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FREQUENCY AND PATHOGENICITY OF *Fusarium* SPECIES IN WHEAT SPIKE: PRELIMINARY RESULTS

María Guadalupe **Gómez-Espinoza**[1*], Alda Alejandra **Arratia-Castro**[2], Miguel Ángel **Guzmán-Altamirano**[3], Raúl **Rodríguez-Guerra**[4]

[1]Universidad Politécnica de Pénjamo. Departamento de Ingeniería Agroindustrial. Carretera Irapuato-La Piedad km 44, El Derramadero, Pénjamo, Guanajuato, Mexico. C. P. 36921.
[2]Universidad Politécnica de Pénjamo. Departamento de Ingeniería en Biotecnología. Carretera Irapuato-La Piedad km 44, El Derramadero, Pénjamo, Guanajuato, Mexico. C. P. 36921.
[3]Tecnológico Nacional de México/ITS Irapuato. División de Ingeniería en Mecatrónica. Carretera Irapuato-Silao km 12.5, Col. El Copal, Irapuato, Guanajuato, Mexico. C. P. 36821.
[4]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Región Noreste. Campo Experimental General Terán. Carretera Montemorelos-China km 31, General Terán, Nuevo Leon, Mexico. C. P. 67400.
* Author for correspondence: g.gomez@uppenjamo.edu.mx

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ABSTRACT

Wheat (*Triticum aestivum*) is a commercially important cereal in Mexico; however, there have been reports of head blight in Guanajuato, Mexico, which have been attributed to *Fusarium* species. This study hypothesized that wheat head blight is caused by several species of *Fusarium*. Therefore, the objective was to evaluate the frequency and pathogenicity of *Fusarium* species in wheat spike in the state of Guanajuato. For this purpose, plots sown with wheat in the 20 wheat-producing municipalities were sampled. The strains were isolated until pure cultures were obtained, and they were morphologically and molecularly characterized; the sequences obtained were registered in GenBank. The pathogenicity and severity of the disease were assessed on flowering wheat plants of the Cortázar S94 variety at the flowering stage. The results were evaluated 10 days after inoculation and the data were analyzed using ANOVA statistical analysis. Ten *Fusarium* species were found, with *F. oxysporum* (35.57 %), *F. verticillioides* (12.5 %), and *F. proliferatum* (11.53 %) being the most common. All *Fusarium* strains were pathogenic, with *F. oxysporum* being the most severe, followed by *F. proliferatum*. In conclusion, it was possible to identify the causal species of wheat head blight in the state of Guanajuato.

Keywords: head blight, Mexico, Guanajuato.

INTRODUCTION

Wheat (*Triticum aestivum*) is a cereal with global importance which has become an important source of food (Igrejas and Branlard, 2020). In Mexico, the states with the highest production are Sonora (43 %), Baja California (11 %), Guanajuato (9.3 %), Sinaloa (8.2 %), Michoacán (9.3 %), and Chihuahua (2.3 %) (Flores-Márgez

et al., 2021). However, it has recently been reported that global wheat production and food security is threatened by *Fusarium* head blight (FHB) (Petronaitis *et al*., 2021). This disease is characterized by early senescence and a decrease in grain quantity and quality. Because this genus of fungi produces mycotoxins, the problem is widespread in the agro-industrial sector (Palacios *et al*., 2021).

Mycotoxins are almost unavoidable in food and are highly dependent on climatic conditions, making control difficult, if not impossible. Mycotoxins can adversely affect both human and animal health (Ekwomadu *et al*., 2021). These metabolites can cause diarrhea, vomiting, leukocytosis, and gastrointestinal bleeding at low doses, but can cause cancer, immunosuppression, endocrine disruption, and death at high and/or chronic doses (Kimura *et al*., 2007). The mycotoxins found in grains depend on the *Fusarium* species; in this sense, it has been reported that the *Fusarium* species causing FHB may vary depending on the region. For example, in Finland, *F. culmorum, F. graminearum, F. poae, F. sporotrichioides, F. langsethiae,* and *F. avenaceum* were found to be responsible for this disease (Hietaniemi *et al*., 2016), whereas in Italy the disease is attributed to *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. avenaceum*, *F. proliferatum,* and *F. sambucinum* (Beccari *et al*., 2018).

In the Mixtec region in Mexico, this disease has been attributed to *F. boothii*, while in the center of the country it is caused by *F. avenaceum* and *F. tricinctum* (Cerón-Bustamante *et al*., 2018). The hypothesis is based on the possibility of isolating multiple species of *Fusarium* in wheat fields in Guanajuato as a causal agent of wheat head blight (FHB). The goal of this work is to evaluate the frequency and pathogenicity of *Fusarium* species in wheat spike in Guanajuato.

MATERIALS AND METHODS

Collection and isolation

During the 2018 cycle, 81 plots with early senescence were sampled from 20 wheat producing municipalities in the state of Guanajuato. The latitude and longitude data were represented on the map (Figure 1) using the QGIS program version 3.30.2"s -Hertogenbosch' (https://qgis.org/es/site/index.html). Subsequently, the fields with the highest incidence of the disease were identified and selected for this study.

For isolation, 10 glumes were removed from each plant, disinfected with 3% sodium hypochlorite for one minute, washed three times with sterile distilled water, and immediately placed on sterile absorbent paper for 10 min. The glumes were seeded in Petri dishes with potato dextrose agar (PDA) medium, and the inoculated dishes were incubated at 28 °C until mycelial growth appeared. To obtain pure cultures, a piece of agar with mycelium was removed from the colony's edge, seeded on clean PDA, and incubated at 28 °C until only one color and one type of mycelium was observed in the culture.

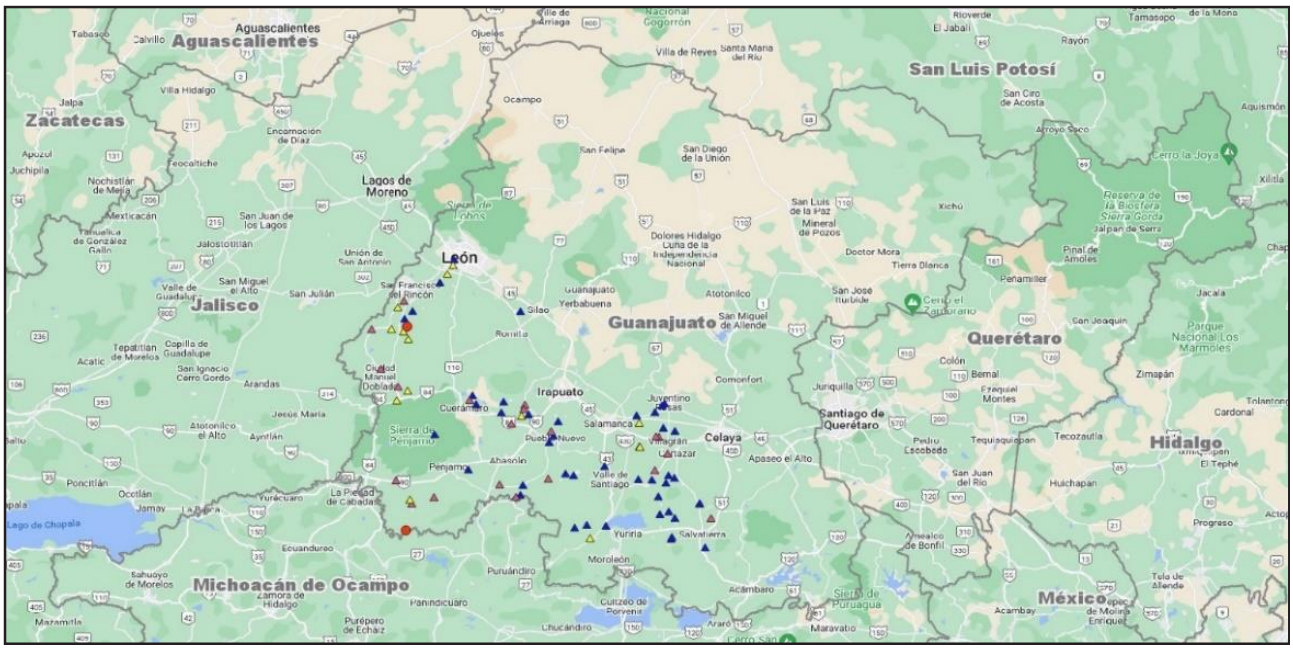

Figure 1. Locations of the 81 wheat spike collection points with symptoms of wheat head blight (FHB). Blue triangle (fields with an incidence of FHB of 1–5 %), brown triangle (fields with an incidence of FHB of 6–10 %), yellow triangle (fields with an incidence of FHB of 11–20 %), and red circle (fields with an incidence of FHB of 21–30 %) (Google Maps, 2023; INEGI, 2023).

Morphological characterization

Individual colonies were transferred to PDA culture medium for morphological characterization. Strains with white, red, or purple peach mycelium were grouped and divided into morphotypes. They were then cultured on Speieller Nährstoffmmarmer agar medium (SNA; 1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4$ $7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, and 20.0 g agar in one liter of distilled water) for microscopic analysis. Characterization consisted of identifying the morphology of macroconidia, microconidia, spores, chlamydospores, and phialides, according to Nelson *et al*. (1983) and Leslie and Summerell (2006).

Molecular characterization

For molecular characterization, the strains were grown on PDA medium at 28 °C for 6 days. The mycelium was recovered and used for genomic DNA extraction following the CTAB method, as described by Zhang *et al*. (1998). DNA quality was assessed using 1 % agarose gel electrophoresis with 1X TAE and stained with ethidium bromide at a concentration of 2 mg μL^{-1}. Concentrations were normalized and DNA was amplified by PCR in the ITS1-5.8S-ITS4 region using the ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) primers (White *et al*., 1990). The PCR reaction was carried in a total volume of 25 µL (50 mM KCl, 10 mM Tris HCl (pH 8.3),

0.2 mM dNTP's, 1.5 mM $MgCl_2$, 1.5 U of Taq polymerase (Invitrogen, catalog number 10342053), 10 pmol of each primer, and approximately 30 ng of DNA). Reaction conditions consisted of 30 cycles with denaturation at 95 °C for 1 min, alignment at 51 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min.

PCR products were visualized using 2 % agarose gel electrophoresis and sequenced using the labeled dideoxynucleotides method on an Applied Biosystems 3130 Genetic Analyzer sequencer (Applied Biosystems). The sequences obtained were identified by comparing them to the NCBI database (nucleotide BLAST) and then deposited in the GenBank database. The sequences were aligned, and a phylogenetic analysis was performed using the UPGMA method with MEGA5 software (version 7.0.1).

Frequency of pathogens

The fungal frequency was calculated using the formula reported by González *et al*. (1998):

$$Fq\ (\%) = \frac{\text{Number of samples containing a genus or species}}{\text{Total number of samples}} \text{ x } 100$$

Pathogenicity and disease severity analysis

For pathogenicity tests, conidial production of the strains was induced by boiling 40 g of black bean in one liter of distilled water for 10 min, after which the supernatant was sterilized and the strains were inoculated in it. The culture was incubated at 28 °C for three weeks. Subsequently, conidia were obtained by centrifugation at 12 000 RPM for 10 min, the volume was adjusted to bring it to the concentration of $1X10^6$ conidia mL^{-1} (Wu *et al*., 2005).

The conidia were inoculated by injection in the spike of 10 wheat plants of the Cortázar S94 variety in flowering stage, which were covered with a plastic bag to maintain humidity. The inoculated plants were kept in a greenhouse (Wang and Cheng, 2017). The results were evaluated 10 days after inoculation. The disease severity was determined using a symptom scale adapted from Fabre *et al*. (2019), which categorizes symptoms as Type-0 for asymptomatic spikelets, Type-1 for spikelets with the first yellowish spot, Type-2 for spikelets with the first brown spot, Type-3 symptoms for completely burned spikelets, and Type-4 symptoms characterized by total desiccation of the spike with mycelium visible outside the plant organ and a decrease in spikelet size (Figure 2), were evaluated using the Minitab program's Anova and Tukey's mean tests.

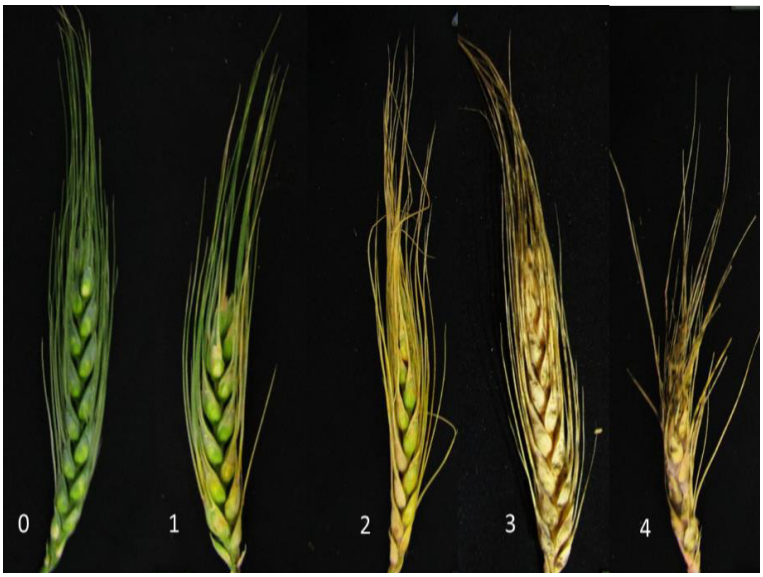

Figure 2. Scale of damage caused by *Fusarium* species on wheat plants variety Cortázar S94 10 days after inoculation with the fungus.

RESULTS AND DISCUSSION

Wheat spikes exhibiting early senescence were collected in the 20 wheat producing municipalities of the state of Guanajuato, and the incidence of the symptoms was distributed as follows: 45 fields (1–5 %), 21 fields (6–10 %), 13 fields (11–20 %), and 2 fields (21–30 %), the latter two located in San Francisco del Rincón and Pénjamo. Twenty-five fields with the highest disease incidence were chosen (Table 1), and 431 isolates were obtained, 327 of which correspond to the genus *Fusarium*, representing a frequency of 75.96 %.

Ten species of this pathogen were identified: *F. oxysporum* (35.57 %), *F. verticillioides* (12.5 %), *F. proliferatum* (11.53 %), *F. redolens* (1.92 %), *F. solani* (0.96 %), *F. cerealis* (1.92 %), *F. polyphialidicum* (4.8 %), *F. chlamydosporum* (0.96 %), and *F. equiseti* (4.8 %). The remaining 24.04 % was divided among the genera *Alternaria* spp. (9.61 %), *Chaetomium olivaceum* (4.81 %), *Coprinopsis urticula* (4.81 %), and *Trichoderma* spp. (4.81 %). *Fusarium* species were identified in 19 of the municipalities sampled; the occurrence of each *Fusarium* species varied across municipalities (Table 2).

The most frequent species were *F. oxysporum* (11 municipalities), *F. proliferatum* (11 municipalities), and *F. verticilliodes* (10 municipalities), with their combination or at least one of the species covering 19 of the 20 municipalities. The municipalities with the greatest number of species were Huanímaro (*F. cerealis, F. chlamydosporum, F. oxysporum, and F. polyphialidicum*), Pénjamo (*F. oxysporum, F. polyphialidicum, F. proliferatum, and F. verticillioides*), and Valle de Santiago (*F. equiseti, F. oxysporum, F. proliferatum, and F. verticillioides*).

Table 1. Information on representative sites of wheat producing fields with wheat head blight (FHB) symptoms in the state of Guanajuato, Mexico.

Municipality	Wheat variety	Latitude	Length	Plot incidence (%)
Abasolo	Cortázar	20.57	-101.52	6–10
Cortázar	Cortázar	20.47	-100.99	6–10
Cuerámaro	Cortázar	20.63	-101.6	1–5
Huanímaro	Cortázar	20.36	-101.54	6–10
Irapuato	WR	20.59	-101.48	11–20
Jaral del Progreso	WR	20.41	-101.04	6–10
Juventino Rosas	Cortázar	20.63	-101.01	1–5
León	Cortázar	21.06	-101.73	11–20
Manuel Doblado	Alondra	20.64	-101.40	11–20
Pénjamo	Cortázar	20.30	-101.85	6–10
	Cortázar	20.41	-101.66	11–20
	Cortázar	20.31	-101.85	11–20
Pueblo Nuevo	Cortázar	20.54	-101.38	6–10
Purísima del Rincón	Alondra	20.88	-101.91	11–20
Salamanca	WR	20.56	-101.09	11–20
Salvatierra	WR	20.25	-100.85	6–10
	WR	20.27	-101.02	1–5
San Francisco del Rincón	Alondra	20.84	-101.85	11–20
	Saturno	20.89	-101.86	21–30
Santiago Maravatío	WR	20.18	-100.98	1–5
Silao	Cortázar	20.94	-101.85	1–5
Valle de Santiago	WR	20.38	101.39	6–10
Villagrán	WR	20.52	-101.02	6–10
Yuriria	WR	20.2284	-101.20	1–5
	WR	20.17	-101.25	11–20

*WR: without record.

These findings are consistent with those of Rangel-Castillo *et al*. (2017) who found that the species with the highest presence in the municipalities of Pénjamo, Abasolo, and Salamanca was *F. proliferatum*, followed by *F. subglutinans* and *F. oxysporum*. Leyva-Mir *et al*. (2017) also reported *F. proliferatum* as the causal agent of wheat shriveling in the Bajío, while Mariscal-Amaro *et al*. (2017), found *F. proliferatum*, F. *verticillioides, F. equiseti*, and *F. moniliforme* as wheat pathogens in the Bajío, with the first three species agreeing with our findings.

Previously, Sandoval-Martínez *et al*. (2012) reported *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. poae*, and *F. verticillioides* as pathogenic species associated with this disease. Morales-Rodriguez *et al.* (2007) identified *F. chlamydosporum*, *F. poae, F. pseudonygamai, F. subglutinans*, and *F. verticillioides* as the

Table 2. Representative sampling points and pathogens associated with wheat head blight (FHB) in the state of Guanajuato, Mexico.

Municipality	A	B	C	D	E	F	G	H	I	J	K
Abasolo				*				*	*	*	
Cortazar						*		*			
Cuerámaro			*					*			
Huanímaro	*	*		*	*					*	
Irapuato				*		*		*			
Jaral del Progreso								*		*	
Juventino Rosas			*	*							
León de los Aldamas						*	*	*			
Manuel Doblado				*		*		*			
Pénjamo				*	*	*		*		*	
Pueblo Nuevo				*						*	
Purísima del Rincón						*					*
Salamanca				*		*					
Salvatierra				*				*			
San Francisco del Rincón	*		*	*							
Santiago Maravatío						*					*
Silao				*		*	*				
Valle de Santiago			*	*		*		*			
Villagrán											*
Yuriria						*					

A: *F. cereales*; B: *F. chlamydosporum*; C: *F. equiseti*; D: *F. Oxysporum*; E: *F. polyphialidicum*; F: *F. proliferatum*; G: *F. redolens*; H: *F. verticillioides*; I: *F. solani*; J: *Alternaria* spp., K: other genus.

species responsible for drying in central Mexico. As observed, *F. proliferatum* and *F. verticillioides* are the two most commonly reported pathogens in the area. *Alternaria* was another genus with a high incidence, found primarily in the southwestern zone (Abasolo, Huanímaro, Pénjamo, and Pueblo Nuevo), as well as in Jaral del Progreso. *Alternaria* has been reported to act as a secondary pathogen invading lesions caused by other pathogens causing leaf blight symptoms (Mata-Santoyo *et al*., 2018). Due to the saprophytic nature of the phytopathogen, which grows best when plant tissues are under stress, colonization is easier when the plant grows weakened and loses its defense mechanisms (Schiro *et al*., 2018). However, the presence of this genus must be confirmed to determine whether it is merely a methodological coincidence or if the genus is indeed related to plant symptoms.

The sequences obtained from the molecular characterization of the selected isolates were compared to the database to confirm genus and species. Sequences were submitted to the Genbank with the following accession numbers for each species: *F. proliferatum* (MN737763), *F. solani* (MN737764), *F. redolens* (MN710478.1), *F.*

thapsinum (MN737762), *F. verticilloides* (MN737769), *F. poliphyalidicum* (MN737767), *F. cerealis* (MN737766), *F. clhamydosporum* (MN737771), *F. oxysporum* (MN737770), *F. equiseti* (MN737772), *Alternaria* spp. (MN944539, MN944540), *Chaetomium olivaceum* (MN944529), *Coprinopsis urticula* (MN944532), and *Trichoderma* spp. (MN944531).

The sequences from the genera *Fusarium* and *Chaetomium* were used to construct the phylogenetic tree (Figure 3), which shows two distinct groups: one for *Fusarium* species and another for *Chaetomium* species. The representative morphotypes of Fusarium species can be seen in the same figure.

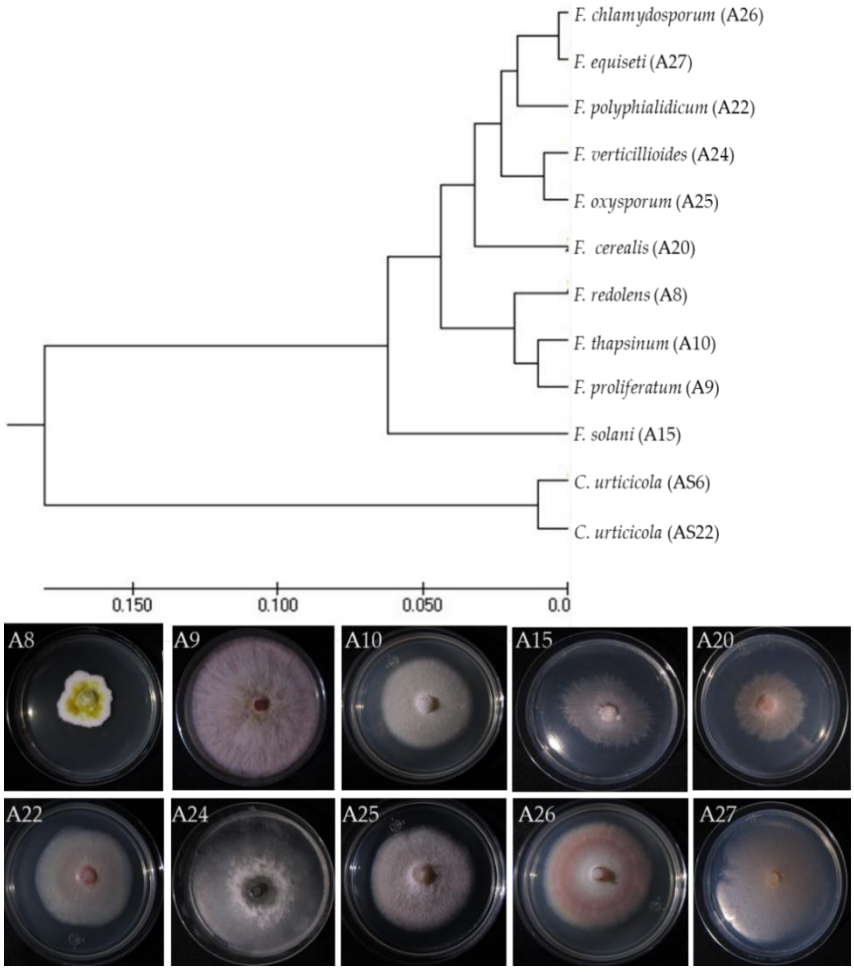

Figure 3. Phylogenetic tree of *Fusarium* species found in wheat fields in the state of Guanajuato constructed using the UPGMA method and elaborated with ITS sequences: *F. cerealis* (A20), *F. chlamydosporum* (A26), *F. equiseti* (A27), *F. oxysporum* (A25), *F. polyphialidicum* (A22), *F. proliferatum* (A9), *F. redolens* (A8), *F. solani* (A15), *F. thapsinum* (A10), *F. verticillioides* (A24), *C. urticicola* (AS6), and *C. urticicola* (AS22).

To further identify this species, additional sequencing of elongation factor 1-alpha (EF) and β-tubulin (TUB) are considered to be carried out soon as an next step to confirm our preliminary results.

The pathogenicity analysis was carried out using the molecularly identified isolates and it was observed that all species were pathogenic. However, the species that triggered the highest level of severity was *F. oxysporum*, which caused senescence throughout the spike, mycelial growth, darkening of the glume, and reduction in spike size. Followed by *F. proliferatum*, which causes yellowing in part or all of the spikelet, these two species are significantly more severe than the other *Fusarium* species associated with the disease (Table 3).

Table 3. Analysis of the severity of pathogenicity of *Fusarium* species found in wheat fields in the state of Guanajuato, Mexico.

Strain Id	Severity level	Group*	Species
C	0	d	
A8	1.2	c	*F. redolens*
A9	1.9	b	*F. proliferatum*
A10	1.2	c	*F. thapsinum*
A15	1.0	c	*F. solani*
A20	1.3	c	*F. cerealis*
A22	1.0	c	*F. polyphialidicum*
A24	1.0	c	*F. verticillioides*
A25	3.8	a	*F. oxysporum*
A26	0.9	c	*F. chlamydosporum*
A27	0.9	c	*F. equiseti*
OG	-		Other genus

*Figures with the same letter are statistically equal (Tukey $p \leq 0.05$). C: Non-inoculated culture.

The above indicates that the species with the highest frequency and that induce greater severity in the wheat spike were *F. oxysporum*, followed by *F. proliferatum*. However, Rangel-Castillo *et al.* (2017) reported that the most aggressive species in the Bajío was *F. proliferatum*. Regarding *F. verticillioides*, we found a high frequency, but the induced severity was low. On the other hand, little is known about *F. oxysporum* as a causal agent of FHB. In this regard, Cosic *et al.* (2007) showed evidence of the presence of this pathogen in the spikes, but it is not shown as an agent that induces high severity of the disease. Meanwhile, Rangel-Castillo *et al*. (2017) demonstrated the presence of *F. oxysporum* in the spike and discovered that *Fusarium* can affect the root system of wheat regardless of the part of other plants from which the inoculum was obtained.

The increase in the incidence of FHB, as well as the frequency and severity of the species, can be attributed to the agronomic practices of the region, including monoculture, the permanence of stubble on the land, the use of flood irrigation, poor land leveling, and fertilization with doses higher than 5 Mg ha^{-1} (Rangel-Castillo *et al*., 2017). When combined with the humidity and temperature of the area (Schiro *et al*., 2018), which are conducive to the development of the genus, these factors predispose the wheat crop to FHB.

Although in terms of molecular identification and phylogenetic analyses of this species by using the ITS region may represent a preliminary output, we are currently planning to perform additional analyses by amplifying and sequencing the EF and TUB regions.

CONCLUSIONS

According to this study, ten species of the genus *Fusarium* were found to be associated with wheat head blight in the state of Guanajuato. The most frequently encountered species were *F. oxysporum*, *F. proliferatum*, and *F. verticillioides*. The most pathogenic species present in Guanajuato were *F. oxysporum* and *F. proliferatum*.

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