FUNGI OF THE BOTRYOSPHAERIACEAE FAMILY CAUSE DIFFERENT LEVELS OF STEM CANKER ON PECAN TREES (*Carya illinoinensis*) IN BRAZIL

Jessica Mengue Rolim[2*], Jéssica Emília Rabuske[3], Lucas Graciolli Savian[3], Clair Walker[4], Janaína Silva Sarzi[5], Júlio Carlos Pereira da Silva[6] and Marlove Fátima Brião Muniz[6]

[1] Received on 12.08.2021 accepted for publication on 08.03.2022.
[2] Universidade Federal de Pelotas, Programa de Pós-Graduação em Ciência e Tecnologia de Sementes, Pelotas, RS - Brasil. E-mail: <eng.jessicarolim@gmail.com>.
[3] Universidade Federal de Santa Maria, Programa de Pós-Graduação em Engenharia Florestal, Santa Maria, RS - Brasil. E-mail: <jessicarabuske@gmail.com> and <lucassavian@hotmail.com>.
[4] Universidade Estadual de Londrina, Departamento de Agronomia, Londrina, PR - Brasil. E-mail: <clairwalker@gmail.com>.
[5] Universidade Federal de Santa Maria, Programa de Pós-Graduação em Agronomia, Santa Maria, RS - Brasil. E-mail: <janainasarzi@yahoo.com.br>.
[6] Universidade Federal de Santa Maria, Departamento de Defesa Fitossanitária, Santa Maria, RS - Brasil. E-mail: <julio.c.silva@ufsm.br> and <marlovemuniz@yahoo.com.br>.
*Corresponding author.

ABSTRACT – Pecan tree [*Carya illinoinensis* (Wangenh.) K. Koch] is a widespread species in the southern region of Brazil and commercially important for nut and wood production. Stem cankers in pecan trees negatively impact the orchard, but the biotic causes of cankers are usually not identified. Symptoms of stem canker were observed in about 60% of pecan trees from orchards in the states of Rio Grande do Sul and Paraná. Therefore, this study aimed to analyze the molecular and morphophysiological characteristics of fungi associated with pecan canker in orchards in southern Brazil and to confirm their pathogenicity in pecan seedlings. Samples from symptomatic stems were collected in different municipalities and possible causal agents were isolated. Molecular identification was performed by the Polymerase Chain Reaction technique for amplification of the EF-1α region and subsequent sequencing. The sequencing, followed by morphophysiological aspects of mycelial growth and colony pigmentation, allowed the identification of a species complex included in the Botryosphaeriaceae family. The isolates were classified as *Lasiodiplodia theobromae*, *Pseudofusicoccum kimberleyense* and *Neofusicoccum parvum*. Pathogenicity was tested by inoculating the isolates into pecan seedlings to evaluate the symptoms. All isolates caused canker in the stem, but there was variation in the severity. Therefore, different species of the Botryosphaeriaceae family are responsible for stem canker occurrence in pecan trees in the southern region of Brazil, but at different levels of injury. Thus, it is crucial to identify and understand the behavior of the fungal isolates to best control cankers in pecan orchards.

Keywords: Fungi morphophysiology; Molecular identification; Elongation factor 1-alpha.

FUNGOS DA FAMÍLIA BOTRYOSPHAERIACEAE CAUSAM DIFERENTES NÍVEIS DE CANCRO DO TRONCO EM POMARES DE PECAN (Carya illinoinensis) NO BRASIL

*RESUMO – A nogueira-pecã [**Carya illinoinensis** (Wangenh.) K. Koch] é uma espécie arbórea amplamente difundida na região sul do Brasil e comercialmente importante devido à produção de nozes e madeira. Cancros do tronco em árvores de noz-pecã impactam negativamente o pomar, mas as causas bióticas dos cancros geralmente não são identificadas. Sintomas de cancro do caule foram observados em cerca de 60% das nogueiras de pomares em municípios do Rio Grande do Sul e Paraná. Assim, este estudo teve como objetivo analisar as características moleculares e morfofisiológicas de fungos isolados de cancros em nogueira-pecã em pomares do sul do Brasil e confirmar sua patogenicidade na planta. Amostras de caules sintomáticos foram coletadas e possíveis agentes causais foram isolados. A identificação molecular foi realizada pela técnica de Reação em Cadeia da Polimerase para amplificação da região EF-1α e posterior sequenciamento. O*

sequenciamento, seguido de aspectos morfofisiológicos do crescimento micelial e pigmentação da colônia, permitiu a identificação de um complexo de espécies incluído na família Botryosphaeriaceae. Os isolados foram classificados como ***Lasiodiplodia theobromae***, ***Pseudofusicoccum kimberleyense*** *e* ***Neofusicoccum parvum***. *A patogenicidade foi testada inoculando os isolados em mudas de nogueira-pecã para avaliar os sintomas. Todos os isolados causaram cancro no caule, mas houve variação na severidade. Portanto, diferentes espécies da família Botryosphaeriaceae são responsáveis pela ocorrência de cancro em tronco de nogueira-pecã na região sul do Brasil, mas em diferentes níveis de dano. Assim, é crucial identificar e compreender o comportamento dos isolados fúngicos para melhor controlar os cancros nos pomares.*

Palavras-Chave: Morfofisiologia de fungos; Identificação molecular; Fator de elongação 1-alfa.

1. INTRODUCTION

Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] is an arboreal hickory tree, whose main product is the seed, usually consumed as nuts or sometimes as an ingredient for different food recipes in many American countries (Martins et al., 2017). Besides, pecan wood has high malleability being easy to handle for crafted furniture, increasing the commercial value of the tree (Gatto et al., 2008). Pecan is also considered a feasible option to be intercropped with other crops in agroforestry systems or included in silvopastoral systems for animal husbandry (Bremm, 2018).

In addition to North America, pecan is cultivated in Brazil, Argentina, Uruguay, Australia, South Africa and China (Fronza and Hamann, 2016). In Brazil, the southern region is the major pecan producer. There was a great expansion of the pecan cultivated area in southern Brazil recently, where climate conditions are favorable to its development. However, with the increase in the cultivated area, the occurrence of diseases caused by biotic pathogens in the orchards has become more common. Species of fungal pathogens have been reported damaging pecan trees in Brazil. Some of these pathogens cause significant losses, resulting in leaf spots (Lazarotto et al., 2012; Walker et al., 2016), root rot (Lazarotto et al., 2014), vascular wilt (Rolim et al., 2020a) and stem canker (Poletto et al., 2016; Rolim et al., 2020b).

Botryosphaeriaceae is a fungal family comprising 22 genera (Phillips et al., 2019) with cosmopolitan species that are mostly distributed in tropical and subtropical regions, occurring in a wide range of habitats (Abdollahzadeh et al., 2010). The species in this family have distinct characteristics, can survive as saprophytes on the plant debris, cause plant diseases or inhabit plant tissue as harmless endophytes without causing damage to plants (Abdollahzadeh et al., 2010; Luo et al., 2019). However, endophytic species may change their harmless condition to a plant-parasitic habit whenever environmental conditions become unfavorable to the host or the host suffers some injury (Phillips et al., 2019). Among the pathogenic genera of this family are *Lasiodiplodia*, *Pseudofusicoccum*, *Neoscytalidium*, *Neofusicoccum*, *Diplodia, Dothiorella* and *Botryosphaeria*, which cause different symptoms in their hosts, such as bud blight, stem cankers, fruit rot and gummy disease (Abdollahzadeh et al., 2010). Therefore, the incidence of these pathogens may jeopardize the yield of some crops, including pecan.

Stem canker caused by *Lasiodiplodia subglobosa* was reported in pecan in Brazil, causing lengthwise cracking in the stem, necrosis of the affected tissues and' formation of cankers, which consequently compromised plant growth and nut production (Poletto et al., 2016). From that first report, similar symptoms have been identified in pecan trees in different orchards of southern Brazil.

Therefore, this study aimed to identify fungal species from cankers in pecan trees by using molecular and morphophysiological techniques and also confirm their pathogenicity in pecan trees. In addition, it was verified the differences in the level of injury caused by the isolates in the stem.

2. MATERIAL AND METHODS

2.1 Sample collection and fungal isolation

Typical stem canker symptoms such as lengthwise cracking in the stem, necrosis of the affected tissues and formation of cankers in the trunk were found in commercial orchards in the municipalities of Santa Maria, Segredo, Pantano Grande, state of Rio Grande do Sul, and in Guarapuava, state of Paraná

(Table 1). According to Köppen's classification, in the municipalities of Rio Grande do Sul the climate is considered subtropical, with warm summers, while in Guarapuava the climate is mesothermal-humid subtropical, with no defined dry season.

The trunks and branches from collected symptomatic trees were deeply chipped, with a sharped sterile knife, into 10-cm fragments and stored in properly identified kraft paper bags. Subsequently, the samples were sent for pathogen isolation to the Phytopathology Laboratory Elocy Minussi, Federal University of Santa Maria, Rio Grande do Sul, Brazil.

Initially, the bark was removed from the sampled fragments to fully expose the infected tissues. Then the tissues were subdivided into samples with dimensions of 4 × 4 cm. Subsequently, the samples were sterilized in a bleach solution (20% v/v) for 2 min, followed by double washing in sterile distilled water. The samples were dried by an aseptic filter paper and transferred to Petri dishes containing potato dextrose agar medium (PDA) with streptomycin. The samples were incubated for seven days at 25 °C under 12 h lighting. Subsequently, fungal growth from the fragments of infected tissues was observed under an optical microscope to confirm the presence of plant pathogens. Each different fungal mycelia were transferred to a Petri dish containing pine needle agar culture medium (agar: 20 g; sterile pine needle ground fragments: 250 g; distilled water: 1,000 ml;), under the same incubation conditions described above, but for 20 days to induce sporulation (Poletto et al., 2016). After the incubation period, the spores from each mycelium were collected and the isolates were purified according to the monosporic culture technique (Fernandes, 1993). The isolates received the identification codes GUA, SEG, SM and PAN (Table 1).

Molecular analysis

For molecular identification by phylogenic test, the isolates were first grown on Potato Dextrose medium (PD). DNA extraction protocol used was the "Mini-prep", according to the protocol adapted from Leslie and Summerell (2006). For DNA extraction, the mycelium was collected and macerated in liquid nitrogen. A 100 µg of mycelium was mixed to 700 µl of CTAB (Cationic hexadecyl trimethyl ammonium bromide) buffer, followed by vortexing for 2-3 s and subsequent heating at 65 °C for 25 min. In sequence, 300 µl of chloroform:isoamyl alcohol (24:1, v/v) was added to each sample, then vortexed and centrifuged for 5 min; 600 µl of the resulting aqueous phase was transferred to new tubes of 1.5 ml, 600 µl of isopropanol was added, followed by manual inversion for mixing the contents. The material rested for 5 min and then was centrifuged for 5 min. The supernatant was discarded and 600 µl of 1 × TE buffer (300 µl of phenol: chloroform: isoamyl alcohol - 25: 24: 1) was added, vortexed and the tubes were placed in a centrifuge where they rested for 5 min and centrifuged at 130 RPM. With a pipette, 500 µl of the liquid was transferred to new tubes and 1 µl of RNAse was added. The tubes were vortexed and incubated at 37 ºC for 30 min. To each tube 500 µl of isopropanol (2-propanol) was added and manually inverted and rested for 5 min at room temperature, resulting in the precipitated nucleic acids. The samples were centrifuged at 130 RPM, for 5 min. for the sedimentation of DNA. The aqueous portion was discarded, and the tube was inverted on a paper towel for 5 min. The DNA pellets were washed twice with 1 ml of cold 70% (v/v) ethanol, then the paper drying process was repeated. The pellets were resuspended with 50 µl of TE buffer and stored at 4 ºC for later use.

Table 1 – Data of fungal isolates collected from stem canker in orchards of pecan trees.
Tabela 1 *– Dados de isolados fúngicos de cancro de tronco coletados em pomares de nogueira-pecã.*

Isolate	County/State	Coordinates (GMS)	Sampled date	Canker Incidence*
GUA	Guarapuava/PR	25º23'23"S/ 51º27'51"W	Jan/2017	65 %
PAN	Pantano Grande/RS	30º19,05'02,1"S/ 52º39,45'36,9"W	Jan/2017	60 %
SEG	Segredo/RS	29º36,09'16"S/ 52º94,50'76"W	Jan/2017	60 %
SM	Santa Maria/RS	29º41'05,12"S/ 53º43'33"W	Jan/2017	60 %

*Estimated by visual estimation of symptomatic trees in the sampled orchards.
**Estimado por estimativa visual de árvores sintomáticas nos pomares amostrados.*

The extracted genomic DNA samples were subjected to the Polymerase Chain Reaction (PCR), for the amplification of the elongation factor 1 - alpha (EF-1α) region with the primers EF1-728F and EF1-928R (Carbone and Kohn, 1999). The PCR reaction consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM from each dNTP, 0.2 µM from each primer, 1 unit of Taq DNA polymerase (Invitrogen, ThermoFisher, California, USA) and 100 ηg of DNA. The reactions took place in a thermocycler, where the initial denaturation was carried out at 94 °C for 3 min.; followed by 40 cycles at 94 °C for 20 s; pairing step at 60 °C for 30 s; extension at 72 °C for 30 s; and final incubation at 72°C for 10 min. The amplifications included control samples, without DNA. The amplified fragments and the control were separated by electrophoresis in 1.5% agarose gel, in 1X TBE buffer (40 mM Tris Base, 40 mM acetic acid and 1 mM EDTA) containing 1 µl of red gel and visualized under ultraviolet light. The PCR products were purified by precipitation with 13% polyethylene glycol 8000 (PEG 8000). Then, the samples were separated again by 1.5% agarose, in 1X TBE buffer (40 mM Tris Base, 40 mM acetic acid and 1 mM EDTA) containing red gel and visualized under ultraviolet light, to confirm the purification. Afterward, the samples were sequenced in ABI-Prism 3500 GeneticAnalyzer equipment (Applied Biosystems, ThermoFisher, California, USA). The GenBank sequences that showed the highest scores (% of similarity) were selected and aligned together with those acquired in the sequencing by the ClustalW algorithm. The sequences were aligned with the BioEdit software. The phylogenetic analysis was performed using the "Neighbor-joining" method with 1,000 replicates by MEGA software version 4 (Tamura et al., 2007). The phylogram was constructed and the similarity of the nucleotide sequences between the isolates was calculated using the Basic Local Alignment Search Tool - BLAST (Altschul et al., 1990). After molecular identification, the isolates were registered on GenBank, under the numbers MT436263, MT533179, MN233653 and MN258734 for GUA, SEG, SM and PAN, respectively. All the isolates were deposited at the Herbarium of the Federal University of Santa Maria under the numbers 18,363; 18,362; 18,364; 18,365 for GUA, SEG, SM and PAN, respectively.

2.2 Morphophysiological aspects of the isolates

For the evaluation of mycelial growth, initially, 5-mm PDA disks containing structures (mycelium) from the fungal monosporic isolates were transferred to the center of 90 mm Petri dishes with PDA. The plates were incubated for five days at 25 ºC under 12 h lighting. Mycelial growth was estimated by measuring the colony diameter every 24 h for five days in two perpendicular directions to compare the isolate's growth over time. On the fifth day, the color of the colonies and aerial mycelium of the isolates was also observed, which were determined according to the Munsell color chart (Munsell Color, 2009).
For each isolate, four replicates were used, consisting of one Petri dish per replicate.

2.3 Pathogenicity test

Pecan seeds, cultivar Barton, were purchased from a nursery located in the municipality of Anta Gorda - Rio Grande do Sul. The seeds were stratified in sterile sand at 4 ºC for 90 days, after which they were sown in 750 ml disposable cups containing sterilized commercial substrate (Florestal 1, MecPlant, Paraná, Brazil). Thirty days after emergence, the seedlings were transplanted to plastic bags (12 cm x 25 cm) with the sterile substrate, for appropriate growth conditions until fungal inoculation. Subsequently, 90 days-old pecan seedlings were inoculated with each fungal isolate. For this, on the stem of each seedling, 12 cm above the root collar, a circular (3 mm) wound was made, using a sterilized scalpel. Then, a 3 mm diameter PDA disk containing the mycelium of each fungal isolate was placed directly in contact with the exposed tissues inside the wound. Also, control seedlings were inoculated with a sterile PDA disk. A small amount of cotton, moistened with sterile distilled water, was placed over the discs, which were protected with plastic film to prevent moisture loss in the wound. The inoculated seedlings were kept in a greenhouse at 25 °C and watered twice a day. The plants were observed for 90 days after inoculation to evaluate visually canker symptoms. At the end of the period, stem injury was evaluated according to the scale proposed by Borges et al. (2018), with minor modifications. This scale ranges from 0 to 4, where: Score 0 = seedlings without visible damage; Score 1 = lesion up to 3 cm long; Score 2 = lesion 3 to 6 cm long; Score 3 = lesion longer than 6 cm, and Score 4

= seedlings with lesion longer than 6 cm, deep lesion, darkening of the vessels, showing leaf fall and death. Then, the isolates were re-isolated from symptomatic tissues to complete Koch's postulates, by comparing the morphophysiological characteristics previously observed in Petri dishes.

Four inoculated seedlings per isolate were used and four non-inoculated seedlings represented the control. The data were analyzed by One-way variance analyses. The score attributed to the canker injury scale was then compared by the posthoc Tukey test ($P<0.05$) using SISVAR 5.3 (Ferreira, 2011).

3. RESULTS

3.1 Molecular identification of pathogens

Based on the phylogenetic analysis in this study and comparing them with those deposited in GenBank, the isolates obtained belonged to three different clades (Figure 1). GUA and SEG isolates were allocated in the *Lasiodiplodia theobromae* clade, with a bootstrap value of 58 and 88%, respectively. SM isolate was allocated in another clade with isolates belonging to the species *Pseudofusicoccum kimberleyense*, while PAN, was in the clade of the species *Neofusicoccum parvum*, with a bootstrap value of 86 % and 100 %, respectively.

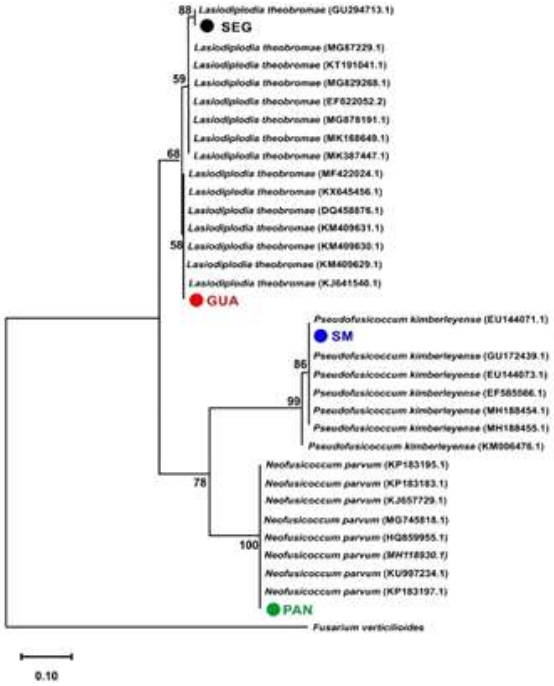

Figure 1 – Phylogram of the fungal isolates generated from elongation factor 1-alpha region sequences of the DNA. Phylogenetic analysis was based on the Neighbor-joining method. The length of the branches is indicated by the scale of the tree base and bootstrap values (1000 repetitions) are indicated in percentages above the internodes. Isolates came from pecan orchards in Guarapuava-PR (GUA), Pantano Grande-RS (PAN), Segredo-RS (SEG) and Santa Maria - RS (SM) in Brazil.

Figura 1 *– Filograma dos isolados fúngicos gerados a partir de sequências da região 1-alfa do fator de alongação do DNA. A análise filogenética foi baseada no método Neighbor-joining. O comprimento das ramificações é indicado pela escala da base da árvore e os valores de bootstrap (1000 repetições) são indicados em porcentagens acima dos entrenós. Os isolados foram coletados em pomares de nogueira-pecã em Guarapuava-PR (GUA), Pantano Grande-RS (PAN), Segredo-RS (SEG) e Santa Maria - RS (SM) no Brasil.*

3.2 Morphophysiological characteristics of isolates

There were differences in the mycelial growth of each isolate. The isolate *L. theobromae* (SEG) filled the entire surface of the Petri dish four days after isolation, while the other isolates required five days to reach 90 mm of colony diameter. The *Neofusicoccum parvum* isolate (PAN) colony was the only one that did not take the whole dish surface (Figure 2). The aerial mycelium of the isolates varied from light gray (SEG and SM), greenish-gray (PAN) to greenish-black (GUA). The colonies ranged from greenish-gray (PAN), very dark greenish gray (SEG and SM) to greenish-black (GUA).

3.3 Pathogenic test and stem canker symptoms

The four obtained isolates proved to be pathogenic when inoculated in pecan seedlings. The first symptoms were observed thirty days after inoculation, starting with lengthwise cracking in the stems, followed by tissue necrosis and lumps in the form of cankers. GUA (*Lasiodiplodia theobromae*) was the first isolate to cause symptoms, thirty days after inoculation. Forty days after inoculation, isolates SEG (*L. theobromae*) and PAN (*N. parvum*) also caused longitudinal cracking in the plants, whereas SM (*P. kimberleyense*) caused symptoms in all replicates only 65 days after inoculation. The control, inoculated only with a PDA disk, did not show any symptoms, confirming that the observed symptoms were caused by the fungal isolates inoculated in the plants (Table 2). Ninety days after inoculation,

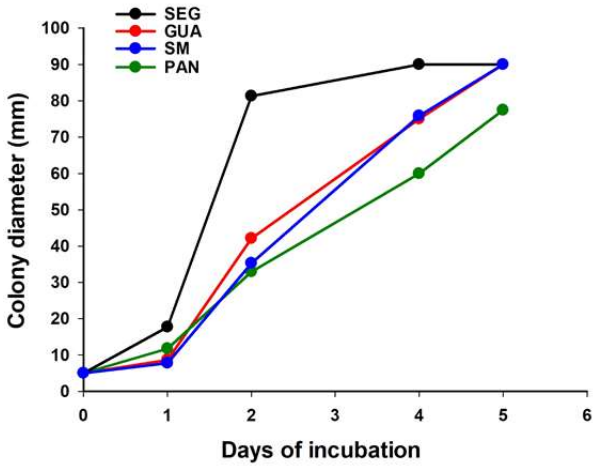

Figure 2 – Mycelial growth of fungal isolates from stem cankers of different pecan orchards. The colony diameter was measured in Petri dishes for five days. Isolates came from pecan orchards in Guarapuava-PR (GUA), Pantano Grande-RS (PAN), Segredo-RS (SEG) and Santa Maria - RS (SM) in Brazil.

***Figura 2** – Crescimento micelial de isolados fúngicos de cancro do tronco de diferentes pomares de nogueira-pecã. O diâmetro da colônia foi medido em placas de Petri por cinco dias. Os isolados foram coletados em pomares de nogueira-pecã em Guarapuava-PR (GUA), Pantano Grande-RS (PAN), Segredo-RS (SEG) e Santa Maria - RS (SM) no Brasil.*

the final evaluation of symptoms was made and the lesions were measured to evaluate the injuries caused by the pathogens. Lengthwise cracking was observed in all plants inoculated with each isolate. Then, the inoculated isolates were re-isolated from symptomatic tissues, confirming Koch's postulates. The reisolated isolates showed the same characteristics as the inoculated isolates in Petri dishes.

There was a significant difference in the injuries and growth speed among the different isolates ($P<0.05$) (Table 2). GUA isolate, was the most aggressive, as it caused symptoms such as deep lesions, darkening of plant vessels, leaf fall and plant death, thus reaching a severity score of 4. However, GUA did not differ significantly from the PAN isolate, which was also potentially aggressive compared to the others. In contrast, the isolates SEG, SM, and PAN did not differ in severity, with lesions up to 6 cm long, presenting severity scores of 2.5, 1.5 and 2.75, respectively. However, all isolates differed significantly from the control treatment, with no symptoms (severity score of zero).

Table 2 – Injury of stem canker caused by different isolates in the pecan tree over the days. Isolates came from pecan orchards in Guarapuava-PR (GUA), Pantano Grande-RS (PAN), Segredo-RS (SEG) and Santa Maria - RS (SM) in Brazil.

***Tabela 2** – Lesão de cancro do tronco causado por diferentes isolados em nogueira-pecã ao longo dos dias. Os isolados foram coletados em pomares de nogueira-pecã em Guarapuava-PR (GUA), Pantano Grande-RS (PAN), Segredo-RS (SEG) e Santa Maria - RS (SM) no Brasil.*

Isolates	First symptom appearance - DAI*	Injury level after 90 days (± ST)**
Lasiodiplodia theobromae GUA	30	4.00 (0.0) a
Neofusicoccum parvum PAN	40	2.75 (0.47) ab
Lasiodiplodia theobromae SEG	40	2.50 (0.28) b
Pseudofusicoccum kimberleyense SM	65	1.50 (0.28) b
Control	-	0.00 (0.0) c

* Days after inoculation.
** Injury level according to canker scale of Borges et al. (2018). Means followed by the same letter do not differ by the Tukey test ($P<0.05$). St represents the standard error of the means.
** Dias após a inoculação.*
*** Nível de lesão de cancro de acordo com a escala de Borges et al. (2018). As médias seguidas da mesma letra não diferem pelo teste de Tukey ($P<0,05$). St representa o erro padrão das médias.*

4. DISCUSSION

Stem canker is a significant disease in the cultivation of pecan nuts, affecting about 60-65% of trees in the sampled orchards, which compromised the development and productivity of the trees (Table 1). All cankered plants presented significant damage in the trees and frequently the death of young plants. Cankers in pecan stems are usually found in the southern region of Brazil, but few studies reported the biotic nature of such symptoms (Poletto et al., 2016; Luo et al., 2019; Rolim et al., 2020b). Here, the symptoms observed in the field were replicated in the pathogenicity test. However, there was a variation in severity among the isolates. A variety of symptoms is commonly found in the Botryosphaeriaceae family as a function of the region and genetic groups as a phenotypic response (Luo et al., 2019). Furthermore, Botryosphaeriaceae isolates may cause various symptoms in the same host (Phillips et al., 2019; Abdollahzadeh et al., 2010). However, for the correct management of any disease, the first step is the identification of the agent causing the symptoms.

The combination of morphophysiological and molecular information is the best way to assure

correct identification of fungi (Hyde et al., 2014; Machado et al., 2014; Correia et al., 2016; Netto et al., 2016). Through amplification and sequencing of the elongation factor, three species causing stem canker were identified: *L. theobromae* (GUA and SEG), *P. kimberleyense* (SM) and *N. parvum* (PAN). *Lasiodiplodia theobromae* is an important canker-causing pathogen and has been reported causing canker in several hosts, such as in *Fraxinus americana* (Chen et al., 2019), *Falcataria moluccana* (Ji et al., 2017) and *Vaccinium* spp. (Borrero et al., 2019). *Pseudofusicoccum kimberleyense* was associated with the canker symptom in *Mangifera indica* and *Eucalyptus* spp. (Sakalidis et al., 2011). *Eucalyptus* (*Eucalyptus* spp.) and pecan (*C. illinoinensis*) have also been reported hosting *Neofusicoccum parvum*, and the presence of the pathogen in eucalyptus has been described in many countries, such as Australia, Chile, China, Ethiopia, Indonesia, South Africa and Uruguay (Rodas et al., 2009). Furthermore, *N. parvarum* has been described in ninety other hosts, mainly wooden species (Sakalidis et al., 2013).

The morphophysiological aspects consolidate the distinction of each isolate, supporting the phylogenetic identification. Initially, the colonies were colored with whitish tones, and over the days they acquired a gray tint and finally, some had a greenish-black color. The characteristics of mycelial growth and color observed in the present study have already been described for fungi of the Botryosphaeriaceae family (Pavlic et al., 2008; Liang et al., 2019; Phillips et al., 2019). Here, the pathogenic isolates grew rapidly in the culture medium. The fact that isolates grow differently may interfere with the management in orchards and even at nursery seedling production, where the strategies should be chosen according to their characteristics. Therefore, despite SEG and GUA grouped in the same clade of *L. theobromae*, they behaved differently in their morphophysiological aspects in *in vitro* tests.

Botryosphaeriaceae isolates commonly change their behavior according to environmental conditions, which may range from causing no symptoms to highly cankered plants (Cipriano et al., 2015; Slippers and Wingfield, 2007). The isolates SEG and GUA were considered *L. theobromae*, but SEG grew faster in Petri dishes, while GUA started to cause symptoms in seedlings 10 days earlier and also injured more the inoculated plants. Meanwhile, *P. kimberleyense* (SM) and *N. parvum* (PAN) caused injuries similar to *L. theobromae* (SEG). The severity of the isolates may be directly related to the incidence of the disease in the orchards. In this sense, *L. theobromae* (GUA) isolate, from an orchard with 65% disease incidence, was considered the most aggressive, since it was the first to cause symptoms and serious severity. In contrast, the other isolates, from orchards with the 60% incidence, caused fewer canker symptoms and severity in all inoculated plants. Usually, in the sampled season, Guarapuava region, where GUA isolate was isolated from, has adequate moisture and temperature conditions for the development of Botryosphaeriaceae members than the other sampled regions (Abdollahzadeh et al., 2010; Phillips et al., 2019). Then, the pathogenic nature of *L. theobromae* (GUA) may be increased in that region. However, in morphophysiological tests in Petri plates, *L. theobromae* (SEG) grew faster than GUA, supporting the importance of performing multiple studies to identify and understand the isolate's behavior. Furthermore, the phylogenetic analysis provided a bootstrap value lower than 80% for GUA to be allocated in *L. theobromae*, which demonstrates that more regions than EF-1α should be sequenced to support the identification of GUA.

Despite their differences, all the species found in this study have been reported causing canker in different levels in other plant species (Borrero et al., 2019; Chen et al., 2019; Ji et al., 2017; Sakalidis et al., 2011). The isolates caused canker symptoms similar to *L. subglobosa* cankers previously reported on pecan trees in Brazil (Poletto et al., 2016). Therefore, all this evidence emphasizes the importance of the correct and early identification of the Botryosphaeriaceae isolates before it significantly damages the pecan tree and interferes with orchard yield.

5. CONCLUSIONS

Morphological and molecular techniques confirmed the isolates from the pecan orchards as *Lasiodiplodia theobromae* (GUA and SEG), *Neofusicoccum parvum* (PAN) and *Pseudofusicoccum kimberleyense* (SM). All the isolates were pathogenic to pecan with different injury levels, where *L. theobromae* (GUA) demonstrated more aggressiveness than all others. Therefore, it is crucial to identify and understand the behavior of the isolates

from different regions to best control canker in pecan orchards in the early stages.

AUTHOR CONTRIBUTIONS

Jessica Mengue Rolim: Data collection and organization, analysis the data, discussion of results, and text writing. Jéssica Emilia Rabuske: Data collection and text writing.Lucas Graciolli Savian: Data collection and text writing.Clair Walker: Writing and reviewing the text. Janaína Silva Sarzi: Data collection and text writing. Júlio Carlos Pereira da Silva: Writing, reviewing and text editing. Marlove Fátima Brião Muniz: Research supervision, technical review, and text review.

6. ACKNOWLEDGEMENTS

The authors acknowledge the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) for financial support – Finance Code 001.

The authors declare that they have no conflict of interest.

7. REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology. 1990;215(3):403-410. doi: 10.1016/S0022-2836(05)80360-2

Abdollahzadeh J, Javadi A, Goltapeh EM, Zare R, Phillips AJL. Phylogeny and morphology of four new species of *Lasiodiplodia* from Iran. Persoonia Molecular Phylogeny and Evolution Fungi. 2010;25(1):1-10. doi: 10.3767/003158510X524150

Borges RCF, Marques E, Macedo MA, Martins I, Silva Filho JGD, Mello SCMD. Biocontrol of teak canker caused by *Lasiodiplodia theobromae*. Revista Árvore. 2018;42(3). doi: doi.org/10.1590/1806-90882018000300004

Borrero C, Pérez S, Avilés M. First Report of Canker Disease Caused by *Lasiodiplodia theobromae* on Blueberry bushes in Spain. Plant Disease. 2019;103(10):2684-2684. doi: 10.1094/pdis-03-19-0473-pdn

Bremm N. Alelopatia de *Carya illinoinensis* (Wangenh.) K. Koch na germinação e desenvolvimento de plântulas de *Lactuca sativa* L. [undergraduate thesis]. Cerro Largo, RS: Universidade Federal da Fronteira Sul; 2018.

Carbone IE, Kohn, LM. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia. 1999;91:553-556. doi: 10.1080/00275514.1999.12061051

Cipriano AKAL, Gondim DMF, Vasconcelos IM, Martins JAM, Moura AA, Moreno FB, et al. Proteomic analysis of responsive stem proteins of resistant and susceptible cashew plants after *Lasiodiplodia theobromae* infection. Joural of Proteomics. 2015;103:90-109. doi: 10.1016/j.jprot.2014.09.022

Correia KC, Silva MA, Morais JRMA, Armengold J, Phillips AJL, Câmara MPS, et al. Phylogeny, distribution and pathogenicity of *Lasiodiplodia* species associated with dieback of table grape in the main Brazilian exporting region. Plant Pathology. 2016;65(1):92. doi: 10.1111/ppa.12388

Color X-RITE M. Munsell soil-color charts with genuine Munsell color chips. USA: Munsell Color; 2009.

Chen F, Zheng X, Zhao X, Chen F. First report of *Lasiodiplodia theobromae* causing stem canker of *Fraxinus americana*. Plant Disease. 2019;103(12):3276-3276. doi: 10.1094/pdis-04-19-0892-pdn

Fernandes MR. Manual para laboratório de fitopatologia. Passo Fundo: EMBRAPA – CNPT; 1993.

Ferreira DF. Sisvar: a computer statistical analysis system. Ciência e agrotecnologia. 2011;35(1):1039-1042. doi: 10.1590/S1413-70542011000600001

Fronza D, Hamann JJ. Técnicas para o cultivo da nogueira-pecã. Santa Maria: Universidade Federal de Santa Maria; 2016. v.1.

Gatto DA, Haselein CR, Santini EJ, Marchiori JNC, Durlo MA, Calegari L, et al. Technological characteristics of the wood of *Luehea divaricata*, *Carya illinoinensis* and *Platanus* x *acerifolia* when submitted to bending. Ciência Florestal. 2008;18(1):121-131. doi: 10.5902/19805098516

Hyde KD, Nilsson RH, Alias SA, Ariyawansa HA, Blair JE, Cai L, et al. One stop shop: backbones trees

for important phytopathogenic genera: I. Fungal Diversity. 2014;67(1):21-125. doi: 10.1007/s13225-014-0298-1

Ji CY, Chen CJ, Wang XR, Liu Y, Liu JY, Zeng BS. A report on canker disease of *Falcataria moluccana* caused by *Lasiodiplodia theobromae* in China. Crop Protection. 2017;91(1):89-92. doi: 10.1016/j.cropro.2016.09.018

Lazarotto M, Muniz MFB, dos Santos RF, Blume E, Harakawa R, Hamann FA. First report of *Fusarium equiseti* associated on Pecan (*Carya illinoinensis*) seeds in Brazil. Plant Disease. 2014;98(6):847-847. doi: 10.1094/PDIS-09-13-0976-PDN

Lazarotto M, Muniz MFB, Poletto T, Dutra CB, Blume E, Harakawa R, et al. First report of *Pestalotiopsis clavispora* causing leaf spot of *Carya illinoensis* in Brazil. Plant Disease. 2012;96(12):1826. doi: 10.1094/PDIS-07-12-0615-PDN

Leslie JF, Summerell BA. The *Fusarium* laboratory manual. Iowa: Blackwell Pub; 2006.

Liang L, Li H, Zhou L, Chen F. *Lasiodiplodia pseudotheobromae* causes stem canker of *Chinese hackberry* in China. Journal of Forestry Research. 2019;31(6):2571-1580. doi: 10.1007/s11676-019-01049-x

Luo Y, Lichtemberg PSF, Niederholzer FJA, Lightle DM, Felts DG, Michaillides TJ. Understanding the Process of latent infection of canker-causing pathogens in stone fruit and nut crops in California. Plant Disease. 2019;103(9):2374-2384. doi: 10.1094/PDIS-11-18-1963-RE

Martins CR, Malgarim M, Bilhava M, de Marco R. Noz-Pecã: A rainha dos frutos secos. Revista Campo & Negócios. 2017;57-61.

Machado AR, Pinho DB, Pereira OL. Phylogeny, identification and pathogenicity of the Botryosphaeriaceae associated with collar and root rot of the biofuel plant Jatropha curcas in Brasil, with a description of new species of *Lasiodiplodia*. Fungal Diversity. 2014;67(1):231-247. doi: 10.1007/s13225-013-0274-1

Netto MSB, Lima WG, Correia KC, Silva CFB da, Thon M, Martins RB, et al. Analysis of phylogeny, distribution, and pathogenicity of Botryosphaeriaceae species associated with gummosis of *Anacardium* in Brazil, with a new species of *Lasiodiplodia*. Fungal Biology. 2016;121(4):437-451. doi: 10.1016/j.funbio.2016.07.006

Pavlic D, Wingfield MJ, Barber P, Slippers B, Hardy GESTJ, Burgess TI. Seven new species of the Botryosphaeriaceae from baobab and other native trees in Western Australia. Mycologia. 2008;100(8):851–866. doi: 10.3852/08-020

Phillips AJL, Hyde KD, Alves A, Liu JK. Families in Botryosphaeriales: a phylogenetic, morphological and evolutionary perspective. Fungal Diversity. 2019;94(1):1-22. doi: 10.1007/s13225-018-0416-6

Poletto T, Gonzatto Maciel C, Muniz MFB, Blume E, Poletto I, Brioso P. First report of stem canker caused by *Lasiodiplodia subglobosa* on *Carya illinoinensis* in Brazil. Plant Disease. 2016;100(5):1016-1016. doi: 10.1094/PDIS-08-15-0948-PDN

Rodas CA, Slippers B, Gryzenhout M, Wingfield MJ. Botryosphaeriaceae associated with *Eucalyptus* canker diseases in Colombia. Forest Pathology. 2009;39(2):110-123. doi: 10.1111/j.1439-0329.2008.00569.x

Rolim JM, Savian LG, Walker C, Blume E, Muniz MFB, Poletto T, et al. First report of fusarium wilt caused by *Fusarium oxysporum* on pecan in Brazil. Plant Disease. 2020a;104(6):1870. doi: 10.1094/pdis-09-19-1956-pdn

Rolim JM, Savian LG, Walker C, Rabuske JE, Sarzi JS, Muniz,MFB, et al. First report of stem canker caused by *Neofusicoccum parvum* and *Pseudofusicoccum kimberleyense* on *Carya illinoinensis* in Brazil. Plant Disease. 2020b;104(11):3067. doi: 10.1094/PDIS-01-20-0082-PDN

Sakalidis ML, Ray JD, Lanoiselet V, Hardy GESJ, Burgess TI. Pathogenic Botryosphaeriaceae associated with *Mangifera indica* in the Kimberley Region of Western Australia. European Journal Plant Pathology. 2011;130(3):379-391. doi: 10.1007/s10658-011-9760-z

Sakalidis ML, Slippers B, Wingfield BD, Hardy GESJ, Burgess TI. The challenge of understanding the origin, pathways and extent of fungal invasions:

Global populations of the *Neofusicoccum parvum*-N. ribis species complex. Diversity and Distribuition. 2013;19(8):873-883. doi: 10.1111/ddi.12030

Slippers B, Wingfield MJ. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. Fungal Biology Reviews. 2007;21(2-3):90-106. doi: 10.1016/j.fbr.2007.06.002

Tamura K, Dudley J, Nei M, Kumar S.MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 2007;24(8):1596-1599. doi: 10.1093/molbev/msm092

Walker C, Muniz MFB, Rolim JM, Martins RRO, Rosenthal VC, Maciel CG, et al. Morphological and molecular characterization of *Cladosporium cladosporioides* species complex causing pecan tree leaf spot. Genetic Molecular Research. 2016;15(3):gmr8714. doi: 10.4238/gmr.15038714