DOI: 10.22620/agrisci.2018.24.002

MICROBIOLOGICAL ASSAY OF MYCOTOXIN-PRODUCING MOLDS

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Abstract

Mycotoxins are secondary fungal metabolites, toxic to humans and animals alike.

Mycotoxins are considered hazardous which requires accurate detection of each toxin. Several screening methods for direct visual determination of mycotoxin production have been reported. In order to reduce the risk for consumers, it is necessary to introduce rapid methods for the detection of toxigenic microfungi isolated from food samples.

For the purpose of the study presented in this paper, we managed to isolate and identify different fungal species belonging to *Fusarium sp*., *Penicillium sp*. and *Aspergillus sp.* from wheat seeds on the basis of their macroscopic and microscopic characteristics.

The primary concern of our study was to use proper morphological methods and selective media with a view to identifying filamentous fungi isolated from wheat grains. As a result, a total of 19 wheat germ molds were isolated, seven of which being *Fusarium sp*., six *Penicillium sp.* and the other six – from the *Aspergillus* species.

Furthermore, we found that 53 percent of the isolated mold species were capable of producing aflatoxins grown in the β-cyclodextrin media.

Another important implication was that the culture media and reagents used in our study were found suitable for mycological laboratory analyses. Significant advantages of such media are considered the following: rapid diagnosis, simple preparation, better availability and relatively low-prices.

Keywords: wheat, storage, fungi, mycotoxins, aflatoxins, rapid detection methods.

INTRODUCTION

Cereal crops are exposed to mold fungus attacks during their growth, harvesting, storage and processing time (Berthier and Valla 1998, Rutz et al., 2002, Kana et al., 2013). Approximately 10% of total world production is polluted with mould fungus, making the produce unfit for consumption (Pitt and Hocking, 2009). They reduce the protein and fat content; degrade organoleptic properties of crops and deteriorate food and feeds qualities (Hogg, 2005).

Contamination of cereal crops with toxic mold fungus and mycotoxins produced by them, even at low, sublingual concentrations, is a prerequisite for hidden mycotoxicosis. The problem of the presence of mycotoxins in foods and the need for their control is due to the high degree of contamination of cereal crops with molds. Secondary metabolites of fungi of the genus *Fusarium sp*., *Aspergillus sp*. and *Penicillium sp*. are commonly found worldwide and their high toxicity is of global importance (Driehuis et al., 2008). The climate conditions in Bulgaria are suitable for the development of molds of the genus *Fusarium sp*., as well as those of the genus *Aspergillus sp*. and *Penicillium sp*. This requires the introduction of monitoring of cereal contamination with molds and the possibility of the rapid diagnosis of potential mycotoxins.

Among the most common mycotoxins are those, produced by *Fusarium*, aflatoxins and ochratoxins, which are of acute toxic and carcinogenic effect (Sivakumar et al., 2014). Their toxicity is accompanied by ubiquitous distribution, and the magnitude of economic losses makes them a global problem (Muhammad et al., 2010).

Mycotoxins have a destructive effect on many systems of the human and animal organism. Digestive, nerve disorders, changes in blood formation, respiratory disturbances, abortions, body temperature increase, etc. are observed (Begum and Samajpati, 2000). Most mycotoxicoses are characterized by a lack of specific clinical symptoms and are not administered to drug therapy (Kana et al., 2013). The most important deleterious properties of the secondary metabolites are: nephrotoxic, cardiotoxic, dermocrotic, carcinogenic, teratogenic and immunosuppressive (Shareef, 2010). This multidimensional mode of action of mycotoxins also determines their widespread clinical symptoms (Whitlow, 2005).

Not all *Fusarium*, A*spergillus* and *Penicillium* species are able mycotoxins and it is important to screen selective nutrient media to

prove their toxin-producing ability, (Saito and Machida, 1999, Fente et al., 2001; Kumar et al., 2007). Davis et al. (1987) used a rapid method by incubating moulds on Coconut agar (CA) to study the ability of microscopic fungi to synthesize aflatoxins. In this method, the aflatoxigenic strains show blue fluorescence on the reverse side of the colonies under ultraviolet light at a wavelength of 365 nm. The same method was later applied by Varga and Samson (2008) for the synthesis of ochratoxin from certain strains of *the Aspergillus* genus. Positive results include colonies that form dark green fluorescence under UV light.

The aim of the present study was to isolate wheat fungal microflora and to identify potential mycotoxin-producing strains by applying different microbiological selective media.

MATERIAL AND METHODS

The experiments were carried out in the Laboratory of the Department of Microbiology and Ecological Biotechnology, at the Agricultural University – Plovdiv. Grain from different varieties of wheat stored in paper bags under laboratory conditions was investigated. The isolation of fungi was done by using the agar dilution method. 20g of kernels were homogenized with 80 ml of buffer peptone water. Fungal isolates were cultured on Potato dextrose agar (PDA), (Biolife, Italy) and chloramphenicol was added (100 mg/l) using the Botton method (1990). Inoculated plates were incubated for 7 days at 25°C. To determine the effect of media on colour change coconut (COA), yeast extract (YEA) and Czapex Dox (CDA) agars were used. Coconut agar (COA) was prepared according to the method of Yazdani et al., (2010). Each media was autoclaved at 121°C for 20 mim. The inoculated plates were cultured in dark at 28°C for 5 to 7 days. The result was determined by the presence of fluorescence under UV light (365 nm). Fungal plate isolation is cultivated on YEA agar by the method of Kumar et al. (2007). The 72 hours fungal culture plates were treated for 10 minutes with ammonium hydroxide. The result is scored as positive from pink to red colour of the colonies and as negative when there is no change or the colour is brown. Plates with YEA agar with 3% methyl β-cyclodextrin developed by Fente et al. (2001) were used to determine the presence or absence of a fluorescence ring under irradiation with UV light (365 nm). To confirm that the colour change was associated with aflatoxin productivity Czapek Dox agar was tested, Hara et al. (1974). The result is determined by the presence or absence of a fluorescence ring under UV light (365 nm) after 7, days of incubation. The presence of fluorescence (green or blue) is an indicator of aflatoxin synthesis.

Isolates were identified by using schemes, based on microscopic observation and culture appearance including colonies, colours, texture, conidia shape and nature of spores. The identification of fungal species was done by BIOLOG semiautomatic system, Inc. USA.

RESULTS AND DISCUSSION

Isolated fungal colonies were identified using traditional microbiological methods. In total 19 strains were isolated on PDA and Capex Dox agar. The morphological evaluation revealed that the seven (7) isolates belonged to the genus *Fusarium*. They form a dense, fluffy and pink-white to grey air mycelium. The width of the microconidia ranges from 1.0 to 3.0 μm and their lengths from 3.0 to 9.0 μm. For macroconidia, the width variation is from 1.5 to 3.0 μm and the length from 3.0 to 9.0 μm. Based on the microscopic examination and phenotypic profile from BIOLOG the identified species are: *Fusarium sporotrichioides*; *Fusarium lateritium (fig.1); Fusarium subglutinan*; *Fusarium oxysporum*; *Fusarium tricinctum; Fusarium coccicicola and Bionectria sesquicill.*

Six species classified under *Aspergillus* genera were isolated – *Aspergillus phoenicis*; *Aspergillus ostianus; Aspergillus tereus; Aspergillus flavus; Aspergillus carbonarius* and *Aspergillus parasiticus*. They are characterized by a wide variety of mycelial growth, including black, orange-green, copper-brown, yellow-green and green mycelium. Conidia are spherical in shape, yellow-brown, with a smooth or rough surface, (fig. 1). The diameter of the conidia varies from 2.5 to 8.0 μm.

Penicillium is a large genus, with more than 200 recognized species, of which 50 or more are of common occurrence (Pitt, 2000). Among the isolates we identified six species: *Penicillium canescens*: *Penicillium thomii: Penicillium brevicompactum; Penicillium griseofulvum* 1; *Penicillium griseofulvum* 2; and P*enicillium aethiopicum*. They are characterized by green mycelium, except for the species *Penicillium thomii*, which has an orange-green colour of the mycelium. Conidia are round to spherical in shape, colourless and with a rough surface (fig. 3) The diameter of the conidia ranges from 1.0 to 3.3 μm.

The presence of more than one genus and type of molds in wheat grains is a prerequisite for the risk of synthesizing mycotoxins from different groups. In order to ensure the quality of the wheat used for human and animal consumption, it is necessary to check for the presence of mycotoxin molds. The results of culturing mold fungi on Coconut media found that 42.00% of the isolates

tested had a toxin-producing ability. Out of a total of 19 molds, only 5 of them are fluorescent green and 3 light blue. The presence of a fluorescent blue-green ring around *Aspergillus tereus*, *Aspergillus ostianus*, *Aspergillus flavus*, *Aspergillus carbonarius* and *Penicillium aethiopicum* has been observed. This blue fluorescence ring around the fungal colonies is evidence of the presence of aflatoxins and ochratoxin A. Only two species (*Penicillium brevicompactum* and Penicillium *griseofulvum* 2) have a positive reaction in a UV light-producing mycotoxin test, where a weak blue fluorescent ring. Only *Fusarium coccicіocola* had a positive result in aflatoxin synthesis. Under the UV light around the mycelium, a blue fluorescent ring is observed.

According to Ciobotaru et al., (2014) a formation of a blue fluorescence ring around the mold mycelium under UV light is associated with the synthesis of aflatoxins, and in the formation of a blue-green ring, there is a synthesis of ochratoxins.

According to the studies of Yazdani et al. (2010), coconut ingredients influence the fluorescence effect due to the presence of pigment in coconut culture. The use of coconut aflatoxin detection media is not always a reliable screening method, because of the high sensitivity of *Aspergillus* molds to the nutrient media components.

Ammonium hydroxide reagent was used to detect aflatoxins produced by fungal isolates. Positive results were reported for *Fusarium sporotrichioides, Fusarium tricinctum, Aspergillus ostianus*, but only *Fusarium tricinctum* showed purple staining after treatment with the reagent. The colour change was visible 7 days after applied ammonium hydroxide. No positive effect was observed in *Bionectria sesquicilli; Penicillium canescens; Penicillium griseofulvum 1; Penicillium griseofulvum 2.* Saito and Machida (1999) and Jefremova et al. (2015) used ammonium hydroxide successfully in order to diagnose aflatoxin-producing molds.

The present study shows that after treatment with the β-cyclodextrin, a fluorescent ring was detected in *A. ostian; A. flavus; A. phoenicis; A. carbonarius; P. canescens; P. brevicompactum; P. griseofulvum 1; P. griseofulvum 2.; Fusarium oxysporum and Fusarium subglutinans.*

A number of authors use β-cyclodextrin to increase the ability of molds to fluoresce more intensively. According to Sudini et al., (2015) the fluorescence of aflatoxin B1 and aflatoxin G1 is significantly improved in the presence of β-cyclodextrin. They are cyclic oligosaccharides consisting of α-D-glucopyranose units (α – 1, 4). Production of aflatoxin B1 in the presence of β-cyclodextrin has been investigated by several authors (Blais et al., 1988; Cepeda et al., 1988; Francis et al., 1988; and Vazquez et al., 1991). However, the exact mechanism of increasing fluorescence of aflatoxin-producing molds in the presence of β-cyclodextrin has not been established (Vazquez et al., 1992).

Table 1. Effect of different media upon potential mycotoxin production

Fungal isolated species	MEDIA			
	COA	YEA + ammonium hydroxide	YEA + β-cyclodextrin	CDA
Fusarium oxysporum	-	-	+	-
Fusarium coccicіocola	+	-	-	-
Fusarium sporotrichioides	-	+	-	-
Fusarium tricinctum	-	++	-	-
Fusarium lateritium	-	-	-	-
Fusarium subglutinans	-	-	+	-
Bionectria sesquicilli	-	-*	-	-
Aspergillus terreus	+	-	-	+
Aspergillus ostianus	+	+	+	+
Aspergillus flavus	+	-	+	+
Aspergillus phoenicis	-	-	+	+
Aspergillus carbonarius	+	-	+	+
Aspergillus parasiticus	-	-	-	+
Penicillium canescens	-	-*	+	-
Penicillium aethiopicum	+	-	-	-
Penicillium brevicompactum	+	-*	+	-
Penicillium griseofulvum 1	-	-*	+	-
Penicillium griseofulvum 2	+	-*	+	-
Penicillium thomii	-	-	-	-

*Legend: "-" without fluorescence, negative result; "+" with fluorescence, positive result; -*brown colour, negative effect*

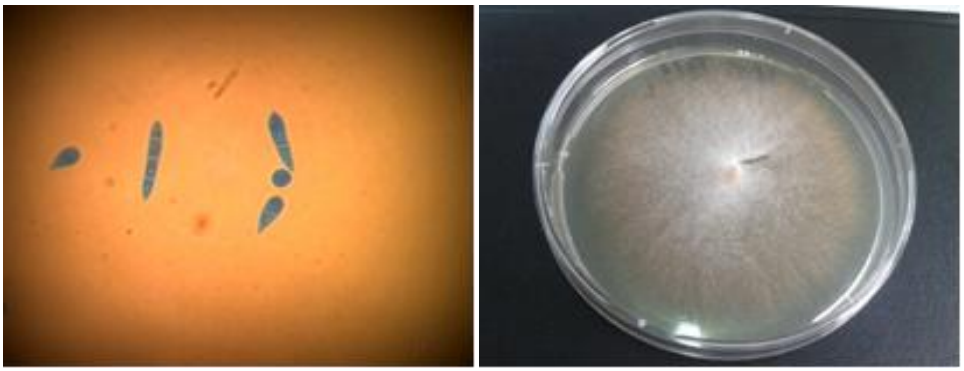

Fig. 1. *Fusarium lateritium, micro- and macroconidia, stained with lactophenol cotton blue*

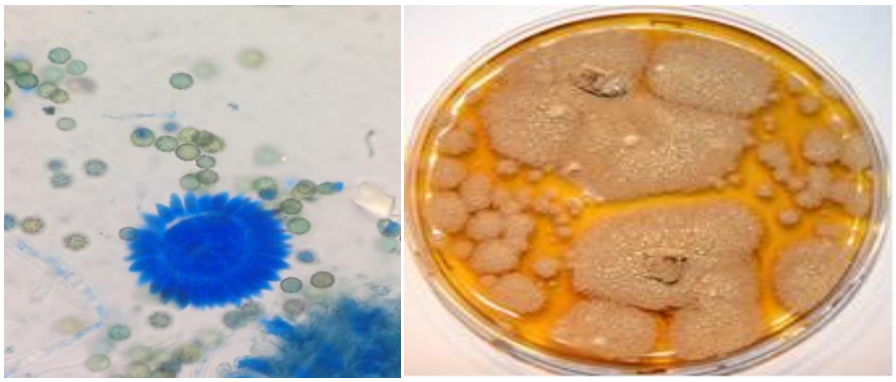

Fig. 2. *Aspergillus tereus, stained with lactophenol cotton blue*

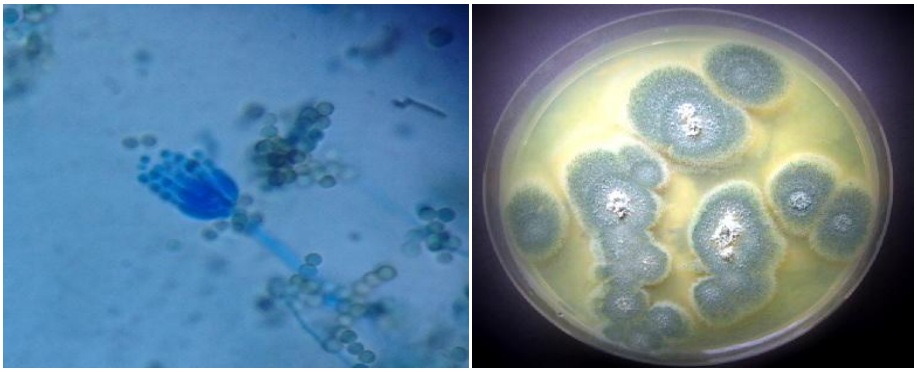

Fig. 3. *Penicillium griseofulvum stained with lactophenol cotton blue*

The experiment with Czapek media shows that all isolated strains of *the Aspergillus* genus have been fluorescence.

We find out that *Aspergillus terreus* fluoresces in blue and species such as *Aspergillus ostianus, Aspergillus flavus, Aspergillus phoenicis, Aspergillus carbonarius* and *Aspergillus parasiticus* fluoresce green. Only *Aspergillus phoenicis* (fig. 4) has the ability to fluoresce blue and green under ultraviolet light at a wavelength of 365 nm.

In ultraviolet photographs, aflatoxin-producing strains are seen as green or blue colonies; therefore these colonies absorb the emitted ultraviolet light.

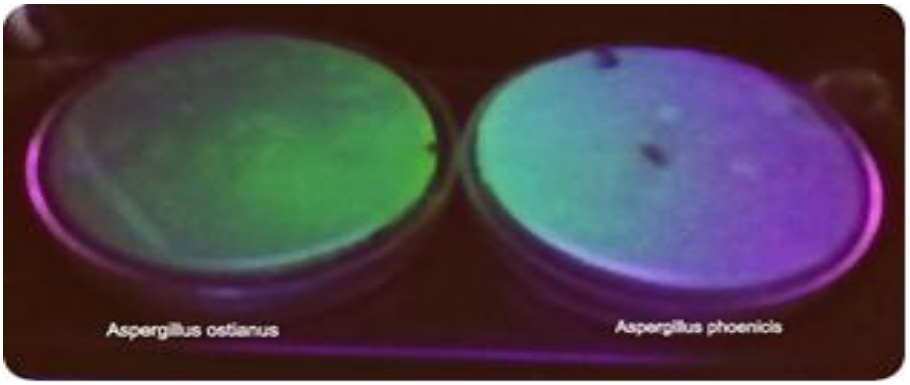

Fig. 4. *Green (Aspergillus ostianus) and blue (Aspergillus phoenicis) fluorescence*

CONCLUSIONS

All of the media, tested in this study is suitable for microbiological diagnosis of toxigenic microfungi.

1. Isolated strains, growth on COA medium visible fluorescence were observed in *F. coccicіocola, A. terreus, A. ostianus, A. flavus, A. carbonarius, P. aethiopicum, P. brevicompactum, P.griseofulvum* 2.

2. Application of ammonium hydroxide reagent increased the pigmentation in the following species: *F. sporotrichioides, F. tricinctum* and *A.ostianus*.

3. β-cyclodextrin culture media showed positive results in *F. oxysporum, F. subglutinans, A. ostianus, A. flavus, A. phoenicis, A. carbonarius, P. canescens, P. brevicompactum, P. griseofulvum1* and *P. griseofulvum 2.* Thus, mold fungus having the ability to produce aflatoxins is about 53% of all isolated species.

4. Green fluorescence was detected in Czapek media in species such as *A. ostianus, A. flavus, A. phoenicis, A. carbonarius and A. parasiticus*, and in *A. tereus* blue.

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