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REGULAR ARTICLE

THE OCCURRENCE OF MICROMYCETES IN APPLES AND THEIR POTENTIAL ABILITY TO PRODUCE MYCOTOXINS

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ABSTRACT

The aim of this study was to determinate microscopic fungi involved in rot of apples in market and to test isolated potentially toxigenic species for ability to produce chosen mycotoxins in conditions *in vitro*. From 30 apples with rotting were isolated and identificated 8 genera (*Penicillium, Monilinia, Botrytis, Aspergillus, Cladosporium, Epicoccum, Fusarium* and *Geotrichum*) of filamentous fungi. The most frequent (40% rot apples) was *Penicillium expansum*, the most important producer of rotting during storage of apples. For the ability to produce mycotoxins *in vitro* were tested isolates, potential producers of mycotoxins. All tested isolates were determinated as producers of mycotoxins: *Penicillium expansum* (patulin and citrinin, 12 isolates), *Penicillium citrinum* (citrinin, 1 isolate), *Penicillium roqueforti* (roquefotin C, 1 isolate) and *Aspergillus versicolor* (sterigmatocystin, 1 isolate)

Keywords: apples, micromycetes, patulin, *Penicillium expansum*

INTRODUCTION

Apples are seasonable products, so it has been necessary to increase the life of the products, thus allowing furnishing the market all along the year. The cold storage is the main technology used **(Morales, *et al.,* 2010)**. However, apple decay by microorganisms cannot be completely avoided. The most common and destructive fungal spoilage agent in pome fruits (apples and pears) is again a *Penicillium*, causing a blue rot, in this case *Penicillium expansum* **(Neri, *et al.,* 2010, Morales, *et al.,* 2010, Baert, *et al.*, 2012)**. The *Penicillium expansum* causes blue mould rot, a serious post-harvest disease of apples and it is the main producer of the mycotoxin patulin **(Baert *et al.*, 2007, Tolaini *et al.*, 2010)**.

The aim of this study was to determine microscopic fungi involving rot of apples in market and to test isolated potentially toxigenic species for ability to produce chosen mycotoxins in conditions *in vitro*.

MATERIAL AND METHODS

In this study, we detected causers of rotting in apples bought in the commercial network in Slovak Republic. We bought 30 apples with the characters of rotting. roducers of rotting were inserted directly on MEA (Malt Extract agar; **Klich, 2002**). Cultivation proceeded for 5 – 7 days in the dark at 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**), CREA (Creatine-Sucrose agar, **Samson *et al.,* 2002**) and YES (Yeast Extract agar; **Samson *et al.,* 2002**), respectively. Members of other genera were identified 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 aspergilla, **Pitt *et* Hocking (2009); Samson *et al.* (2002); Samson *et* Frisvad (2004)** for penicillia and **Samson *et al.* (2002)** for species of other genera. *Monilinia* sp. was determined according to characteristics on rotting apple and micromorfological characteristics. Mycotoxins screening was done by a modified agar plug method. 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) was carried out on YES and for intracellular (sterigmatocystin) on CYA; conditions of cultivation: 14 days in the dark at 25 ± 1 °C. In each tested isolate, 3 pieces of mycelium together with cultivation medium of approximately size 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. Further, 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: patulin by spraying with 0.5 % methylbenzothiazolone hydrochloride (MBTH, Merck, Germany) in methanol, heated at 130 °C for 8 min and then detected as a yellow-orange spot. Directly under UV light (365 nm) was citrinin (yellow-green spot) and sterigmatocystin (reddish spot) visualised.

RESULTS AND DISCUSSION

From the 30 apples with rot we determined 8 genera of moulds (Tab 2). Blue mold in apple is caused by *Penicillium* spp., primarily *Penicillium expansum* and *Penicillium solitum* **(Sanderson *et* Spotts, 1995)**. Species of genus *Penicillium* were isolated from 18 samples (60 %). From 12 (40 %) apples was isolated *Penicillium expansum*. Blue mold decay caused by species of *Penicillium expansum* is the most important disease of stored apples **(Sholberg et al., 2005)**. **Sanderson *et* Spotts (1995)** hypothesized that some weak pathogens (other penicillia), especially *Penicillium solitum*, could function as predisposing agents that would allow entry of aggressive pathogens such as *Penicillium expansum* to cause a more destructive decay. From apples affected by rotting were isolated other species of *Penicillium* like *Penicillium solitum*, *Penicillium citrinum* and *Penicillium funiculosum*.

The second most frequent genus isolated from rotten apples was *Monilia*. This genus was on the four apples (Fig. 2) also. Brown rot was caused by *Monilinia* spp. (*Monilinia laxa*, *Monilinia fructigena*) **(Casals, *et al.,* 2010a, 2010b)**. *Botrytis cinerea* was isolated from one apple. Gray mold caused by *Botrytis cinerea* is a common postharvest disease of pome fruit **(Zhao *et al.*, 2010)**.

Table 1 Microscopic fungi causing rot of apples

Number of sample	Variety	Determinated pathogen
1.	Braeburn	*Botrytis cinerea*
2.	Braeburn	*Penicillium expansum*
3.	Without variety determination, sold as "red loose"	*Penicillium expansum*
4.	Jonagold	*Monilinia* sp.
5.	Red Chief	*Penicillium expansum*
6.	Idared	*Penicillium expansum*
7.	Rubín	Yeast
8.	Jonagold	*Monilinia* sp.
9.	Jonagold	Yeast
10.	Jonagold	Yeast
11.	Golden Delicous	*Penicillium expansum*
12.	Red Delicous	*Penicillium expansum* *Penicillium roqueforti*
13.	Galla	*Penicillium expansum* *Penicillium citrinum* *Fusarium* sp.
14.	Braeburn	Yeast
15.	Braeburn	*Penicillium expansum* Yeast
16.	Fugi	*Penicillium expansum* yeast *Alternaria* sp.
17.	Glosten	*Penicillium expansum*
18.	Granny Smith	no fungus
19.	Without variety determination, sold as "red loose"	*Penicillium expansum* *Cladosporium* sp.
20.	Fugi	*Geotrichum* sp. *Monilinia* sp.
21.	Fugi	*Monilinia* sp. *Aspergillus versicolor*
22.	Idared	*Penicillium funiculosum*
23.	Without variety determination, sold as "red loose"	yeast *Penicillium solitum*
24.	Idared	*Penicillium citrinum*
25.	Without variety determination, sold as "red loose"	*Penicillium solitum*
26.	Glosten	*Epicoccum nigrum* *Cladosporium* sp.
27.	Red Delicous	*Penicillium expansum*
28.	Galla	*Penicillium* sp.
29.	Rubín	*Penicillium solitum*
30.	Golden Delicous	*Cladosporium* sp.


Figure 1 Apple rot – *Penicillium expansum* **Figure 2** Apple rot – *Monilinia* sp.

From apples were 15 strains of four potentially toxigenic species of genera *Aspergillus* and *Penicillium* isolated. These isolates were tested for ability to produce chosen mycotoxins in conditions *in vitro* using TLC method (thin-layer chromatography) (Tab 2). All tested strains were producing mycotoxins in conditions *in vitro*. *Penicillium expansum* is known as the main producer of the mycotoxin patulin **(Pitt *et* Hocking, 2009)**. Patulin is frequently found as a contaminant in apples and apple products **(Moake *et al.,* 2005).** Authors referred to the occurrence for patulin in 47.2 % of samples 100 % apple juice **(Spadaro *et al.*, 2007)**, 35 % of apple juice, baby food and mixed juice **(Zaied et al., 2012)**. Almost 100 % of *Penicillium expansum* isolates are patulin producers **(Morales et al., 2008)**. In our study also all isolates were producers of patulin. All isolates of *Penicillium expansum* produced mycototoxin citrinin, also. We have isolated and identified the producers of other mycotoxins *Penicillium citrinum* (citrinin), *Penicillium roqueforti* (roquefortin C) and *Aspergillus versicolor* (sterigmatocystin). As referred above, all tested strains produced mycotoxins *in vitro*.

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

Species	Number of tested isolates	Detected toxin	Evaluation	
			+	-
Aspergillus versicolor	1	sterigmatocystin	1	0
Penicillium citrinum	1	citrinin	1	0
Penicillium expansum	12	patulin	12	0
	12	citrinin	12	0
Penicillium roqueforti	1	roquefortin C	1	0

Legend: + confirmed production of mycotoxin, - not detected production of mycotoxin

CONCLUSION

Microscopic fungi are the most important spoilage factor of stored apples. Only from one rot apple was not isolated microscopic fungus. From 83 % rotting apples were isolated moulds. The most important spoilage species *Penicillium expansum* was isolated from 43 % rot apples. All tested isolates of *Penicillium expansum* were detected as producers of mycotoxin patulin *in vitro*.

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