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Зборник Матице српске за природне науке / Matica Srpska J. Nat. Sci. Novi Sad, № 133, 95—104, 2017

UDC 633.15:582.281/.282
https://doi.org/10.2298/ZMSPN1733095N

Milica V. NIKOLIĆ[*], *Slavica Ž. STANKOVIĆ*, *Iva J. SAVIĆ*

Maize Research Institute "Zemun Polje"
Laboratory of Phytopathology and Entomology
Slobodana Bajića 1, Zemun Polje – Belgrade 11185, Republic of Serbia

COMPARISON OF METHODS FOR DETERMINATION OF THE TOXIGENIC POTENTIAL OF *Aspergillus parasiticus* Sp. AND *Aspergillus flavus* L. ISOLATED FROM MAIZE

ABSTRACT: Maize is considered one of the most susceptible crops to mycotoxins worldwide. Compared to other mycotoxins, the greatest attention has been paid to aflatoxins, due to their potential carcinogenicity and due to significant and longstanding problems they can cause in humans and animals. *A. flavus* and *A. parasiticus* produce aflatoxins in many economically significant crops in both fields and storages. Because of the potential aflatoxin contamination of maize grain, the toxigenic potential of *A. flavus* and *A. parasiticus* isolates, originating from Serbia, was tested in the present study. Furthermore, various applied methods for detection of these mycotoxins were compared in the study. Cultural, serological and analytical methods for the detection of mycotoxins were compared in the course of the experiment by the direct extraction of aflatoxins from the nutrient medium. The cultural methods for the detection of aflatoxin production were applied to 20 isolates of *A. flavus* (MRIZP Af18-20) and *A. parasiticus* (MRIZP Ap1-17). These methods are based on the yellow pigment formation in mycelia and nutrition media, occurrence of fluorescence on PDA (potato dextrose agar), agar containing β-cyclodextrine (CD-PDA), as well as on the red pigment formation after adding ammonium hydroxide to the existing medium. The ELISA was used to check quantitative and qualitative analyses of total aflatoxins (B1, B2, G1, G2) while the HPLC method was applied to establish ability of isolates to synthesize aflatoxins B1, B2, G1, G2. The yellow pigment formation, fluorescence and colony color changes of isolates into red, as a proof of toxigenicity of isolates, were confirmed in all cases by ELISA. A high potential of total aflatoxin production was determined in the majority of observed isolates. The ability of *A. parasiticus* isolates to synthesize aflatoxins G1 and G2 was confirmed by the HPLC method. This was essential for a better understanding of the key role of the suitability of cultural methods for preliminary evaluation of a large number of isolates. Our goal was to employ rapid biochemical approaches to prevent aflatoxin contamination of crops, and to reduce human and animal exposure to foodborne mycotoxins.

KEYWORDS: *A. flavus*, *A. parasiticus*, maize, toxigenic potential

[*] Corresponding author. E-mail: mnikolic@mrizp.rs

INTRODUCTION

The majority of aflatoxins producing fungi are members of the genus *Aspergillus* classified into the section *Flavi* (Frisvad *et al*. 2004). These fungi are often isolated from warm-humid areas. Among 22 closely related species in *Aspergillus* section *Flavi*, *Aspergillus flavus* and *A. parasiticus* are commonly encountered in a variety of agricultural products. The two species are responsible for the majority of aflatoxin contaminations, but *A. flavus* is the most common one (Varga *et al.* 2011).

Numerous studies have shown that the mycotoxigenic potential and profile of *A. flavus* is far more variable, with some isolates producing little or no aflatoxins. *A. parasiticus* typically produces more consistent and higher concentrations of aflatoxins. Razzaghi-Abyaneh *et al*. (2006) reported 100%, i.e. 27.5% of aflatoxigenic *A. parasiticus*, i.e. aflatoxigenic *A. flavus* strains, respectively. Similarly, Rodrigues *et al*. (2009) detected 77% of atoxigenic isolates in *A. flavus*, while all *A. parasiticus* isolates were found to be aflatoxigenic. *A. flavus* typically produces AFB and can be most frequently isolated from the aboveground plant parts (leaves, flowers), while *A. parasiticus* produces AFG1 and AFG2, as well as AFB1 and AFB2 and is more adapted to soil environments (EFSA, 2007). Due to their high toxicity and carcinogenic potential, they are of high concern for the safety of food worldwide (Ellis *et al.* 1991).

There are many highly specialised and sensitive methods that can be applied to the determination of the aflatoxin concentration in commodities or in cultures, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), and fluorescence polarisation assay (Seitz, 1975; Trucksess *et al.* 1994; Whitaker *et al*. 1996; Maragos and Thompson, 1999; Stroka and Anklam, 2000; Nasir and Jolley, 2002; Sobolev and Dorner, 2002; Abbas *et al.* 2004). These methods are usually expensive and time-consuming. Commercially available ELISA kits provide a relatively simple assay for quantification of the total aflatoxin concentration, but do not provide the identification of individual aflatoxins present in the sample (Abbas *et al.* 2004).

Considering the significance of the problems caused by aflatoxins, simple, rapid, and reliable cultivation methods were necessary to determine aflatoxins tested in this study. Several screening methods have been developed for the direct visual determination. Aflatoxin production by *Aspergillus* isolates can be visualised under long-wave UV light (365 nm) when grown on media suitable for aflatoxin production, such as potato dextrose agar and coconut agar (Gupta and Gopal, 2002). The fluorescence emission of AFB1 and AFG1 is substantially improved when treated with enhancer agents, such as cyclodextrins (CDs). Fente *et al*. (2001) showed that adding ß-CD to a suitable agar medium enhanced detection of aflatoxin production by *A. flavus* and *A. parasiticus* in the blue fluorescent zone under 365 nm. A second cultural method involves yellow pigment production by *A. flavus* colonies that correlates with aflatoxin production (Lin and Dianese, 1976). Aflatoxin production can be

detected with ammonia vapour as it changes the colour of toxigenic colonies from yellow to pink upon exposure (Saito and Machida, 1999).

The major objective of this study was to screen aflatoxin producing fungi in the maize samples based on the different cultural methods and to compare them with analytical methods. Because of the potential aflatoxin contamination of maize grain, the toxigenic potential of *A. flavus* and *A. parasiticus* isolates, originating from Serbia, was tested in the present study. Furthermore, various methods applied to detect these mycotoxins were compared in the study. Cultural, serological and analytical methods for the detection of mycotoxins were compared in the course of the experiment by the direct extraction of aflatoxins from the nutrient medium.

MATERIALS AND METHODS

Aspergillus strains

The trial with a total of 20 isolates of *A. flavus* and *A. parasiticus*, isolated from Serbian maize, was carried out in the period 2013–2015. In order to identify fungi and to perform morphological studies, fungi were cultured on Czapek yeast agar (CYA) and malt extract agar (MEA). *A. flavus* and *A. parasiticus* were identified according to the standard identification keys (Raper *et al*. 1973; Klich and Pitt 1988; Samson *et al*. 2007; Samson *et al*. 2014). The obtained pure cultures of *A. flavus* and *A. parasiticus* were maintained in the fungal collection of the Maize Research Institute "Zemun Polje" and were then used to obtain monospore cultures. Twenty isolates were selected for further toxicological analyses: 17 *A. parasiticus* isolates and three isolates of *A. flavus*.

Sample preparation for the mycotoxin assessment

The point inoculation of isolates was done in the center of the plate by using a dense conidial biomass. Isolates were grown on potato dextrose agar (PDA) and PDA enriched with 0.3% ß-CD (CD-PDA) (Acros, Organics, China) as single colonies in the 9-cm Petri dishes. Cultures were incubated for 5 days at 28±1 °C in the dark (Abbas *et al.* 2004). All strains were tested for aflatoxin production according to the following methods:

Cultivation methods and observation of fluorescence

Rapid techniques for the detection of aflatoxigenic and non-aflatoxigenic *Aspergillus* were investigated in this study using a yellow pigment formation in mycelium and media (Lin and Dianese, 1976; Gupta and Gopal, 2002) ammonia vapour (Saito and Machida, 1999; Kumar *et al.* 2007) and methylated ß-cyclodextrine (Fente *et al*. 2001) as indicators of *Aspergillus* colonies grown

on PDA and CD-PDA. In the course of the application of these methods, aflatoxigenic strains were developed on the reverse side of colonies of purple reddish colour, when subjected to ammonium vapour and fluorescence under UV light.

Yellow pigmentation

The yellow pigment formation in mycelia and media is a basis for the diagnostic determination of aflatoxigenic isolates (Abbas *et al.* 2004a; Shier *et al*. 2005; Odhiambo *et al*. 2014). The degree of yellow pigmentation is proportional to blue fluorescence in culture media (Lin and Dianese, 1976).

Methyl-ß-cyclodextrin test

The presence or absence of fluorescence in the agar surrounding the colonies grown on PDA and CD-PDA was determined under UV radiation (365 nm) and assayed as positive or negative. All strains were analysed with ELISA and HPLC to confirm the correlation between fluorescence and aflatoxin production.

Ammonia vapour test

After five-day growth of colonies, Petri dishes were inverted over 3 drops of 28–30% ammonium hydroxide (Acros Organics, USA). Toxicity of isolates was determined on the basis of a change in colour of the nutrient medium. The color change occurred after the colony was in contact with ammonia vapour. In the non-toxic isolates there was no change of colour.

Determination of aflatoxins by ELISA and HPLC

The contents of PDA and CD-PDA plates were scraped into a tube to collect fungal biomass. Fungal biomass (mycelia, conidia heads, conidia) was placed in glass scintillation vials (20 mL) and fresh weights were recorded (typically 0.5–1 g). Methanol-water (70:30, *v*/*v*) was added (10:1, *v*/*m*) to vials, and the vials were shaken for 30 min at high speed with a reciprocal shaker. A 1-mL aliquot of extract was removed and centrifuged at 12 000 *g* for 10 min with a MicroSpin 12S centrifuge. The supernatant was assayed for the presence of total aflatoxins (B1, B2, G1, and G2), using ELISA kits (Elabscience Biotechnology Co., Ltd). The obtained strains were analysed by high-pressure liquid chromatography (HPLC) with fluorescence detection to confirm the presence of aflatoxins B1, B2, G1, and G2. Prior to the separation by HPLC, fungal biomass had to be extracted with a solvent mixture acetonitrile-dd H2O

(90:10, *v/v*) and was added (100:1, *v/m*) to vials (Abbas *et al.* 2004). All experiments were repeated three times.

RESULTS AND DISCUSSION

A large number of fungal colonies showing positive results after 5 days of growth on PDA and CD-PDA were determined with one of the methods stated below. Agreements and minor divergences are shown in Table 1.

Yellow pigmentation

A. parasiticus cultures producing aflatoxin formed yellow pigmentation in almost all strains used in our experiments. The isolate MRIZP – AP17 was an exception as it did not produce yellow pigment in mycelium, but it produced toxins. The degree of yellow pigmentation was in concordance with fluorescence in all isolates producing toxins. Non-aflatoxigenic isolates did not produce yellow pigments (MRIZP – AF1, MRIZP – AF2, and MRIZP – AF3). This is in agreement with results obtained by Lin and Dianese (1976) who indicated that the degree of yellow pigmentation was proportional to fluorescence in all media tested.

Ammonium hydroxide vapour test

A. parasiticus isolates MRIZP – AP1, MRIZP – AP2, MRIZP – AP10, and MRIZP – AP14 produced purple reddish pigmentation, while *A. parasiticus* isolates MRIZP – AP3, MRIZP – AP4, MRIZP – AP5, MRIZP – AP6, MRIZP – AP7, MRIZP – AP8, MRIZP – AP9, MRIZP – AP11, MRIZP – AP12, MRIZP – AP13, MRIZP – AP15, and MRIZP – AP16 produced moderate purple reddish pigmentation on the reverse side of colonies with the ammonium hydroxide test. *A. flavus* isolates MRIZP – AF1, MRIZP – AF2, and MRIZP – AF3 did not show color change in any of the experiments, because they are non-aflatoxigenic strains. The isolate MRIZP – AP17, which did not produce yellow pigment in mycelium, showed red pigmentation, which was unusual. There was toxin confirmation when this isolate was tested with ELISA and HPLC. Nevertheless, it can be concluded that the ammonium hydroxide test was a reliable test for detection of aflatoxins. Saito and Machida (1999) reported that the ammonium hydroxide vapour test gave 11% false positive and 6% false negative results for aflatoxigenicity. Kumar *et al.* (2007) reported 92% efficacy for the ammonium vapour test having 8% false negatives. Abbas *et al.* (2004) used yellow pigmentation combined with the ammonium hydroxide vapour test and thus false negatives were reduced to 7%.

The number of positive colonies detected using this methodology also followed a similar pattern as exhibited by the results obtained from colony fluorescence.

Methyl-ß-cyclodextrin test

Results gained with the methyl-ß-cyclodextrin test are in accordance with the findings discovered with the ammonium hydroxide vapour test. The only exception was the isolate MRIZP – AP17 as toxins were produced in it, which was indicated by results of ELISA and HPLC. Moreover, false positives were obtained by the absence of blue fluorescence in the CD-PDA media under UV. Our findings showed that the methyl-ß-cyclodextrin test was sensitive enough to detect aflatoxins.

ELISA and HPLC

Aflatoxin production abilities tested previously by fluorescence under UV light of strains cultivated on potato dextrose agar were in concordance

Table 1. Agreement between three methods for culturing, with ELISA and HPLC quantities from *A. flavus* and *A. parasiticus*

Isolates	Yellow pigmentation		Fluorescence		Ammonia vapour		ELISA (total aflatoxins) ng/g	HPLC G1/G2 (ng/g)
	PDA	CD-PDA	PDA	CD-PDA	PDA	CD-PDA		
MRIZP Ap 1	+	+	+	+	+	+	2,110	3.123,01/ ND
MRIZP Ap 2	+	+	+	+	+	+	4,140	5.059,26/114,57
MRIZP Ap 3	+	+	+	+	±	±	4,970	5.746,97/217,83
MRIZP Ap 4	±	+	±	±	±	±	5,595	6.449,07/92,02
MRIZP Ap 5	±	+	±	±	±	±	2,350	3.437,81/306,65
MRIZP Ap 6	+	+	+	+	±	±	2,525	3.461,35/260,47
MRIZP Ap 7	±	+	±	+	±	±	3,215	7.122,59/395,18
MRIZP Ap 8	±	±	±	±	±	±	2,300	8,03/ ND
MRIZP Ap 9	±	±	±	±	±	±	2,695	554,22/47,69
MRIZP Ap 10	+	+	+	+	+	+	6,655	7.421,58/374,93
MRIZP Ap 11	±	±	±	±	±	±	2,510	3.285,56/188,75
MRIZP Ap 12	±	+	±	+	±	±	4,595	634,80/ ND
MRIZP Ap 13	±	+	±	+	±	±	2,175	4.976,12/109,58
MRIZP Ap 14	+	+	+	+	+	+	7,260	7.095,32/102,61
MRIZP Ap 15	±	±	±	±	±	±	5,075	5.124,99/128,02
MRIZP Ap 16	±	±	±	±	±	±	7,490	6.240,54/201,47
MRIZP Ap 17	–	–	–	–	+	+	2,435	6.518,88/90,52
MRIZP Af 1	–	–	–	–	–	–	ND	ND
MRIZP Af 2	–	–	–	–	–	–	ND	ND
MRIZP Af3	–	–	–	–	–	–	ND	ND

ND – not detected

with those obtained by ELISA and HPLC determination. A high potential of total aflatoxin production was established in the majority of tested isolates. In our study, all *A. parasiticus* strains produced aflatoxins. On the other hand, none of *A. flavus* strains produced aflatoxins. Their ability to synthesize a high concentration of aflatoxins G1 and G2 was confirmed by the HPLC method. *A. parasiticus* isolates showed a high production potential of B- and G-type aflatoxins in previous researches (Vaamonde *et al.* 2003; Rodrigues *et al.* 2009; Baquião *et al.* 2013).

The fluorescence on CD-PDA had the highest degree of conformity for the identification of toxigenic isolates with the ELISA assay. Abbas *et al.* (2004) achieved similar results with the strongest correlation between the cyclodextrin-enchanced fluorescence test and ELISA among the tested methods of culturing and they showed that these methods were reliable in screening aflatoxin production by *Aspergillus* strains.

CONCLUSIONS

The present study shows the adequacy of the culturing method for the preliminary isolate evaluation. In case of limited resources, this method is suitable for the detection of aflatoxins due to the cost-effectiveness of application.

Although all culturing methods applied in this study could be used as preliminary indicators, the ELISA test, i.e. HPLC is confirmed to be a reliable method for the toxin detection in *A. flavus* and *A. parasiticus*. Having in mind that aflatoxin contamination, as a major threat to human health and to the world's food supply, will be an issue in the foreseeable future, further insight might lead to the development and improvement of methods for its continuous analysis.

As global climate changes continue to affect regions of Europe, the potential impact of aflatoxin contamination may increase beyond regions with tropical climate, which is already happening. The ability to estimate climate changes and the relation between these changes and fungal infection and subsequent aflatoxin contamination will help in predicting and dealing with this emerging risk.

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КОМПАРАЦИЈА МЕТОДА ЗА УТВРЂИВАЊЕ ТОКСИГЕНОГ ПОТЕНЦИЈАЛА *Aspergillus parasiticus* Speare И *Aspergillus flavus* Link ИЗОЛОВАНИХ СА КУКУРУЗА

Милица В. НИКОЛИЋ, Славица Ж. СТАНКОВИЋ, Ива Ј. САВИЋ

Институт за кукуруз „Земун Поље“,
Лабораторија за фитопатологију и ентомологију,
Слободана Бајића 1, Земун Поље – Београд 11185, Република Србија

РЕЗИМЕ: Кукуруз се широм света сматра једним од усева најподложнијих за контаминацију микотоксинима. Афлатоксинима се, у поређењу с другим микотоксинима, придаје највећа пажња због њихове потенцијалне канцерогености, значајних и дугорочних проблема које изазивају код људи и животиња. Врсте *A. flavus* и *A. parasiticus* могу продуковати афлатоксине код многих економски значајних култура у пољима и складиштима. Због потенцијалне контаминације зрна кукуруза афлатоксинима у овом раду је испитан токсигени потенцијал изолата управо ове две врсте пореклом из Србије и упоређене су различите методе детекције ових микотоксина. Током експеримента упоређене су одгајивачке, серолошке и аналитичке методе детекције микотоксина, директном екстракцијом афлатоксина из хранљиве подлоге. Истраживања су базирана на примени одгајивачке методе за одређивање продукције афлатоксина код 20 изолата *A. parasiticus* (MRIZP Ap1-17) и *A. flavus* (MRIZP Af18-20) пореклом из Србије. Одгајивачке методе су биле засноване на формирању жутог пигмента у мицелији и хранљивој

подлози, на појави флуоресценције на PDA (кромпир декстрозни агар) и подлози која садрзи β-циклодекстрин (CD-PDA), као и на образовању црвеног пигмента у подлози након додавања амонијум хидроксида. ELISA тест је коришћен за проверу квантитативних и квалитативних садржаја укупних афлатоксина Б1, Б2, Г1 и Г2, док је HPLC методом утврђена концентрација појединачних афлатоксина Б1, Б2, Г1 и Г2. Образовање жутог пигмента, флуоресценција и промена боје колоније изолата у црвену, као доказ токсигености изолата, потврђена је у свим случајевима и ELISA тестом. Код већине изолата установљен је висок потенцијал продукције укупних афлатоксина. HPLC методом потврђена је и способност синтезе афлатоксина Г1 и Г2 од стране изолата *A. parasiticus*. Циљ експеримента био је да се испита ефикасност употребе брзих тестова за детекцију афлатоксина, како би се спречила контаминација усева и изложеност људи и животиња афлатоксинима.

КЉУЧНЕ РЕЧИ: *Aspergillus parasiticus*, *Aspergillus flavus*, кукуруз, токсигени потенцијал