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Original Article

Silviculture

The Causal Agent of Damping-off in *Pinus patula* (Schiede) and *Pinus tecunumanii* (Schwerdtf.)

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ABSTRACT

Damping-off is considered one of the most serious risks for production of *Pinus* seedlings due to the significant losses it can cause in forest nurseries. In Colombia there is little information about the etiology of this limiting disease. Different species of the genus *Fusarium* have been reported as causal agents, which makes the study of the pathogenicity of this genus relevant. In this study, strain 001F of *Fusarium oxysporum* was obtained from the diseased tissues of *P. patula* seedlings. The identity of the species was determined by sequencing the internal transcribed spacer (ITS) regions. Pathogenicity tests confirmed the ability of strain 001F to cause damping-off symptoms in *P. patula* and *P. tecunumanii*. Thus, *F. oxysporum* strain 001F represents a significant risk to produce tree species in Colombia and in other parts of the world.

Keywords: *Fusarium* sp., forest nurseries, pathogenicity.

1. INTRODUCTION

Colombia is the seventh country in the world in area covered with natural forests with about 49% of its terrestrial area (Sánchez-Cuervo et al., 2012). 24 million hectares are suitable for commercial reforestation, however, only 450.000 ha have been planted and for 2038 it is projected to increase to 1.5 million ha (UPRA, 2018). The genus *Pinus* (L.) predominates commercial reforested area in Colombia, with 108.060 ha, followed by *Eucalyptus* (L'Hér), with 57.447 ha, *Acacia* (Mill) with 36.986 ha and other genera (PROFOR, 2017). *P. tecunumanii* (Schwerdtf. ex Eguiluz and Perry) excels within *Pinus* species, mainly due to its excellent phenotypic and silvicultural characteristics, fast growth and arguably, the best shank from all tropical *Pinus*, which confers a great potential for reforestation in tropical and subtropical regions (Kanzler et al., 2014; Claros Cuadrado et al., 2017). *P. patula* (Schiede ex Schltdl. and Cham) is another important species with potential for production in Colombia because of its high content of cellulose and hemicellulose (Gómez et al., 2012). However, for sustainable production of commercial forests diseases such as damping-off should be appropriately managed. Damping-off is considered one of the biggest phytosanitary risks affecting the future sustainability of the wood industry (Seseni et al., 2015; López-López et al., 2016). This is because it significantly decreases the possibility of obtaining healthy nursery seedlings suitable for the field (Vivas et al., 2012). This disease can progress rapidly and cause significant losses in forest productivity (García Díaz & Cibrián Tovar, 2011).

This pathology, common in seeds and seedlings of many tree species, is caused by various pathogens such as *Phytophthora* spp. (de Bary), *Pythium* spp. (Pringsh), *Rhizoctonia* spp. (DC.) and *Fusarium* spp. (Link ex Grey), among others (Dar et al., 2011). These pathogens have been reported as causal agents of damping-off in seedlings of several *Pinus* species, such as *P. nigra* (Arnold), *P. pinea* (L.), and *P. patula* (Machón et al., 2009; Herrón et al., 2015). In Colombia, there are currently few studies aimed at understanding forest pathologies despite being one of the most limiting factors to produce seedlings of several *Pinus* species used for commercial reforestation.

Fusarium spp. are cosmopolitan fungi that have pathogenic forms and are capable of parasitizing different gymnosperm and angiosperm species (Bosland, 1988). It has been identified as a causal agent of rot and wilt symptoms (Dean et al., 2012; Zuo et al., 2015; Carrasco et al., 2016), causing important economic losses in more than 200 crops around the world (Pérez et al., 2017). *Fusarium* species are frequently the causal agents responsible for damping-off in forest nurseries, and sometimes they cause important damage in very young plantations in the field (Vivas et al., 2012; Martín-Rodrigues et al., 2015; Fajardo-Mejía et al., 2016). Stewart et al. (2012) found that isolates of *F. oxysporum* (Schltdl.) and *F. commune* (Skovgaard, O'Donnell and Nirenberg) caused damping-off in Douglas fir seedlings under greenhouse conditions. Similar results point to *F. circinatum* (Nirenberg and O'Donnell) as the causal agent of damping-off in seedlings of *P. maximinoi* (Moore) and *P. tecunumanii* (Maphosa et al., 2016; Martínez-Álvarez et al., 2016; García Díaz et al., 2017; Flores-Pacheco, 2017; Swett et al., 2018).

In Colombia, Steenkamp et al., (2012) found that *F. circinatum*, besides being related to the symptoms of damping-off in *P. patula*, *P. maximinoi*, and *P. tecunumanii* seedlings, is also related to shoot dieback and outbreaks of resinous stem canker on the plantation trees.

To carry out an adequate management of the problem, it is necessary to start with timely and precise identification of the causal agent of the disease because an incorrect diagnosis could cause the disease control to fail, increasing costs and the risk of losses in the plantation. In this research, the causal agent of damping-off was determined for *P. patula* and *P. tecunumanii* seedlings, two of the most important species for commercial reforestation in Colombia. Ultimately, this finding is vital for management of the disease, reducing the risks posed by its causal agents both in the nursery phase and in field plantations.

2. MATERIAL AND METHODS

2.1. Microorganism isolation

Isolates were obtained from *P. patula* seedlings harvested in a commercial nursery, with symptoms typical of damping-off, such as wilting or basal rot of the stem and root. The tissue of the diseased plant was disinfected in 1% hypochlorite for 1 minute, rinsed

for 1 minute with sterile distilled water, followed by one-minute disinfection with 70% ethanol, and a final wash with sterile distilled water. Then, small pieces of tissue containing lesions were cut, seeded directly on potato-dextrose-agar culture medium (PDA, Difco USA), incubated at approximately 28°C and observed daily. Fungal isolates were grown in petri dishes containing PDA medium at 28°C, for 14 days for further work.

2.2. Morphological and molecular identification

Colony description was made based on the color, the diameter, and characteristics of the aerial mycelium. Observation and description of the reproductive structures were performed using a Nikon SMZ1000 optical microscope coupled to a digital camera. Morphological characteristics of conidia were studied based on the shape, the septation and the mean of dimensions (width and length) calculated from 30 measurements performed in the light microscope (Fourie et al., 2011; Santos et al., 2019).

2.3. DNA extraction

DNA was extracted from 14-day cultures of fungal isolate in Petri plates containing PDA medium. To do so, mycelium was scraped directly from the surface of PDA plates. The mycelium obtained was lyophilized and pulverized with liquid nitrogen using a mortar and a pestle until obtaining a fine powder, which was kept at –20 °C until further use. Nucleic acids were extracted from 100 mg of mycelium powder using the CTAB, phenol:chloroform (1:1) method (CTAB 3%, 100 mM Tris HCl pH 8.0, 1.4 mM NaCl, 20 mM EDTA pH 8.0, supplemented with 150 µL of SDS (20%)), and precipitated with cold isopropanol and 3M sodium acetate. Nucleic acids were resuspended in 50 µL of TE buffer (Tris-HCl 10 mM pH 8.0). The RNA was digested with RNase (10 $\mu g \cdot mL^{-1}$) and the DNA solution was stored at –20 °C for further use. The purified DNA was analyzed and quantified by spectrophotometry using a NanoDrop equipment (Thermo Scientific) and electrophoresis in 0.8% agarose gels in TBE 0.5 X pH 8.0 (0.09 M Tris-borate, 0.002 M EDTA, Amresco) visualized under UV by staining with EZ-96 VISION® (Thermo Scientific, Waltham, MA. USA), following the manufacturer instructions.

2.4. Sequence analysis of the Internal Transcribed Spacer (ITS) regions of the fungal rDNA

The molecular identification was carried out by sequencing the ITS regions of the rDNA. These regions were amplified by PCR using the universal primers ITS1 (5 'TCC GTAGGT GAA CCT GCG G 3') and ITS4 (5 'TCC TCC GCTTATTGATAT GC 3') (White et al., 1990). For PCR amplification, 10X KCl buffer, 25 mM MgCl2, 2.5 mM dNTP, 10 µM primers, Taq DNA Polymerase and 3 ng of the purified DNA were used in a final volume of 30 µl. The program started with a temperature of 95°C for 3 minutes, followed by 35 cycles of amplification (denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute) and a final extension at 72°C for 10 minutes. The PCR products were visualized by electrophoresis in 1.2% agarose gels, stained with EZ-VISION®, following the manufacturer's instructions (Thermo Scientific). The bands of the expected size (500 to 600 bp) were purified using the GenJet kit (Thermo Scientific), following the manufacturer's instructions and sent for sequencing. The sequences obtained were assembled, edited and aligned manually, using the BioEdit software. The sequences were compared with the National Center for Biotechnology Information (NCBI) databases, using the BLAST package (NCBI, 2019).

The species identification included 16 ITS nucleotide sequences from *Fusarium* spp. retrieved from NCBI database (Genbank code between parentheses). These sequences were selected for comparison with some species of *Fusarium* distant from *F. oxysporum* and reported as causing disease in *Pinus* spp.: 11 sequences of *F. oxysporum* (MG966527.1, MG798785.1, KY855514.1, KY855512.1, KY855511.1, KY855508.1, KY855507.1, KY855506.1, KY855505.1, MG670446.1 and KF313101.1), 1 of *F. commune* (KT982281.1), 1 of *F. proliferatum* ((Matsush) Nirenberg ex Gerlach and Nirenberg) (GU074010.1), 1 of *F. circinatum* (KC464617.1), 1 of *F. bactridioides* (Wollenw.) (KC464615.1) and 1 of *F. fujikuroi* (Nirenberg) (MF356517.1) (Herrón et al., 2015; Stewart et al., 2012; González et al., 2008).

A dendrogram was constructed by using the Maximum Likelihood method based on the Jukes-Cantor model (Tuffley et al., 2012). A bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less

than 50% bootstrap replicates were collapsed. Analyses were conducted in MEGAX (Kumar et al., 2018).

2.5. Obtaining the inoculum

The spore inoculum was prepared using an isolate that was grown for 14 days under the previously described conditions. To do so, sterile distilled water was added to the Petri dishes and the mycelium was scraped to form a suspension. Subsequently, the inoculum was quantified in a Neubauer chamber, to adjust the concentration to $1x10^6$ spores/ml. Additionally, another source of inoculum was prepared for the pathogenicity tests from the macerate of diseased *P. tecunumanii* seedlings obtained approximately 15 days after sowing. This timing was chosen because at 15 days the seedlings showed a higher incidence of the disease compared to the non-inoculated plants. The macerate was obtained by liquefying diseased plantlets covered in their entirety with sterile distilled water, until a homogeneous suspension was obtained (approximately 3 minutes). The suspension was decanted by gravity and the supernatant was recovered, which was used as a source of inoculum of the disease.

2.6. Pathogenicity tests

Sexual seeds of *P. patula* and *P. tecunumanii* were sown in germinating trays of 25 plants each. The isolate obtained was evaluated to determine its ability to cause disease in *P. patula* and *P. tecunumanii* seedlings. The Koch postulates were fulfilled. The tests were carried out under laboratory conditions using plastic trays disinfected with 1% hypochlorite, then filled with autoclaved soil. Three trays were used per each treatment for a total of 75 plants per treatment. Seeds were provided by the Santa Elena forest nursery and INAFOR (National Forest Institute, Nicaragua), respectively. *Pinus* seeds were embedded in an inoculum suspension at a concentration of $1 X 10^6$ spores ml^{-1} for 12 hours. Then, the soil substrate was inoculated with 5 mL of the spore suspension at the same concentration described and treated seeds were sown. A plant showing damping-off symptoms was considered as diseased. Plants were monitored daily for symptom appearance. The incidence of the disease (%) was recorded as the number of diseased plants over the total number of plants in the experimental unit multiplied by a hundred. Plants treated only with sterile distilled water were used as the negative control. Incidences were contrasted based on the Score method, using the software R (Tippmann, 2015), to determine if there was a significant effect of inoculation on the incidence of damping-off.

The fungal isolate of *F. oxysporum* obtained from diseased plants of *P. patula*, from now on named strain 001F, was the only isolate that reproduced the original symptoms of damping-off, therefore was selected for pathogenicity tests. Its pathogenicity in *P. tecunumanii* plants was determined by nursery test. To do so, a comparison was made between three groups of plants sprouted 1) seeds inoculated with macerated diseased plants, 2) seeds inoculated with strain 001F spores as described, and 3) control plants from seeds without inoculum. Inoculation was performed one day before planting the seeds of *P. tecunumanii*. Briefly, 1.5 ml of a suspension of $1x10^6$ spores/ml of strain 001F or 2 ml of a suspension from macerated diseased plants, was applied per each seed (Ownley & Trigiano, 2016). Trays for germination of plants were disinfected with 1% hypochlorite and filled with autoclaved pine bark. Each experimental unit consisted of 25 *P. tecunumanii* plants in a tray, with three replications, for a total of 75 plants per each treatment. The incidence was recorded daily for 28 days. Data obtained were subjected to repeated measures analysis, using the SAS MIXED procedure, version 9.00. Before fitting the model, angular transformation was applied to the incidences.

3. RESULTS

3.1. Morphological characterization of fungal isolate

Five fungal isolates were obtained from symptomatic plant tissues. When re-inoculated, one isolate reproduced the original symptoms of damping-off observed in plants, the strain 001F. The remaining isolates did not produce symptoms. In the macroscopic observations, the pathogenic colony was characterized by the presence of an aerial mycelia that was initially white and changed to a purple-color over time. The colony grew fast covering the whole Petri dish in a period of 10 days and maintaining a radial pattern of growth (Figure 1). Under the microscope, a straight to slightly curved septate mycelium and relatively thin and thin-walled macroconidia with mean dimensions of 36 (standard deviation (SD) ± 3.6) µm X 3.2 (SD ± 0.8) µm and with

three to five septa, were observed. Abundant oval shaped microconidia, without septa, with mean dimensions of 8 (SD ± 2.3) µm X 2.3 (SD ± 0.5) µm, straight to curved, were also observed. In 14-days cultures, the presence of terminal and interspersed chlamydospores was observed (Figure 1). The macroscopic and microscopic observations were consistent with characteristics of the *F. oxysporum* species complex of fungus (van Dam et al., 2018; Fourie et al., 2011).

3.2. Sequence analysis of the ITS regions and identification

The amplification with universal ITS primers and the subsequent visualization of the PCR products by electrophoresis in agarose gel, revealed a 520 bp fragment. The sequence obtained from the amplicon of strain 001F was deposited in NCBI-GenBank with the submission number SUB5357489 and accession number MK680158. Comparison of the sequence with those deposited in the NCBI database revealed a 99% similarity with the accession identified with code KT271765, corresponding to *F. oxysporum*. In addition, the sequence obtained clearly grouped with other 11 sequences of *F. oxysporum* retrieved from Genbank in the dendrogram made using the Maximum Likelihood method; meanwhile ITS sequences of other species of *Fusarium* distant from *F. oxysporum* and causing disease in *Pinus* spp. such as *F. commune*, *F. proliferatum*, *F. circinatum*, *F. bactridioides* and *F. fujikuroi*, formed different clusters (Figure 2). Together, morphological and molecular results confirm that our strain 001F is within the *F. oxysporum* species complex.

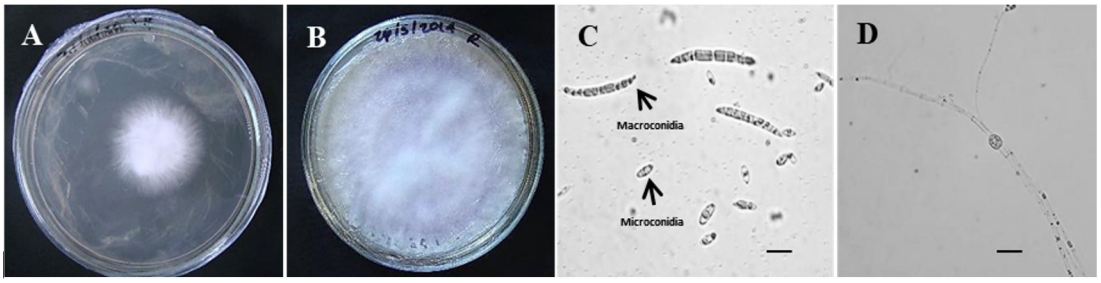

Figure 1. Morphological characterization. (A) The colony 3 days after seeding on agar plates; (B) The colony after 10 days of incubation; (C) Macroconidia and microconidia (100X); (D) Chlamydospores and hyphae with septa (60X). Fungal incubation was performed in Petri plates containing PDA at 28°C. Scale bar: 10 µm.

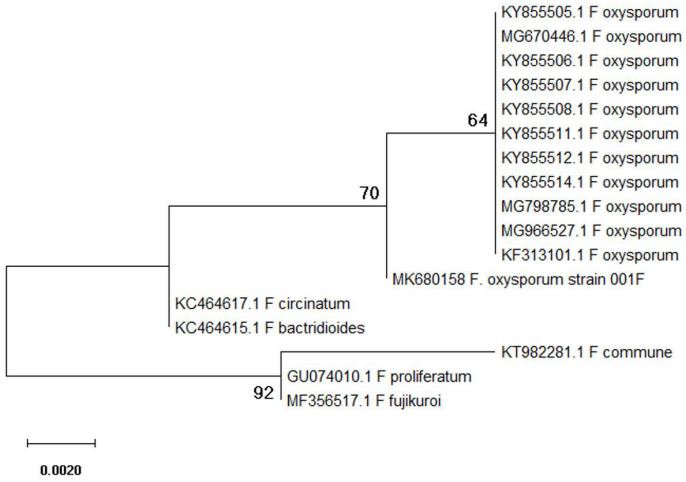

Figure 2. Sequence analyses of ITS regions of pathogenic isolate. Dendogram of *Fusarium* sequences was constructed by Maximum Likelihood method. Sequence codes correspond to Genbank accession number retrieved from NCBI using the Blastn algorithm. Numbers in branches mean Bootstrap values using 1000 iterations. Bootstrap values below 60 were deleted. Analyses were performed in MEGA X.

3.3. Plant symptomatology

3.3.1. Pathogenicity tests

During the 23 days evaluated, no incidence of damping-off was observed in the control seedlings of *P. patula* not inoculated and treated only with sterile distilled water (Figure 3). In clear contrast, in the treatment of plants inoculated with the strain 001F, disease incidence gradually increased until reaching 85% after 23 days (P <0.0001) (Figure 4). Together, these results suggest that *F. oxysporum* is the causative agent of the damping-off symptoms in *P. patula* (Figure 3 and 4). In this study, strain 001F was re-isolated from inoculated and diseased tissues of *P. patula*, fulfilling the Koch postulates (Figure 3).

Similarly, plants of *P. tecunumanii* inoculated with the strain 001F showed basal stem strangulation, light brown basal necrosis with an ascending tendency in the neck of the plant, and wilted needles with a tendency to present total necrosis, symptoms typical of damping-off (Figure 5). Damping-off in the 001F-inoculated plants, reached an incidence of 41.3% 23 days after inoculation (Figure 6).

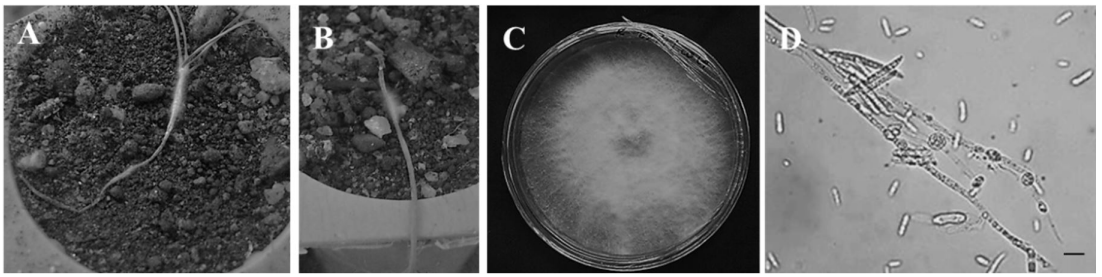

Figure 3. Koch postulates for the causal agent of damping-off in *P. patula*. (A) and (B) Disease symptoms in *P. patula* seedlings inoculated with the 001F isolate of *F. oxysporum*; (C) Colony obtained by re-isolation of 001F isolate from symptomatic tissues of inoculated *P. patula* seedlings; (D) Macroconidia, microconidia and chlamydospores of re-isolated 001F isolate of *F. oxysporum* (60X). Photos are representative of results obtained from three replicates of 25 plants each for a total of 75 plants per treatment. Scale bar = 10μm.

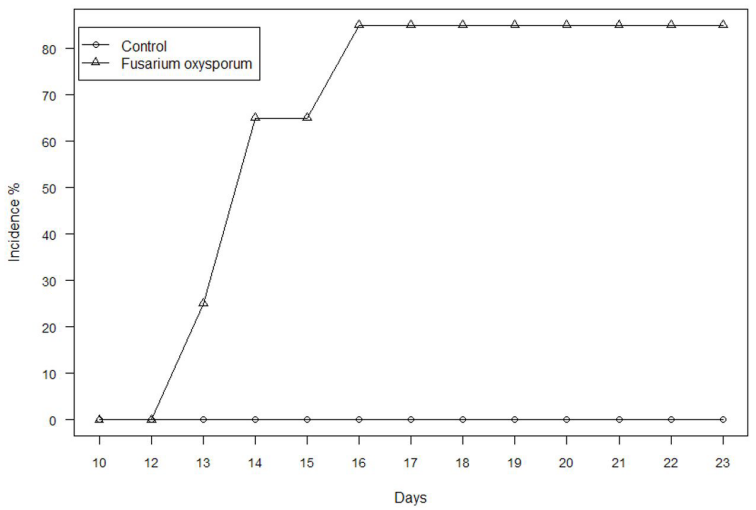

Figure 4. Incidence in percentage of damping-off in *P. patula* seedlings inoculated with the 001F isolate of *F. oxysporum*. Each point represents the percentage of disease incidence calculated as the number of diseased plants divided by total plants multiplied by 100. A total of three replicates of 25 plants each for a total of 75 plants were used per treatment. Circular points correspond to non-inoculated seedlings used as control. Triangular points represent plants inoculated with the 001F isolate of *F. oxysporum*. Data obtained were subjected to repeated measures analysis, using the SAS MIXED procedure, version 9.00. Before fitting the model, angular transformation was applied to the incidences.

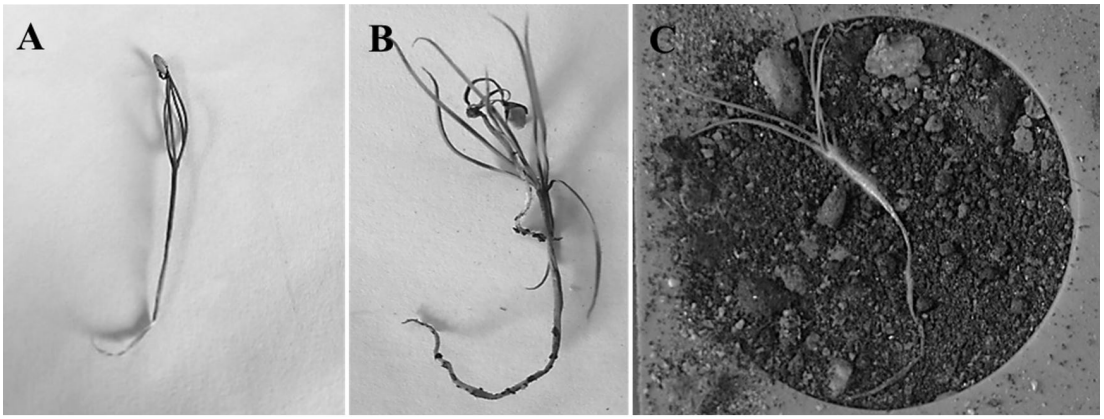

Figure 5. Symptoms of damping-off in *P. tecunumanii* seedlings. (A) Basal stem strangulation; (B) Basal stem rot; (C) Basal stem rot and sporulation. Photos are representative of results obtained from three replicates of 25 plants each for a total of 75 plants per treatment.

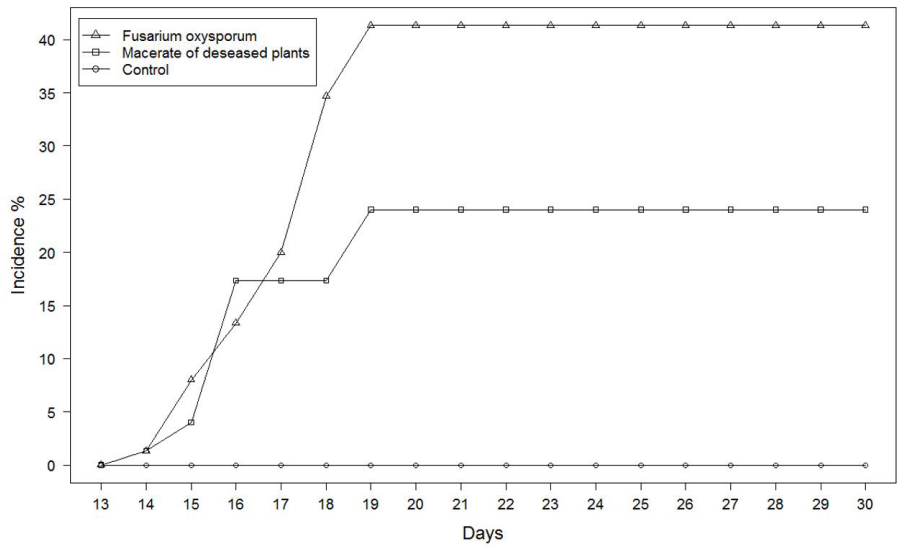

Figure 6. Incidence in percentage of damping-off in *P. tecunumanii* seedlings inoculated with the 001F isolate of *F. oxysporum*. Each point represents the percentage of disease incidence calculated as the number of diseased plants divided by total plants multiplied by a 100. A total of three replicates of 25 plants each for a total of 75 plants were used per treatment. Circular points correspond to non-inoculated seedlings used as control. Triangular points represent plants inoculated with the 001F isolate of *F. oxysporum*. Data obtained were subjected to repeated measures analysis, using the SAS MIXED procedure, version 9.00. Before fitting the model, angular transformation was applied to the incidences.

The control seedlings of *P. tecunumanii* irrigated with sterile distilled water only did not present symptomatology of the disease. There was a highly significant difference in the incidence of damping-off ($P = 0.0004$) between the plants inoculated with strain 001F and the control plants (Figure 6). The strain 001F of *F. oxysporum* was isolated repeatedly from seedlings with symptoms of damping-off produced under these commercial production conditions. No other pathogens were isolated using this procedure.

4. DISCUSSION

The development of this work allowed us to isolate the strain 001F of the fungus *F. oxysporum* and identify it as a causal agent of damping-off in two

Pinus species in Colombia. In support of our findings, there are numerous reports of this pathogen affecting other forest species (Morris et al., 2014; Herrón et al., 2015; Gordon et al., 2015; Fajardo-Mejía et al., 2016). We identified this species by morphological and molecular criteria (Fourie et al., 2011; van Dam et al., 2018). Additionally, pathogenicity tests were performed.

The pathogenicity tests for the two pine species evaluated showed conclusively that the strain 001F is capable of producing the symptoms of the disease in *P. patula* and *P. tecunumanii* seedlings. Similar results were found by Stewart et al. (2012), where they found that different isolates of *F. oxysporum* and *F. commune* obtained from healthy and diseased seedlings of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and western white pine (*Pinus monticola* (Douglas ex Don) were able to reproduce the symptoms of damping-off in Douglas fir seedlings under greenhouse conditions. Similarly, Herrón et al., (2015) were able to isolate *F. oxysporum* and *F. parvisorum* (Herron, Marinc. and Wingf.) from diseased nursery seedlings of the genus *Pinus*. Additionally, other researchers found that different isolates of the genus *Fusarium*, obtained from pine seedlings, are pathogenic for seedlings of *P. patula* (Fitza et al., 2013).

On the other hand, the seedlings of *P. tecunumanii* inoculated with the macerate of diseased plants presented a lower incidence of the disease. Damping-off is a disease caused by a complex of pathogens (Lazreg et al., 2014; Weiland et al., 2014; Gordon et al., 2015) and when inoculating with the macerate of diseased plants it is probable that other pathogenic and not pathogenic microorganisms that may compete between them, be involved in the effect observed. A question remains to be answered about if this is the only causal agent or maybe there are others. In the present work, only strain 001F of *F. oxysporum* was isolated, however, as mentioned, damping-off may be caused by several plant pathogenic microorganisms. Therefore, other causal agents of damping-off should not be discarded when managing this disease in plantations around the country and accurate diagnosis should be performed in each particular case.

Phytosanitary analysis revealed that the inoculum of the disease can have various sources of origin such as seeds, irrigation water, and the substrate used, where pathogenic fungi such as *F. oxysporum* were present (Fajardo-Mejía et al., 2016; Lamichhane et al., 2017). Additionally, *F. oxysporum* produces a large amount of chlamydospores that allow it to survive in the soil for long periods of time, as well as in dead tissue of the host plant, and in plant remnants in the soil in the absence of a host plant, making it difficult to control (Gordon et al., 2015). In addition, Jones et al. (2014), found that high concentrations of inoculum caused higher mortality levels in *P. patula* seedlings. For these reasons, the handling of damping-off should be focused on avoiding the arrival of infectious propagules of the pathogen to the growth substrates used in the nursery, as well as in an early detection.

Several investigations found that conifer trees obtained from seedlings that presented damping-off caused by *Fusarium* spp. can suffer significant damage from resinous canker in the field (Stewart et al., 2012; Steenkamp et al., 2012; Jones et al., 2014). This could be attributed to the fact that the pathogen can be latent in the transplanted seedling; therefore, an early detection of these pathogens would be crucial to avoid sending them to the field (Jones et al., 2014).

In our study a single species was identified causing disease, however it is very important because little information is known about causal agents of *Pinus* diseases in Colombia. Therefore, this knowledge is crucial for proper management. Future research including more areas and nurseries for *Pinus* spp. production are needed to identify the different pathogens that affect the conifers and other gymnosperms native to Colombia and to study the susceptibility to these pathogens, allowing the development of adequate control strategies. This will require detailed knowledge about the distribution, host range and pathogen ecology.

In Colombia, the identification of strain 001F of *F. oxysporum* as the causal agent of damping-off in *P. patula* and *P. tecunumanii* is just the beginning of what is necessary to aid in the making of phytosanitary recommendations and control strategies adjusted especially for the traditional nursery management of seedlings. Of particular importance is the precise identification of the different disease-causing agents, since, for example, some chemical products that are effective in oomycete microorganisms, are not effective in fungi. For this reason, an incorrect identification of the causal agent entails an increase in costs, due to the application of control measures that are not effective

and also pollute the environment and can affect public health. This work may be used as a model for causal agent identification in forestry plantations in Colombia.

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