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Chromatographic characteristics of secondary metabolites of micromycetes detected on vegetables and grains

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The selection of toxin-producing micromycetes isolated from stored vegetables and grains, as well as from the dust and air of the storehouses was performed. Two micromycetes, characterised by secondary metabolism were isolated from the dust of a vegetable storehouse, one micromycete from the storehouse air, three micromycetes were isolated from stored vegetables and four micromycetes from stored grains.

Micromycete *Penicillium aurantiogriseum* most abundantly produced pigments into CYA and YES media.

The chromatography method on silica gel revealed the following: *Fusarium proliferatum* was producing T2 toxin, *F. tricinctum* – zearalenon, *Penicillium aurantiogriseum* – citrinin, *P. verrucosum* – patulin, *P. corymbiferum* – cytochalozin and sterigmatocistin, *Scytalidium lignicola* – tenuazonic acid, and *Aspergillus flavus* – kojic and cyclopiazonic acids.

Key words: vegetables, grains, micromycetes, chromatography, secondary metabolites

INTRODUCTION

The physiological adaptation peculiarities of micromycetes are their ability to react towards physical, chemical, biological factors in the constantly changing environment and to assimilate, due to labile biochemical processes, a high variety of natural and artificial substrates and produce specific secondary metabolites, enzymes, vitamins, acids, antibiotics, mycotoxins.

The ability of micromycetes to excrete toxic substances formed in the course of secondary metabolism is one of the most important adaptational properties that ensure their survival in the competitive environment. Filtenborg et al. (2000) indicate that about 400 mycotoxins are presently known. The amounts of the excreted mycotoxins, other biologically active substances (antibiotics, growth stimulators, etc.) and their proportions depend not only on the species and strains of fungi, but also on the chemical composition of the decomposed product and the whole complex of ecological conditions. A relation among micromycete growth, morphological differentiation, and secondary metabolism has been noticed (Betina, 1995).

Interest in the products of micromycete secondary metabolism, mycotoxins, is increasing worldwide; new toxic substances produced by microscopic fungi are determined, new diseases caused by them are diagnosed (Brera et al., 1998; Kuhls et al., 1999; Piecková, Jesenská, 1999; Dutkiewicz et al., 2001; Lugauskas et al., 2002; Parshikov et al., 2002).

To avoid the risk caused by mycotoxins to human health, in the majority of European countries the contamination of food and fodder with mycotoxins is monitored (Brera, Miraglia, 1996; Weigert et al., 1997; Egmond, 2000). It has been stated that about 25% of the yield worldwide is contaminated with mycotoxins; contamination of grains and fodder with toxins produced by fungi of the genus *Fusarium* is an urgent problem (Fink-Gremmels, 1999). It is expected that in future the research on mycotoxins would be directed towards investigation of their chemical composition and functional activities (Samson et al., 2000; Hussein, Brasel, 2001).

The aim of the present research was to select toxin-producing micromycetes detected on vegetables and grains and to carry out chromatographic analyses of their secondary metabolites.

MATERIALS AND METHODS

Micromycetes isolated from vegetables and grains taken from a storehouse were used in the research. The selection of toxin-producing micromycetes was carried out by cultivating them on agar media: Czapek yeast agar (CYA) and yeast extract-sucrose agar (YES). The evident changes in colonies and abundant excretion of

pigment into CYA and YES media as compared with the growth on standard Czapek agar show that micromycetes of the study strains distinguish themselves by secondary metabolism, and they are potential producers of mycotoxins.

For mycotoxin quality analysis, the micromycetes on CYA and YES agar media were cultivated for 20 days. Then the obtained micromycete biomass was extracted with methyl alcohol, centrifuged and purified.

The obtained alcohol extracts of individual micromycetes were chromatographed on silica gel 60 with an UV 254 fluorescence indicator, 20×20 cm, 0.2 mm thick.

The following systems of solvents were used:

1. Toluene – ethyl acetate – formic acid (5:4:1)
2. Chloroform – methanol (98:2)
3. Chloroform – ethanol (97:3)

Fungal metabolites were identified according to Cole & Cox (1981).

RESULTS AND DISCUSSION

In our study, the following micromycetes were producing toxins: *Fusarium proliferatum* (Matsusch.) Nirenberg; *Fusarium tricinctum* (Corda) Sacc.; *Fusarium semitectum* Berk. et Rav.; *Penicillium aurantiogriseum* Dierckx; *Penicillium verrucosum* Dierckx; *Trichoderma viride* Pers; *Scytalidium lignicola* Pesante; *Penicillium corymbiferum* Westling; *Aspergillus niger* Tiegh.; *Aspergillus flavus* Link.

The tests showed (Table 1) that micromycete *Penicillium aurantiogriseum* most abundantly excreted pigments into CYA and YES media. *Fusarium proliferatum, F. tricinctum, F. semitectum, Scytalidium lignicola* and *Penicillium corymbiferum* abundantly excreted pigments into the YES medium; less abundant excretion of pigments into the CYA medium was characteristic of *F. tricinctum, Scytalidium lignicola,* and *P. corymbiferum*. The least amount of pigments into the CYA medium was excreted by *P. verrucosum* and *Trichoderma viride*.

Several systems of solvents were used for chromatography of compounds on silica gel. The best distribution of compounds was observed in the: toluene – ethyl acetate – formic acid (5:4:1) system of solvents (Table 2).

The research showed that in the chromatogram of *Fusarium proliferatum* alcohol extract seven compounds forming dark purple fluorescence in the presence of UV light were present. Their Rf in the above mentioned system of solvents ranged from 0.125 to 0.79. The compound with Rf 0.40 in the chloroform–methanol system of solvents (98:2), after comparison with the standard, was identified as T2 toxin. For the detection of the compound, concentrated H_2SO_4 was used.

In the chromatogram of *Fusarium tricinctum* alcohol extract (Fig. 3), three compounds were recorded. Their Rf in the first system of solvents ranged from 0.69 to 0.83. The compound with Rf 0.83 in the first system of solvents and 0.49 in the third system of solvents, λmax 236 nm (MeOH), was identified as zearalenon. The compound with Rf 0.69 in the first system of solvents was close to citrinin and the compound with Rf 0.71 to cytochalasin. Due to a small amount of these compounds they could not be properly identified.

In the chromatogram of *Fusarium semitectum* alcohol biomass extract (Fig. 1) in the system of solvents toluene – ethyl acetate – formic acid (5:4:1) three compounds were detected, their Rf ranging from 0.55 to 0.73 with purple fluorescence. Their Rf did not match the available standards.

In the chromatogram of *Penicillium aurantiogriseum* alcohol extract (Fig. 2) in the first system of solvents five compounds were detected. Their Rf ranged from 0.32 to 0.72. They formed dark purple fluorescence in the presence of UV light. The compound with Rf 0.72

Table 1. **Excretion of micromycete toxins into Czapek-yeast extract (CYA) and yeast extract-sucrose agar (YES) media**

Micromycete	Medium		Source
	CYA	YES	
Fusarium proliferatum (Matsush.) Nirenberg	+++	+++++	Dust. Onion storehouse
Fusarium tricinctum (Corda) Socc.	++++	+++++	Air. Potato storehouse
Penicillium aurantiogriseum Dierckx	+++++	+++++	Germinated wheat grain. Storehouse
Penicillium verrucosum Dierckx	+	+++++	Potatoes ‘Apart’. Storehouse
Fusarium semitectum Berk. et Rav.	+++	+++++	Barley. Mill
Trichoderma viride Pers.	++	++++	Dust. Carrot storehouse
Scytalidium lignicola Pesante	++++	+++++	Surface leaves of cabbages. Storehouse
Penicillium corymbiferum Westling	++++	+++++	Summer barley. Storehouse
Aspergillus niger Tiegh.	+++	++++	Carrots. Automated storehouse of an individual farm
Aspergillus flavus Link	+++	++++	Dust. Grain storehouse

+++++ – high excretion of pigment into media;
+++ – medium excretion of pigment into media;
++ – low excretion of pigment into media.

Table 2. **Chromatographic characteristic of micromycete toxins isolated from cereals and vegetables**

Micromycete	Number of compounds	Rf	System of solvents	$Y\varsigma\lambda_{max}$ nm	Detection	Identification
Fusarium proliferatum	7	1. 0.125 2. 0.178 3. 0.208 4. 0.488 5. 0.586 6. 0.708 7. 0.791	I Toluene-ethyl acetate-formic acid (5:4:1)	–	Dark purple fluorescence in the presence of UV light	–
		0.40	II Chloroform-methanol (98:2)	–	Spraying with H_2SO_4 and heating at 110°C for 5 min.	T2 toxin
Fusarium tricinctum	3	1. 0.69 2. 0.71 3. 0.83	I Toluene-ethyl acetate-formic acid (5:4:1)	–	–	–
		0.49	III Chloroform-ethanol (97:3)	236	Dark purple fluorescence in the presence of UV light	Zearalenone
Penicillium aurantiogriseum	5	1. 0.32 2. 0.41 3. 0.53 4. 0.69 **5. 0.72**	I Toluene-ethyl acetate-formic acid (5:4:1)	319	Dark purple fluorescence in the presence of UV light	Citrinin
Penicillium verrucosum	6	1. 0.75 2. 0.70 3. 0.66 **4. 0.60** 5. 0.55 6. 0.52	I Toluene-ethyl acetate-formic acid (5:4:1)	275	Brown colour after spraying with 50% H_2SO_4 and heating at 110 °C for 5 min.	Patulin
Fusarium semitectum	3	1. 0.55 2. 0.62 3. 0.73	I Toluene-ethyl acetate-formic acid (5:4:1)	–	Dark purple fluorescence in the presence of UV light	–
Trichoderma viride	8	1. 0.05 2. 0.08 3. 0.136 4. 0.178 5. 0.22 6. 0.47 7. 0.529 8. 0.678	I Toluene-ethyl acetate-formic acid (5:4:1)	–	Dark purple fluorescence in the presence of UV light	–
Scytalidium lignicola	5	1. 0.23 2. 0.44 3. 0.58 4. **0.62** 5. 0.83	I Toluene-ethyl acetate-formic acid (5:4:1)	215 and 277	Red brown colour after spraying with 2% $FeCl_3$ butanol	Tenuazonic acid
Penicillium corymbiferum	7	1. 0.136 2. 0.25 3. 0.36 4. 0.56 **5. 0.68**	I Toluene-ethyl acetate-formic acid (5:4:1)		Dark purple fluorescence in the presence of UV light	
		6. 0.76		220		Cytochalasin
		7. 0.85		208 and 235		Sterigmatocistin

Table 2 (continued)

Aspergillus niger	10	1. 0.10 2. 0.17 3. 0.33 4. 0.39 5. 0.46 6. 0.53 7. **0.565** 8. 0.61 9. 0.80 10. 0.89	I Toluene-ethyl acetate-formic acid (5:4:1)	–	Dark purple fluorescence in the presence of UV light	Patulin?
Aspergillus flavus	6	1. 0.26 **2. 0.32** 3. 0.53 **4. 0.57** 5.0.60 6. 0.9	I Toluene-ethyl acetate-formic acid (5:4:1)2	268 225	Dark purple fluorescence in the presence of UV light	Kojic acid Cyclopiazonic acid

Rf – path, which compound runs from the start line/path, which solvent runs.

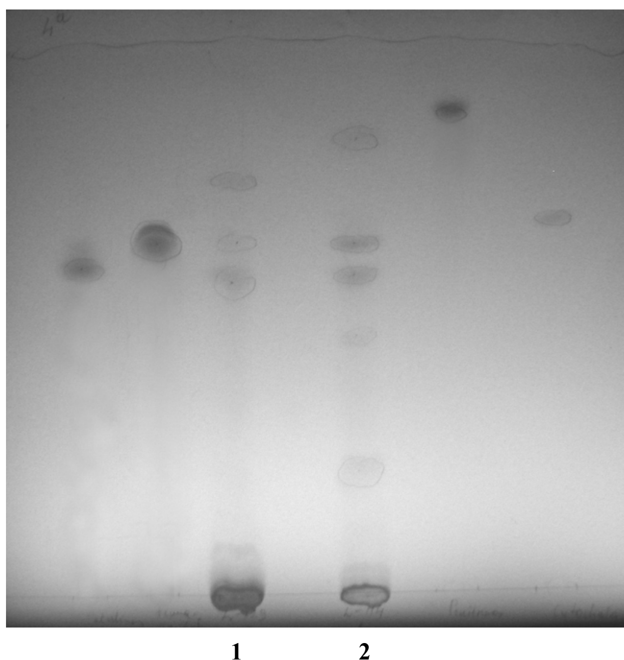

Fig. 1. Chromatogram of mycotoxins: 1 – *Fusarium semitectum*, 2 – *Scytalidium lignicola*

in the first system of solvents, λmax 319 nm (EtOH), after comparison with the standard was identified as citrinin.

In the chromatogram of *Penicillium verrucosum* (Fig. 2), six compounds fluorescenting purple colour in the presence of UV light were detected. Their Rf varied from 0.52 to 0.75. The compound with Rf 0.60 in the first system of solvents, λmax 275 nm (EtOH), after comparison with the standard was identified as patulin.

In the chromatogram of *Trichoderma viride* alcohol extract, eight compounds forming dark purple fluorescence in the presence of UV light were detected. Their Rf in the first system of solvents varied from 0.05 to 0.678. The compounds were not identified because of the non-existence of standards.

In the chromatogram of *Scytalydium lignicola* (Fig. 1), in the first system of solvents five compounds were detected. Their Rf in the first system of solvents varied from 0.23 to 0.83. The compound forming dark purple fluorescence in the presence of UV light, red-brown colour after spraying with ethanolic $FeCl_3$ with Rf 0.62, λmax 217 and 277 nm (EtOH), was identified as tenuazonic acid.

In the chromatogram of *Penicillium corymbiferum* (Fig. 4), seven compounds were detected; their Rf in the first system of solvents varied from 0.136 to 0.85. The compound with Rf 0.68, forming dark purple fluorescence in the presence of UV light, having λmax 220 nm (EtOH), was identified as cytochalasin, and the compound with Rf 0.85, λmax 208 and 235 nm (EtOH), was identified as sterigmatocistin.

In the chromatogram of *Aspergillus niger* (Fig. 3), 10 compounds were detected; their Rf varied from 0.10 to 0.89. The compound with Rf 0.56 was close to patulin.

In the chromatogram of *Aspergillus flavus* (Fig. 2), six compounds were detected. The main compound with Rf 0.32 in the first system of solvents, λmax 268 nm (HOH), and forming dark purple fluorescence in the presence of UV light was identified as kojic acid, and the compound with Rf 0.57, λmax 225 nm (EtOH), coinsided with cyclopiazonic acid.

The ability of micromycetes to synthesize toxic secondary metabolites is an important ecological property which allows them to establish in various ecological niches and to compete with other biota for nutritional sources and space. Micromycetes can synthesize and excrete into the environment compounds of different chemical origin (terpenes, steroids, various phenolic compounds, organic acids and other complex derivatives)

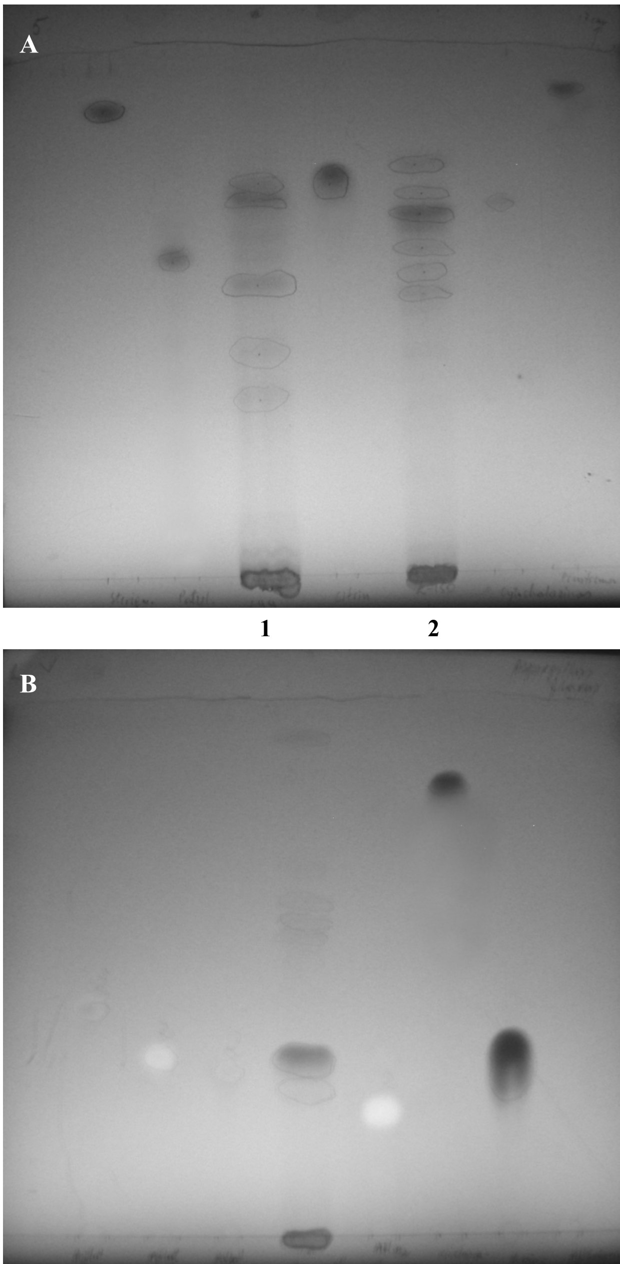

Fig. 2. Chromatogram of mycotoxins: **A**: 1 – *Penicillium aurantiogriseum,* 2 – *Penicillium verrucosum*; **B**: *Aspergillus flavus*

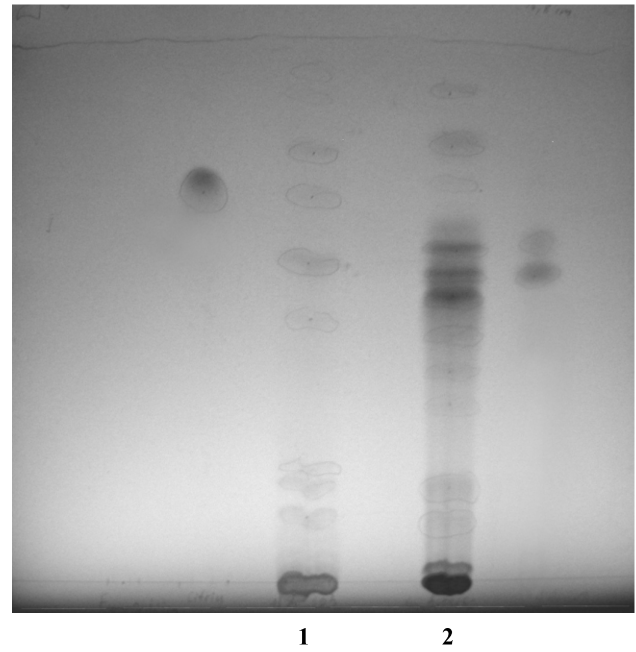

Fig. 3. Chromatogram of mycotoxins: 1 – *Fusarium tricinctum*, 2 – *Aspergillus niger*

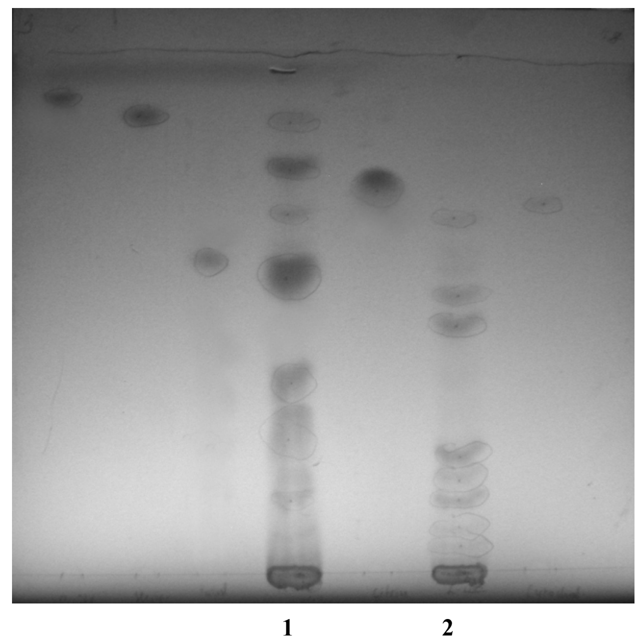

Fig. 4. Chromatogram of mycotoxins: 1 – *Penicillium corymbiferum*, 2 – *Trichoderma viride*

which change essentially the composition of soil and other substrata where fungi develop.

CONCLUSIONS

1. The micromycete *Fusarium proliferatum*, isolated from dust of a vegetable storehouse, was distinguished for the secondary metabolism and T_2 toxin production.

2. The micromycete isolated from dust of a grain storehouse produced kojic and cyclopiazonic acids.

3. *Fusarium tricinctum* isolated from the indoor air of a vegetable storehouse produced zearalenone.

4. *Penicillium verrucosum*, *Scytalidium lignicola* were isolated from vegetables stored in storehouses, and these fungi synthesized patulin and tenuazonic acid.

5. *Penicillium aurantiogriseum* and *P. corymbiferum* were isolated from stored grains, and they produced citrinin together with citochalasin and sterigmatocistin, respectively.

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ANT DARŽOVIŲ IR GRŪDŲ APTINKAMŲ MIKROMICETŲ ANTRINIŲ METABOLITŲ CHROMATOGRAFINĖ CHARAKTERISTIKA

S a n t r a u k a

Atlikta atranka mikromicetų – toksinų producentų, išskirtų nuo sandėliuojamų daržovių ir grūdų, taip pat iš jų sandėlių dulkių ir oro. Nustatyta, kad 2 mikromicetai, kuriems yra būdingas antrinis metabolizmas, buvo išskirti iš daržovių sandėlio dulkių, 1 iš sandėlio oro; 3 mikromicetai buvo išskirti nuo sandėliuose saugomų daržovių ir 4 nuo sandėliuojamų grūdų.

Gausiausiai pigmentus į CYA ir YES terpes gamino mikromicetas *Penicillium aurantiogriseum.*

Chromatografijos ant silikagelio metodu nustatyta, kad *Fusarium proliferatum* gamino T2 toksiną; *F. tricinctum* – zearalenoną; *Penicillium aurantiogriseum* – citrininą; *P. verrucosum* – patuliną; *P. corymbiferum* – citochaloziną ir sterigmatocistiną; *Scytalidium lignicola* – tenuazono rūgštį ir *Aspergillus flavus* – kojo ir ciklopiazoninę rūgštį.

Raktažodžiai: daržovės, grūdai, mikromicetai, chromatografija, antriniai metabolitai