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REGULAR ARTICLE

THE OCCURRENCE OF MICROMYCETES IN THE BREAD SAMPLES AND THEIR POTENTIAL ABILITY PRODUCE MYCOTOXINS

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ABSTRACT

The aim of this study was to determinate microscopic fungi that can cause occurrence of mould in bread. We used breads from experimental baking with different addition of walnuts (0 - 15%) as model samples. Bread samples were stored in the fridge, in plastic bags and in the bread box. After three days of storage 25% of samples were moldy. The middle parts of breads (4 pieces), that were not moldy, were stored on DRBC and cultured at 25±1°C for three days. All the colonies of micromycets were inserted on identification agar. Molding of bread samples was caused by species of genera: *Aspergillus, Penicillium, Cladosporium* and *Rhizopus*. 28 strains of potentially toxigenic species of genera *Aspergillus* and *Penicillium* were tested by TLC method for the ability to produce chosen mycotoxins in conditions in vitro. We discovered the production of cyclopiazonic acid, penitrem A and roquefortin C using mentioned method.

Keywords: bread, micromycetes, mycotoxins

INTRODUCTION

Bakery products are an important part of a balanced diet. However, bakery products, like many processed foods, are subject to physical, chemical and microbiological spoilage **(Smith *et al.*, 2004).** The major problem for long-term shelf life of baked goods is contamination with fungi **(Coda *et al.*, 2011).** The most widespread species of fungi that contaminate bakery products belong to the genera *Eurotium*, *Aspergillus*, *Monilia*, *Mucor*, *Cladosporium*, *Fusarium* and *Rhizopus* **(Coda *et al.*, 2008)**. Besides the visible growth, fungi may be responsible for off-flavors and synthesize mycotoxins and allergenic compounds.

The aim of this study was to determinate microscopic fungi involved in molding of bread and to test isolated potentially toxigenic species for ability to produce chosen mycotoxins in conditions *in vitro*.

MATERIAL AND METHODS

In this study we tested mycological 12 samples of bread prepared by experimental baking. The main substance used for preparing the bread was wheat flour T-650 with different ratio (0 – 15%) of walnuts. After baking the bread samples were three days stored at room temperature in a plastic bag, in the bread box and in the fridge at temperature 4 - 8°C. After that we detected contamination of samples by microscopic filamentous fungi with a goal to gain overview about the range of fungi that occur in used types of bread in these ways of storage, which are the most used at our homes. Fungi that created visible colonies on the surface of the bread were inserted directly on MEA (Malt Extract agar; **Klich, 2002**). All the bread samples (the middle part) were cut to cubes of sides 1.5 x 1.5 x 1.5 cm and were given in number of four pieces directly on plates with DRBC medium (Dichloran Rose Bengal Chloramphenicol agar; Merck, Germany). This was repeated twice. Cultivation proceeded for three days in the dark 25±1°C. Members of genera *Aspergillus* and *Penicillium* were consequently isolated on diagnostic media of CYA (Czapek Yeast Extract agar; **Klich, 2002**), MEA (Malt Extract agar; **Klich, 2002**), CY20S (Czapek Yeast Extract agar with 20% Sucrose; **Klich, 2002**) and CYA, MEA, CREA (Creatine-Sucrose agar, **Samson *et al.*, 2002**), YES (Yeast Extract agar; **Samson *et al.*, 2002**; 1000 ml of distilled water), respectively. Members of other genera were isolated on diagnostic medium MEA. In all cases, cultivation proceeded for 5 – 7 days in the dark at 25 ± 1°C. To determine particular species, diagnostic literature was used as follows: **Pitt, 1985**, **Klich, 2002**, **Samson *et al.*, 2002** for aspergilli and

Pitt *et* Hocking (2009); Samson *et al.* (2002); Samson *et* Frisvad (2004) for penicillia and **Samson *et al.* (2002)** for species other genera. Ability of selected isolates of potentially toxigenic species to produce relevant mycotoxins in *in vitro* conditions was screened by means of thin layer chromatography (TLC) according to **Samson *et al.* (2002)** modified by **Labuda *et* Tančinová (2006)**. Cultivation for screening of extracellular metabolites (patulin, citrinin, aflatoxin B_1, ochratoxin A) was carried out on YES and for intracellular (cyclopiazonic acid, penitrem A, roquefortin C) on CYA; conditions of cultivation as described above. In each tested isolate, 3 pieces of mycelium together with cultivation medium of area of approximately 5 x 5 mm were cut from colonies and extracted in 1000 ml of chloroform-methanol (2:1, v/v) on vortex for 2 minutes. 20 µl of liquid phase of extracts along with standards (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system toluene:ethylacetate:formic acid (5:4:1, v/v/v/). Visualisation of extrolites was carried out as follows: cyclopiazonic acid directly in daylight after spraying with the Ehrlich reagent (violet-tailed spot); patulin by spraying with 0.5 % methylbenzothiazolone hydrochloride (MBTH, Merck, Germany) in methanol, heated at 130 °C for 8 min and then detectable as a yellow-orange spot; penitrem A after spraying with 20 % $AlCl_3$ in 60 % ethanol, heated at 130 °C for 8 min and then detectable as a dark green to black spot on daylight; roquefortin C after spraying with $Ce(SO_4)_2$ x 4 H_2O visible as orange spot. Directly under UV light (365 nm) were visualised following mycotoxins: aflatoxin B (blue spot), aflatoxin G (green), citrinin (yellow-green, griseofulvin (blue), ochratoxin A (bluish-green).

RESULTS AND DISCUSSION

In three samples (25%) after three days of cultivation micromycetes created colonies on the surface of a crust (Tab 1). All visibly moldy samples were stored in plastic bags at room temperature. We identified samples responsible for molding as *Penicillium chrysogenum* (3 samples), *P. aurantiogriseum* (1 sample), *P. aethiopicum* (1 sample) a *Cladosporium sphaerospermum* (1 sample). Even 78% of pieces taken from different non moldy midlle parts of bread stored on DRBC were after three days of cultivation covered with microscopic filamentous fungi (Tab 2), but to molding leaded all variants of samples. The most frequent contaminants of samples were *Aspergillus flavus*, *Cladosporium sphaerospermum*, *Penicillium crustosum* and *P. chrysogenum*. Similar mycocenosis was

described also by **Coda *et al.* (2008)**. But in our study we did not isolate members of genus *Eurotium*.

Table 1 Microscopic fungi causing mould in bread samples

Species	**Growth of fungi on the bread samples (n=12)**	
	Number of positive sample during storage (3 days)	Number of positive sample during cultivation piece of bread on DRBC
Aspergillus flavus		7
Aspergillus section *Nigri*		1
Aspergillus sp.		1
Cladosporium sphaerospermum	1	5
Penicillium aethipicum	1	1
Penicillium aurantiogriseum	1	1
Penicillium chrysogenum	3	5
Penicillium crustosum		5
Penicillium glabrum		2
Penicillium roqueforti		1
Penicillium sp.		3
Rhizopus stolonifer		2

Legend: DRBC - Dichloran Rose Bengal Chloramphenicol agar

From analysed samples 28 strains of six potentially toxigenic species of genera *Aspergillus* and *Penicillium* were tested for ability to produce chosen mycotoxins in conditions in vitro using TLC method (thin-layer chromatography) (Tab 2). Totally by 27 strains (96%) we discovered the ability to produce minimally one mycotoxin. All tested strains of *A. flavus* were producing cyclopiazonic acid, but the ability to produce aflatoxins was not detected. All tested strains of *P. roqueforti* (1), *P. chyrosgenum* (8), *P. crustosum* (8) and *P. expansum* (1) were producing roquefortin C in conditions in vitro. Strains of *P. crustosum* were also producing penitrem A. We tested strains of *P. expansum* for ability to produce other mycotoxins, patuline and citrinine. Production of these mycotoxins was not detected. Mycotoxins are extrolites produced by filamentous microfungi that can cause disease in vertebrate animals when introduced via a natural route: ingested, absorbed through the skin or inhaled **(Frisvad et al., 2007)**. Molding of the bread does not represent just a danger by its consummation, but also by inhalation, contact with it.

Table 2 *In vitro* production of mycotoxins by aspergilli and penicillia isolated from breads tested by means of thin layer chromatography

Species	Number of tested isolates	Detection toxin	Evaluation	
			+	-
Aspergillus flavus	7	AFB_1, AFG_1		7
	7	CPA	7	
Aspergillus section *Nigri*	1	OTA		1
Penicillium chrysogenum	9	ROC	9	
Penicillium crustosum	8	ROC	8	
	9	PA	9	
Penicillium expansum	1	PAT		1
	1	ROC	1	
	1	CIT		1
Penicillium roqueforti	1	ROC	1	

Legend: AFB1 aflatoxin B_1, AFG1 – aflatoxin G1, CPA – cyklopiazonic acid, OTA – ochratoxin A, PA – penitrem A, PAT- patulin, ROC roquefortin C, + production of mycotoxin confirmed, - production of mycotoxin was not detected

CONCLUSION

Filamentous microscopic fungi are the most frequent cause of bread decay. Although the bread is included in commodities with short durability, there is an effort to prolong it. After three days of storage visible destruction of bread by growth of fungi was found in bread samples stored in plastic bag at room temperature. During this time fungi did not start to grow just on a sample with walnut addition. We detected *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *P. aurantiogrisem* and *P. aethiopicum* as a cause of molding. By cultivation of samples on DRBC other species were responsible for molding: *Aspergillus flavus*, *Aspergillus* section *Nigri*, *Penicillium chrysogenum*, *P. glabrum, P. crustosum, P. roqueforti* and *Rhizopus stolonifer*. 27 out of 28 tested potential strains produced minimally one mycotoxin.

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