

Morphology and pathogenicity of *Fusarium* isolated from pine in nurseries in Durango

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Abstract

Damping-off is a disease that occurs in forest nurseries during the establishment stage of seedlings and is mainly caused by fungi. The objective was to carry out the isolation, morphological characterization, and pathogenicity tests of fungi in five forest nurseries in the state of Durango in 2018. Fungal isolates were obtained from seedlings of *Pinus engelmannii*, *P. durangensis*, *P. cooperi*, and *P. arizonica* approximately 45 days old, which were selected based on the presence of symptoms characteristic of the disease. Samples were seeded in Petri dishes containing PDA supplemented with antibiotics. Macroscopic and microscopic characteristics were recorded from colonies and structures produced in PDA, CLA and SNA media. The pathogenicity was evaluated by inoculating a suspension of conidia in the seed, and evaluating the percentage, index, and speed of germination, as well as the severity of the disease, survival analysis with Kaplan-Meier curves and the fit of epidemiological models of the progress of the disease was also determined. It was found that the fungal isolates that cause the damping-off problem in a pre-emergent and post-emergent way, obtained from seedlings of *Pinus* spp. in the forest nurseries of the state of Durango, were morphologically similar to isolates belonging to the *F. fujikuroi* and *F. oxysporum* species complexes. In addition, differences were observed in the severity of the disease produced by the isolates and they fitted the Gompertz model and the monomolecular model.

Keywords:

F. fujikuroi, *F. oxysporum*, *Pinus* spp., germination, survival.

Introduction

Growing seedlings in a nursery is one of the most important activities of reforestation programs. To consider a seedling suitable for such programs, the National Forestry Commission (CONAFOR, for its acronym in Spanish) considers certain characteristics that must comply with the Mexican standard NMX-AA-170-SCFI-2016 (Secretaría de Economía, 2016). In Durango, in 2018, 8 291 544 plants were produced and 4 680.8 ha were reforested (CONAFOR, 2019a, 2019b), which corresponded to 9.6% of the area and 11% of the plant produced in the country, making it the state with the largest number of plants produced and the largest reforested area.

In a study conducted by Prieto *et al.* (2009), they reported that 100% of the nurseries studied presented problems caused by phytopathogenic fungi that cause damping-off.

The disease occurs in two ways: the first is called pre-emergent, where seeds in the process of germination are affected, and post-emergent, which attacks young seedlings that have not lignified the stem (Landis *et al.*, 1989; Dar *et al.*, 2011). This disease frequently occurred in seeds and seedlings of many forest species and other crops and is caused by a complex of microorganisms which includes oomycetes and fungi that see their development favored mainly by high moisture conditions (Fajardo-Mejía *et al.*, 2016).

The most common genera of fungi that cause the damping-off problem are *Fusarium* and *Rhizoctonia*, of which *Fusarium* is the most important for its ubiquity (Dumroese and James, 2005). One of the main goals of phytopathology is to gain knowledge of the agents that cause diseases in cultivated plants and, through the application of appropriate techniques and tools, reduce the severity and incidence of diseases and thus reduce the losses caused by them (Solano-Bonilla and Brenes-Chacón, 2012).

The present study aimed to isolate fungi from pine seedlings with symptoms of the disease in forest nurseries in the state of Durango in order to identify them and characterize them by using brightfield optical microscopy and through the use of pathogenicity tests, and to determine the damage caused to the plant, the survival function, and the fit to epidemiological models.

Materials and methods

Five nurseries distributed in the state of Durango were sampled in September 2018. INIFAP Nursery (23° 59' 23.2" north latitude and 104° 37' 26.2" west longitude, 1 879 masl), General Francisco Villa nursery (25° 58' 19.7" north latitude and 104° 35' 51.6" west longitude, 1 878 masl), nursery of the Faculty of Forest Sciences of the Juárez University of the State of Durango (24° 00' 48.3" north latitude and 104° 41' 03.2" west longitude, 1 899 masl) and two nurseries located in the municipality of Santiago Papasquiaro: General Emiliano Zapata Nursery (25° 03' 40" north latitude and 105° 26' 17" west longitude, 1 756 masl) and San Nicolás Nursery (24° 56' 57" north latitude and 105° 25' 02.8" west longitude, 1 806 masl).

The sampling consisted of extracting seedlings approximately 45 days old with damping-off symptoms; they were homogenized with phosphate buffer solution pH 7.2; serial decimal dilutions were performed and from dilutions 10^{-5} and 10^{-6} , they were sown with the plate extension technique in potato dextrose agar (PDA) culture medium in duplicate. The plates were incubated at 28 °C for seven days in an incubator (Quincylab® 12-140). At the end of the incubation period, the aspect of fungal colonies was recorded (Gabrekiristos *et al.*, 2020). To obtain axenic culture, consecutive transfers were performed by hypha tip in PDA.

To observe the overall microscopic characteristics, each isolate was cultured using the PDA microculture technique (Harris, 1986) and the way of inoculation proposed by Rosana *et al.* (2014). Microscopic observations were made with the 40x objective of the microscope (Leica® CME) and microconidia were measured with the AmScope® software version 3.7 (calibrated with a Euromex® AE 1110 micrometer with 0.01 mm reticle). The identification of each fungal isolate at the species complex level was carried out with the descriptions and keys reported by Leslie and Summerell (2006).

To observe the specific microscopy characteristics of the isolates, monosporic cultures were prepared with the adaptation of the technique applied by Obanor *et al.* (2013). Finally, mycelial discs from the edge of an isolated colony were transferred to Petri dishes with PDA, and they were incubated at room temperature for seven days with continuous light (Nelson *et al.*, 1983) in SNA culture medium, and incubated at 25 °C for seven days (Leslie and Summerell, 2006), and also in carnation leaf agar (CLA) medium prepared with the technique described by Peluola *et al.* (2020) and incubated at 25 °C for seven days, with a photoperiod of 12 h of ultraviolet light (365 nm) and 12 h of white light (Britz *et al.*, 2001).

The shape and size of microconidia, macroconidia, apical cell shape, base cell shape, length, width, dorsiventral curvature, and number of septa were recorded. One hundred measurements of length and width of macroconidia and microconidia produced by each isolate were taken, as suggested by Robles-Yerena *et al.* (2016); García-Díaz *et al.* (2017); Rentería-Martínez *et al.* (2019).

A nursery trial was carried out to verify the pathogenicity of the isolates (Koch's postulates). The experiment was carried out in the forest nursery of INIFAP-Durango. We used rigid polyethylene containers with 98 cavities filled with a mixture composed of 50% peat moss, 25% expanded perlite, and 25% vermiculite with Osmocote® slow release (8 months) fertilizer of formulation 18-6-12 (N-P-K) at a dose of 4 g L⁻¹ of prepared substrate.

The size of the experimental unit was 18 cavities; two treatments (absolute control and inoculated treatment) with five replications were evaluated, and the design applied was completely randomized. Each fungal isolate was evaluated on the pine species from which it was originally obtained. Prior to sowing, the seed received a flotation treatment, was disinfested in a commercial sodium hypochlorite solution, and rinsed with sterile distilled water and left to dry for 10 min (Aoudou *et al.*, 2012).

The absolute control was sown after its pre-germination preparation by placing a seed 1 cm deep in each cavity and covered with substrate mixture. Regarding the seed for the inoculated treatment, the inoculation was carried out following the methodology of Morales-Rodríguez *et al.* (2018) with some modifications. Afterwards, one seed was deposited per cavity at a depth of 1 cm and covered with a mixture of substrate.

Daily germination counts were made from the first emerged seedling to 50 days after sowing (das). The variables evaluated were: germination percentage and germination index, with the following formulas (González-Zertuche and Orozco-Segovia, 1996).

$$GP = \frac{S_g}{S_s} * 100$$

$$GI = \frac{\sum (n_i t_i)}{N}$$

And

$$GS = \sum \left(\frac{n_i}{t} \right).$$

Where: GP= germination percentage; sg= germinated seeds; ss= seeds sown; GI= germination index; ni= number of seeds germinated at day i, ti= number of days after sowing and N= total seeds sown; GS= germination speed; and t= germination time from sowing to germination of the last seed. Also, from the time the first seed germinated, seedlings killed by damping-off were counted daily for 180 days. These damaged seedlings were removed from the growth cavity for transfer to the laboratory and transfer to PDA culture medium.

From the developed colonies, they were transferred to plates prepared for microculture using the methodology of Harris (1986); Rosana *et al.* (2014) and were incubated at 25 °C for six days. Microscopic observations were made to corroborate the growth and verify Koch's postulates and thus determine the percentage of damage in seedlings.

Germination and damage data were subjected to statistical analysis using the Wilcoxon test; the Log-Rank test was performed based on the Kaplan-Meier curves to determine the survival differences between treatments at 180 das. With the data on the percentage of damage of the isolates that presented significant differences compared to the control, disease progress curves and their fit to epidemiological, Gompertz, logistical, exponential, and monomolecular models were determined (Campbell and Madden 1990) in order to define the parameters of the equation and define the incidence of the disease. The statistical analysis was performed with the help of the InfoStat[®] software version 2017 (Di Rienzo *et al.*, 2017) and graphs in SigmaPlot[®] version 10.0.

Results and discussion

Seedlings of these species were found with the symptoms characteristic of damping-off: prostrate with generalized loss of turgor, constriction, and necrosis in the neck. Fungal isolates were obtained from these seedlings, and seven of the isolates showed macroscopically the colonial morphology of the genus *Fusarium*: dry surface and cotton-like mycelium and production of pale pink to purplish-pink pigments (Nelson *et al.*, 1983; Watanabe, 2010).

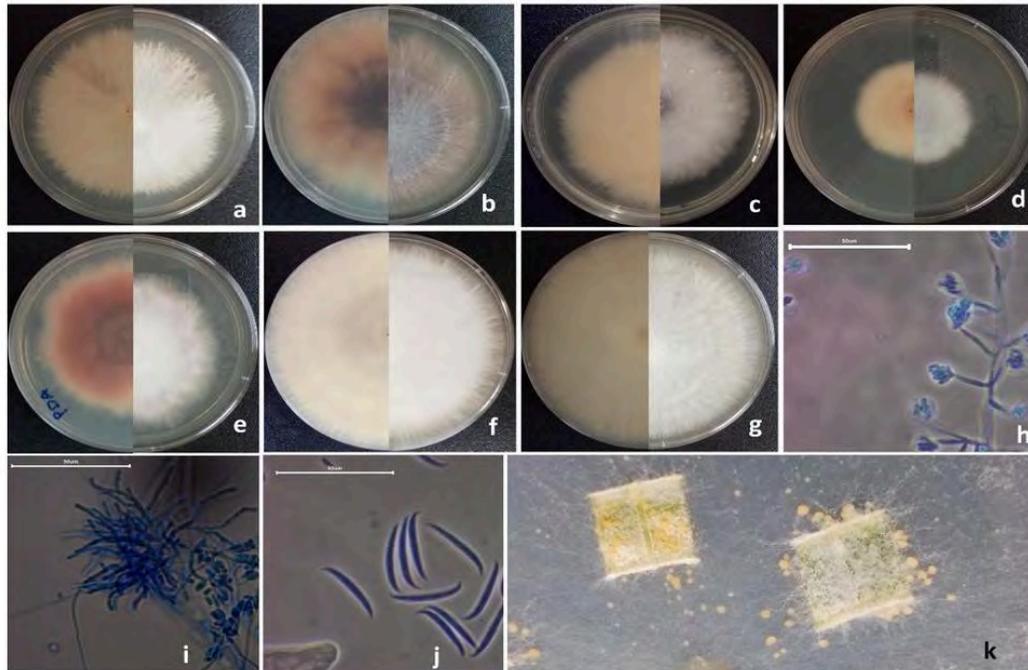
The isolates obtained from the cultivated species during the sampling of the present study coincide with the findings of Menkis *et al.* (2006), in which fungi of the genus *Fusarium* were found colonizing roots of seedlings of *Pinus sylvestris* and *Picea abies* and no species of the genera *Rhizoctonia*, nor oomycetes of the genera *Pythium* and *Phytophthora* were found.

There were variations between these isolates, such as elevation of the colony (flat, umbonate, elevated, and convex) and the arrangement of the hyphae in the mycelium (inclined, slightly inclined, and erect). It was decided to separate these fungal isolates and assign them an ID according to these differences and the species of pine from which they were isolated. Isolate 1 was obtained from *Pinus engelmannii* (FPe1), isolate 2 from *Pinus engelmannii* (FPe2), isolate 3 from *Pinus engelmannii* (FPe3), isolate 4 from *Pinus durangensis* (FPd1), isolate 5 from *Pinus arizonica* (FPa1), isolate 6 from *Pinus arizonica* (FPa2), and isolate 7 from *Pinus cooperi* (FPc1).

When the isolates were cultured in SNA medium, the formation of ovoid-shaped microconidia without septa was observed, and these produced in false heads on monophialides and polyphialides (Figure 1). The average size of the microconidia was 6.54 μm long by 2.13 μm wide. Of these isolates, FPe1, FPe3 and FPd1 stand out for the development of coiled hyphae that did not develop in the other isolates. Isolates FPe2, FPa1, FPa2 and FPc1 produced terminal and intercalary chlamydospores when cultured in PDA, a detail that was not present in the other isolates.



Figure 1. a) isolate 1; b) isolate 2; c) isolate 3; d) isolate 4; e) isolate 5; f) isolate 6; g) isolate 7; from a to g, the left side is the reverse side and the right side is the front of the plate; h) conidiophores produced by isolate 3 in SNA medium; i) coiled hyphae produced by isolate 4 in SNA medium; j) macroconidia produced by isolate 6; scale bar 50 μm ; and k) sporodochia produced by isolate 7.



In the CLA medium, three isolates produced yellow to orange sporodochia (FPa1, FPa2, and FPc1), spindle-shaped macroconidia were visualized, with moderate dorsiventral curvature (Figure 1), with the base cell in the shape of a foot and the apical cell in a curved shape, with two and three septa in FPa1, FPa2, and three to four septa in FPc1. The size of the macroconidia of FPa1, FPa2 was 37.62 μm long by 3.3 μm wide, in FPc1 the size of the macroconidia was 23.63 μm long by 2.53 μm wide.

Given the macroscopic and microscopic characteristics of the isolates obtained and their morphological similarities with two *Fusarium* species complexes mentioned in the *Fusarium* laboratory manual (Leslie and Summerell, 2006), it was suggested that isolates FPe1, FPe3 and FPd1 corresponded to the characteristics of the *Fusarium fujikuroi* species complex and for isolates FPe2, FPa1, FPa2 and FPc1, they corresponded to the *Fusarium oxysporum* species complex.

The studies reported by Robles-Yerena *et al.* (2016); García-Díaz *et al.* (2018) are those that have been carried out in Mexico on the finding of *Fusarium* species that produce wilting problems in pine seedlings in the nursery stage and some are similar to some isolates in the present research.

After 1998, the description of new species increased, based on the recognition that several traditional morphospecies are species complexes composed of many species (Summerell and Leslie, 2011); for example, the species complex of *F. fujikuroi* contains 65 accepted species and includes some species that can only be identified based on their phylogenetic inference by molecular biology (Yilmaz *et al.*, 2021); in addition, as mentioned by Crous *et al.* (2021) other lineages of Nectriaceae produce conidia with morphology similar to *Fusarium* and therefore the recognition of *Fusarium* species only with morphological traits is very complicated, which is why in the present work only concordances in the morphology of the obtained isolates to the *F. fujikuroi* (FFSC) and *F. oxysporum* (FOSC) complexes are suggested.

In most of the treatments inoculated with the isolates, there were no differences compared to the control in the variables of germination, germination index and germination speed, so it is confirmed that they did not cause pre-emergent damage and there was an average germination between 80 and 95% in them, only in the species *P. arizonica*, although there was no pre-emergence damage

compared to the control, the average germination was 34 to 45%, possibly because the cultivation of this particular species is carried out in the north of the state of Durango, a location of more northern latitude than in the place where the nursery experiment was carried out.

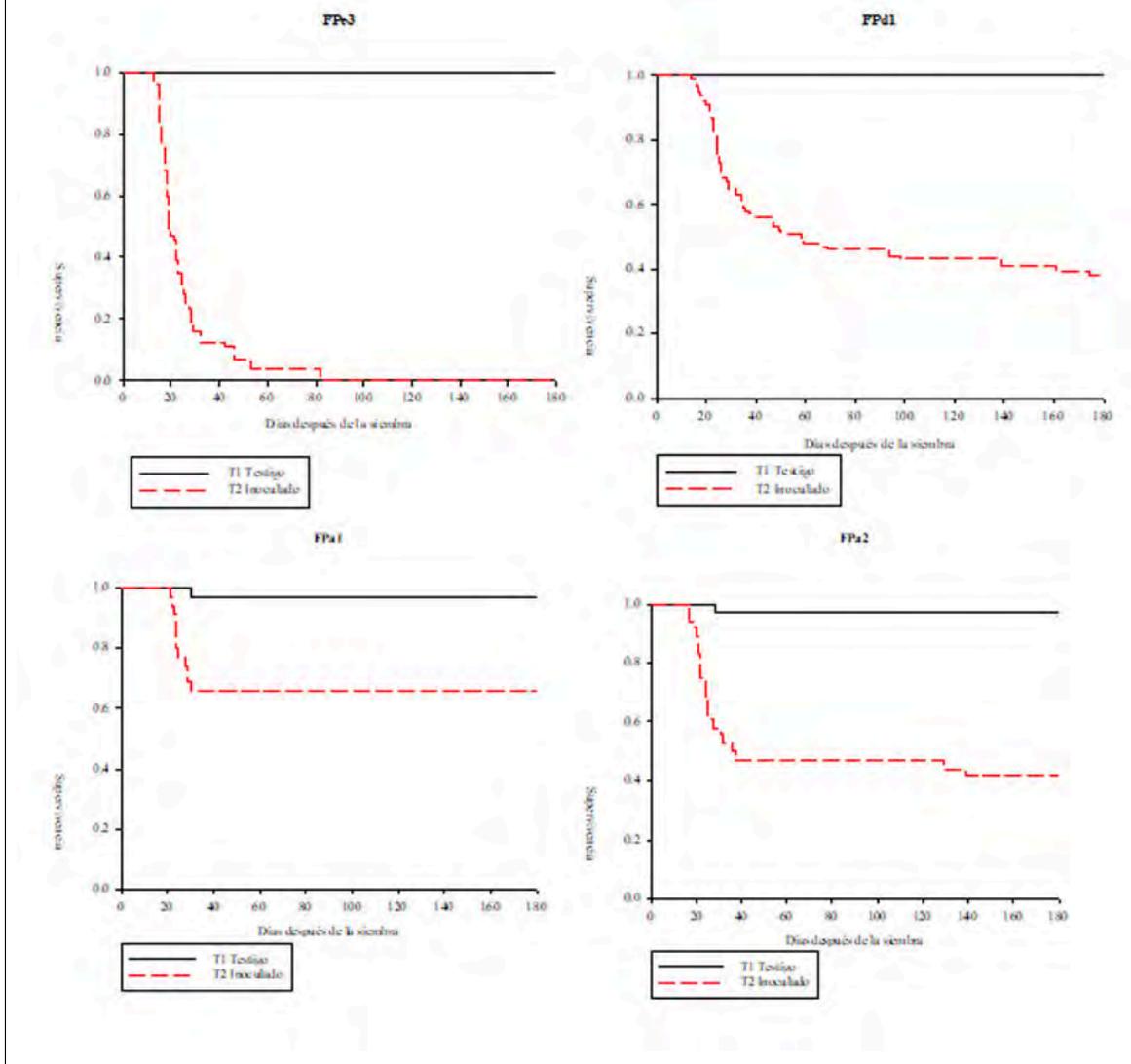
In germination, there were only differences in a treatment inoculated in the species *P. engelmannii* (FPe3). In this, the germination percentage in the control was 90% and in the inoculated treatment it was 63%; that is, germination decreased by 27%, indicating that this isolate was the only one that caused pre-emergent damage and this was confirmed by the results obtained in the germination index analysis, since it also presented significant differences in this variable. Only in *P. durangensis* were there differences in the germination speed variable between the control and the inoculated treatment.

The accumulation of damaged seedlings was concentrated from 13 das to 40 das (week 2 to week 7). In the damage variable, the treatments inoculated with isolates that did not present differences compared to the control were FPe1, FPe2 and FPc1, so that they are considered to be the least virulent.

On the other hand, the treatments inoculated with the isolates that caused significant damage compared to the control, that is, the highest percentage of post-emergent damage in ascending order were: FPa1 (32.2%), FPd1 (60.7%), FPa2 (61.1%), and FPe 3 (100%). Isolate FPe3 (isolate 3) is considered to be the most virulent since it accumulated the largest number of damaged seedlings in the shortest time (Figure 2).



Figure 2. Kaplan-Meier survival curves of *Fusarium* isolates that showed significant differences compared to the control.



These results coincide with those described in other studies that have analyzed the pathogenesis of *Fusarium* in other pine species, such as those of Davydenko *et al.* (2018), who found that *F. circinatum* caused the death of almost all seedlings of *Pinus sylvestris* of different origins and *F. oxysporum* caused considerable damage, of 62-66% by day 21 after inoculation.

On the other hand, Mateos *et al.* (2017) found that *F. oxysporum* presented a higher index of damage compared to *F. verticillioides* in *Pinus sylvestris* and *Pinus pinea*. On the other hand, Lazreg *et al.* (2014) found that *F. redolens* and *F. solani* cause the most damage in pine seedlings, followed by *F. tricinctum* and *F. oxysporum* in *Pinus halepensis*.

In this essay, the first few weeks were key to pathogen development and seedling death. This period is the critical one to carry out the preventive and curative phytosanitary treatments necessary to stop the development of the disease. This is related to what was expressed by Davydenko *et al.* (2018), who indicated that greenhouses with plants infected with *F. circinatum* are the first point of entry of the disease and are a source of transmission of pine pitch canker in commercial forest plantations.

The epidemiological model that presented the best fit for the behavior of isolate FPe3 was the Gompertz model, represented by the expression $\text{damage} = \alpha \cdot \exp(-\beta \cdot \exp(-\gamma \cdot \text{DAS}))$, in which the parameters made a significant contribution ($p < 0.05$) and its parameters were α 98.36, β 15.36 and γ 0.15. Also for isolate FPd1, the best fit was shown by the Gompertz model and its parameters were α 55.78, β 7.27 and γ 0.08.

On the other hand, for the isolates obtained from *P. arizonica*, that is FPa1 and FPa2, the model that presented the best fit was the monomolecular model, represented with the expression $\text{damage} = \alpha \cdot (1 - \beta \cdot \exp(-\gamma \cdot \text{DAS}))$; for FPa1, the parameters were α 55.78, β 7.27 and γ 0.08; and for FPa2, the parameters were α 55.78, β 7.27 and γ 0.08.

In this context, Ocamb *et al.* (2002) mentioned that variation in *Fusarium* populations can be influenced during handling and sowing and this fungus can be introduced during seed storage or handling and they recommend pathogenicity tests to really know the potential for damage of *Fusarium* species isolated from seedlings.

Conclusions

It was possible to isolate fungi from seedlings with damping-off symptoms in nurseries in the state of Durango and these corresponded to isolates of the genus *Fusarium* from the *F. fujikuroi* and *F. oxysporum* species complexes according to their morphological traits. The most virulent isolates were obtained from *Pinus engelmannii*, *P. durangensis* and they fitted the Gompertz model, while the isolates obtained from *P. arizonica* fitted better the monomolecular model. It is necessary to carry out molecular identification of these isolates in order to know in detail the species of *Fusarium* that cause damping-off in pine seedlings that are produced in nurseries in the state of Durango.

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