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Lasiodiplodia theobromae associated with seeds of *Pinus* spp. originated from the northwest of Rio Grande do Sul, Brazil

Lasiodiplodia theobromae associada a sementes de *Pinus* spp. oriundas do noroeste do Rio Grande do Sul, Brazil

Caciara Gonzatto Maciel[1], Marlove Fátima Brião Muniz[2], Ricardo Mezzomo[1] e Lia Rejane Silveira Reiniger[2]

Resumo

Lasiodiplodia theobromae é um fungo comumente encontrado associado a sementes, com potencial para ser transmitido para as plântulas. As coníferas de uma maneira geral, quando expostas a condições de stress tornam-se vulneráveis ao ataque deste patógeno. O presente trabalho tem como objetivo avaliar a qualidade sanitária e fisiológica de lotes de sementes de *Pinus* spp.; verificar a patogenicidade de isolados de *Lasiodiplodia* sp. obtidos a partir do teste de sanidade e transmissão, e então identificar esses isolados em nível de espécie. O teste de sanidade foi realizado pelo método do "blotter test" e o teste de patogenicidade através do contato das sementes e a cultura fúngica, por 48 horas. *Lasiodipodia* foi identificado com base em características morfológicas e no seqüenciamento da região ITS, mostrou-se patogênico a *P. elliottii* e *P. taeda*, causando podridão de sementes e "*damping-off*".

Palavras-chave: patogenicidade, morfologia, ITS.

Abstract

Lasiodiplodia theobromae is a fungus commonly found associated with seeds, with the potential to be transmitted to seedlings. Generally, conifers become vulnerable to attack by the pathogen when exposed to stress conditions. This study aims to assess the sanitary and physiological quality of *Pinus* spp. seed lots; to verify pathogenicity of *Lasiodiplodia* sp. obtained from the transmission and sanitary tests, and then to identify these isolates at the species level. The sanitary test was performed through a "blotter test" and the pathogenicity test by contact of the seeds with the fungal culture for 48 hours. *Lasiodiploda theobromae* was identified based on morphological characteristics and sequencing of the ITS region and proved to be pathogenic to *P. taeda* and P. *elliottii*, causing seed rot and "damping-off".

Keywords: patogenicity, morphology, ITS.

INTRODUÇÃO

The percentage of forest seeds contaminated by saprophytic or pathogenic fungi grows constantly. According to Machado (2000), once installed in the seed, they are considered the most active pathogens with greater ability to directly penetrate and colonize the plant tissue.

Lasiodiplodia theobromae (Pat.) Griffon and Maubl. (syn. *Botryodiplodia theobromae* Pat.) is a fungus considered to be cosmopolitan, polyphagous and opportunistic (PUNITHALINGAM, 1980). This pathogen is commonly transmitted via seeds, with conifers being very susceptible to attacks (CILLIERS et al. 1993). The association of *Botryodiplodia* with *Pinus* spp. was shown by Ress (1988) and Shayesta e Rahman (1985). The pathogen causes different symptoms in annual and perennial species, such as death, gummosis and stem rot in *Mangifera indica* (SHAHBAZ et al. 2009); dry rot in *Malpighia emarginata* (LIMA et al. 2012); dry rot of leaves and basal rot in post harvest of *Cocus nucifera;* dieback in *Persea americana*, *Annona muricata* L. and *Spondias tuberosa* Arruda; death of the peduncle in *Carica papaya* (LIMA et al. 2013).

Effective control of this pathogen becomes difficult because of characteristics of the fungus, for being cosmopolitan and polyphagous and the wide variety of susceptible hosts. Kristensen et al. (2005) argued that verification of pathogenicity, geographical location of the affected plant, morphological and biological

[1]Doutorando(a) em Engenharia Florestal. UFSM - Universidade Federal de Santa Maria - Departamento de Defesa Fitossanitária. Av. Roraima, 1000 – Camobi - 97105-900 - Santa Maria, RS.

[2]Professor Doutor. UFSM - Universidade Federal de Santa Maria, Centro de Ciências Rurais. Av. Roraima, 1000 – Camobi - 97105-900 - Santa Maria, RS.

characterization of the isolate are all careful and fundamental steps for identifying needs in terms of species, which have the goal of supplementing the molecular phylogeny.

Given the above, this study had the following objectives: to survey the sanitary quality of seeds of *Pinus* sp.; to evaluate the pathogenicity of *Lasiodiplodia* sp. in seeds of *Pinus* sp.; and to identify isolates of *Lasiodiplodia* spp. at the species level, using morphological and molecular tools.

MATERIALS AND METHODS

The seeds of *Pinus elliottii* and *Pinus taeda* (Lot 2013) used in the study had their origin in the municipality of Ijui (28°23'16"S e 53°54'53"O), located in the northwest region of Rio Grande do Sul state, Brazil. Four lots were used: two of *P. elliottii* (Lot1 and Lot2) and two of *P. taeda* (Lot3 and Lot4). The seeds used in the tests remained stored in a freezer (3-5 ° C) for two weeks until their use, as a method for breaking dormancy (BRASIL, 2009).

Blotter test: performed with four replicates of 200 seeds, incubated at 25 ± 2 ° C and 12 hour photoperiod; the assessment was conducted after seven days of incubation, with the aid of a stereoscopic microscope and by optical means. *Transmission test:* performed with four replicates of 200 seeds, incubated at 25 ± 2 ° C and 12 hour photoperiod; sand was used as a substrate, autoclaved at 120 ° C and 1 atm, for two periods of 60 minutes with an interval of 24 hours; weekly evaluations were performed with observation of symptoms. When structures of the pathogen were visualized in emerging and non-germinated seeds in a blotter test or a transmission test, isolation and purification of the colony were performed for morphological and molecular characterization. Purification of the colony was carried out from the technical monosporic culture (FERNANDES, 1993).

For morphological characterization, the transfer of the isolates proceeded from the pure colony to a culture medium of potato-dextrose-agar (BDA), plus sterilized pine needles (*Pinus* sp.) (LIMA et al., 2013). They were incubated for 25 days at a temperature of 25 ± 2 ° C and 12 hours photoperiod, for scaling and photography of reproductive structures. 30 conidia were evaluated by isolates of *Lasiodiplodia* sp., from which length and width measurements were considered.

To determine the average mycelial growth of isolates, we proceeded to transfer mycelium disks and the culture medium (12 mm) derived from pure cultures of the isolates to the center of a Petri dish containing PDA medium. The plates were incubated at 25 ± 2 º C with 12 hours photoperiod. The mycelial growth was observed by measuring the diameter of the colony every 24 hours with the aid of a digital caliper. Two measures were executed in diametrically opposed directions. Then, the average growth for each plate (cm / day) was determined. After 15 days the color of the colony was determined using the Rayner color chart (RAYNER, 1970).

For molecular characterization, fungal mycelium and spores of the pathogen were collected from cultures grown on PDA for two weeks at 20 °C in the dark. DNA extraction of the pathogen occurred with the CTAB method (cetyltrimethylammonium bromide) (DELLAPORTA et al., 1983). Samples of extracted genomic DNA were subjected to Polymerase Chain Reaction (PCR) for the amplification of regions: ITS rDNA with primer pairs ITS1 and ITS4 (WHITE et al., 1990). Each PCR reaction contained approximately 1 µL of DNA, 10 L of 5X GoTaq Reaction Buffer (Promega, EUA), 1 µL of mix of dNTPs, 1 µL of each primer, 0.2 µL GoTaq DNA polymerase (Promega, EUA) and water MiliQ autoclaved to complete the final reaction volume of 50 µL. The reactions were performed in a thermocycler GeneAmp PCR System 2400 (Perkin Elmer, EUA) under the following thermal conditions: 94 ° C for 2 min, 40 cycles of denaturation at 94 ° C for 30 s, annealing at 50 °C for 2 min and elongation at 72 °C for 1 min. A final extension was performed at 72 °C for 4 min. At the end of the reaction, the PCR products were kept cold at a temperature of 4 ºC. A negative control without DNA was included in the PCR amplifications. The amplified fragments and the control were visualized by electrophoresis on a 0.8% agarose gel stained with ethidium bromide (1 mg L^{-1}) in 1X TAE buffer (Tris-

Table 1. Isolates, origin, date and accession number of isolates of *L. theobromae*. Santa Maria, 2013.
Tabela 1. Isolados, origem, data e número de acesso de isolados de *Lasiodiplodia theobromae*. Santa Maria, 2013.

Isolate	Origin	Date of colection	Accession number in Genbank
Bot1UFSM	Seeds of *P. taeda* (Lot4)	04/2013	KF924398
Bot2UFSM	Seeds of *P. taeda* (Lot3)	04/2013	KF924398

acetate 0.04 M + 1 mM EDTA), and visualized under ultraviolet light. The marker 1 kb Plus DNA Ladder (Invitrogen, USA) was used as molecular weight marker. The PCR products obtained were purified following the protocol described by Schmitz e Riesner (2006) using polyethylene glycol 6000 (PEG 6000).

To compare sequences, sequences of *Lasiodiplodia theobromae* and *Botryosphaeria rhodina* (teleomorfh) were used, all available in Genbank. The sequences from GenBank that showed the highest "scores" were selected and aligned with sequences obtained by sequencing the ClustalW algorithm. Furthermore, phylogenetic analysis was conducted adopting the "Neighbour-joining" method with 1000 replicates in the MEGA program version 4 (TAMURA et al., 2007). The similarity of nucleotide sequences between isolates was calculated using the Basic Local Alignment Search Tool (BLAST).

For pathogenicity, initially, the seeds were sterilized with a solution of 70% alcohol (v / v) for 30 seconds, and then with a solution of sodium hypochlorite (1% v / v) for 1 minute. Afterwards, they were washed with sterile distilled water, and the seeds were then dried on sterile filter paper. Each treatment used 100 seeds, divided into four replicates of 25.

After the incubation period of the fungus (seven days at a temperature of 25 ± 2 °C with a photoperiod of 12 hours), inoculation was carried out by the method of contact of the seeds with the culture of the fungus for 48 hours, at a temperature of 25 ± 2 °C and 12 hours photoperiod. The control consisted of exposure of the seeds only to the medium culture PDA under the same conditions. After inoculation procedures, the test of emergence in sand was held, in which the seeds were placed in plastic boxes (11 x 11 x 3,5 cm) with sifted sand as substrate, sterilized in autoclave for two hours (with interval of 24 hours) at 1 atm and 120 ºC. The material remained incubated in a temperature-controlled room with a temperature of 25 ± 2 ºC and with manual irrigation where necessary.

The variables evaluated were: a) speed of emergence index (SEI): daily count of emerged seedlings, considered when the hypocotyls were bigger than 1.0 cm. To calculate the speed of emergence index (SEI), the equation suggested by Popinigis was used (1977); b) seedling emergence: counting the number of seedlings at 28 days; c) abnormal seedlings symptomatic: seedling with symptoms caused by the fungus *Lasiodiplodia thaeobromae* were checked; d) non-germinated seeds: count of the seeds with rotted aspect and of those that had not started the germination process. For all variables, except the SEI, the results were expressed as percentages. The test was monitored for forty days. When the presence of damping-off was detected, the seedlings were collected and incubated in a moist chamber or placed in Petri dishes with PDA culture medium, with the goal of determining whether the damage was caused by the inoculated fungus, and then re-isolation was performed.

The Bot1UFSM and Bot2UFSM isolates were inoculated in four lots of *Pinus* sp. seeds. Means comparison was done by Tukey test at 5% probability; the software used was SISVAR 5.3 (FERREIRA, 2008).

RESULTS AND DISCUSSION

The identified fungal genera associated with the different batches of seeds were: *Lasiodiplodia*, *Trichoderma*, *Penicillium*, *Fusarium* and *Aspergillus* (Table 2). The pathogen *Lasiodiplodia* spp. showed a higher incidence, especially in lots of *P. taeda* (Lot4 and Lot3), and therefore was used for pathogenicity tests.

The isolates of the *Lasiodiplodia* studied showed culture coloration gray/lead; conidial measurements ranged from 24.4 x 12.25 µm (length / width ratio 1.97) to 23.97 x 12.86 µm (length / width ratio 1.92) for Bot1UFSM and Bot2UFSM, respectively (Table 3). The conidia were characterized by an ellipsoid oval form, with thin walls, ranging between 0 and 1 septum (Figure 1C). According to Lima et al.

Table 2. Health characteristics of seed lots of *Pinus* sp. used in the study. Santa Maria, 2013.
Tabela 2. Características sanitárias de lotes de sementes de *Pinus* sp. utilizadas no estudo. Santa Maria, 2013.

Origin	Fungal genera (%)				
	Lasiodiplodia	Trichoderma	Penicillium	Fusarium	Aspergillus
P. taeda - Lot4	33 a*	2,0 b	4,0 b	1,0 a	6,0 a
P. taeda - Lot3	43 a	13 a	12 b	2,0 a	1,0 a
P. elliottii – Lot1	9,0 a	0,0 b	83 a	0,0 a	0,0 a
P. elliottii – Lot2	15 a	3,0 b	7,0 b	0,0 a	0,0 a

* Means followed by the same letter in the column do not differ by Tukey test at 5% significance. Lot 1-4: (Lots of seeds of *Pinus* sp.)

(2013) the length / width ratio expresses the shape of the conidia, where the higher the value of this variable, the more tapered (ellipsoid) is the structure, and the lower this value, the more rounded (oval) is the conidium. These authors found a variation of conidia measures between 18.10 to 30.94 µm x 10.64 to 15.86 µm and length / width ratio of 1.52 to 2.26 for isolates of *Lasiodiplodia theobromae* obtained from tropical fruit. Alves et al. (2008) found (19)21-31(32.5) × (12)13-15.5(18.5) µm as measures of conidia of the *L. theobromae*. Only the anamorphic phase was observed.

The average growth rates in the middle of the BDA culture (25 ± 2 ° C) were 39 and 36.5 mm.d^{-1} for isolates Bot1UFSM and Bot2UFSM, respectively. Gure et al. (2005) found values of 17 mm.d^{-1} for *Botryosphaeria parva* (malt extract agar (MEA) culture medium and 25 º C). Lima et al. (2013) observed a variation between 30 and 65.4 mm.d^{-1} for different isolates of *L. theobromae* associated with tropical fruit, under the same conditions as this study. Halfeld-Vieira et al. (2007) found 36.2 mm.d^{-1} of medium mycelial growth of *L. theobromae*, isolated from *Citrus* sp., *Coco nucifera*, *Acacia mangium*; Abdollahzadeh et al. (2010) found a mean increase of 40 mm.d^{-1} for isolates of *L. theobromae* obtained from *Mangifera indica*, *Eucalyptus* sp., *Citrus* sp., *Salvadora persica*, *Juglans* sp., and *Terminalia catapa*, in MEA culture.

The pycnidium of black color could be viewed both on the surface of pine needles and internally in the culture medium (Figure 1B). The growth of pycnidium varied among isolates. The Bot1UFSM isolate presented lower development of these structures in the culture medium and hence lower sporulation. On the other hand, the Bot2UFSM isolate showed higher pycnidium development and sporulation. This difference did not affect the pathogenicity test, since both isolates are pathogenic for *Pinus* sp. seed (Table 4).

From the analysis of the ITS region of DNA from Bot1UFSM and Bot2UFSM isolates, it was concluded that both belong to the same species, *Lasiodiplodia theobromae* (anamorph *Botryosphaeria rhodina*) with a 99 bootstrap (Figure 2). Other authors have also based on the ITS region to identify the *Botryospharaceae* family (PAVLIC et al., 2004; SMITH; SANTOSZ, 2001). Abdollahzadeh et al. (2010) agree that it is possible to distinguish *Lasiodiplodia* phylogeny species based on DNA, but in conjunction with a morphological analysis of the culture.

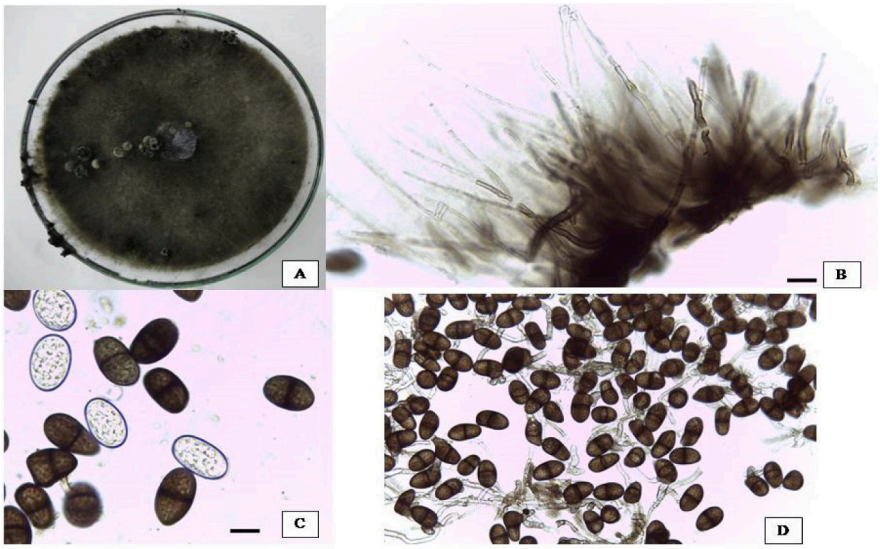

Figure 1. Morphological characterization of *Lasiodiplodia theobromae*. PDA (A); paraphyses (B); immature hyaline conidia and mature conidia (C); mature conidia, dark-walled, one-septate (D). Scale bars = 10 µm.
Figura 1. Caracterização morfológica de *Lasiodiplodia theobromae*. BDA (A); parafises (B); conídios hialinos imaturos e conídios maduros (C); conídios maduros, parede escura e um septo (D). Barra = 10 µm.

Table 3. Morphological characteristics of isolates *Lasiodiplodia* sp. obtained from seeds of *Pinus* sp. Santa Maria, 2013.
Tabela 3. Características morfológicas de isolados de *Lasiodiplodia* sp. oriundos de sementes de *Pinus* sp. Santa Maria, 2013.

	Bot1UFSM	Bot2UFSM
Color of colony[1]	gray/lead	gray/lead
Measure of conidia[2]	(20) 24,4 (32,5) x (10) 12,25 (15) µm	(20) 23,97 (30) x (10)12,86 (20) µm
Sporulation	+	++
Presence of picnidia[1,2]	+	++

[1] Potato dextrose agar culture medium, 25 ± 2 °C and a photoperiod of 12 hours; [2] Acicula of pine-agar culture medium, 25 ± 2 °C and a photoperiod of 12 hours; (+): until five picnidia on each Petri dish; (++): more than ten picnidia on each Petri dish.

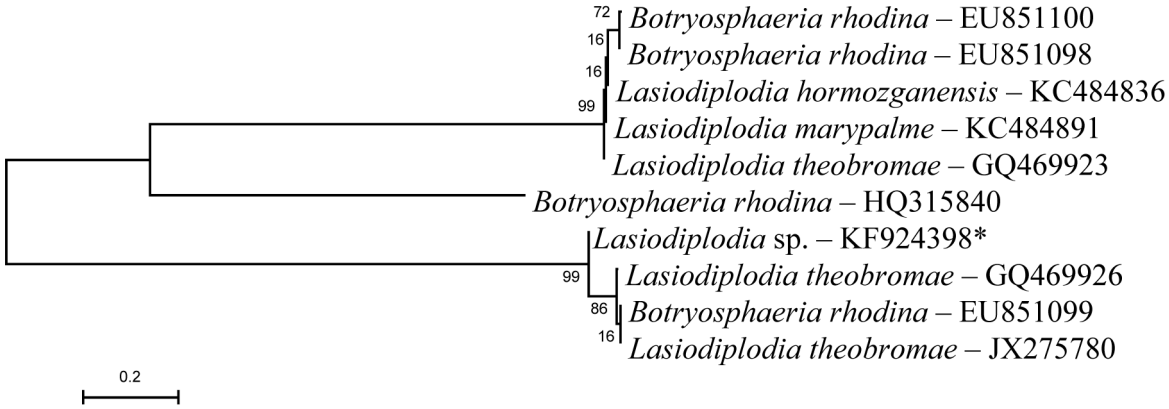

Figure 2. Phylogenetic dendrogram based on the neighbor-joining method from the DNA sequences of the ITS region. The numbers on the branches indicate the percentage of bootstrap replications of the analysis in which the repeats were observed (1000 replications). * Isolate of *Lasiodiplodia* sp. obtained in the present study.

Figura 2. Dendrograma filogenético baseado no método Neighbor-joining a partir de sequências de DNA da região ITS. Os números sobre os ramos indicam a porcentagem de repetições da análise de bootstrap na qual as repetições foram observadas (1000 repetições). * Isolados de *Lasiodiplodia* sp. obtidos no presente estudo.

Table 4. Mean values of speed of emergence index (SEI), emergency (E), normal seedlings (NS), abnormal seedlings (AS) and non-germinated seeds (NGS) of *Pinus* sp. after inoculation with *Lasodiplodia* sp. Santa Maria, 2013.

Tabela 4. Valores médios do índice de velocidade de emergência (SEI), emergência (E), plântulas normais (NS), plântulas anormais (AS) e sementes não germinadas (NGS) de *Pinus* sp. após inoculação de *Lasodiplodia theobromae*. Santa Maria, 2013.

Lot x Isolated	**Variables**				
	SEI	**E**	**NS**	**AS**	**NGS**
P. elliottii Lot1 X Bot1UFSM	9,05 b*	49,0 b	41,0 b	7,0 b	52,0 a
P. elliottii Lot1 X Bot2UFSM	6,64 b	39,0 b	34,0 b	5,0 ab	61,0 a
P. elliotii Lot1	16,3 a	78, 0 a	76,0 a	2,0 a	22,0 b
C.V. (%)	8,94	9,97	12,06	26,61	12,98
Lot x Isolated	**Variables**				
	SEI	**E**	**NS**	**AS**	**SNG**
P. elliottii Lot2 X Bot1UFSM	10,7 ab	66,0 a	62,0 ab	4,0 a	34,0 a
P. elliottii Lot2 X Bot2UFSM	9,3 b	57,0 a	48,0 b	8,0 a	44,0 a
P. elliotii Lot2	15,3 a	75,0 a	72,0 a	3,0 a	25,0 a
C.V. (%)	11,36	9,49	9,36	33,99	17,61
Lot x Isolated	**Variables**				
	SEI	**E**	**NS**	**AS**	**NGS**
P. taeda Lot4 X Bot1UFSM	33,9 a	41,0 b	38,0 b	3,0 a	59,0 b
P. taeda Lot4 X Bot2UFSM	2,8 c	25,0 c	25,0 c	1,0 a	74,0 a
P. taeda Lot4	13,8 b	73,0 a	72,0 a	1,0 a	27,0 c
C.V. (%)	9,11	6,23	5,85	54,55	5,04
Lot x Isolated	**Variables**				
	SEI	**E**	**NS**	**AS**	**NGS**
P. taeda Lot3 X Bot1UFSM	7,0 a	48,0 ab	34,0 b	6,0 a	49,0 a
P. taeda Lot3 X Bot2UFSM	5,37 a	47,0 b	36,0 b	11,0 a	51,0 a
P. taeda Lot3	9,65 a	64,0 a	60,0 a	4,0 a	36,0 a
C.V. (%)	14,86	7,83	10,92	38,77	9,65

*Means followed by the same letter in the column do not differ by Tukey test at 5% significance. Where: Bot1UFSM and Bot2UFSM are isolates of *Lasiodiplodia theobromae* obtained from seeds of *Pinus taeda* Lot4 and Pinus taeda Lot3, respectively, and CV: coefficient of variation.

The results shown in Table 5 indicate that the isolate Bot1UFSM is pathogenic to *Pinus taeda* (Lot4) and *P. elliottii* (Lot1) seeds, since it is statistically different from the control for the variables seedling emergence and normal seedlings. In the case of Bot2UFSM isolate, the pathogenicity was observed for seeds of *P. taeda* (Lot4), *P. taeda* (Lot3) and *P. elliottii* (Lot4), considering the seedling emergence variable. When the percentage of normal seedlings was observed, this isolate also differed from the control for the lot of *P. elliottii* (Lot2). Symptoms observed in the pathogenicity test were hypocotyl rot, malformation of root and

shoot and seedling damping-off (Figure 3A e 3B); in more advanced stages it was possible to observe the development of the reproductive structures of the pathogen on the seedling, both in the caulicle and the shoot (Figura 3C).

Reports of transmission of the pathogen *L. theobromae* via seed, for different species of conifers, has been described in the scientific environment. Among them, Cilliers et al. (1993) presents a literature review that includes associations of this fungus with seeds of *P. elliottii* and *P. taeda* (CARNEIRO, 1986); *P. caribea*, *P. resinosa* and *P. thumberghii* (WATANABE, 1988); *P. caribea* in Nicaragua (RESS, 1988) and *P. elliottii* U.S. (FRAEDRICH et al., 1994). These studies state that the pathogen is commonly found in the internal structures of the seed, causing a reduction in germination potential and seedling death.

Owolade et al. (2001) found elevated incidences of *Botryodiplodia theobromae* causing "blackned" in seeds of *Zea mays* (maize). Cardoso et al. (2006) reported the occurrence of this pathogen in seeds of *Annona muricata* L. (soursop), causing a reduction in germination potential.

This study relates the first occurrence of *Lasiodilodia theobromae* in seeds of *Pinus* sp. from Rio Grande do Sul state.

CONCLUSION

- Two isolates of *Lasiodiplodia* were pathogenic to the seeds of *P. elliottii* and *P. taeda* causing reduction in germination potential and seedling damping-off.
- The isolates were identified as *Lasiodiplodia theobromae*, according to morphological characteristics and sequencing of the ITS region.

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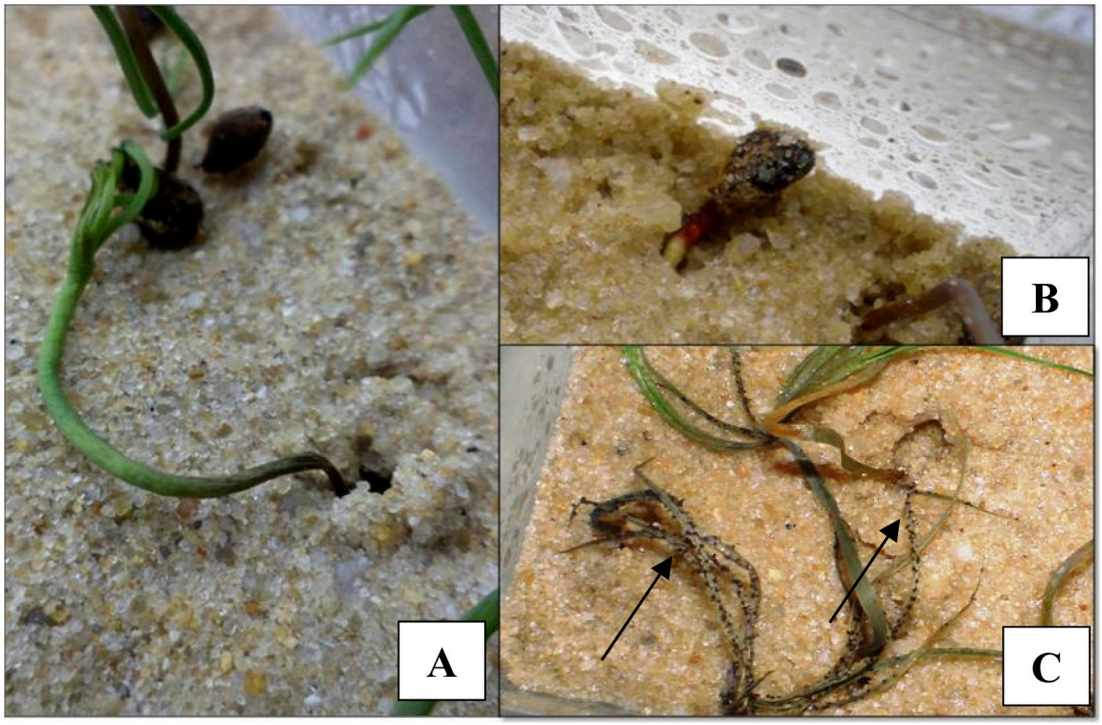

Figure 3. Pathogenicity test of *Lasiodiplodia theobromae* in *Pinus* sp. Seeds. "Damping-off" in *P. elliottii* (A); damping of pre-emergence in *P. elliottii* (B) and reproductive structures of the pathogen in seedlings of *P. taeda* (C).

Figura 3. Teste de patogenicidade de *Lasiodiplodia theobromae* em sementes de *Pinus* sp. Damping-off em *Pinus elliottii* (A); damping - off em pré-emergência em plântulas de *P. elliottii* (B) e estruturas reprodutivas do patógeno em plântulas de *P. taeda* (C).

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