

Regional Distribution of *Fusarium verticillioides* in Mexico and Its Implications in Animal, Human Nutrition and Health

Carlos Muñoz-Ruiz¹, Cudberto Contreras-Perez², Rene Rosiles-Martinez³,
Patricia Gutierrez-Garcia⁴, Claudia Rios-Rosas⁵, Jose D. Mendez^{6*}

¹Interdisciplinary Research Center for Regional Integral Development, National Polytechnic Institute. Michoacán, Mexico.

^{2,4,5}Mycology Laboratory. Institute of Epidemiological Diagnosis and Reference, Ministry of Health. Mexico City, Mexico.

³Toxicology Laboratory, School of Veterinary Medicine and Zootechnics, National Autonomous University of Mexico. Mexico City, Mexico.

⁶Medical Research Unit in Metabolic Diseases. Cardiology Hospital. Mexican Institute of Social Security. Mexico City, Mexico.

Abstract— This study was designed to investigate the presence of *Fusarium* species in Mexican corn. Maize samples from 26 States were analyzed. Corn kernels were cultivated following a sequence of cultivation methods until obtaining spores which were transferred to carnation leaf agar medium. Taxonomic identification of fungi was carried out by microscopic examination. To evaluate the *in vitro* production of fumonisin B1, it was experimentally induced in un-contaminated maize. The quantitative determination of fumonisin B1 in the maize samples was performed by thin layer chromatography. Quality control and sensitivity were established using a standard solution of commercial origin whose purity was corroborated by both thin-layer chromatography and high-performance liquid chromatography.

Thirty-eight strains were isolated; 29 corresponded to *Fusarium verticillioides* and 9 to *Fusarium subglutinans*. Strains of *Fusarium verticillioides* exhibited a variable behavior in fumonisin B1 production. 4 strains produced fumonisin B1 in a range of 3.12 to 6.57 ppm.

In conclusion, two species of *Fusarium*; *Fusarium verticillioides* and *Fusarium subglutinans* were found in maize from 26 States of Mexico, their distribution is regionalized. Strains found in five States produced fumonisin B1 in concentrations that can be considered clinically relevant.

Keywords— Corn, Fumonisin B1, *Fusarium subglutinans*, *Fusarium verticillioides*, Mycotoxins.

I. INTRODUCTION

The fungi of the *Fusarium* genus are considered important food pollutants due to the deleterious effects they produce *in vivo* and the economic losses they cause during grain harvest and storage, both by reducing the food quality and by the production of mycotoxins (1). *Fusarium verticillioides* (*moniliforme*) and *Fusarium proliferatum* are two species that occur worldwide and are associated with infections in corn (2). Both species are known to produce the mycotoxins fumonisin B1, fumonisin B2 and fumonisin B3 (3), which have been stated to be associated with several diseases in animals and in humans (4-12). Fumonisin B1 is the major mycotoxin produced by *Fusarium verticillioides* (13) and related species (14) and is found most frequently in corn.

Some researchers have referred to the fumonisin B1 as the cause of equine leukoencephalomalacia with neurotoxic effects and hepatic damage (1). In addition, maize infected with *Fusarium verticillioides* that has been included in the diet or injected in animals in an experimental way exhibits cancer-promoting activity in rats (8) and in other animals such as swine, poultry, and rabbits causing pulmonary edema, hepatic necrosis and bone marrow cells disorder syndromes, respectively (15-18), in chicken embryos severe hemorrhages were evident after exposure to fumonisin B1 (19). *Fusarium verticillioides* has been suggested to be associated with esophageal cancer. It has been isolated frequently from some regions of China and southern Africa, where the highest incidences of human esophageal cancer have been reported (9-11).

Fumonisin B1 has been detected in corn, corn forage, mash and tortillas from several states of Mexico, which may represent health risk for humans or animals (20-23). In addition, it has been found that the isolated strains of corn from the northwest region are strains that produce high fumonisin B1 levels (24). During the last 10 years, veterinarians from several academic institutions in Mexico have observed gross and microscopic lesions of equine leukoencephalomalacia in horses suffering from a nervous syndrome. In Oaxaca, horses that ingested corn containing fumonisin B1 died from equine leukoencephalomalacia (25). Such findings suggest the possibility of distribution of *Fusarium* fungi and fumonisin B1 throughout Mexico.

This study was designed to investigate the presence of *Fusarium* species in Mexican corn and to evaluate the *in vitro* production of fumonisin B1 by this species of fungi.

II. MATERIAL AND METHODS

2.1 Corn samples

Five-hundred gram samples of randomly selected corn kernels (34 total; 12-14 % moisture) from 2007 and 2008 were obtained from 26 states of Mexico and sent to the Institute of Diagnostic and Reference Epidemiological in 2009. Some states sent several samples from different locations at different times; each sample was considered for analysis. Hybrid or native varieties of corn used for human consumption were employed. Corn samples were subdivided to sub-samples of eight kernels each. Each sub-sample was surface-treated with 0.5 % sodium hypochlorite for 3 min, rinsed in distilled water, and blotted dry on paper towel. These kernels were cultured on synthetic nutrient agar and potato dextrose agar medium plates and incubated at 25 °C for six days. *Fusarium* species that grew from kernels were then grown on potato dextrose agar. Afterwards, a cellular suspension was prepared and inoculated in agar water (1.5 %). After 24 hours of incubation at 25 °C, single spores were found and transferred to carnation leaf agar medium. Taxonomic identification of fungi was carried out after macroscopic and microscopic examination as per Burgess (26).

2.2 *In vitro* production of fumonisin B1 by *Fusarium* spp

Isolated strains of *Fusarium* spp. from corn were prepared as described by Logrieco (27). A fumonisin production assay was conducted in flasks, containing 25 g of toxin (FB1)-free corn kernels with 12-14% humidity and 13 mL of distilled water and autoclaved twice for 30 min. at 121 °C. After the corn was cooled, it was inoculated with 1 mL of a water suspension of conidia with 1×10^6 cell / mL from the carnation leaf agar culture and incubated in darkness at 25 °C for 21 days. Control corn was treated in the same way, except that it was not inoculated. To avoid clump formation, the cultures were hand-shaken every week. Control corn meal was produced in the same way, except that it was not inoculated.

2.3 Extraction and detection of fumonisin B1

Samples were prepared as described previously (11, 28, 29). Briefly, toxins were extracted with 50 mL methanol / water (3: 1) in a laboratory blender for 30 seconds and then filtered through to Whatman filter paper No. 4. An aliquot (4 mL) of the filtered extract was applied to a strong anion-exchange cartridge for separation (Varian, Harbor City, CA) previously humidified by the passage of 0.5 mL methanol / water (3: 1), then centrifuged at 500x g for 1 min. This cartridge was washed twice by centrifugation at 250x g with 3 mL of methanol / water (3: 1), then three times each with methanol and 5 % acetic acid in methanol to elute the mycotoxin. The eluate was evaporated to dryness at 40 °C, under a moderate stream of nitrogen and stored dry at -20 °C until analysis.

The residue after clean-up was re-dissolved in 100 µL of methanol for a thin layer chromatography analysis. An aliquot 20 µL of this solution was placed on a thin layer of chromatoplate reverse phase, previously activated at 120 °C by 10 min. At the same time 10 µL of fumonisin B1 standard solution (Sigma Chemical Co. No. F1147, 1 ppm) was placed. Thin layer chromatography analysis was carried out in a stationary silica gel reverse phase and a mobile phase of methanol / demineralized water (7: 3) allowing up to 90 % plate coverage. The plate was dried by airflow and atomized with vanillin 0.5 % solution in pure sulfuric acid (97 %) / absolute ethanol mixture 4: 1. Plates were dried again with airflow and kept 3 min at 80 °C in a stove. Fumonisin B1 was identified as a purple band with the same running front (R_f) as the standard. Some strains were analyzed by high-performance liquid chromatography showing at 20 % variation coefficient. Analysis for fumonisin B1 was carried out previous to derivatization with o-phthaldialdehyde using an 18-C column as a stationary phase and methanol 0.1 mol / sodium phosphate (80:20), adjusted to pH 3.3 with orthophosphoric acid as the mobile phase. Mobile phase flow rate phase was 1 mL / min. A fluorescent detector at 335 nm excitation and 440 nm emission wavelength was used. Results of sample analysis were plotted in tables for frequency and geographic location.

III. RESULTS

Table 1 shows data obtained from 34 corn samples analyzed. The presence of *Fusarium* spp was noted in 32 samples (94 %). *Fusarium verticilloides* was also isolated from 29 samples (85 %), of which six also contained *Fusarium subglutinans*. *Fusarium subglutinans* alone was isolated from three (9%) samples (Table 1). *Fusarium* spp. was not detected in samples from Baja California Sur and Michoacán. Of the thirty eight total strains isolated, 16 strains of *Fusarium verticilloides* and one strain of *Fusarium subglutinans* (45 % total) produced fumonisin B1. The strains with the highest production of

fumonisin B1, were found in samples from the States of Morelos (6.6 µg / g), Oaxaca (4.9 µg / g), Colima (3.1 µg / g) and Jalisco (3.1 µg / g) (Table 1).

Figure 1 shows the geographical distribution of *Fusarium verticilloides* and *Fusarium subglutinans* in México. Thirteen (50 %) of the 26 states sending samples had fumonisin B1-producing strains. These States were Colima, Chihuahua, Jalisco, Durango, Nayarit, Morelos, Tamaulipas, Oaxaca, Sonora, San Luis Potosí, Zacatecas, Querétaro and Sinaloa. The States of Yucatán, Veracruz and Puebla did not participate in this study. Baja California Norte, Coahuila and Mexico City States, are not corn producers. In the rest of the States, although samples containing *Fusarium* were found, the strains isolated did not produce fumonisin B1.

TABLE 1
STATES OF MEXICAN REPUBLIC, NUMBER OF *FUSARIUM* FUNGI STRAINS ISOLATED FROM CORN AND FUMONISIN B1 PRODUCTION OF CULTURED MATERIAL.

Sample	State	Code	Strains	Type of strain	µg/g FB ₁
1	Aguascalientes	A	2	<i>Fusarium verticilloides</i> <i>Fusarium subglutinans</i>	Nd*
2	Baja California Sur	B	0		
3	Campeche	Ca	1	<i>Fusarium verticilloides</i>	Nd
4	Colima	Co	1	<i>Fusarium verticilloides</i>	Nd
5	Colima	Co	2	<i>Fusarium verticilloides</i> <i>Fusarium subglutinans</i>	2.35
6	Colima	Co	2	<i>Fusarium verticilloides</i> <i>Fusarium subglutinans</i>	3.12 1.90
7	Colima	Co	1	<i>Fusarium verticilloides</i>	Nd
8	Chiapas	C	1	<i>Fusarium verticilloides</i>	Nd
9	Chihuahua	Chi	1	<i>Fusarium verticilloides</i>	2.47
10	Chihuahua	Chi	2	<i>Fusarium verticilloides</i> <i>Fusarium subglutinans</i>	2.23
11	Durango	D	1	<i>Fusarium verticilloides</i>	2.74
12	Durango	D	1	<i>Fusarium verticilloides</i>	Nd
13	Durango	D	1	<i>Fusarium verticilloides</i>	Nd
14	Estado de México	E	1	<i>Fusarium subglutinans</i>	Nd
15	Guanajuato	Gu	1	<i>Fusarium subglutinans</i>	Nd
16	Guerrero	G	1	<i>Fusarium verticilloides</i>	Nd
17	Hidalgo	H	1	<i>Fusarium subglutinans</i>	Nd
18	Jalisco	J	1	<i>Fusarium verticilloides</i>	3.12
19	Jalisco	J	1	<i>Fusarium verticilloides</i>	2.87
20	Michoacán	Mi	0		
21	Morelos	Mo	1	<i>Fusarium verticilloides</i>	6.57
22	Nayarit	N	1	<i>Fusarium verticilloides</i>	Nd
23	Nayarit	N	1	<i>Fusarium verticilloides</i>	2.35
24	Nuevo León	NL	1	<i>Fusarium verticilloides</i>	Nd
25	Oaxaca	O	2	<i>Fusarium verticilloides</i> <i>Fusarium subglutinans</i>	4.85
26	Querétaro	Que	1	<i>Fusarium verticilloides</i>	2.45
27	Quintana Roo	QR	1	<i>Fusarium verticilloides</i>	Nd
28	San Luis Potosí	S	2	<i>Fusarium verticilloides</i> <i>Fusarium subglutinans</i>	2.06
29	Sinaloa	Si	1	<i>Fusarium verticilloides</i>	2.01
30	Sonora	So	1	<i>Fusarium verticilloides</i>	2.19
31	Tabasco	T	1	<i>Fusarium verticilloides</i>	Nd
32	Tamaulipas	Tam	1	<i>Fusarium verticilloides</i>	2.71
33	Tlaxcala	Tl	1	<i>Fusarium verticilloides</i>	Nd
34	Zacatecas	Z	1	<i>Fusarium verticilloides</i>	2.08

*Nd. Not detectable.

Figure 2 shows the presence of highest amount fumonisin B₁ revealed by thin layer chromatography. Samples were from Morelos and Oaxaca States. R_f value is identical to the Sigma fumonisin B₁ standard located in the middle lane of the silica gel plate. Figure 3 shows the chromatogram of analytical standard of fumonisin B₁ derivatized with phtaldialdehyde diluted reagent with 5.3 min as retention time.

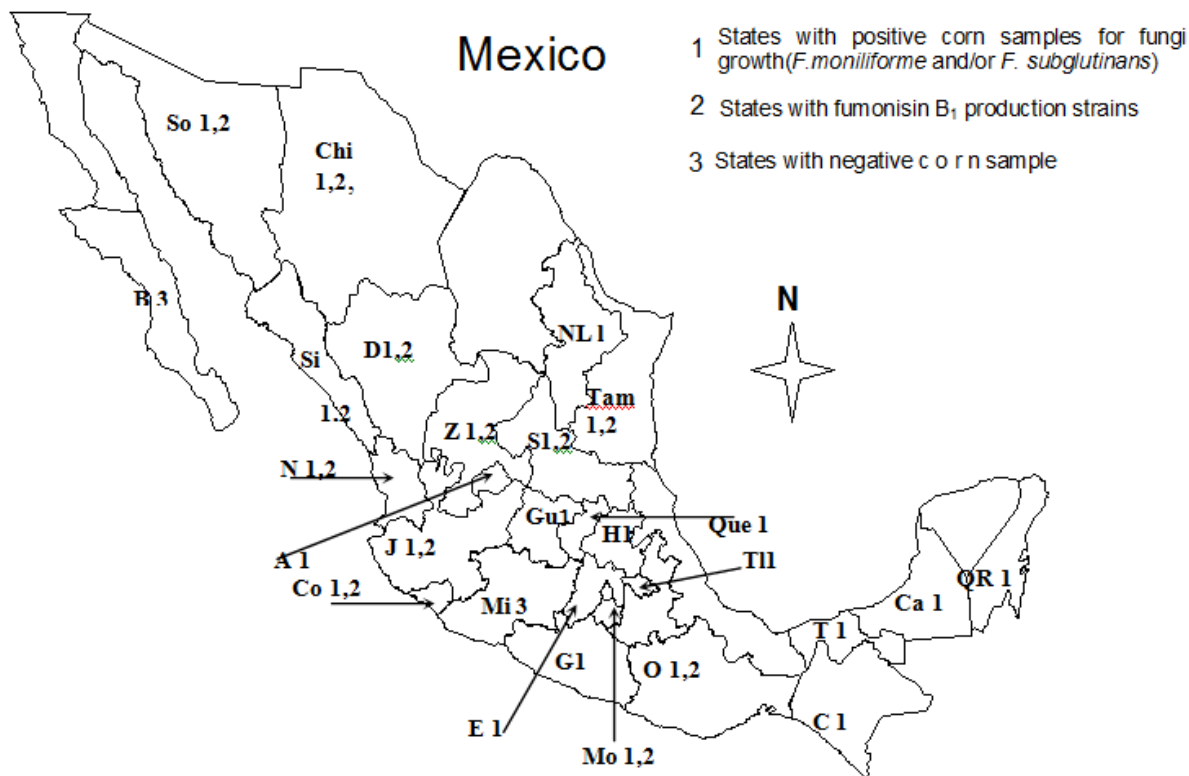


FIGURE 1. Geographical distribution of *Fusarium verticilloides* and *Fusarium subglutinans* in México (See Table 1 for Code).

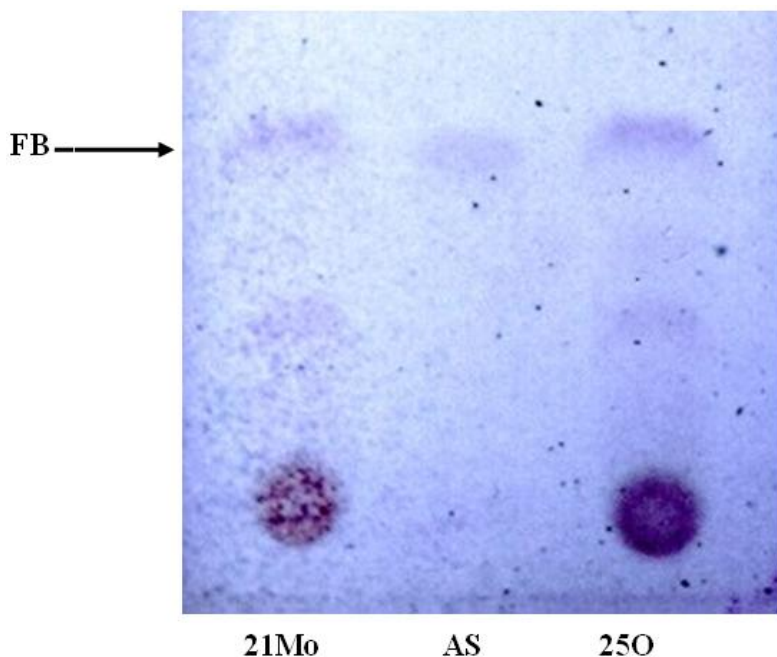


FIGURE 2. Fumonisin B₁ produced by strains of *Fusarium verticilloides* isolated from corn of some regions of México. 21Mo. *Fusarium verticilloides* isolated from maize of Morelos (Mo), México. FB₁= 6.5 µg/g. AS. Analytical standard, Sigma Chemical Co. N° F1147. FB₁= 1 µg. 25O. *Fusarium verticilloides* isolated from maize of Oaxaca (O), México. FB₁ = 4.85 µg/g.

IV. DISCUSSION

This study shows that *Fusarium* spp is widely distributed in Mexico. The presence was found in 94 % of analyzed samples, a higher percent than that previously reported oscillating between 61.8 and 80.6 % (20, 24). Regarding the species involved, in Sonora State the species *Fusarium oxysporum*, *Fusarium solani*, *Fusarium proliferatum* and *Fusarium subglutinans* were found (23). In this study, the species *Fusarium proliferatum*, specie commonly associated with maize plants, was not isolated. *Fusarium subglutinans* in our study registered an incidence of 24 % and was detected in samples from Chihuahua, Aguascalientes, Colima, Guanajuato, San Luis Potosi, Hidalgo, State of Mexico and Oaxaca. *Fusarium subglutinans* has been shown to be a contaminant of maize, mostly in warm regions (27, 30). The strain isolated in Colima was a producer of fumonisin B1 in low concentration (1.9 ppm). In previous studies in Mexico, only *Fusarium verticillioides* isolates have been able to produce fumonisin B1 (20, 24). Consequently, this is the first report of fumonisin B1 production by *Fusarium subglutinans*. The natural occurrence of FB1 in corn and the capability of *Fusarium subglutinans* to synthesize this toxin cause that it could be a significant food and grain contaminant.

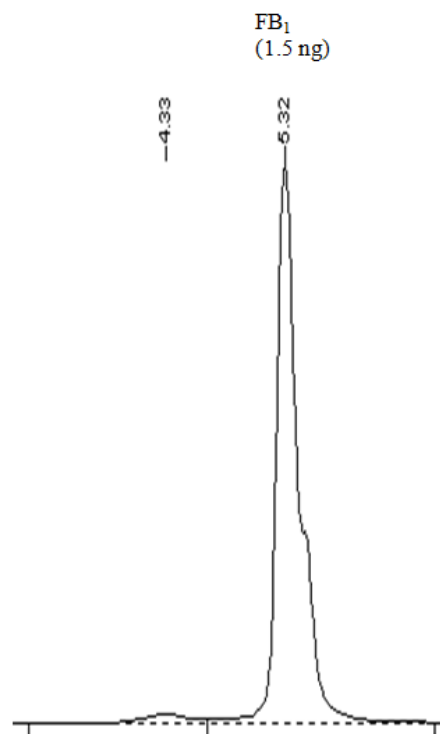


FIGURE 3. HPLC chromatogram of FB₁ (1.5 ng) by fluorescence detection of the o-phthalaldehyde derivative. Standard solution (Sigma Chemical Co. N° F1147).

Most studies in Mexico have investigated the direct presence of fumonisins and only two studies have investigated the *in vitro* production of toxins. Desjardins et al (20) found that 33 of 34 isolated strains of corn from Nuevo León produced fumonisin B1 in a range of 10 to 9000 µg / g. Sánchez-Rangel et al (24), isolated 67 strains in Sonora, of which 60 behaved as high producers of fumonisin B1, while that those isolated in the State of Mexico were low producers. The results obtained in this work differ in the northeast region, where in five states; Baja California Sur, Chihuahua, Durango, Sinaloa and Sonora, the strains isolates were considered low producers of fumonisin B1. The strains obtained from Baja California Sur did not produce toxin. Also in the south central region, where the strains studied behave as low producers of fumonisin B1. In this region, the isolated strain in Morelos was the one that produced the highest quantity of toxin, 6.57 ppm. This observation correlates with the study conducted in 4 municipalities of Morelos State (12) where the consumption of tortilla was evaluated as a source of exposure to fumonisins. The results indicated that some subjects had an estimated intake of 23 µg / Kg / d, which is greater than what is reported in the United States. At concentrations higher than 1 ppm of fumonisin B1, a woman weighing 60 kg who consumes 120 g / d of tortilla could exceed the tolerable daily intake (12).

Among the States that produce the largest amount of corn are Jalisco, Sinaloa, Chiapas and the State of Mexico; followed by Guerrero, Michoacán, Puebla and Oaxaca. In these States, only the strains isolated in Jalisco and Oaxaca were producing fumonisin B1 (3.12 and 4.85 ppm), while the strains isolated in Sinaloa, Chiapas, State of Mexico and Guerrero did not produce toxins.

The high contamination of maize by *Fusarium verticillioides* found (76 %) and additionally 55 % of the isolated strains produce fumonisin B1, are a determining fact that occurs in Mexican corn, in the field and during storage. The subsequent fumonisin contamination maize-based products for humans has become a worldwide chronic phenomenon, known to cause deleterious effects in animals and is suspected to be a carcinogen and toxic health agent for humans (31-34).

Although fumonisin B1 and esophageal cancer in humans have been linked elsewhere, this relationship may be less relevant in Mexico. In 2010, esophageal cancer does not appear as one of the main causes of death in men and women (35), however, the National Institute of Medical Sciences "Salvador Zubirán" recorded 134 cases during the period from 1977 to 2006. Highlights two characteristics, the increase in its incidence and its higher mortality (36). Regionally, the states of Morelos and Oaxaca, which contributed maize samples with fumonisin that produce high levels of fumonisin B1 production, also have a relatively low frequency of esophageal cancer in Mexico (35).

3.1 Beneficial actions of corn nixtamalization for human consumption

Corn "nixtamalization" (cooking corn with calcium hydroxide), a process which significantly reduces the fumonisin B1 amount in maize for human consumption (37), may help prevent even contaminated corn from causing health effects in humans. In Mexico, most maize for human consumption receives this treatment; therefore, the health risks from this toxin may be greatly reduced among humans, as well as in corn-mash and tortillas (0.79 ppm). According to the available information on the fumonisin B1 toxicity, several authors have concluded that these estimated intakes are unlikely to possess a health risk on the population (34).

3.2 Relevance of fumonisin B1 in animal health

The fumonisin B1 presence in corn-stocks for animal feeding is likely to be more important. Detected levels in corn-stocks samples from Nayarit (mean 4.5 mg / Kg) are very close to the toxic threshold (5 mg / Kg) that some authors described as toxicogenic for horse (22). The results of our study support this observation, since one of the two strains of *Fusarium verticillioides* isolated in the State of Nayarit was a producer of fumonisin B1 at a concentration of 2.35 ppm. It is important to consider that the development of *Fusarium* and the production of fumonisin B1 can occur in the field before harvesting, during the storage of the forage or during the process and storage of food for different species of animals. So it should alert on the presentation of any symptoms related to the effect of mycotoxins.

On the other hand, related to fumonisin quantitation, thin layer chromatography technique has been used by several researchers (9, 20, 25, 33). The modification used by us is very useful in Mexico for basic Toxicology Laboratories. This method is simple, economical and reliable to evaluate the presence of fumonisins during maize harvest and storage. The detected sensitivity of 1 ppm is quite acceptable below the value established by the FDA (2-4 ppm) for maize destined for the production of tortillas or masa (12).

Due to the growing importance of corn contamination by *Fusarium* species, it is convenient to establish Microbiological Standards to prevent contamination of agricultural areas by the use of contaminated seeds, including that which could be imported from other countries. In Mexico, corn is the most cultivated agricultural product, but it is also one of the most imported products. The national consumption in 2017 stood at 38.7 million tons (40). The States of Sinaloa and Jalisco concentrate 34.3% of the national production. In both States the isolated strains were producers of fumonisin B1. The results of the study justify a greater investigation of the contamination of corn in our country.

V. CONCLUSION

In conclusion, two species of *Fusarium*; *Fusarium verticillioides* and *Fusarium subglutinans* were found in maize from 26 States of the Mexican Republic. About 50 % of the isolated strains produced toxins variable degree. Four States of the northeast region have strains with low production of fumonisin B1, which contrasts with previous results where this region was considered a high producer of this toxin. In addition, it was also found that in nine States there were non-producing strains of fumonisin B1. However, the south-central and southwestern regions, considered to be of low production, also betray an important change, since isolated strains in Oaxaca and Morelos produced fumonisin B1 in quantities of public health importance that exceeds that established by the FDA for corn intended for human consumption.

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