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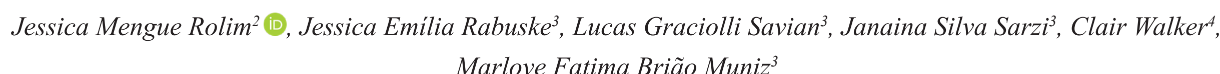

Characterization of *Fusarium* spp. causal agent of wilt in pecan[1]

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ABSTRACT

One of the limiting factors for pecan production is the incidence of disease caused by *Fusarium* spp. which are a threat to orchards in the south of the country. Therefore, the study aimed to confirm the pathogenicity and evaluate the morpho-physiological and molecular characteristics of *Fusarium* spp. associated with pecan in Rio Grande do Sul and Paraná. For this, samples of symptomatic plant material were collected, and potentially pathogenic isolates were evaluated for pathogenicity, by immersing the roots of the plants in a spore suspension of *Fusarium* spp. In addition, the severity of the disease was assessed using a rating scale according to the symptoms expressed by the plants. The isolates were also analyzed for morphophysiological variability, through evaluations of mycelial growth, colony and aerial mycelium pigmentation, and characteristics of reproductive structures. Molecular characterization was performed which amplify the region of the 1-alpha elongation factor and sequencing. Nineteen isolates were obtained, which were considered pathogenic, however, there was variability in their aggressiveness and morphophysiological characteristics. In addition, the sequencing was used to identify different species. Therefore, species of Fusarium are responsible for the occurrence of wilt in pecan in southern Brazil.

Keywords: *Carya illinoinesis*; elongation factor 1-alpha; fungi morphophysiology; genetic variability.

INTRODUCTION

The pecan (*Carya illinoinensis* (Wangenh) C. Koch) is a species belonging to the family Juglandaceae, native to North America and Mexico (Wells, 2013). In Brazil, the species was introduced by North American immigrants, who established the first orchards in the state of São Paulo (Gomes, 1976). Being a native of temperate climate, the walnut tree adapted very well when it was implanted in the southern states of Brazil and in the mountainous regions of Rio de Janeiro and Minas Gerais.

In the southern region, pecan has a very important role in increasing the income of several producers, mainly in Rio Grande do Sul, which has a higher agricultural yield, with more than 6,500 hectares of planted area (SEAPDR, 2020).

However, although the activity is constantly expanding, there is still a lack of information about the implantation of orchards from the production of seedlings, nutritional needs of plants and the management of pests and diseases which affect the development and production of trees.

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Diseases are responsible for a large part of the reduction in orchard production and many of them have already been observed and described for the species. In this sense, the wilt caused by *Fusarium* spp. appears as a new threat to orchards in the southern region of the country, where trees show symptoms such as yellowed, withered and dry leaves, in addition to darkening of the vascular system, compromising the production of plants and can lead to death quickly (Rolim *et al*., 2020).

In this context, the present study aimed to confirm the pathogenicity, as well as to analyze the morphophysiological and molecular characteristics of *Fusarium* spp. associated with pecan wilt in orchards in Rio Grande do Sul and Paraná.

MATERIAL AND METHODS

Obtaining and purifying isolates of Fusarium spp.

Symptomatic fragments of pecan plants were collected in orchards located in municipalities in the states of Rio Grande do Sul and Paraná. All collection points were georeferenced using GPS equipment. The isolates obtained came from pecan plants, which showed symptoms such as yellowing followed by wilting and leaf necrosis, in addition to darkening of vascular tissues (Figure 1).

Figure 1: Pecan plants with symptoms of wilt. A: yellowing and dry leaves; B: trunk fragment with darkening of vascular tissues.

Table 1 shows the codes of the isolates obtained, origin of the isolates, geographic coordinates of the places of origin and the dates of collection or receipt of the materials. All isolates referred to in the same collection municipality come from plants located in a single orchard.

Table 1: Code, municipality of origin, geographical coordinates and date of collection of Fusarium spp. obtained from pecan trees with symptoms of wilting

Code	Origin	Coordinates Geographical (GMS)	Date of Collection
GUA	Guarapuava – PR	25º23’23” – 51º27’51”	Jan/2017
PAN1	Pantano Grande – RS	30º09’41,1” – 52º32’17,3”	Jan/2017
PAN2	Pantano Grande – RS	30º09’39” – 52º32’18”	Jan/2017
PAN3	Pantano Grande – RS	30º09’36” – 52º32’28”	Jan/2017
S1	Segredo – RS	29º20’07” – 52º58’52”	Feb/2017
S2	Segredo – RS	29º20’07” – 52º58’53”	Feb/2017
S3	Segredo – RS	29º20’06” – 52º58’52”	Feb/2017
S4	Segredo – RS	29º20’07” – 52º58’54”	Feb/2017
S5	Segredo – RS	29º20’08” – 52º58’52”	Feb/2017
SL	Santana do Livramento – RS	31º00’11” – 56º06’08”	Mar/2017
SSP1	São Sepé – RS	30º09’49” – 53º34’17”	Mar/2017
SSP2	São Sepé – RS	30º09’50” – 53º33’52”	Mar/2017
VN1	Vila Nova do Sul – RS	30º20’28” – 53º53’05”	Jan/2017
VN2	Vila Nova do Sul – RS	30º20’26” – 53º53’15”	Jan/2017
VN3	Vila Nova do Sul – RS	30º20’23” – 53º53’08”	Jan/2017
VN4	Vila Nova do Sul – RS	30º20’26” – 53º53’10”	Jan/2017
VN5	Vila Nova do Sul – RS	30º20’26” – 53º53’11”	Jan/2017
VN6	Vila Nova do Sul – RS	30º20’26” – 53º53’07”	Jan/2017
VN7	Vila Nova do Sul – RS	30º20’27” – 53º53’10”	Jan/2017

After the collection was carried out, the plant materials were sent to the phytopathology laboratory Elocy Minussi of the Department of Phytosanitary Defense of the Rural Sciences Center of the Federal University of Santa Maria - RS. First, the vegetative samples were subjected to superficial asepsis with immersion in sodium hypochlorite in a 4: 1 ratio for 2 minutes, followed by double washing in sterile distilled water. Subsequently, the fragments were incubated in Potato-Dextrose-Agar (PDA) culture medium at 25 °C with a 12-hour photoperiod for five days. After the incubation period, fragments of mycelium which grew from the plant material were observed under an optical microscope to verify the presence of fungal structures. After the confirmation of the presence of structures characteristic of the pathogen, portions of mycelium were transferred to Petri dishes containing PDA culture medium under the same incubation conditions described above (Alfenas *et al.*, 2007). After seven days of growth, the isolates were purified according to the monosporic culture technique, described by Fernandez (1993).

Pathogenicity of Fusarium spp.

The pathogenicity test was performed with pecan seedlings from seeds of the Barton cultivar. The seeds were stratified in alternating layers of sterile sand at 4 ± 0.5 °C for ninety days. Subsequently, sowing was performed on sterile substrate (Florestal 1 - MecPlant®) in transparent disposable cups with a capacity of 750 ml. For the pathogenicity test, plants were selected 15 to 20 days after emergence, height varying between 20 and 25 cm and with the first pair of real leaves present. The roots of the plants were immersed for 30 minutes in a spore suspension of each isolate, with 14 days of cultivation or in sterile distilled water for the control treatment. In order to standardize the test, the spore suspension was adjusted to 1×10^6 spores mL^{-1}, with the aid of the Neubauer chamber. After immersion, the plants were transplanted into plastic bags (17 x 25 cm) containing commercial substrate. Four repetitions were performed, each composed of a plant, which were randomly distributed in a greenhouse with air conditioning at 25 °C and irrigated daily.

The inoculated plants were observed up to 120 days, to verify the expression of symptoms. In order to confirm pathogenicity, the pathogen was re-isolated in an PDA culture to complete the Koch Postulates. In addition, the severity of the disease was assessed according to the scale proposed by Pastrana *et al.* (2017). The scale consists of grades from 1 to 5, where 1 = no symptoms; 2 = dryness and death of older leaves; 3 = older symptomatic leaves accompanied by young leaves wilting and / or stunting, 4 = severe leaf symptoms and stem darkening and 5 = dead plants.

Morphophysiological characterization of Fusarium spp.

First, mycelial growth was evaluated. Thus, to determine the mycelial growth rate, 6 mm diameter mycelium discs, with seven days of incubation, were transferred to the center of the Petri dishes, containing PDA culture medium. The isolates were incubated at 25 °C with a 12-hour photoperiod. Four repetitions were performed and mycelial growth was measured every 24 h, by measuring the colonies in diametrically opposite directions, with the aid of a digital caliper (Maciel *et al.*, 2017).

Sporulation was determined using isolates with 10 days of incubation. For that, 20 ml of sterile distilled water were added to each Petri dish and then the colonies were scraped and sieved in a double layer of gauze. The concentration of conidia (conidia. mL^{-1}) was estimated using the Neubauer chamber.

The pigmentation of the colonies and aerial mycelium of the isolates was determined after seven days of incubation in PDA medium at 25 ºC and a photoperiod of 12 hours, using the Munsell color booklet (Munsell Color, 2009).

In order to characterize the reproductive structures, the presence or absence of sporodochia, sporodochia staining when present, size of macro and microconidia, form of microconidia, number of macroconidia septa and presence or absence of chlamydospores were observed (Leslie & Summerel, 2006; Nelson *et al.*, 1983). For the dimensioning of the structures, the length and width of 30 macroconidia and 30 microconidia were measured, with the aid of a WHO eyepiece, coupled to the optical microscope at a magnitude of 40 X. For this, the cultures were grown for 10 days at 25 °C, with a 12h photoperiod, in a clove-agar leaf culture medium.

Molecular characterization

For DNA extraction the isolates were grown in potato dextrose liquid medium at 25 °C with a 12 h photoperiod for 5 days. Subsequently, the "Mini-prep" DNA extraction protocol (based on the protocol adapted by AmgadSaleh and Kurt Zeller (Leslie & Summerell, 2006) was performed.

The total DNA concentration was estimated by spectrophotometric reading (NanoDrop 2000, Thermo Scientific®)

by absorbance at 260 nm (A260) (Pilo *et al*., 2022).

The extracted genomic DNA samples were subjected to polymerase chain reaction (PCR) for amplification of the 1-alpha elongation factor region, with the EF1 (5'-ATG-GGTATAGA (A / G) GACAAGAC-3 ') primer pair and EF2 (5'-GGA (G / A) GTACCAGT (G / C) ATCATGTT-3 ') (O'Donnell *et al.*, 1998). The PCR reaction consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.1 mM dNTP each, 0.2 mM from each primer, 1 unit of Taq DNA Polymerase (Invitrogen) and 100 ng of DNA. The reactions were performed in a thermal cycler under the following thermal conditions: 94 °C for 3 min, followed by 40 cycles at 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s and final incubation at 72 °C for 10 min also included in the amplifications was a negative control without DNA.

Amplified and control fragments were separated by electrophoresis in 1.5% agarose gel in TAE 1X buffer (40 mM Tris Base, 40 mM acetic acid and 1 mM EDTA) containing gel-red and visualized under ultraviolet light, in order to confirm purification. The PCR products were purified by precipitation with 13% polyethylene glycol (PEG) and subsequently the samples were sequenced. The sequenced fragments were analyzed with the aid of the BioEdit software (Hall, 1999). The nucleotide sequences obtained were compared with those of GenBank for the isolates and the GenBank sequences that demonstrated the highest "scores" were selected and aligned with sequences acquired in the sequencing by the ClustalW algorithm. Phylogenetic analysis was performed using the "Neighbor – joining" method with 1000 replicates using the MEGA version 4 program (Tamura *et al.*, 2007). The similarity of nucleotide sequences between the isolates was calculated using the Basic Local AlignmentSearch Tool - BLAST (http://blast.ncbi.nlm.nih.gov).

After molecular identification, the isolates were registered in GenBank, under the numbers DQ016271, MT792866, MT533180, MT759637, MN258735.1, KJ920414, for S1, S2, S4, SL, VN4 and VN6 respectively.

Statistical analysis

For the pathogenicity test, scores attributed to the wilt severity scale in pecan plants were compared by Scott Knott's test with a 5% probability of error. Quantitative data on the morphophysiological characterization of *Fusarium* spp. were submitted to the multivariate analysis technique to group the isolates using the GENES program. The Euclidean distance matrix was calculated as a measure of dissimilarity and used for grouping the isolates by the Unweighted Pair Group Method with Arithmetic Mean - UPGMA, by the GENES 2015 5.0 program (Cruz, 2008).

RESULTS

All *Fusarium* spp. were considered pathogenic to pecan. The first typical symptoms of the disease, such as yellowing and withering of some leaves, started at 75 days after inoculation (Figure 2 - A). After 10 days, the symptoms progressed to marginal necrosis, drought and leaf fall, and death of some plants, characterizing the disease's progression (Figure 2 - B, C and D). The VN2 isolate from the municipality of Vila Nova do Sul - RS, was the first to cause symptoms, causing leaf dryness and subsequent death of all plants. After five days, leaf symptoms expressed by the plants inoculated with the PAN2, S4 and VN3 isolates were observed, whereas the PAN3 isolate, in addition to severe leaf symptoms, also caused the death of all plants.

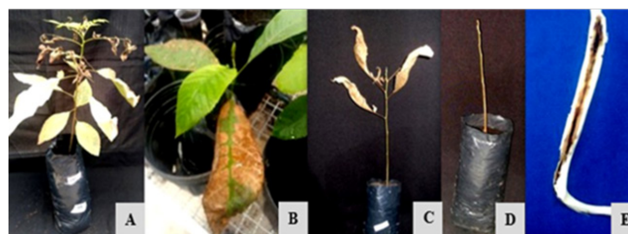

Figure 2: Symptomatic pecan plants after inoculation with Fusarium spp. A: yellowing and withering of leaves; B: marginal necrosis; C and D: dry; E: darkening of vascular tissue.

At 120 days, the final evaluation of the symptoms was performed by performing a longitudinal cut in the plants where the darkening of the tissues was observed in at least one of the replicates inoculated with each isolate (Figure 2 - E). Subsequently, the pathogens were isolated from symptomatic tissues, completing Koch's postulates.

Regarding the severity of the disease, the isolate from the municipality of Santana do Livramento (SL) was considered the least aggressive, causing only leaf symptoms in three of the four inoculated repetitions and severe leaf symptoms accompanied by darkening of vascular tissues in only one plant, reaching 2.5 on the disease severity scale and not significantly differing from the control treatment, which reached a score of 1, corresponding to no symptoms (Table 2). The other isolates differed significantly from the control treatment, with the PAN1, PAN2, S1 and VN3 isolates having an average score ranging from 3.25 to 3.75, while the other isolates reached scores between 4 and 5 on the scale. Severity, which corresponds to the manifestation

of severe leaf symptoms and tissue darkening until the death of plants (Table 2).

For the morphophysiological characterization of *Fusarium* spp., evaluating mycelial growth, differences were found between the isolates, with isolate S2 being the one that obtained the fastest mycelial growth, completely reaching the size of the Petri dish on the seventh day of incubation. The isolate also had the highest daily mycelial growth rate, reaching 11.43 mm / day. On the other hand, the S5 isolate showed the lowest mycelial growth after seven days of incubation, reaching 40.03 mm, in addition to the lowest M.G.R, reaching 5.72 mm / day (Table 3). The color of the aerial mycelium of the isolates varied between white, reddish yellow and light pink, while the color of the colonies varied between shades of yellow, pink and red (Figure 3).

Table 2: Notes attributed to the severity of wilt in pecan caused by isolates of Fusarium spp

Isolated	Note
GUA	4.50 a*
PAN1	3.75 a
PAN2	3.50 a
PAN3	5.00 a
S1	3.75 a
S2	4.50 a
S3	4.75 a
S4	4.00 a
S5	4.50 a
SL	2.50 b
SSP1	4.00 a
SSP2	4.25 a
VN1	4.75 a
VN2	5.00 a
VN3	3.25 a
VN4	4.50 a
VN5	4.00 a
VN6	4.25 a
VN7	4.75 a
CONTROL	1.00 b
CV (%)	26.06

* The means followed by the same letter do not differ in the column by the Scott-Knott test with a 5% probability of error.
Where: GUA: isolate from Guarapuava - PR; PAN1, PAN2 and PAN3: isolates from Pantano Grande - RS; S1, S2, S3, S4, and S5: isolates from Segredo - RS; SL: isolate from Santana do Livramento - RS; SSP1 and SSP2: isolates from São Sepé - RS; VN1, VN2, VN3, VN4, VN5, VN6 and VN7: isolates from Vila Nova do Sul - RS.

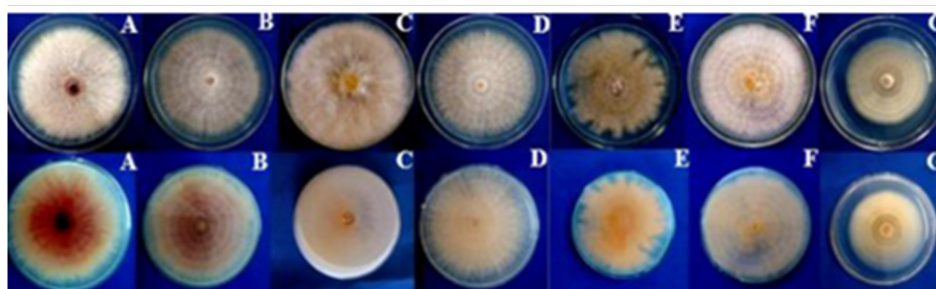

Figure 3: Colony coloration and aerial mycelium, in PDA medium after 10 days of incubation, of *Fusarium* spp. isolates. obtained from pecan plants with wilt symptoms. Upper images indicate the coloration of the aerial mycelium and the lower colonies. Upper face: A: light pink; B: white; C: reddish yellow; D: white; E: reddish yellow; F: light pink; G: White. Bottom face: A: Red; B: pale red; C: yellowish red; D: pink; E: reddish yellow; F: reddish yellow; G: pale yellow.

Table 3: Daily mycelial growth (mm) during seven days of incubation and daily mycelial growth rate (mm / day) (M.G.R) potato-dextrose-agar culture medium (PDA) from Fusarium spp. obtained from pecan plants with wilt symptoms

Isolated	Days							M.G.R
	Daily mycelial growth							
	1	2	3	4	5	6	7	
GUA	6.59	17.20	32.05	41.80	52.70	61.39	71.38	10.20
PAN1	6.56	13.41	23.82	30.73	38.27	45.64	54.49	7.78
PAN2	7.48	16.78	28.86	38.25	47.53	53.40	65.01	9.29
PAN3	7.75	18.80	32.49	41.02	50.75	56.58	68.37	9.77
S1	7.37	19.21	31.02	40.61	47.08	54.33	66.00	9.43
S2	16.42	35.69	58.97	69.62	73.53	78.96	80.00	11.43
S3	10.80	19.16	27.63	37.88	44.92	53.93	58.98	8.43
S4	6.51	15.71	23.49	30.73	36.05	47.99	55.89	7.98
S5	6.25	12.80	18.38	23.96	27.32	33.81	40.03	5.72
SL	6.68	15.80	25.43	37.92	46.64	53.32	65.05	9.29
SSP1	8.59	19.70	29.95	42.05	51.64	58.70	67.24	9.61
SSP2	9.77	25.04	35.76	46.98	54.41	69.65	73.60	10.51
VN1	8.22	16.65	26.30	37.31	44.61	52.74	58.79	8.40
VN2	7.60	16.34	27.04	35.86	44.42	49.63	60.29	8.61
VN3	8.83	15.18	23.86	33.71	41.82	48.74	52.53	7.50
VN4	7.99	25.67	37.60	52.08	59.40	69.22	70.51	10.07
VN5	6.78	21.53	31.55	43.68	49.97	63.74	70.92	10.13
VN6	7.56	16.41	26.44	37.04	45.56	53.28	59.73	8.53
VN7	7.48	19.32	30.37	44.73	56.42	67.14	73.09	10.44

As for sporulation, the GUA isolate was the one that stood out the most, as it obtained an average of 27.00 x 10^6 spores / mL (Table 4). In contrast, isolate S2 did not produce spores in PDA medium (Table 4). As for the sizing of reproductive structures, the analyzed isolates showed variation in the size of macro and microconidia (Figure 4). The length of the macroconidia oscillated between 5.85 µm in the VN2 isolate and 17.13 µm for the S5 isolate. The width varied between 1.02 µm obtained by the GUA isolate to 2.73 µm, expressed by the VN3 isolate. The number of septa varied from 1 to 3, in the vast majority of isolates (Table 4).

For microconidia, the length varied between 2.29 µm of the VN2 isolate to 5.26 µm for the PAN1 isolate. The width oscillated between 0.85 and 1.76 µm expressed by the conidia of isolates SSP1 and VN3, respectively (Table 4). In addition, it is worth noting that the S4 isolate, when grown in clove-leaf agar culture medium, did not present macroconidia, whereas the SSP2 and VN4 isolates did not express the presence of microconidia (Table 4). The S2 isolate did not produce spores in the culture medium. The microconidia of all isolates had a reniform shape. The production of chlamydospores and sporodochia also varied among the isolates. The sporodochia when present, showed a cream, yellow or orange color (Table 4).

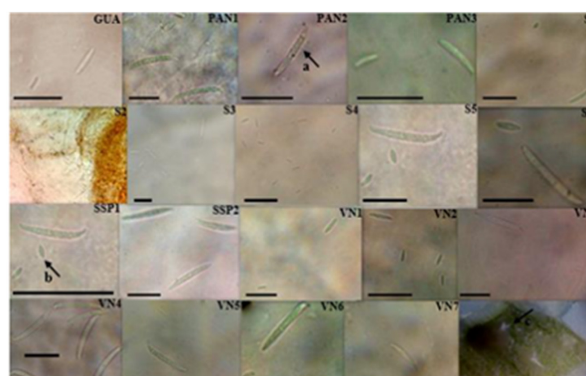

Figure 4: Reproductive structures (macro and microconidia) and formation of sporodochia in the medium of clovers-leaf-ágar (CLA), from *Fusarium* spp. isolates. obtained from pecan plants with wilt symptoms.
Macroconidia (a); microconidia (b); Sporodochia (c). The bar in each image corresponds to the measurement of 10 µm.

Table 4: Morphological characteristics of *Fusarium* spp. obtained from pecan plants with wilt symptoms

Isolated	Spor. (x10⁶)[1]	Macroconids[2] Length (µm)	Macroconids[2] Width (µm)	Septa Nº[2]	Microconids[2] Length (µm)	Microconids[2] Width (µm)	Sporodochia[2]	Color Sporod.[2]	Chlamyd.[2]
GUA	27.00	6.66	1.02	1-2	2.94	0.92	-	-	+
PAN1	3.29	17.06	1.98	2-3	5.26	1.53	+	Cream	+
PAN2	1.37	11.53	1.59	2-3	3.33	1.16	-	-	+
PAN3	3.27	7.23	1.25	1-2	3.40	1.11	-	-	+
S1	10.72	7.68	1.34	2-3	2.86	0.87	+	Yellow	+
S2	-	-	-	-	-	-	-	-	-
S3	4.07	10.80	1.24	0-1	3.54	0.86	-	-	+
S4	6.46	-	-	-	2.86	1.06	-	-	-
S5	4.49	17.13	1.49	2-3	3.30	1.07	+	Cream	-
SL	2.48	15.56	1.55	2-4	5.06	1.30	+	Cream	+
SSP1	2.52	7.40	1.14	1-2	2.62	0.85	-	-	+
SSP2	9.32	15.73	1.70	3-4	-	-	+	Orange	-
VN1	11.71	6.01	1.22	1-2	2.44	0.96	-	-	+
VN2	7.61	5.85	1.27	1-3	2.29	0.95	-	-	+
VN3	4.08	16.03	2.73	1-3	4.22	1.76	+	Cream	+
VN4	5.03	15.17	1.95	3-4	-	-	+	Orange	-
VN5	2.56	12.75	1.90	1-2	3.31	1.03	-	-	+
VN6	19.68	8.13	1.37	2-3	3.33	1.00	-	-	+
VN7	2.37	11.13	1.42	0-1	3.41	0.91	+	Yellow	+

Where: Spor. corresponds to sporulation; Sporod. corresponds to sporodochia; and chlamyd. corresponds to chlamydospores. 1 = Potato-dextrose-agar culture medium; 2 = Clovers-leaf-ágar culture medium. GUA: isolate from Guarapuava - PR; PAN1, PAN2 and PAN3: isolates from Pantano Grande - RS; S1, S2, S3, S4, and S5: isolates from Segredo - RS; SL: isolate from Santana do Livramento - RS; SSP1 and SSP2: isolates from São Sepé - RS; VN1, VN2, VN3, VN4, VN5, VN6 and VN7: isolates from Vila Nova do Sul – RS.

After the characterization of the isolates, the data related to sporulation, mycelial growth (M.G.R), length and width of macro and microconidia were submitted to the UPGMA clustering method and through the constructed dendrogram, the isolates were grouped by similarity between the means of the characters. Looking at the dendrogram (Figure 5), it can be seen at 55% of dissimilarity, the formation of six large groups. Group 2 also presented isolates with less than 40% dissimilarity. Group 3, however, is composed only of isolate S4, which possibly had characteristics that distinguished it from the others.

The fourth group allocated isolates with dissimilarity greater than 50%, in this case, the S5 isolate seems to have characteristics that differentiate it from the other isolates in the group, however, the isolate still remains in the same clade, considering that the objective of the analysis is define groups of isolates. The group 5 isolates also showed similarity, with a percentage of dissimilarity below 20%, but dissimilarity greater than 80% when compared to the other groups. The S2 isolate, belonging to the sixth group, was completely different from the other isolates, with 100% dissimilarity.

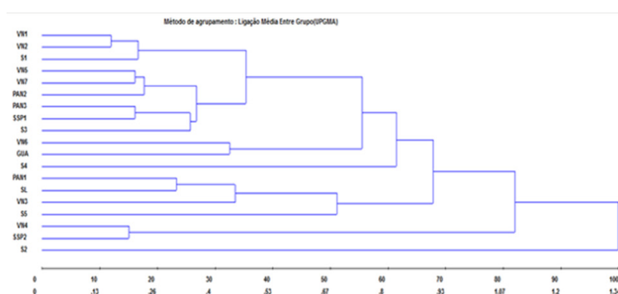

Figure 5: Dendrogram showing the percentage of dissimilarity among the 19 isolates of *Fusarium* spp. The dendrogram was obtained by the UPGMA technique, from the analysis of the Euclidean distance matrix with six morphological characters (sporulation, mycelial growth rate, length and width of macroconidia and length and width of microconidia).

In addition to segregating isolates into groups, the UPGMA method also provides the relative contribution of the characters to the divergence between isolates. Thus, it was observed that sporulation was the most influential charac-

teristic in the differentiation of the isolates, contributing with 58.12%, followed by the length of the macroconidia, responsible for 35.98%, while the other characters hardly influenced the grouping.

After performing the morphophysiological characterization, one isolate from each group generated by the UPGMA (S1, S2, S4, SL, VN4 and VN6) was randomly selected for molecular identification (Figure 6). Thus, it was observed that the S1 and VN6 isolates were associated with *Fusarium oxysporum*, although they were allocated in different clades, which demonstrates a certain genetic divergence between them. This distinction was also verified during the analysis of morphophysiological characteristics, which separated the isolates into different groups. The VN6 isolate was allocated to a 74% bootstrap clade, while the S1 isolate was grouped into a 68% bootstrap clade.

Although these values are not considered extremely high, when compared to the other *F. oxysporum* sequences, the bootstrap value increased to 89% and 85% for the VN6 and S1 isolates, respectively. Isolate S4 was identified as *Fusarium fujikuroi*, grouping with sequences of this species with 98% bootstrap. In addition, it was found proximity between the clades that grouped *F. oxysporum* and *F. fujikuroi*, however, analyzing the morphophysiological characteristics of each isolate it was possible to confirm the results generated through molecular identification.

The S2 isolate was considered the most distinct morphophysiologically from the other isolates, and in the phylogenetic dendrogram it was allocated to an 87% bootstrap clade with *Fusarium graminearum*. The VN4 isolate was identified as *Fusarium incarnatum*, reaching 97% bootstrap. In addition, in the CLA environment, the isolate showed characteristics that confirmed the identification of the species, such as the formation of orange colored sporodochia, absence of microconidia and chlamydospores.

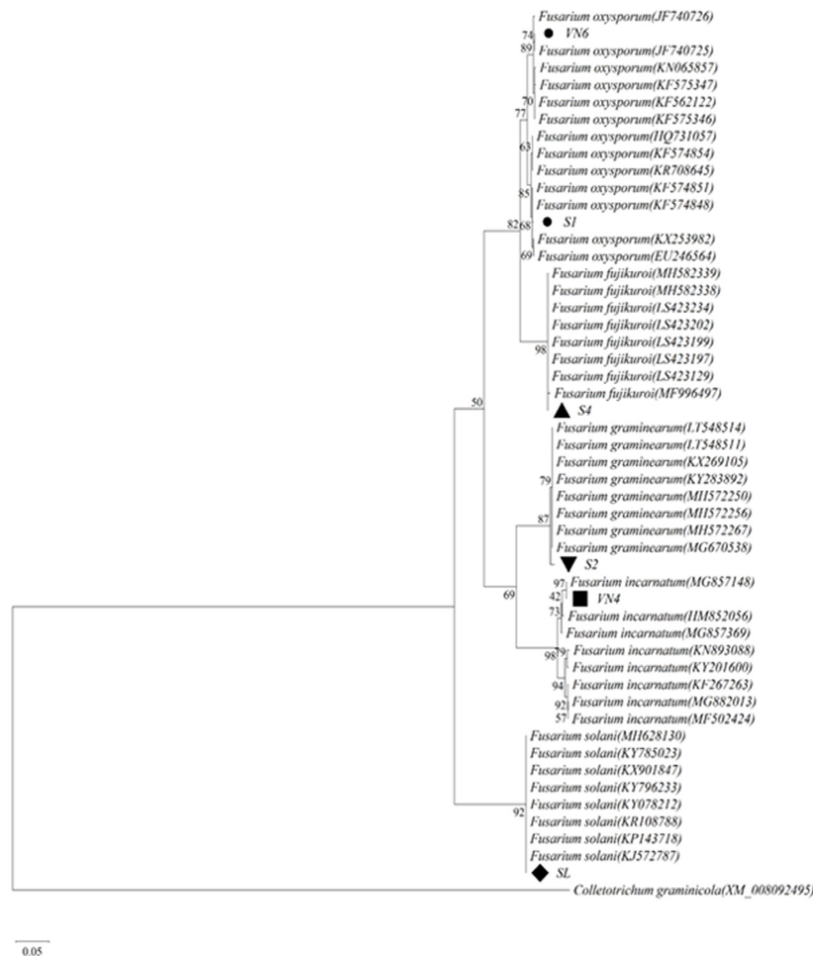

Figure 6: Phylogenetic dendrogram based on the Neighbor-joining method based on DNA sequences from the region of the 1-α elongation factor of *Fusarium* spp. isolates, based on 1000 bootstrap replicates. The number on the branches represents the bootstrap number.

The SL isolate was identified as *Fusarium solani*, allocated in a clade with 92% of the bootstrap support with sequences of the species. Like the others, it also presented characteristics similar to those expressed by fungi of this species, such as a light colored colony and the presence of a cream colored sporodochia.

DISCUSSION

The tests used to isolate and identify fungi do not prove the pathogenicity of the isolates or indicate the level of virulence of these organisms, being necessary for this, pathogenicity tests that aim to prove the occurrence of the disease, thus being essential for certain studies (Elliott, 2018). Therefore, tests with this objective confirmed the occurrence of *Fusarium* species causing vascular wilt in agronomic and forestry crops of economic interest, being a problem considered difficult to manage when installed in certain areas.

In this sense, *Fusarium* wilt caused by *Fusarium decemcellulare*, *Fusarium lateritium* and *Fusarium solani* has been described as a new threat to coffee plantations (*Coffea canephora*) in Brazil, directly affecting crop productivity (Belan *et al.*, 2018). In teak (*Tectona grandis*), one of the forest species considered of greatest economic importance in the central-west and Amazon regions, isolates of *Fusarium oxysporum* were identified causing wilt in plantations in western Mato Grosso, thus compromising the production and quality of wood from of culture (Borges *et al.*, 2018).

Regarding the severity of the disease, several studies confirm the existence of variability in the aggressiveness of *Fusarium* spp. In this sense, Jarek *et al.* (2018) evaluating the aggressiveness of five *Fusarium* spp. in peach palm (*Bactris gasipa*es Kunth var. Henderson), they observed the existence of variability in aggressiveness between isolates of the *F. oxysporum*, *F. verticillioides* species complex, *F. solani* species complex and *Gibberella fujikuroi* species complex. Silva *et al.* (2017) also confirmed the existence of different levels of severity among isolates of *Fusarium* spp. inoculated in *Pinus taeda*.

Regarding the morphophysiological characterization, it can be said that mycelial growth, although considered a secondary characteristic, is an important evaluation for comparison between *Fusarium* species, because although there is variation between isolates of the same species, some species grow considerably faster than others, which allows this separation (Leslie & Summerell, 2006). In addition, colony coloration and aerial mycelium of *Fusarium* species is also a characteristic that must be analyzed together with other characters, due to the occurrence of color overlap in different species or because isolates of the same species express different colors, even when grown uniformly.

The sporulation process is considered quite complex, in which the reproductive cells can be affected by changes resulting from morphological, physiological and biochemical factors (Castro & Coelho, 2000). In addition, characteristics such as the production of chlamydospores and sporodochia, size and shape of macro and microconidia contribute to the identification and characterization of *Fusarium* species.

Currently, due to the modifications presented by fungi of the genus *Fusarium*, the identification of species based on morphological characters is not routinely found in the literature (Barreto, 2018). However, morphological identification manuals such as those produced by Nelson *et al.* (1983) and Leslie & Summerell (2006) are still used for purposes of characterization and comparison between isolates and species.

When it comes to molecular identification, it can be said that this is an essential technique for studies aimed at the characterization of pathosystems, as it allows the correct identification of pathogens, which facilitates the construction of control strategies. In this sense, *Fusarium oxysporum* is considered a species of great economic importance, as it is responsible for the occurrence of wilt in several cultures in addition to being reported as harmful to humans and animals (Nelson *et al.*, 1981; Vartivarian *et al.*, 1993). *Fusarium fujikuroi* is also considered the causative agent of diseases in several plant species. Carmo (2017) amplified the elongation factor 1-alpha region and sequenced 29 isolates of *Fusarium* spp. pathogenic to *Pinus* spp. Through phylogenetic analysis, the author identified species belonging to four distinct complexes, 13 of which corresponded to the *F. fujikuroi* complex. In addition, as in the present study, the morphophysiological characteristics were related to the results obtained in identification, attributing more reliability to the work.

Fusarium graminearum is known to cause fusariosis or wheat gibberellas, considered one of the most dangerous diseases in the world. The pathogen produces extremely toxic mycotoxins, which affect grains and pose a risk to the health of humans and animals (Goswami & Kistler, 2004). Therefore, if lodged in the fruits, *Fusarium* spp. it can become a threat to pecan production. *Fusarium incarnatum* and *F. solani* also fit species considered to be potentially pathogenic to a wide range of hosts. The amplification and

sequencing of the elongation factor 1-alpha region allowed the identification of *Fusarium incarnatum* as a pathogen in Morus Alba (Chen *et al.*, 2017). Yang *et al.* (2017) used the sequencing of the ITS, β-tubulin and 1-alpha elongation factor regions to identify *Fusarium solani*, responsible for the wilt in *Plukenetia volubilis*.

Regions such as ITS, β-tubulin and 1-alpha elongation factor are commonly amplified and sequenced to identify fungal species. In this sense, the 1-alpha elongation factor has been the most used region for the identification of *Fusarium* spp. mainly because it is considered a highly informative gene, which allows the separation of related species (Geiser *et al.*, 2004). In addition, the morphophysiological characterization must be used as an auxiliary tool, aiming at the correct identification of the species. Thus, the correlation between the two analyzes provides veracity to the results and reliability to the study.

CONCLUSION

The results obtained in the present study allowed us to conclude that *Fusarium* spp. pecan are pathogenic, but there is variability in aggressiveness.

Fusarium oxysporum (S1 and VN6), *F. fujikuroi* (S4), *F. incarnatum* (VN4), *F. graminearum* (S2) and *F. solani* (SL) are causal agents of wilt in pecan.

The sporulation and the length of the macroconidia are the characters that most influenced the morphophysiological characterization of *Fusarium* spp.

The sequencing of the elongation factor 1 - alpha region is efficient in identifying *Fusarium* species pathogenic to pecan.

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