Revista Brasileira de Ciências Agrárias
ISSN (on line) 1981-0997
v.13, n.2, e5514, 2018
Recife, PE, UFRPE. www.agraria.pro.br
DOI:10.5039/agraria.v13i2a5514
Protocolo 5514 - 13/10/2017 • Aprovado em 06/02/2018

Morphological, physiological and molecular characterization of *Fusarium lacertarum* causal agent of damping-off in *Casuarina equisetifolia*

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ABSTRACT: Casuarina (*Casuarina equisetifolia* L.) is a species used like wind break, in streets ornamentation, soils conservation and dunes fixation. The main form of propagation of this species is by seeds, although, in nursery, the fungus *Fusarium lacertarum* have been cause *damping-off* in seedlings. Thus, the objective of this work was to characterize morphologically, physiologically and molecularly the pathogen. To molecular characterization, pure colonies were cultivated in PDA and LCA culture media, and for physiological characterization, colonies were cultivated in PDA, SNA, Mathur and V8 culture media. The genomic segment most appropriated to identify this species was the *tef1-α*. The causal agent of *damping-off* in *Casuarina equisetifolia* seedlings was confirmed like *F. lacertarum*, and the most efficient genomic region to identification is *tef1-α*. The best incubation condition to conidia production of *F. lacertarum* was in Mathur medium and the largest daily average growth was in SNA medium. The morphological characteristics are equivalent to the characterization of *F. incarnatum-equiseti* species complex isolated.

Key words: ITS; nursery; phytopathogens; *tef1-α*

Caracterização morfofisiológica e molecular de *Fusarium lacertarum* agente causal de tombamento em *Casuarina equisetifolia*

RESUMO: A casuarina (*Casuarina equisetifolia* L.) é uma espécie utilizada como quebra-ventos, na ornamentação de ruas, conservação de solos e fixação de dunas. Sua principal forma de propagação é seminal, entretanto, em viveiros, o fungo *Fusarium lacertarum* tem causado tombamento em plântulas. Dessa forma, o objetivo deste trabalho foi caracterizar morfofisiológica e molecularmente o patógeno. Para a caracterização molecular, colônias puras foram cultivadas nos meios de cultura BDA e FCA, e para caracterização fisiológica, colônias foram cultivadas nos meios BDA, SNA, Mathur e V8. O segmento genômico mais apropriado para identificação da espécie foi o *tef1-α*. O agente causal de tomabamento em plântulas de *Casuarina equisetifolia* foi confirmando como *F. lacertarum*, sendo a região genômica mais eficiente para identificação a *tef1-α*. A melhor condição de incubação visando à produção de conídios de *F. lacertarum* foi em meio Mathur e o maior crescimento médio diário foi em meio SNA. As características morfológicas condizem com a caracterização de isolados do complexo de espécies *F. incarnatum-equiseti*.

Palavras-chave: ITS; viveiro; fitopatógeno; *tef1-α*

Introduction

Casuarina (*Casuarina equisetifolia L.*) is a species of the Casuarinaceae family, native to tropical coastal regions of Australia and Southeast Asia (Parrota, 1993). Due to its versatility of adaptation, it has been widely cultivated outside its natural area, mainly in subtropical and tropical countries (Ayin et al., 2015). The species is widely used as windbreaks, in the ornamentation of streets, conservation of soils and fixation of dunes. Also, its wood has high calorific value, used mainly as fuel and in interiors of houses as decorative furniture (Ferreira, 2004; Lin et al., 2016; Ayin et al., 2015). Its roots have nitrogen-fixing bacteria and its deep root system allows being efficiently used to improve soil physical characteristics (Ferreira, 2004; Zhang et al., 2010).

The propagation of Casuarina is carried out through the seeds kept in seeding until being peeled into individual packages. Because it is a generally warm and humid environment, the nursery provides favorable conditions for the development of diseases, mainly caused by fungi. Parisi & Santos (2011) highlighted the pre and post-emergence damping-off and root rot caused mainly by the pathogens of the genus *Fusarium*, *Rhizoctonia*, and *Pythium*. These diseases affect the plants at the beginning of their development, which hinders the production of seedlings with phytosanitary and physiological quality.

Poletto et al. (2015) reported the fungus *Fusarium lacertarum* causing damping-off on Casuarina seedlings in Brazil for the first time. In the sowing, the symptoms of the disease were observed in clusters, and the seedlings presented necrotic lesions in the stems and roots, which later progressed in yellowing of the leaves, stagnation of growth, tipping, and death, with losses of up to 80% of nursery seedlings.

Fusarium lacertarum is one of the species of the *Fusarium incarnatum-equiseti* complex (O'Donnell et al., 2009). The fungal species belonging to this complex have similar characteristics and are mostly differentiated only by genomic DNA analysis. These fungi have been reported pathogenically associated with other cultures. *Fusarium equiseti* was reported causing root rot in *Carya illinoinensis* in Brazil (Lazarotto et al., 2014), damping-off in *Pinus halepensis* seedlings in Algeria (Lazreg et al., 2014), and in *Vigna unguiculata*, causing damping-off and root rot in Georgia (Li et al., 2017).

When a new pathosystem is established, the correct diagnosis of the disease is essential, given by the description of the morphological characteristics (morphology of the colonies, mycelia, conidia, sporulation, mycelial growth) and molecular characteristics (sequencing of regions of genomic DNA) since they provide subsidies for the development of disease control and prevention strategies, mainly related to genetic resistance.

Despite the importance of the *F. lacertarum* - *Casuarina equisetifolia* pathosystem, studies addressing the morphophysiological characterization of this pathogen were not found in the literature, studying the culture media that provide better in vitro development and detailing the regions of the genome that best characterize the pathogen. Thus, the objective of this work was to characterize the damping-off pathogen in *C. equisetifolia* seedlings by morphological and molecular characters.

Material and Methods

Morphophysiological characterization of the pathogen

For the morphological characterization, colonies of the pathogen obtained in previous studies by Poletto et al. (2015) and stored in the mycology collection of the "Elocy Minussi" Phytopathology Laboratory of the Department of Plant Protection of the Center of Rural Sciences, Federal University of Santa Maria, identificated like CAS, were transferred to petri dishes containing PDA medium (potato-dextrose-agar) and incubated for seven days at 25 ± 2 °C with photoperiod of 12 hours.

For the evaluation of the reproductive structures, it was incubated in petri dishes containing leaf clove agar medium (LCA), for 7 days at 25 ± 2 °C with photoperiod of 12 hours (Nelson et al., 1983). This medium allows *Fusarium* species to sporulate and present conidia with uniform and clear forms, not obtained in PDA culture medium (Poletto et al., 2006).

The presence of microconidia and chlamydospores in the aerial mycelium, length, width, number of septa and shape of the basal cell of the macroconidia, and conidiogenous structure were evaluated. The dimensions of the macroconidia (length and width) were obtained under an optical microscope at the 40x objective, with the aid of an OSM eyepiece attached to the microscope. Thirty macroconidia were randomly selected and measured.

The morphological identification of the fungus was performed according to the classification keys of Nelson et al. (1983), Leslie & Summerell (2006) and Gerlach & Nirenberg (1982). The colony staining was evaluated at the seventh day of growth on PDA medium and visually determined with the aid of the Munsell color primer (2009).

Development in culture medium

Mycelial discs (7 mm) from colonies grown on PDA medium for seven days and transferred to petri dishes to evaluate the development of *F. lacertarum* on the following culture media: PDA (200 g of potato, 20 g of dextrose, 15 g of agar, q.s.p. 1000 mL of distilled water); Mathur (2.8 g of dextrose, 2.5 g of $MgSO_4.7H_2O$, 2.7 g of KH_2PO_4, 1 g of peptone, 0.8 g of yeast extract, 15 g of agar, q.s.p. 1000 mL of distilled water); SNA (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 20.0 g agar, q.s.p. 1000 mL of distilled water) and V8 (100 mL of V8, 2 g of $CaCO_3$, 20 g of agar and 900 mL of distilled water). Subsequently, they were incubated at 25 ± 2 °C with photoperiod of 12 hours.

Daily mycelial growth was evaluated until the eighth day by two measurements of the colony in diametrically

opposite directions. Subsequently, the average daily growth was determined, dividing the total growth by the days of measurement. The growth data were also used to calculate the Mycelial Growth Rate Index (MGRI) of the fungus, using the Maguire formula (1962): MGRI =Σ((D-Da)/N), where: MGRI - mycelial growth rate index; D - current mean diameter of the colony; Da - the mean diameter of the colony of the previous day; N - number of days after inoculation.

There were 10 mL of sterilized distilled water added to each petri dish for the evaluation of sporulation on the eighth day and after, the scraping of the colony surface was done with the help of the Drigalski loop to release the conidia. The suspension was pipetted and filtered in a double gauze layer for retention of mycelial fragments and culture medium, and the spore concentration count was performed with the Neubauer chamber.

Molecular characterization

Portions of mycelium of the pathogen were removed from monosporic culture grown on PDA medium for five days using the CTAB method for the extraction of the genomic DNA (Doyle & Doyle, 1991). The extracted genomic DNA samples were submitted to the Polymerase Chain Reaction (PCR) for the amplification of the regions: rDNA transcribed internal spacer (ITS) with the oligonucleotide primers ITS1 (5' TTCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990); elongation factor - 1 alpha (*tef*1-α), with the pair of primers EF1-T (ATGGGTAAGGARGACAAGAC) and EF1-1567R (ACHGTRCCRATACCACCRATCTT) (Rehner & Buckley, 2005). The amplified fragments and control were separated by 1.2% agarose gel electrophoresis in 1X TBE buffer (10.8 g of tris base, 5.5 g of boric acid, 4 mL of 0.5 M EDTA and 4 mL of distilled water) containing ethidium bromide and visualized under ultraviolet light. PCR products were purified with 13% PEG 8000 for further sequencing.

The sequenced fragments were analyzed using the BioEdit software. The obtained nucleotide sequences were compared with those already existing in *GenBank* for the isolated pathogen. The *GenBank* sequences with the highest "scores" were selected and aligned along with the sequences obtained in the ClustalW algorithm sequencing, and the phylogenetic analysis was conducted by adopting the "Neighbor-joining" method with 1000 replicates by the MEGA software version 4. The similarity of the nucleotide sequences in the isolates was calculated using the *Basic Local Alignment Search Tool* (BLAST).

Statistical analysis

The experimental design was completely randomized and the daily average growth (DAG), mycelial growth rate index (MGRI), total growth (TG) and spore concentration in the different treatments were submitted to analysis of variance and comparison of means of the treatments by the Tukey test to 5% of probability of error. The statistical software used was SISVAR, version 5.3 (Build 77) (Ferreira, 2011).

Results and Discussion

Morphophysiological characterization

In the morphological characterization, colonies of the pathogen presented aerial mycelium of white coloration (Figure 1A) and in the lower part of the mycelium plate, they were orange-brown, resembling the results observed by Poletto et al. (2015), which identified the same staining on PDA culture medium at seven days of incubation.

For *Fusarium equiseti*, Lazreg et al. (2014) observed in the lower part of the plate beige coloration and white aerial mycelium, while Lazarotto et al. (2014) reported brownish-yellow coloration on the underside and the aerial mycelium was whitish to peach coloration. Ramdial et al. (2016) observed for *Fusarium incarnatum*, white and dense aerial mycelium, and staining of the lower part of the plate ranging from cream to salmon.

On LCA culture medium, the colonies showed abundant sporozoites of dark orange color (Figure 1B). Regarding to macroconidia, the mean for the length was 47.3 ± 11.65 µm and 4.8 ± 1.03 µm for the width. The number of septa varied from four to seven and the basal cells presented the foot format (Figure 1C). However, microconidia in the aerial mycelium were not present, only chlamydospores that were in abundance (Figure 1D).

In a study by Poletto et al. (2015), dark orange sporodocchi, macroconidia with septal numbers ranging from five to seven, and basal cell in foot format were also

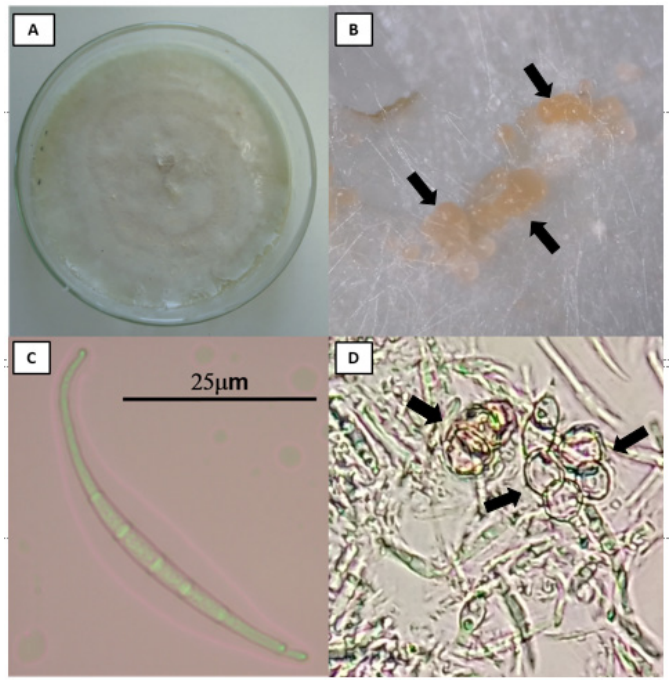

Figure 1. A. Colony of *Fusarium lacertarum* grown on PDA medium. B. Sporodoquios of dark orange staining in *Fusarium lacertarum* in leaf-clove-agar (LCA) medium. C. Conidium of *Fusarium lacertarum* with five septa and basal cell in foot format. D. Chlamydospores in the mycelium formed on LCA medium.

observed in *F. lacertarum*. However, the size of macroconidia differed, ranging from 12.5 to 17.5 µm in width and from 60 to 82.5 µm in length.

Ramdial et al. (2016) observed macroconidia in *Fusarium incarnatum* with four to five septa, measuring between 28 and 31 µm in length and 3 to 5 µm in width and presence of microconidia. In *Fusarium equiseti*, Lazreg et al. (2014) observed the presence of macroconidia with five to six septa, length ranging from 31 to 45 µm and basal cell in foot format, and no microconidia were observed, as Lazarotto et al. (2014) verified macroconidia mostly with 5 septa and absence of chlamydospore.

Growing in culture media

For total mycelial growth (TMG), the only means that differed statistically were SNA and V8, and the mycelial growth in SNA medium was 24.4% higher than in V8. The same trend was observed for the variable mycelial diary (DAG), while in SNA medium, the fungus grew about 3 mm more than in V8 (Table 1). However, for the culture media PDA and Mathur, there was no statistical difference between the other media tested. SNA media is a medium considered to be poor in nutrients and this may favor the growth and development of the fungus because, in nature, nutrient shortages can induce reproduction (Leslie & Summerell, 2006).

The mycelial growth rate index (MGRI) did not differ between the culture media (Table 1). Therefore, Silva & Teixeira (2012) observed that PDA medium favored mycelial growth, while PDA and PSA media favored induction of sporulation of *Fusarium solani*.

Fusarium fungi present different behavior when submitted to different culture media, as observed by Nurbaya et al. (2014), where isolates of *F. ambrosium*, when tested in different culture media, obtained a daily average growth ranging from 6.38 to 12.38 mm day^{-1}.

For the sporulation variable, the Mathur medium differed statistically from V8, in which spore production was not stimulated. The SNA, PDA and Mathur media did not differ, but the Mathur medium promoted superior sporulation (Table 1). For *Colletotrichum gloeosporioides*, the Mathur medium was also able to provide greater sporulation when compared to other means (Lopez & Lucas, 2010).

Table 1. Statistical analysis of the obtained values of TMG, MGRI, DAG, and sporulation of *F. lacertarum* in different culture media.

Treatment	TMG (mm)	MGRI	DAG (mm day^{-1})	Sporulation (x10^4 conidia mL^{-1})
SNA	90.00 a	25.70 a	12.86 a	1.8 ab
MATHUR	83.43 ab	24.75 a	11.92 ab	27.56 a
BDA	79.12 ab	23.71 a	11.30 ab	10.25 ab
V8	68.86 b*	23.43 a	9.84 b	0 b
CV (%)	8.74	5.1	8.07	>30

* Means followed by the same lowercase letter in the column do not differ statistically by the Tukey test at 5% error probability. TMG: total mycelial growth; MGRI: mycelial growth rate index; DAG: daily average growth; CV: coefficient of variation

Isolated from *Fusarium* spp. presented presented difference in sporulation when tested in liquid culture media, a *Fusarium solani* isolate when tested in coconut water medium showed sporulation of 3.3x10^8, while in potato medium and modified dextrose the sporulation was 3.7x10^7 (Nurbaya et al., 2014).

Molecular characterization

The most similar accessions and sequence coverage obtained from the blast research carried out on the *GenBank* website (http://www.ncbi.nlm.nih.gov) were used to construct genetic similarity dendrograms.

Figure 2 shows the phylogenetic dendrogram made for the transcribed internal spacer segment (ITS) of the *Fusarium* isolate together with the comparison sequences. From this genomic segment, it was not possible to allocate the *Fusarium lacertarum* isolate in a specific clade along with the comparison sequences for the species. The bootstrap support value was 3 for the clade, indicating that this DNA region alone was not efficient for discrimination of the species.

The phylogenetic dendrogram made with the sequencing of the elongation factor 1-α region (*tef*1-α) (Figure 3) presented the bootstrap value of 85% for the clade, where the sequence of the *Fusarium lacertarum* isolate (CAS) was allocated together with two other sequences of the same pathogen (JF740828 and GQ505593). Also, the comparison sequences had high percentages of coverage and similarity, increasing the reliability of alignment and discrimination of the species.

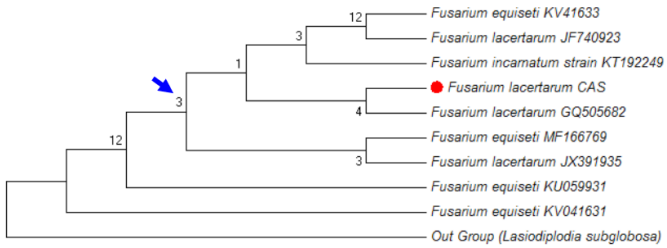

Figure 2. Phylogenetic dendrogram based on the Neighbor-joining method from DNA sequences of the ITS region. The numbers on the branches indicate the percentage of repetitions of the bootstrap analysis in which the repetitions were observed (1000 repetitions).

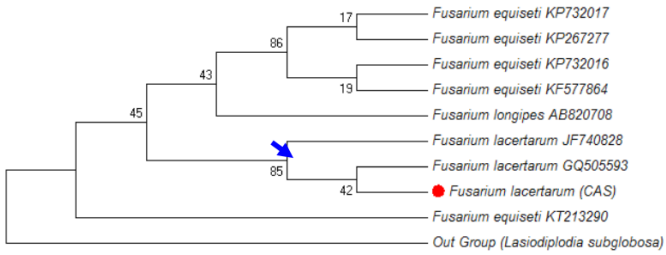

Figure 3. Phylogenetic dendrogram based on the Neighbor-joining method from *tef*1-α region DNA sequences. The numbers on the branches indicate the percentage of replicates of the bootstrap analysis in which the replicates were observed (1000 replicates).

Molecular analysis confirmed the results found by Poletto et al. (2015), as *F. lacertarum* being the damping-off agent in Casuarina, and the genomic region *tef*1-α was the most indicated for species differentiation. Li et al. (2014) also used only the *tef*1-α DNA region for the identification of the *F. equiseti* species that causes sheath rot of corn in China, and Garibaldi et al. (2017) identified *F. equiseti* causing leaf spot in *Eruca sativa* through the sequencing of the ITS regions and *tef*1-α

Conclusion

The causal agent of damping-off in seedlings of *Casuarina equisetifolia* was confirmed as *F. lacertarum*, where *tef*1-α was the most efficient genomic region for identification.

For the morphophysiological characterization, the Mathur culture medium was efficient in the production of conidia and the SNA medium in the mycelial growth.

Morphological analyzes are consistent with the characterization of isolates of the *Fusarium incarnatum-equiseti* Species complex.

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