Detection of mycotoxins produced by *Fusarium* species in Colombia

Detección de micotoxinas producidas por especies de *Fusarium* en Colombia

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ABSTRACT

The genus *Fusarium* produces mycotoxins that are metabolites of low molecular weight that affect the quality of crops, and even more importantly, they affect the health of humans and animals. Among those that cause damage to health are trichothecenes, fumonisins, and zearalenones. The objective of this study was to quantify the mycotoxins produced by species of the genus *Fusarium* from a population of isolates obtained from different crops and locations in Colombia. From 206 isolates, only 14 amplified to regions associated with mycotoxins deoxynivalenol (DON) belonging to the group of trichothecenes, fumonisin (FUM) and zearalenone (ZEA) using PCR. Each isolate with the presence of mycotoxins was conserved in potato dextrose agar (PDA) medium. Eight days after seeding in corn kernel medium, the samples were processed to perform the quantitative analysis of DON, ZEA and FUM using an ELISA kit based on enzyme-linked immunosorbent assays. The results show that mycotoxins were present in the evaluated isolates and their levels were above the standards regulated by Mercosur and the European Union. The use of immunosorbent assays using the ELISA technique becomes a useful tool to detect and quantify mycotoxins of species of the genus *Fusarium* that affect different crops in Colombia.

Key words: fumonisins, trichothecenes, zearalenone, ELISA.

RESUMEN

El género *Fusarium* produce micotoxinas que son metabolitos de bajo peso molecular que afectan la calidad de los cultivos y especialmente la salud de humanos y animales. Entre las que causan daños a la salud se encuentran los tricotecenos, las fumonisinas y las zearalenonas. El objetivo de este estudio fue cuantificar las micotoxinas producidas por especies del género *Fusarium* de una población de aislamientos obtenida de diferentes cultivos y localidades de Colombia. De 206 aislamientos, sólo 14 amplificaron regiones asociadas a las micotoxinas deoxinivalenol (DON), que pertenece al grupo de los tricotecenos, fumonisina (FUM) y zearalenona (ZEA) usando PCR. Cada uno de los aislamientos con presencia de micotoxinas se conservaron en medio papa dextrosa agar (PDA). Ocho días después de la siembra en medio de granos de maíz, las muestras se procesaron para realizar el análisis cuantitativo de DON, ZEA y FUM utilizando un kit de ELISA basado en ensayos de inmunoabsorción ligados a una enzima. Los resultados revelan la presencia de micotoxinas en los aislamientos evaluados cuyos niveles se encuentran por encima de los estándares regulados por Mercosur y la Unión Europea. El uso de ensayos de inmunoabsorción por medio de la técnica de ELISA se convierte en una herramienta útil para detectar y cuantificar micotoxinas de especies del género *Fusarium* que afectan diferentes cultivos en Colombia.

Palabras clave: fumonisinas, tricotecenos, zearalenona, ELISA.

Introduction

The study of mycotoxins has been an object of interest for the world in the last twenty years, not only because of the large losses in crop and animal productivity but also because of their deleterious effects. Mycotoxins are considered the secondary metabolic product of a large number of fungi that in small amounts are capable of triggering alterations and pathological conditions in both humans and animals. The intake of food contaminated with mycotoxins may cause acute or chronic mycotoxicosis. Depending on the degree of toxicity, the central nervous, digestive, cardiovascular, and pulmonary systems can be affected. Mycotoxins can also trigger cancerous diseases, produce mutagenic and teratogenic damages and, in some cases, can behave as immunosuppressants (Bennett and Klich, 2003; Bertero *et al*., 2018).

Mycotoxins are produced by fungi capable of growing on a wide variety of substrates, so they often contaminate food. The biosynthesis is performed by a few precursors derived from intermediates of its primary metabolism. Production occurs during the stationary phase of growth with little or no production of these mycotoxins during the active growth phase (Berthiller *et al*., 2013; Pitt and Miller, 2017).

Received for publication: 9 January, 2019. Accepted for publication: 23 July, 2020 Doi: 10.15446/agron.colomb.v38n2.77176

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Since mycotoxins were recognized as a public health problem, quantification by analytical techniques has continuously improved. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the most used analytical techniques today. These techniques determine several trichothecenes simultaneously with low concentration levels (ng g^{-1}) of evaluated samples, requiring high stringency, especially in the cleaning processes. The ELISA technique allows a large number of samples to be measured routinely with high precision compared to CG and HPLC methods, which are expensive and take longer to get results (Escobar and Fragas, 2004; Pleadin *et al*., 2012).

One of the first reports on food contamination by *Fusarium* mycotoxins in human and animal health mentions the T-2 toxin produced by alimentary toxic aleukia (ATA), which was reported in Russia in the 1940s and fumonisins that cause equine leukoencephalomalacia, reported in several countries in the 1980s (Pitt and Miller, 2017).

Mycotoxin production is stimulated by oxidative and nutritional stress, environmental factors such as pH, temperature, water activity, fungicides and secondary metabolites produced in these environments (Martínez *et al.,* 2010). Evaluations in some regions of Europe showed that 80-100% of grains were contaminated with trichothecenes (Reverberi *et al.,* 2010). These high incidences occurred in regions where crops were affected by drought, infested by insects, or inadequately maintained using inappropriate harvesting equipment or storage facilities (Pohland, 1993).

In the case of *Fusarium*, which is essentially regarded as a field pathogen, mycotoxins are synthesized mainly during plant infection; in the case of cereals they can accumulate in the grain during the production, harvesting and storage stages (Desjardins and Hohn, 1997). Species of this genus, which are contaminants of these plants, can produce a large variety of mycotoxins. The mycotoxigenic profile may vary at inter- and intra-specific levels and often includes a wide range of mycotoxins. Fumonisins and trichothecenes are the most frequent and toxic mycotoxins produced by *Fusarium*, although this genus can produce other important mycotoxins such as zearalenone, enanthines, and moniliformina (Chakrabarti, 2013; Zhang *et al*., 2013).

Environmental conditions, susceptible hosts, and virulence of the pathogen are the factors that promote fungus growth and, consequently, the production of toxins. Therefore, agricultural products affected by these microorganisms may be susceptible to contamination in the production, harvesting, transport, and storage processes (Nelson *et al*., 1993; Desjardins and Hohn, 1997; Desjardins and Proctor, 2007; Zhang *et al*., 2013).

In Colombia, resolution 4506 of October 30 establishes the maximum levels of food contaminants intended for human consumption (Ministry of Health and Social Protection, 2013). The maximum permissible limit for deoxynivalenol (DON) is 100-1750 μg kg^{-1}, for fumonisins (FUM) it is 200-4000 μg kg^{-1} and for zearalenone (ZEA) it is 20-350 μg kg^{-1}. These ranges depend on the food type, the size of particles generated in the industrial process, and the age of the consumers. These values are remarkably similar to those managed by the European Union (EU) and Mercosur (WHO/FAO, 2003).

The overall objective of this study was to quantify the mycotoxins present in *Fusarium* isolates from a collection of samples belonging to the Universidad Nacional de Colombia located in Palmira (Valle).

Materials and methods

This study was conducted in the laboratory of Plant diagnostics of the Universidad Nacional de Colombia located in Palmira.

Fourteen isolates previously evaluated by PCR (Tab. 1) with the trichothecene (*TRI)*, fumonisin (*FUM)* and zearalenone (*PKS)* genes were selected and amplified for trichothecene (DON), fumonisin (FUM) and zearalenone (ZEA) detection (Tab. 2).

TABLE 1. Primers used to detect mycotoxins in *Fusarium* isolates from the collection of the Universidad Nacional de Colombia - Palmira campus.

Mycotoxin	Primer	Sequence 5'-3'	Amp (bp)+	°T hybridization	Genotype	Reference
Deoxynivalenol	TRI 5 F TRI 5 R	5´-AGCGACTACAGGCTTCCCTC -3´ 5´-AAACCAATCCAGTTCTCCATCT-3´	545	59	*TRI*	Li *et al*., 2005
Fumonisin	FUM 1F FUM 4R	5´-GAGGCCCGAGCGAGCACTGG-3' 5´-CCAGCCGCGGAAATTAGGGATGTG-3'	1456	58	*FUM*	Bluhm *et al*., 2004
Zearalenone	PKS13F PKS13R	5'-CCCAGCCAAGCCCAGTACGC-3´ 5´-ACAGCGGCTGACCTGGGTCA-3´	532	59	*PKS*	Stępień *et al*., 2011

Amp (bp)+ = expected amplicon size in base pairs.

TABLE 2. Mycotoxin detection present in isolates obtained by phylogenetic analysis from the collection of the Universidad Nacional de Colombia - Palmira campus.

Isolate	Species	Host	Mycotoxin	Locality
32	*F. graminearum*	Corn	TRI-ZEA	Las piedras/Tolima
38	*F. verticillioides*	Corn	FUM	Tierralta/Cordoba
40	*F. verticillioides*	Corn	FUM	San Agustin/Huila
42	*F. napiforme*	Corn	FUM	Palmira/Valle
61	*F. proliferatum*	Corn	FUM	Bolivar/Valle
67	*F. graminearum*	Corn	TRI-ZEA	Versalles/Valle
73	*F. verticillioides*	Corn	FUM	Cerrito/Valle
75	*F. verticillioides*	Corn	FUM	Guacari/Valle
80	*F. verticillioides*	Corn	FUM	Riofrio/Valle
83	*F. verticillioides*	Corn	FUM	Palmira/Valle
113	*F. equiseti*	Melon	TRI-ZEA	Roldanillo/Valle
132	*F. verticillioides*	Grape	FUM	Roldanillo/Valle
144	*F. verticillioides*	Blackberry	FUM	Ginebra/Valle
202	*F. oxysporum*	Pepper	FUM	Yumbo/Valle

*TRI: Trichothecene; FUM: Fumonisin; ZEA: Zearalenone.

Mycotoxin quantification methods

The isolates were cultured in Petri dishes with potato dextrose agar (PDA) medium. Then, to determine the amount of mycotoxin, the isolates were introduced to corn kernel medium with a concentration of 1 x 10^8 ml^{-1} conidia. These isolates were preserved and incubated with a photoperiod of 12 h of light and 12 h of darkness. Once the mycelium reached growth for 15 d, the conidia count was carried out in a Neubauer chamber at a concentration of 1 x 10^6 ml^{-1} conidia; then, it was separated with a stirring rod adding 10 ml of 70% methanol. The mix was then filtered with Watman No. 1 paper and 100 µl of the solution was added with a micropipette to the controls and samples following the protocol of the NEOGEN® Veratox commercial kit according to the established concentration for each mycotoxin. Finally, the reading was performed in a Neogen 4700 microwell reader 9303 (NEOGEN© Corporation, Lansing, MI, USA) with a length of 650 nm wave. Calibration curves for the quantification of deoxynivalenol, zearalenone and fumonisins were performed with the controls established for each kit (Tab. 3).

Statistical analysis

The absorbance values and the concentration of the samples were performed using the regression formula established by the Elisa reader program (Eq. 1) (NEOGEN© Corporation, Acumedia, Lansing, MI, USA) through the optical densities of both the controls and the samples to be evaluated.

$$Y = \log i\ t\ (Abs)\ X = \log (Conc) \quad (1)$$

where X and Y are the axes.

Results and discussion

Deoxynivalenol

The results show that the evaluated isolates (32, 67, and 113) have concentrations ranging between 0.6 and 2 mg L^{-1}, corresponding to *F. graminearum* and *F. equisetti* (Fig.1). This method demonstrates there is a high sensitivity for the test that is the most used for trichothecene detection in different foods for both human and animal consumption (Meneely *et al*., 2011).

TABLE 3. Values of controls used in mycotoxin quantification assays.

Mycotoxin	Concentration	C1	C2	C3	C4	C5
Deoxynivalenol	mg L^{-1}	0	0.5	1	2.0	5.0
Fumonisin	mg L^{-1}	0	1.0	2	4.0	6.0
Zearalenone	μg L^{-1}	0	25	75	150	500

C= X=log (Conc).

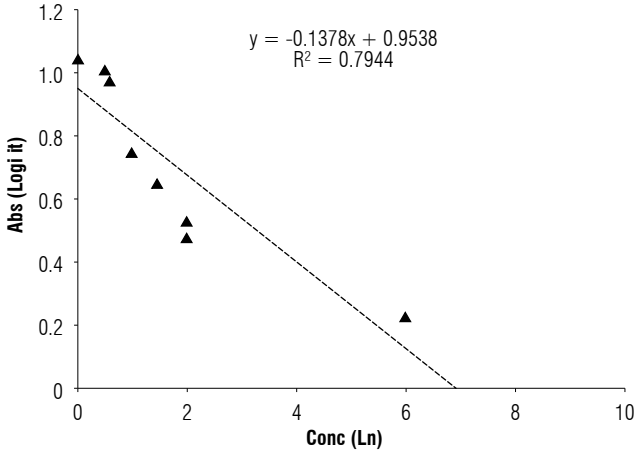

FIGURE 1. Concentration in mg L^{-1} and absorbance values of deoxynivalenol produced by isolates.

Studies of trichothecene quantification are performed using methods universally accepted by the International Official Association of Analytical Chemists (AOAC International), recognized by the Food and Drug Administration (FDA) for the determination of mycotoxins (Yoshizawa *et al*., 2004).

The results obtained in this evaluation indicate that the values for the presence of trichothecenes in food for human consumption are above the levels allowed in Colombia and the EU (Rojas and Wilches, 2011). Values greater than 750 µg kg^{-1} (0.75 mg kg^{-1}) found in *F. graminearum* isolates in corn indicate a high mycotoxin concentration in the evaluated samples. In Argentina corn samples with a high incidence of DON contamination of 0.93 mg kg^{-1} have been found (Pacin *et al*., 1997). In Brazil, the values of this toxin did not exceed 0.6 mg kg^{-1} (Furlong *et al*., 1995). There are no universal values for measuring the damage caused by DON in foodstuffs for human consumption. However, each country or group of countries, such as the EU and Mercosur, manage ranges between 300-2000 µg kg^{-1} with the lowest values being those of the EU (Pleadin *et al*., 2012).

In Colombia, studies on the detection and quantification of DON have been recorded since 1995 (Diaz and Cespedes, 1997; Duarte and Villamil, 2006; Rojas and Wilches, 2011; Rojas *et al*., 2015). These studies show a high concentration of DON in foodstuffs for human consumption, especially processed products from corn and wheat. This fact is worrying since to date controls and application of normativity are not carried out in the Colombia despite the resolution of the Ministry of Health and Social Protection issued in 2014 (Ministry of Health and Social Protection, 2013).

Risks caused by DON are initiated when it enters the food chain, and it is transmitted to humans directly through consumption of cereals and cereal products. Foods such as corn and wheat are the most susceptible to be contaminated with DON. The toxin can remain in processed foods made from contaminated cereal, such as bread, pasta, cookies, etc. Toxicity may occur by contact (causing skin, eye and throat irritation) or by direct ingestion. Depending on the amount ingested, it may cause vomiting, diarrhea, tachycardia, leucopenia (immunosuppressive effect due to leukocyte reduction), or teratogenic effects (associated with congenital diseases) (Sudakin, 2003; Pietsch *et al*., 2014; Yang *et al*., 2014).

On the other hand, it is important to highlight that trichothecenes such as DON are translocated in the plant tissues before any symptoms of the disease and signs of the fungus occur. Metabolites act by decreasing the plant protein synthesis and are able to suppress or delay the defense response. It has been found that isolates that do not produce trichothecenes are less pathogenic and affect the productive development of the plant to a lesser extent (Desjardins and Hohn, 1997; Desjardins, 2006).

Zearalenone

In isolates 32 and 67 (*F. graminearum*) and 113 (*F. equiseti*) amplified with the *PKS* gene by PCR, there was also the presence of mycotoxin zearalenone through the serological ELISA test (Fig. 3). *F. graminearum* predominates in environments with temperatures of 25°C and relative humidity greater than 88%. In contrast, *F. equiseti* develops in tropical and subtropical areas affecting several crops, appearing mainly in harvest and post-harvest time. Several studies show that zearalenone production in stored food increases with water activity, i.e., the amount of free water that is available for microorganisms' growth. The optimum amount is 0.98 mg kg^{-1} which facilitates toxin biosynthesis (Lacey and Magan, 1991, Velluti *et al*., 2000).

The maximum permissible values of this mycotoxin in human food are 0.1 mg kg^{-1} (Ministry of Health and Social Protection, 2013). However, the values found in the evaluated isolates are above the established ranges for Colombia (Fig. 2).

It is observed that the DON and ZEA mycotoxins appear simultaneously in the species *F. graminearum* and *F. equiseti*, and this condition is further aggravated due to the possible synergistic effects associated with ingestion of food contaminated with various mycotoxins. In Kenya, the simultaneous ocurrence of DON, ZEA, OTA (ochratoxin)

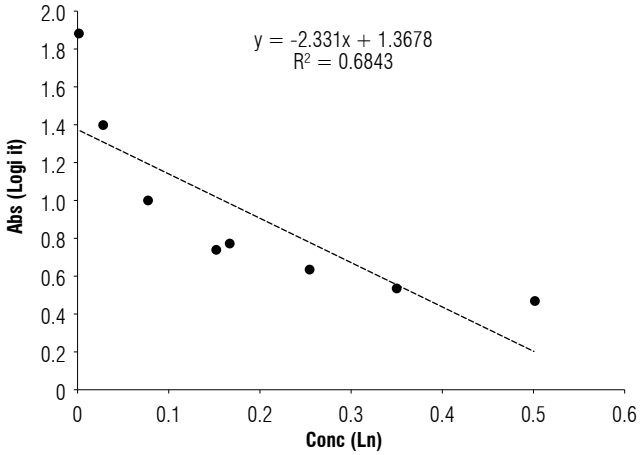

FIGURE 2. Concentration in mg L^{-1} and absorbance values of zearalenone produced by isolates.

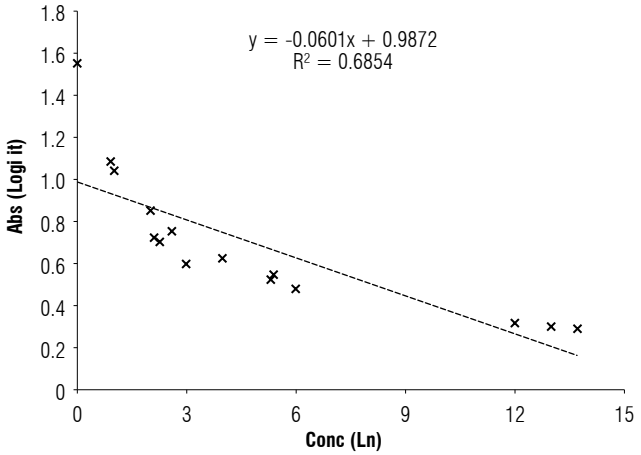

FIGURE 3. Absorbance values and mg L^{-1} concentration of isolates producing fumonisins.

and AFs (aflatoxins) obtained from wheat crops has been reported (Muthomi *et al*., 2008; Li *et al*., 2014).

In studies conducted by Rojas *et al*. (2015) on mycotoxin co-occurrence in food for infants, it was found that ZEA shows high concentrations between 421.34 and 1518.22 μg kg^{-1} (0.421-1.5 mg kg^{-1}), which exceed the legal values established for Colombia and the EU (20 μg kg^{-1}). In a study carried out in 1999 (Díaz, 2005), values ranging from 35 to 134 μg kg^{-1} (0.035-0.134 mg kg^{-1}) were found in corn, which are above the current ranges, as well as those found in this study.

The zearalenone toxin is thermostable which allows it to survive under adverse conditions. Once it enters the body it is rapidly metabolized, producing estrogenic substances such as α-zearalenol and β-zearalenol. These substances have in their structure a lactone that initiates estrogenic activity capable of competing and interacting with estrogen receptors which activate and deactivate metabolic pathways (Li *et al*., 2014).

Information about damages caused by ZEA in humans is poor. However, studies concerning high ZEA concentrations in foods and the emergence of diseases associated with estrogen such as precocious puberty and breast cancer have led to the belief that this toxin triggers a series of events that cause the formation of cancer cells and also affects the immune system (EFSA, 2011).

The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) categorize ZEA and DON produced by *F. graminearum* into group 3, i.e. they are not classified as carcinogenic to humans (IARC, 2016). However, damage caused by these toxins to humans is reflected in estrogenic damage (Rojas and Wilches, 2011).

Fumonisin

High concentrations of FUM were found in *F. verticillioides* isolates 38, 40, 73 and 75 from corn exceeding the control values of 6 mg kg^{-1} (Fig. 3). In Colombia, the permitted limits for this toxin are 3 mg kg^{-1}; however, for the EU the values range from 0.2 to 4 mg kg^{-1}. For the species *F. proliferatum* values also exceed the permitted ranges. In the species *F. napiforme, F. oxysporum and F. verticillioides* (isolates 80, 83 and 144), the concentration of FUM does not exceed those ranges.

Since corn is a daily food in Colombia, concentrations found in culture medium are high compared to the estimated ranges for direct and processed food. Studies carried out in Mexico, where FUM concentration in corn both under field and controlled conditions were evaluated, a high amount of fumonisins (5 mg kg^{-1}) is found above the established standards, becoming a potential risk for human and animal health (Gallardo-Reyes *et al*., 2006). In a study conducted in Brazil by Ono *et al*. (1999) high relative humidity and temperature are key to fungus growth and subsequent fumonisin contamination in the field. The period close to cob maturity or grain filling generates the highest FUM levels (Martínez *et al*., 2010). Dry periods before and during the grain filling also promote the disease severity and increase the fumonisins accumulation (Munkvold, 2003).

Fumonisins are also produced by some species of the *F. fujikuroi* species complex (FFCS) such as *F. proliferatum* and *F. napiforme* and the *F. oxysporum* species complex, with the toxin amount playing an important role in plant pathogenicity (Winter *et al*., 1996; Braun and Wink, 2018). FUM formation is caused by the expression of the *FUM* gene (Waalwijk *et al*., 2004; Stępień et *al*., 2011). The *FUM*

gene cluster is highly correlated between *F. oxysporum*, *F. verticillioides* and *F. proliferatum* given by number of genes, orientation and order (Proctor *et al*., 2008). FUM FB1 amount depends on the substrate, the genotype and the environmental conditions; these conditions play an important role in the transcription factors of the *FUM* gene (Jurado *et al*., 2008).

The species *F. verticillioides* is well known worldwide as the largest FUM producer. In addition, toxicity in corn has been documented for more than 100 years (Desjardins and Hohn, 1997). Studies about FUM toxicity were initiated in 1988 when a devastating disease occurred affecting farm animals especially horses, donkeys, mules and rabbits. Research in South Africa determined that *F. verticillioides* was the causal agent of leukoencephalomacia. This research led to conduct relevant studies with this mycotoxin, demonstrating that oral and intravenous doses lead to the disease development in horses and liver cancer in rats (Marassas, 1988).

The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) categorize this mycotoxin into group 2B, i.e., possibly carcinogenic. However, it is believed that there is a relationship between the occurrence of *F. verticillioides* and esophageal cancer (IARC, 2016; WHO/FAO, 2019). Epidemiological studies conducted in Italy, Iran, Zimbawue, China, the United States and Brazil relate this type of cancer to high concentrations of FUM B1, B2 produced by *F. verticillioides*. Additionally, the high intake of corn and wheat in diets with low content of minerals such as manganese, molybdenum, selenium, folate and vitamins A, C, E and B12 may also promote disease development (Cao *et al*., 2013). In regions such as China, South Africa, and the Texas-Mexico border, neural tube defects are associated with high corn intake (Mutchinick *et al*., 1999; Guéant-Rodriguez *et al*., 2006).

For Colombia, mycotoxin detection studies are required in food for direct consumption, raw materials and processed products to generate the necessary information to determine the safety of the food consumed.

The ELISA technique used in this study allowed the quantification of mycotoxins DON, ZEA and FUM in the isolates previously detected by PCR.

The high levels of mycotoxins detected and quantified with the extraction method used and the immunoabsorption test allowed the obtention of permissibility levels above those established for Colombia.

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