DNA was included in the pCR amplifications. The amplified fragments and the control were separated by electrophoresis in agarose gel at 1.2%, in TBE 1x buffer (10.8 g tris base, 5.5 g boric acid, 4mL EDTA at 0.5 M and 4 mL of distilled water), containing ethidium bromide and observed under ultraviolet light.

Sequencing was performed in the Mega BACE 500 sequencer (Amersham). The sequenced fragments were analyzed using the BioEdit software (HALL, 1999). The nucleotide sequences obtained were compared with those already existing in the GenBank to the isolated pathogens. The GenBank sequences which presented the highest scores were selected and aligned with the sequences obtained in the sequencing by the ClustalW algorithm. The phylogenetic analysis was conducted using the Neighbor-joining statistical method with 1.000 replicates, by MEGA software version 4 (TAMURA et al., 2007). The similarity of the nucleotides sequences between isolates was calculated through the Basic Local Alignment Search Tool procedure - BLAST (<http://blast.ncbi.nlm.nih.gov>).

Pathogenicity Test

The *Fusarium* spp. isolates selected in the pathogenicity pilot test and used in the molecular characterization were multiplied in maize grains, and incorporated into the substrate, following the procedures described below. The grains of maize were soaked in water at ambient temperature for approximately eight hours, then, the excess water was discarded.

Approximately 80 g of these grains were placed in 100 mL glass bottles and autoclaved for 40 min, twice, in an interval of 24 hours. After cooling, five culture medium discs of 7 mm in diameter, containing the pathogen mycelium, were transferred to each flask. The flasks were incubated at 24°C, with 12h of photoperiod, during 14 days, according to the methodology of Klingelfuss et al. (2007) modified by Lazarotto et al. (2014). For the control, five discs of culture medium without the pathogen were placed in flasks, and the other procedures were performed as described previously. Subsequently, plastic pots (approximate 3.6 L capacity), with holes in the bottom, were filled with commercial substrate. Then, 80 cm³ of inoculum produced in maize grains were mixed to the substrate and moistened with sterile distilled water (Lazarotto et al., 2014). After 15 days, the yerba-mate seedlings were transplanted and divided in four replicates of three seedlings, where they were kept in a greenhouse for 180 days or until the manifestation of symptoms such as seedlings damping off, foliar necrosis and roots rotting in each isolate with subsequent pathogen re-isolation in PDA for proof of the Koch's Postulate.

Statistical designs and analyzes

The quantitative data of morphological characterization (sporulation, mycelial growth rate, macroconidia length and width) were submitted to multivariate analysis for grouping of *Fusarium* spp. isolates, executed Genes (Version 2009.7.0) software. The array of standardized Euclidean distance (D2) was calculated as a measure of dissimilarity and used for grouping the isolates by the "Unweighted Pair Group Method with Arithmetic Mean" - UPGMA method (Cruz, 2008).

RESULTS

Thirty-nine isolates were obtained from yerba-mate root necrosis (one isolate by collected symptomatic plant). Isolates were also obtained from seedlings with damping off symptoms, collected in producer nurseries (AV, PF, and M7). Differences in the mycelial growth rate

(M.G.R.) during the morphological characterization of *Fusarium* spp. isolates were observed. The I2AR1 isolate showed the slowest growth (4.85 mm·day⁻¹), on the other hand, the I5AR1 isolated showed the fastest rate of 11.15 mm·day⁻¹ (Table 2).

For the sporulation variable, the highlight was for the I1AR2 isolate, with an average of 69.66x10⁶ spores·mL⁻¹, while the M4AR2 isolate had the lowest average number of spores·mL⁻¹ (2.19x10⁶).

The colony pigmentation on PDA medium was decisive for the isolates separation in groups. Thus, these were gathered into seven categories, in accordance with the varying shades of pink and light pink, purple, yellow, yellow-tan, greenish yellow and olive yellow, brown and pale brown, and dark brown. It can also be observed in Table 2 that in all isolates the aerial mycelium exhibited a white color.

In the macroconidia, large variations of length and width dimensions were observed. At length, the averages varied from 24.02 to 58.90 µm in the I1AR1 and I4 isolates, respectively. At width, averages ranged from 2.90 (I1AR2) to 6.35 µm (PF2). In their great majority, the macroconidia were detailed containing three septa, reaching up to seven. Among the morphologically characterized isolates, only the I5AR1 and M7C3 isolates did not present the formation of chlamydospores in CLA medium (Table 2).

From the morphological characterization of the 39 isolates, the data related to the sporulation, mycelial growth, length and width of the macroconidia were submitted to the UPGMA clustering method and, through the generated dendrogram, the isolates were grouped by the similarity between the average of the characters. By examining the dendrogram (Figure 1) the formation of the described seven major groups may be observed (Table 3).

Also in the dendrogram (Figure 1), note that the dissimilarity among the Group 7 isolates was greater than 70%, which shows significant differences between the parameters used in the analysis, however, they were allocated in the same clade since the objective of the analysis is the separation of the isolates in large groups. The lesser dissimilarity was observed in groups 1 and 2 (below 10%), followed by Group 3 with less than 20%. Groups 4, 5 and 6 presented a dissimilarity among the isolates exceeding 20%. Another data supplied by multivariate analysis (UPGMA) is the relative contribution of the characters for divergence, which means each used variable contributed to the grouping of the isolates in major clades. Sporulation was the variable that contributed the most, with 72.22%, followed by the macroconidia length

TABLE 2 Morphological characteristics of isolates of *Fusarium* spp. obtained from symptomatic plants of *Ilex paraguariensis*.

Isolated	Spo. (x106) l	M.G.R. ¹	Macroconids ¹			Colony pigmentation ¹		Clamyd. ^{2,3}
			Length. (μ m)	Width. (μ m)	Septa N ^o .	Colony	Aerial mycelium	
I1AR1	4.02	6.69	24.02	4.95	3 or 4	Dark yellow	White	+
I1AR2	21.03	9.29	36.03	2.90	3 or 4	Purple	White	+
I2AR1	4.59	4.85	48.90	4.70	5 or 6	Brown	White	+
I2AR2	69.66	9.95	37.10	4.65	3	Purple	White	+
I3AR1	3.36	5.95	39.60	5.00	3-4-5	Purple	White	+
I3AR2	7.54	9.62	36.10	4.82	3 or 4	Purple	White	+
I3AR3	5.52	10.31	40.96	5.38	4 or 5	Olive green	White	+
I4	16.30	6.57	58.90	5.20	5-6-7	Yellow	White	+
I5AR1	14.35	11.15	31.00	4.05	3	Purple	White	-
I5AR2	3.09	7.39	42.90	4.22	5	Yellow	White	+
I6AR2	45.96	10.02	33.10	4.10	3	Purple	White	+
I6AR3	19.85	7.23	55.70	4.60	5-6-7	Yellow tan	White	+
I7	2.57	7.74	37.70	6.15	3-4-5	Yellow	White	+
I8AR1	6.08	7.05	37.80	4.90	3 or 4	Yellow tan	White	+
I8AR2	11.32	6.36	39.70	3.85	3 or 4	Olive yellow	White	+
ATAR1	5.70	7.20	40.80	5.15	3 or 4	Pale brown	White	+
ATAR2	5.32	7.47	51.00	4.65	4 or 5	Yellow tan	White	+
PF1	17.05	9.39	28.40	3.55	3	Purple	White	+
PF2	4.80	8.87	39.80	6.35	3-4-5	Yellow	White	+
AVF1	11.32	7.45	38.20	4.40	3 or 5	Pale brown	White	+
AVF2	4.68	6.96	30.90	5.75	3	Olive yellow	White	+
AVF3	2.75	8.40	40.00	5.05	3	Olive yellow	White	+
M1AR1	6.64	6.72	38.40	4.70	4 or 5	Dark brown	White	+
M1AR2	6.39	7.30	47.90	4.95	5	Dark brown	White	+
M2	2.73	7.92	38.40	4.93	3 or 4	Yellow	White	+
M3AR1	8.00	7.90	45.50	5.03	3 or 4	Yellow	White	+
M3AR2	6.39	7.30	46.30	5.10	3 or 4	Yellow	White	+
M3AR3	8.72	7.48	41.50	5.12	3-4-5	Yellow tan	White	+
M4	15.90	9.05	43.60	4.68	3 or 4	Yellow tan	White	+
M4AR2	2.19	9.03	42.50	5.05	3-4-5	Light yellow	White	+
M5AR1	2.60	6.95	31.80	4.75	3 or 4	Dark yellow	White	+
M5AR2	14.84	6.48	42.40	4.10	3-4-5	Dark nut-brown	White	+
M5AR3	9.16	7.32	40.90	4.95	3 or 4	Yellow	White	+
M5V	6.45	8.38	32.50	4.28	3 or 4	Yellow	White	+
M6AR1	13.85	8.49	40.60	5.05	3 or 4	Yellow	White	+
M6AR2	11.88	7.66	44.20	5.10	3-4-5	Yellow	White	+
M7C1	5.72	7.41	55.40	4.75	4 or 5	Dark brown	White	+
M7C2	11.88	6.99	48.40	4.70	4 or 5	Dark brown	White	+
M7C3	7.62	9.46	30.14	5.12	4 or 5	Dark brown	White	-

Where: Spo.: Sporulation at seven days; Mycelial Growth Rate (M.G.R.) (mm/day); Macroconid.: macroconids; (*) form of macroconids; length: length, macroconids width: width; Clamyd.: chlamydospore; 1: culture medium Potato-Dextrose-Agar; 2: culture medium clover-leaf-agar; 3: Presence (+) and Absence (-). Mean values for each isolate.

(26.77%); the remaining variables practically did not contribute to the divergence of the isolates.

The phylogenetic dendrogram was constructed by comparing the alignment of TEF-1 α sequences of the isolates of this study with the sequences deposited at GenBank. The sequencing of the TEF-1 α (Figure 2) identified the isolated I1AR1, I8AR1, M3AR2, M4, M5AR2, M7C1 as *F. solani* and I6AR2 as *F. oxysporum*.

The high values of the bootstrap support, 99 and 100% respectively, increased the reliability of the alignment.

The sequencing of the TEF-1 α region promoted a more extensive intraspecific differentiation since the *F. solani* isolates were distributed in different branches. The ones obtained in Machadinho (except M7C1) were assigned to the closest clades. Among the ones collected in Ilópolis, the similarity was 88%.

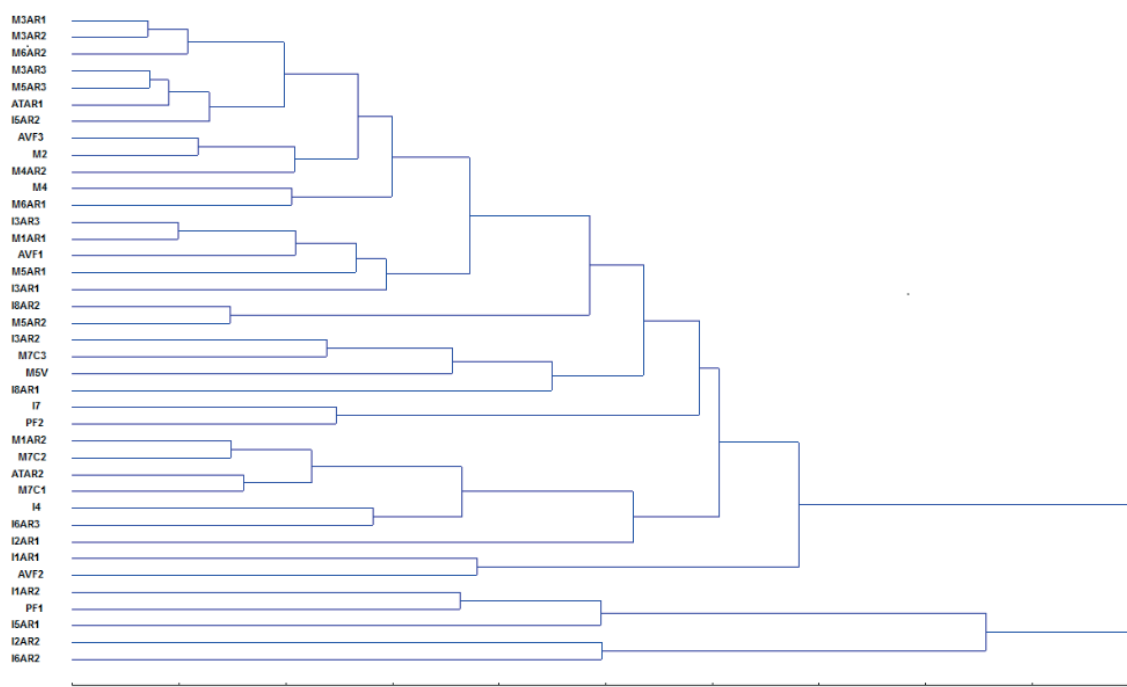


FIGURE 1 Dendrogram showing the percentage of dissimilarity among 39 *Fusarium* spp. isolates. The dendrogram was obtained by UPGMA technique, from the analysis of the Euclidean distance matrix, with the four morphological characters (sporulation, mycelial growth, macroconidia length and width).

TABLE 3 Groups of *Fusarium* spp. isolates based on the UPGMA clustering method.

Groups	Isolates
1	M3AR1, M3AR2, M6AR2, M3AR3, M5AR3, ATAR1 and I5AR2
2	AVF3, M2, M4AR2, M4 and M6AR1
3	I8AR2, M5AR2, I8AR1, M1AR1, AVF1, M5AR1 and I3AR1
4	I3AR2, M7C3, M5V and R3AR3
5	I7, PF2, I1AR1 and AVF2
6	M1AR2, M7C2, ATAR2, M7C1, I4, I6AR3 and I2AR1
7	I2AR2, I6AR2, I1AR2, PF1 and I5AR1

The main symptoms observed were the yellowing of the apex and leaves, yellowing followed by shrouding, yellowing accompanied of shrouding and subsequent leaf necrosis and leaf yellowing and necrosis. Within 30 days of the pathogenicity test, with the emergence of reflex symptoms, a stagnation of seedlings growth was perceived. In some cases, additionally to the growth stagnation, leaf fall and wither, it was possible to verify the death of plants. When removed from the substrate, the roots showed to be little evolved and dark, suggesting the intimate relationship between the reflex symptoms and the root rotting caused by the attack of *F. oxysporum* and *F. solani* present in the substrate (Figure 3). All *Fusarium*

spp. isolates used in the seedlings of the yerba-mate pathogenicity test were re-isolated, confirming their pathogenicity and complying with the Koch's Postulate.

DISCUSSION

In the taxonomy of fungi, it is of fundamental importance the stability of morphological characters in changing environmental conditions (TAYLOR et al., 2001). Yet, the morphological identification of species of *Fusarium*, in addition to being a task of difficult execution, can generate ambiguity due to the variability of phenotypic characters used in the taxonomic classification of this genus (NELSON et al., 1983; LEWIS, 2000). However, Lima and Furtado (2007) emphasize that the situation above does not prevent the species identification to continue being made in a phenotypic approach, based on morphological and cultural characteristics. Poletto et al. (2006) succeeded in identifying species of *Fusarium* using morphological characters, such as macroconidia shape and size, microconidia and chlamydospores formation, following the methodology described by Lewis (2000).

Furthermore, in the present study, the physiological and morphological characters were relevant in the separation of the isolates into groups of similarity, mainly, sporulation and length of tuberculate. Lazarotto et al. (2014) considered valid the morphological characters of

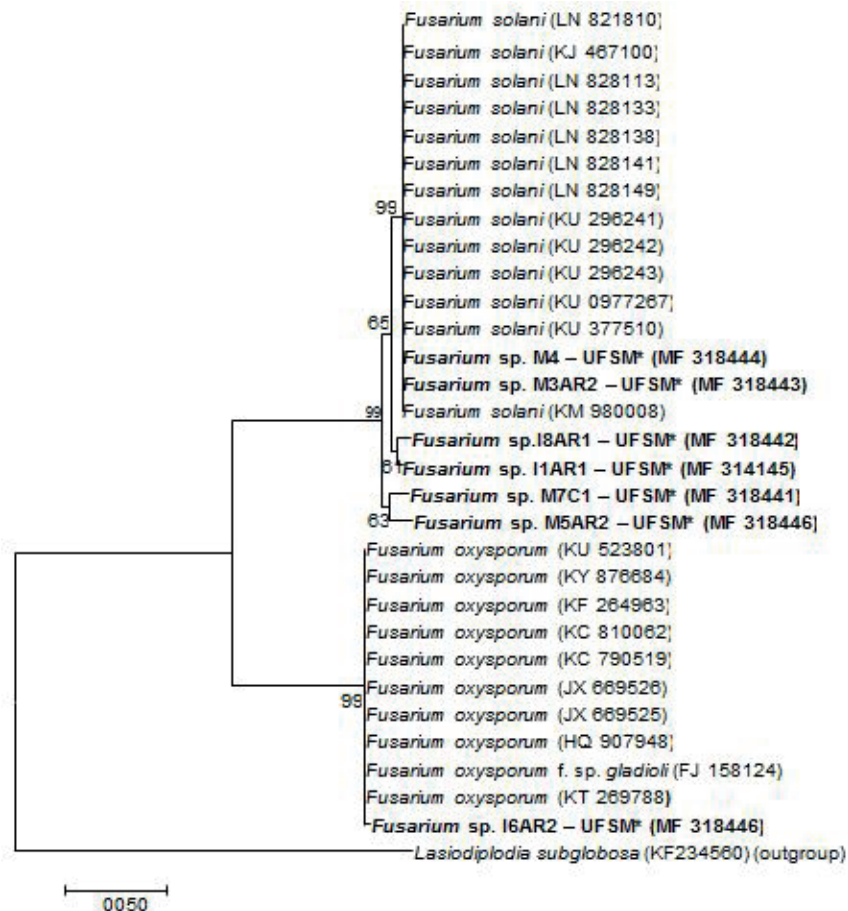


FIGURE 2 Phylogenetic dendrogram based on the neighbor-joining method from DNA sequences in the elongation factor 1- α (TEF-1 α) region. The numbers on the branches indicate the bootstrap analysis percentage of repeats in which the repetitions were observed (1,000 replications). (*): *Fusarium* spp. isolates obtained in the present study. A sequence of *Lasiodiplodia subglobosa* was used as an outgroup.

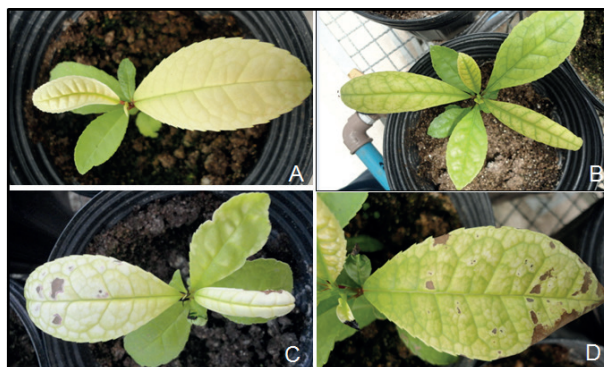


FIGURE 3 Pathogenicity test in yerba-mate seedlings, 180 days after contact with substrate inoculated with *Fusarium* spp.: Reflex symptoms: (A) yellowing; (B) yellowing + shrouding (C) yellowing + shrouding + necrosis; (D) yellowing + necrosis.

colony diameter, sporulation, macroconidial width and length, for the grouping of pathogenic isolates of *Fusarium* spp. to pecan nut (*Carya illinoensis*). The same authors

observed the formation of three groups among 12 isolates used in the study. The authors also reported that the macroconidia width was responsible for 99.98% of the divergence between the *Fusarium* spp. isolates. According to Walker et al. (2016), the multivariate analysis UPGMA was efficient in the separation of 40 isolates of *Cladosporium* spp. obtained from pecan nut into 5 groups with 30% of dissimilarity. According to the authors mentioned above, among the characters used in the analysis (mycelial growth, sporulation, conidia and ramoconides width and length) sporulation was responsible for the greatest relative contribution to the divergence (96.13%) followed by the mycelial growth (2.37%).

In the present study, the sequencing of the TEF-1 α supported by high bootstrap values gave reliability to the identification at the species level of pathogenic isolates of *Fusarium* spp. The diffusion of the TEF-1 α use may be associated to its high degree of information at the species level and to the fact that there have been

reports about the appearance of non-orthologue copies, as well as by the availability of 'universal primers' based on this gene (Geiser et al., 2004). For O'Donnell et al. (1998) and Seifert and Levesque (2004), studies involving genomic regions, performed with *Fusarium* spp. demonstrated that the region of TEF-1 α may be applied as an acceptable tool to distinguish fungus species, possessing a correlation of the *Fusarium* genus with mycotoxigenic capacity. Kristensen et al. (2005) emphasize the importance of the TEF-1 α phylogenetic analysis because they have concluded that this is able to identify species of monophyletic *Fusarium*.

According to Du et al. (2012), the region TEF-1 α was efficient in the classification, into four distinct groups of species, when they sequenced 41 isolates of *Fusarium* since the similarity indices were higher than 95%. Hsuan et al. (2011) were successful in the identification of isolates of *Fusarium* sp., belonging to the complex *Gibberella fujikuroi* obtained from maize (*Zea mays*), rice (*Oryza sativa*) and sugarcane (*Saccharum officinarum*) in Malaysia. According to the authors cited, the sequencing of the TEF-1 α consolidates itself as an important tool for the differentiation of the species of the *Fusarium* genus, supported by high bootstrap values. The polymorphism revealed by the analysis of the TEF-1 α was sufficient to discriminate even closely related species of *Fusarium*. Furthermore, the resulting dendrogram was generated in a clear way, with good structure and resolution in branches distribution (Chandra et al., 2011; Nitschke et al., 2009).

Grigoletti Junior and Auer (2001) reported, for the first time, the root rotting caused by *Fusarium* spp. in mature plants of yerba-mate from plantations in the State of Paraná, Brazil. According to the authors, the trees had symptoms such as yellowing and loss of leaves, stagnation of growth, dark roots, and in more severe cases, death. Similar symptoms were mentioned by Poletto et al. (2006) and Poletto et al. (2007) where pathogenic isolates demonstrated, initially, yellowing and leaf fall, evolving to drought and death of plants. Still, according to the authors, the symptoms are typical in coppices distributed through the planting area. Poletto et al. (2015) observed that *Fusarium* spp. isolates pathogenic to yerba-mate produced signs of necrotic spots on the root system and darkening at the tips of the roots. Lazarotto et al. (2014) obtained similar results to this study, with similar reflex symptoms in the plants analyzed. According to the authors, isolates of *Fusarium* spp. pathogenic to *Carya illinoensis* triggered symptoms such as damping off, foliar necrosis, wilt of the aerial

part and plant death, consolidating the typical root rotting symptoms. Silva et al. (2011) exemplify that the colonization of the black pepper roots (*Piper nigrum*) by *Fusarium solani* f. sp. *piperis* reflects on the aerial part, the leaves become yellowish and flaccid, causing premature fall and as the disease progresses, the death of the plant.

CONCLUSIONS

The macroconid length and sporulation morphological characters are effective in differentiating *Fusarium* spp. isolates in yerba-mate, into groups. The isolates IIARI, I8ARI, M3AR2, M4, M5AR2, M7C1, and I6AR2 are pathogenic to yerba-mate. The TEF-1 α region is efficient for identification at the species level. In this study, we identified *Fusarium solani* and *Fusarium oxysporum* as the cause the root rotting in *Ilex paragariensis* in Rio Grande do Sul state.

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