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Etiology of rhizome rot of asparagus (*Asparagus officinalis*) in Atenco, Mexico State

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ABSTRACT

Objective/Background. The objective of this research was to identify the causal agent of asparagus rhizome rot, as well as evaluate different inoculation methods and the severity of the isolates.

Materials and methods. Sampling was carried out in five producing plots Atenco, Edo. from Mexico. Five isolates of *Fusarium* spp. were selected. (one per plot) to perform pathogenicity tests. Three isolates were selected for their colonization characteristics for severity tests with different inoculation methods: Immersion for 12 h, immersion for 30 min and inoculation by contact with absorbent paper soaked in 1 mL of inoculum. Concentrations of $1x10^6$ conidia mL-1 were used. 10 rhizomes were used per treatment and 10 rhizomes without inoculation. To determine the severity, photographs (in GIMP®) of the rhizome were analyzed seven days after inoculation. The isolates were molecularly identified with ITS4/ITS5, EF688/EF1521 and TUBT1/BT2B.

Results. *Fusarium prolifetatum* was morphologically and molecularly identified in the three isolates. The P3DR isolate was the most severe (14.6%), followed by P5DR (13.9%) and P1SIR (11.6%).

Conclusion. The most effective inoculation method was immersion for 30 min. They were registered in the NCBI Gene Bank with accessions ON738484 (P3DR), ON973801 (P5DR) and ON738483 (P1SIR). This is the first report of *F. prolifetatum* in the Edo. from Mexico.

Keywords: *Fusarium proliferatum*, asparagus, severity, inoculation.

Introduction

The asparagus (*Asparagus officinalis*) is a vegetable, originally from Eastern Europe, Caucasus and Siberia (Faostat, 2019). However, it is a cash crop, since more than 90% of the production of this vegetable is exported to the United States of America and Europe. The economic value of the national production in 2018 rose to 7,7 billion pesos, after producing 206, 225 t (SIAP, 2020). Sonora (129,808 t), Guanajuato (27,146 t), Baja California (27,070 t), Baja California Sur (16,917 t) and Querétaro (5,284 t) are the main producers of this vegetable and they concentrated 99% of the national export supply (SIAP, 2022).

Asparagus is a perennial, diploid monocotyledonous plant, whose commercial part is the shoots that develop from the buds located in the apical part of the rhizome (Moreno-Pinel *et al*., 2021). Once the shoots reach a commercial length, they are cut with a blade by making an incision between the base of the shoot and the rhizome, causing wounds on the plant, making it vulnerable to the entrance of pathogens (Moreno-Pinel *et al*., 2021). Within the *Asparagus* genus, only this species is economically important (Faostat, 2019).

Despite its importance, the crop is affected in all its phenological stages by the rotting of the rhizome caused by species of the *Fusarium* genus. The incidence of this pathogen is reflected in the development of shoots (Quilambaqui *et al*., 2004). *Fusarium proliferatum* is the most important species reported as a pathogen worldwide, and its importance lies in the severity with which it attacks the rhizome of susceptible genotypes (Elmer, 1996; Moreno-Pinel *et al*., 2021). In addition, *Fusarium* spp. Has been reported as a producer of toxins found in marketable asparagus shoots. The presence of these toxins such as B1, B2 and B3 Fumonisins poses a risk to human health and are found in shoots produced in diseased plants (Seefelder *et al*., 2002; Liu *et al*., 2007).

In the *ejido* center of the municipal area of Atenco, State of Mexico, the asparagus crop has gained significant importance due to its profitability and adaptation to the soil and climate conditions of the area. The crop currently covers 20 hectares, all of them with varying ages, levels of technology and management practices. However, in all the production areas, problems have arisen regarding the rotting of the rhizome, resulting in direct economic losses for farmers. Atenco is an area with the potential to expand asparagus cultivation, and due to the increase in the incidence of this disease, this study was undertaken with the aim of identifying the causal agent of the rotting of the asparagus rhizome, evaluating its pathogenicity with different inoculation methods and evaluating the severity of the isolates obtained.

Collecting plant tissue. Five plants were sampled in every plot (across five plots) that displayed symptoms of decline, wilting and yellowing during the flowering-fruiting phenological stage in the municipal area of Atenco, State of Mexico.

Samples were collected using a shovel disinfested with alcohol at 70%, partially unearthing the rhizome and cutting approximately 50 to 10 cm in diameter. All sampling sites had the same genotypical variables and low technological inputs. A handheld Garmin Reach Explorer® GPS navigator was used to georeference each sampling point. The samples were labelled, registered with an I.D. (Table 1), packed in poly-paper bags and transported in a cooler for processing in the Laboratorio de Fitosanidad – Fitopatología (Plant Health – Plant Pathology Lab) of the Colegio de Postgraduados *Campus* Montecillo, State of Mexico.

Table 1. Asparagus plots sampled with symptoms of decline and wilting with low technology (gravity irrigation and without mulching) inorganic agronomic management and the phenological stage of flowering-fruiting, in Atenco, State of Mexico.

I.D.	Plot	Coordinates	Age (years)	Genotype
P1SIR	San Indalecio	19° 33” 24′ N 98° 55” 54′W	3	Sulken
P2DR	Diamante	19° 33” 01′ N 98° 56” 14′ W	5	Sulken
P3DR	Diamante	19° 33” 02′ N 98° 56” 15′ W	6	Sulken
P4DR	Diamante	19° 33” 03′ N 98° 56” 15′ W	6	Sulken
P5DR	Diamante	19° 33” 10′ N 98° 56” 10′W	6	Sulken

Isolation and purification. The rhizome tissues were washed with sterile demineralized water to eliminate any excess soil. Each rhizome sample was cut into 1 × 1 cm pieces using a previously disinfested scalpel with the aid of a Iroscope® YZ-6 microscope. Subsequently, the tissue was disinfested with 1% sodium hypochlorite for 2 min, washed with demineralized water three times and transferred to Petri dishes with Potato-Dextrose-Agar (PDA) from Difco Laboratories®. For every plant (five plants per plot), six pieces of rhizome were planted per dish. The Petri dishes were incubated for two days at 28 °C in a Riossa® Series-33 incubator. When mycelial growth was observed, they were purified using the hyphal tip method in new Petri dishes with PDA medium. From the mycelial growth using the hyphal tip method, monosporic cultures were obtained. By scraping with a curved dissection needle, the mycelial growth from the hyphal tip was transferred to a 50 mL Falcon tube with sterile demineralized water, shaken using a Vortex WiseMix® and the conidia were counted using a Neubauer hematocytometer. Later, dilutions were carried out, up to 1 × 10^6 conidia mL^{-1}. From the last suspension, 100 µL were taken and poured into a Petri dish with PDA and distributed with a sterile Digralsky

loop, incubated at 28 °C until individual mycelial growth was observed, to obtain monosporic growths.

Morphological identification. The morphological identification was carried out using the taxonomic keys reported by Leslie and Summerell (2006) and Booth (1988). The isolates were planted in a Carnation-Agar culture for the production of macroconidia. Later, fixed preparations were made to be observed under a compound microscope (10x and 40x Velab VE-B2) integrated with the Program Motic Images Plus v2.0. The mycelial coloring was established, along with the conidial morphology and morphometry in 60 conidia per isolate aged 7 days after planting.

Pathogenicity tests. Inoculations were carried out in 3-month-old Sulken asparagus seedlings. Five *Fusarium* spp. inoculates were isolated at a concentration of $1x10^6$ conidia mL^{-1} with three repetitions per treatment. There were non-inoculated rhizomes (controls) from each isolate. Three inoculation methods were established:

Immersion in test tubes 1. The root areas of the seedlings were submerged in test tubes containing 50 mL of 1×10^6 of a conidial suspension for 30 min. (Modified from Farahani-Kofoet *et al.*, 2020).

Immersion in test tubes 2. The same methodology described above was carried out, but in a volume of 10 mL of a conidial suspension and it was introduced in the rhizome for 12 h.

Inoculation by filter paper. Whatman No. 4 filter paper circles, each one measuring 5 mm in diameter, were soaked in a 1×10^6 conidial suspension for 2 min. Subsequently, the filter paper circles were placed on the apical area of the rhizome without lesions. The rhizomes were evaluated 7 days after inoculation. The culture was then observed under a stereoscopic microscope and only three isolates were reisolated which, due to their colonization characteristics, were evaluated to determine severity.

Severity evaluation. The same inoculation methodology described above was followed, with 10 repetitions per treatment and control. Photographs were taken of the most affected area of each repetition. The images were taken using a Canon® (Reflex EOS REBEL T7, 20.2 mp) camera. A total of 120 photographs were processed in GIMP® v2.10, to quantify the total area in pixels (At) and damaged area (Ad) to determine the severity. The percentage of severity was obtained using the following area:

$$\text{Severity} = \frac{(Ad)\ (100)}{At}$$

The data were analyzed using an ANOVA in the SAS 9.4 software to determine the most severe isolate and the most effective inoculation method, according to the means analysis using Fisher's LSD method (Abramoff *et al*., 2004).

Molecular identification and phylogenetic analysis. DNA was extracted from three isolations using the CTAB technique at 2% (Minas *et al*., 2011). The concentration and purity of the nucleic acids were determined using spectrophotometry with NanoDrop 2000 (Thermo Fisher Scientific 2000, U.S.A.). The species was confirmed molecularly by PCR with the primers ITS 4/ITS5, EF688/EF1521 and TUB T1/BT2B (Table 2) in a T-100 (BioRad) thermocycler with a final sample volume of 15 µL composed of: 7.86 µL of HPLC water, 3 µL of 5X PCR buffer, 0.6 µL of dNTP´s, 0.18 µL of the primers (10 µM) (Sigma-Aldrich, U.S.A.), 3 µL of genomic DNA (20 ng) and 0.18 µL (2U) of GoTaq® polymerase DNA (Promega, U.S.A.) (Table 2). The amplified fragments were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and viewed with UV light in a

Table 2. Primers used, sequence, region of the amplified gene, amplicon size, and PCR conditions for the genomic identification of *Fusarium* species.

Primers	Sequence	Region	pb	PCR conditions	Reference
ITS 4 ITS 5	5´-TCCTCCGCTTATTGATAT 5´-GGAAGTAAAAGTCGTAAC	Internal transcribed spacer region rRNA	565	[z]ID: 95 °C 4 min 35 cycles D: 95 °C 1min A: 58 °C 1min E: 72 °C 2min EF:72 °C 10 min	White *et al*., 1990
FE688 FE1521	5´-CGGTCACTTGATCTACAA 5´-CCTCGAACTCACCAGTAC	Translation elongation factor 1-α	650	ID: 95 °C 3 min 35 cycles D: 95 °C 35 s A: 59 °C 55s E: 72 °C 1:30 min EF:72 °C 10 min	Alves *et al*., 2008
TUB T1 BT2B	5´AAYATGATIACIGGIGCIGCI 5´-ACCCTCAGTGTAGTGACC	β-tubulin Constitutive protein	560	ID: 95 °C 5 min 35 cycles D: 95 °C 1 min A: 58 °C 1 min E: 72 °C 1:30 min EF:72 °C 5 min	O'Donnell *et al*.,1998

[z] Initial denaturalization (ID), Denaturalization (D), Alignment (A), Extension (E) and Final extension (FE).

photo documenter (UVP, Biolmaging Systems, Epi Chemi II Darkroom). The PCR products were sent to be sequenced to Macrogen®, Korea. The sequences were cleaned and edited with the program BIOEDIT® for comparison in the National Center for Biotechnology Information (NCBI) database.

For the phylogenetic analysis, a classification was performed using UPGMA (Unweighted Pair-Group Method with Arithmetic), using the standard MEGA 1 parameters. The sequences of *F. verticillioides* (EU364864.1) y *F. fujikuroi* (CP023090.1) were chosen as reference species for the creation of the phylogenetic tree.

Morphological identification. The symptoms observed on the field were general wilting and yellowing (Figure 1 A-B). When making a transverse cut at the base of the stem, necrosis and blockage of vascular bundles were observed (Figure 1 D-F). A similar growth was observed in all the rhizome tissue plantations in the five plots; therefore, one isolation was selected from every field. In a PDA medium, isolations P1SIR and P5DR displayed a radial growth with whitish-yellow shades in the front and yellowish-brown tones on the back. Isolation P3DR displayed a mycelium with a white tone, cottonlike characteristics and radial growth. Ten days after planting, all isolates displayed the presence of monophialides and polyphialides, along with false heads or aggregates of microconidia with flat bases, and chains of microconidia without chlamydospores were also observed. The macroconidia proliferated in the Carnation-Agar medium, where the morphometry was 26.47 µm x 4.43 µm (P1SIR), 25.97 x 4.92 µm (P3DR) and 26.21 µm x 4.76 µm in length and width (P5DR). All macroconidia displayed a curved apical cell and a foot-shaped basal cell, with three to five septa. According to Leslie and Summerell (2006) and Booth (1988), this fungus complies with the characteristics of *Fusarium proliferatum,* and it was reported as a facultative parasite, causing similar symptoms to those reported by Seefelder *et al*. (2002) y Liu *et al*. (2007).

Molecular identification. The products of the DNA extraction obtained concentration thresholds of 186.8 - 544 ng μL^{-1} and purity of 1.38 - 2.22 nm. The alignment of the three sequences in the GenBank of the NCBI confirmed the identification of *F. proliferatum* with a homology of 99 - 100%. The isolations were registered in the GenBank with accessions ON738483 (P1SIR), ON738484 (P3DR) y ON973801 (P5DR). The presence and prevalence of *F. proliferatum* as the main pathogen in asparagus has been reported in the main asparagus producing states (Quilambaqui *et al*., 2004; Camacho-Aguiñiga *et al*., 2016). The incidence of this pathogen in the rhizome, as well as its prevalence in production areas in the municipal area of Atenco may be due to the mobilization of propagational material for the establishment of new asparagus producing plots.

Figure 1. Symptoms of wilting in 6-year-old asparagus crop. A) Asparagus crop with symptoms of generalized decline; B) Plant with symptoms of generalized wilting; C) Plant with symptoms of initial wilting; D) Rotting of the rhizome caused by *Fusarium proliferatum*; E) Basal part of the stem with symptoms of F) Basal part of the stem with vascular bundle blockage.

On the other hand, the creation of the phylogenetic tree helped group the three sequences in one same group, according to the homology in them, and left the sequence of *F. verticillioides* (EU364864.1) as an external clade. This species was distant from the main node of *F. proliferatum* as opposed to *F. fujikuroi* (CP023090.1), which appeared in the same clade (Figure 2). The distance and organization between clades coincide with the proposal by Aoki *et al*. (2014) and Mendoza-Ramos *et al*. (2021) since, in the phylogenetic tree that these authors presented, *F. proliferatum* belongs to the *F. fujikuroi* complex. However, it is

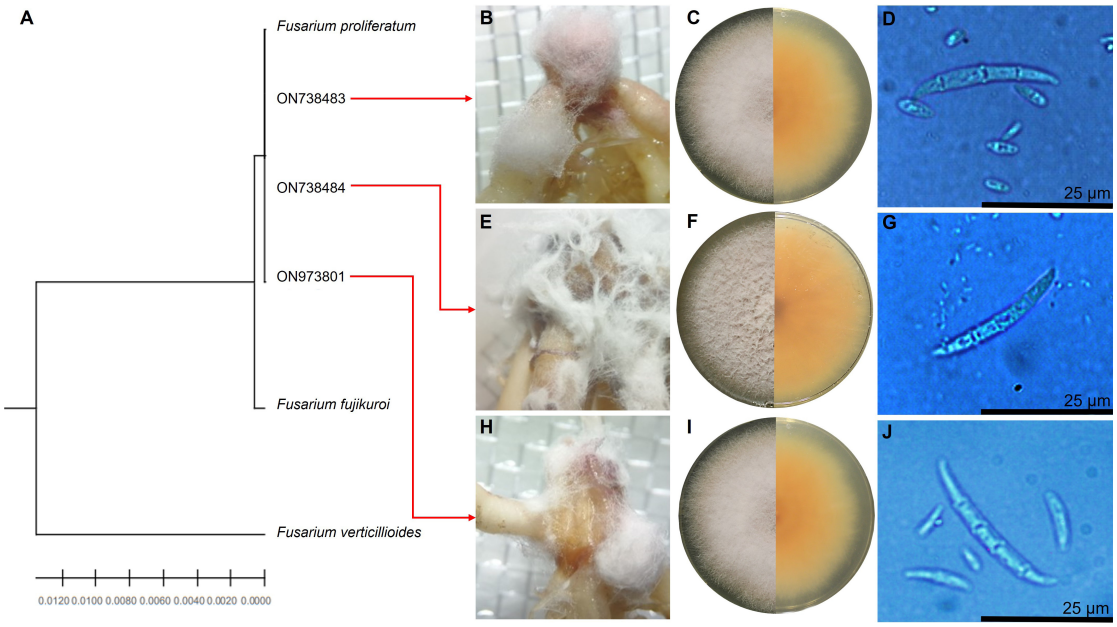

Figure 2. *F. proliferatum* isolations used for the pathogenicity tests via different inoculation methods. A) Concatenated phylogenetic tree of isolates ON738483 (PISIR), ON738484 (P3DR) and ON973801 (P5DR) with the primers ITS 4-ITS5, EF688-EF1521, TUB T1-BT2B. B) Rhizome colonization by isolate P1SIR, C) Mycelial growth of *Fusarium* on the front and back of PDA medium after 7 days of growth, D) Macro and microconidia at 40X. E) Rhizome colonization by isolation P3DR, F) Mycelial growth of *Fusarium* on the front and back of PDA medium after 7 days of growth, G) Macro and microconidia at 40X. H) Rhizome colonization by isolate P5DR, I) Mycelial growth of *Fusarium* on the front and back of PDA medium after 7 days of growth, J) Macro and microconidia at 40X.

important to point out that *F. proliferatum* makes up a diverse evolutionary group, therefore further studies may be required (Leslie and Summerell, 2006).

Pathogenicity tests. With the inoculation method using filter paper, the inoculant was found to be distributed only in the area in which the rhizome was in contact with the paper (Figure 3 B). This result coincides with reports by Cardona-Piedrahita and Castaño-Zapata (2019), who used agar discs with *Fusarium oxysporum* f. sp. *lycopersici* mycelial growth in contact with the root of *Solanum lycopersicum*. The inoculation method by immersion for 30 min displayed an even mycelial growth with whitish tones concentrated in the rhizome, since this tissue is an important reservoir of carbon sources with a high content of carbohydrates, thus explaining the colonization of *F. proliferatum* concentrated in the rhizome since, despite the nutrients found in the root, the rhizome, because it is an organogenesis site, contains higher levels of carbohydrates and amino acids (Farahani-Kofoet *et al*., 2020).

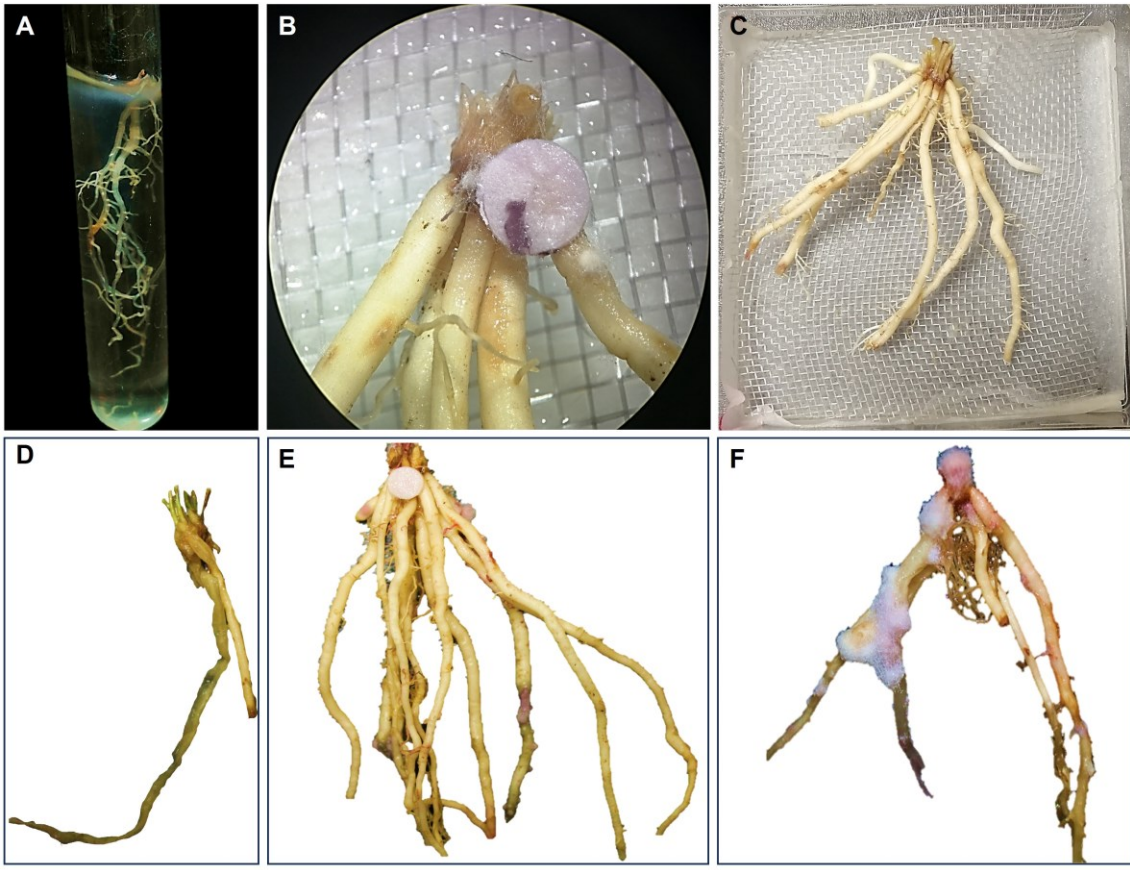

Figure 3. Inoculation methods with *Fusarium* isolations in asparagus rhizomes. A) Immersion of rhizome for 12 h; B) Inoculation with filter paper; C) Immersion of rhizome for 30 min, showing the rhizome suspended by disinfected mesh and moist filter paper inside a sterile square box; D) Rhizome by the immersion method for 12 h E) Rhizome by the inoculation method with filter paper F) Rhizome by the immersion method for 30 minutes.

In the method of immersion in test tubes for 12 h, the same rhizome-centered colonization took place. However, it presented a fermentation activity derived from the absence of oxygen and therefore a loss in the structure of the rhizome's cell wall due to hydrolysis (Corrales *et al*., 2015), leading to a maceration of the epidermis. In this method, maceration interfered with the colonization of the *F. proliferatum* isolates (Figure 3 D).

Determining the severity. The comparison of the means of the different inoculation methods showed that the presence of necrosis and colonization of the rhizome was expressed in the method of inoculation for 12 h (17.5%), followed by the method of immersion for 30 min (16.1%), filter paper (6.5%) and control (0.3%). The latter

method presented a natural oxidation of the rhizome due to a lack of an edaphic medium (Figure 4).

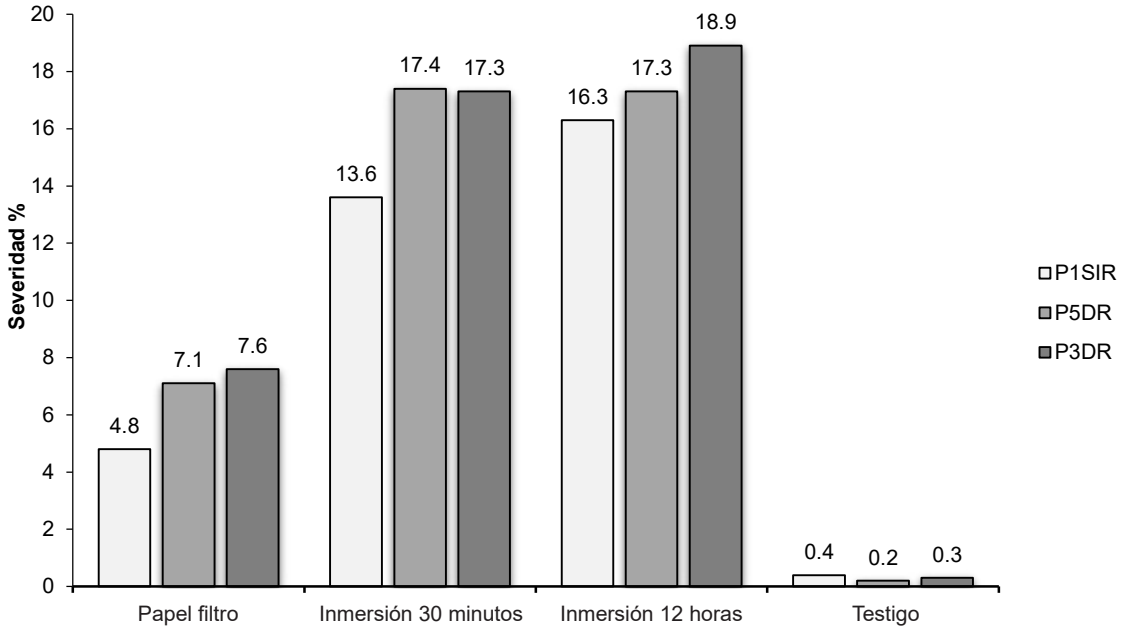

Figure 4. Percentage of severity of three *F. proliferatum* isolates in asparagus under three inoculation methods, and a control, not inoculated.

The results indicated that isolation P3DR was the most severe (7.6% in filter paper and 18.9% by the method of immersion for 12 h) out of all of the methods evaluated, surpassing P5DR and P1SIR by an average of 3.0 and 0.7% (Figure 4). Ángel-García *et al*. (2018), evaluated different *Fusarium solani* f. sp. *passiflorae* inoculation methods in passionfruit seedlings (*Passiflora edulis*), where they obtained 100% severity of passionfruit damping-off 6 days after inoculation using the immersion method. In this investigation, the severity obtained was below 19%. However, the potential for the isolations to inoculate and cause symptoms was confirmed, thus confirming the pathogenicity of the three *F. proliferatum* isolations gathered from asparagus producing plots in Atenco, State of Mexico. The colonization in the asparagus rhizomes in this experiment coincided with reports by Stephens and Elmer (1988) and Farahani-Kofoet *et al*. (2020).

According to the analysis of means with Fisher's LSD method, the immersion treatments (30 min and 12 h) were grouped in one block and the treatment with filter paper and the control on another one. On the other hand, among the *F. proliferatum* isolates, P5DR and P1SIR were grouped in one block, whereas P3DR

was placed in another group. This is confirmed in Figure 4, where a lower severity is confirmed for the latter isolate in comparison with the P5DR and P1SIR isolates. The variation in severity has been reported as a pathogenesis factor, mentioning that this component is closely related to the production of enzymes that degrade the cell walls of the plant as a pathogenesis factor to be in contact with the carbon sources of the rhizome (Farahani-Kofoet *et al*., 2020).

Isolate P3DR was determined to be the most severe, according to the colonization and determination of pixels observed in the experiments of inoculation methods. Although the inoculation methods used in this study induced the colonization of *F. proliferatum* in the rhizomes, the method of immersion of rhizomes for 30 min was the most effective.

The isolates gathered in this study were registered with accession ID numbers ON738484 (P3DR), ON973801 (P5DR) and ON738483 (P1SIR) in the NCBI gene bank.

Applying morphological, genomic and pathogenic criteria *Fusarium proliferatum* was identified as the causal agent of the rotting of the asparagus (*Asparagus officinalis*) rhizome in the genotype Sulken in the municipal area of Atenco, State of Mexico. This is the first report of this fungus in the State of Mexico. This information will help operate under the principle of prevention and implement management measures in asparagus-producing plots in the area.

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