YU ISSN 0352-4906 UDK 5/6 (05)

ЗБОРНИК

МАТИЦЕ СРПСКЕ ЗА ПРИРОДНЕ НАУКЕ

MATICA SRPSKA PROCEEDINGS FOR NATURAL SCIENCES

ЗБОРНИК МАТИЦЕ СРПСКЕ ЗА ПРИРОДНЕ НАУКЕ 108

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NOVI SAD
2005


МАТИЦА СРПСКА
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МАТИЦА СРПСКА
ОДЕЉЕЊЕ ЗА ПРИРОДНЕ НАУКЕ

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MATICA SRPSKA
DEPARTMENT OF NATURAL SCIENCES
PROCEEDINGS FOR NATURAL SCIENCES

Покренут 1951 / First published in 1951.

Published as *Научни зборник*, серија природних наука until the tenth issue (1955), as the Series for Natural Science from the eleventh issue (1956) — *Зборник за природне науке*, and under its present title since the sixty-sixth issue (1984)

Главни уредници / Editors-in-Chief

Miloš Jovanović (1951), Branislav Bukurov (1952—1969),
Lazar Stojković (1970—1976), Slobodan Glumac (1977—1996), Rudolf Kastori (1996—)

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YU ISSN 0352-4906 UDK 5/6 (05)

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INTRODUCTION

Fungi and their toxic metabolites — mycotoxins — are of extraordinary significance for the living world. In their constant circling in nature from soil, water and air, through plants and animals, they reach man, often causing drastic effects with unpredictable consequences. Toxic fungi and mycotoxins cause different diseases in plants, animals and people in all parts of the world, including our country. They are frequent causes of healthy unsafe food-stuffs and animal feed, thus directly influencing the health of people and animals. For those reasons all new knowledge about toxigene and pathogene fungi and their metabolites is very useful.

However, in that variety of fungi there are also some species useful for man. Using them since past times and improving technological methods in which these microorganisms take part, year by year in a modern world there are produced great variety of products which make life easier, nicer and richer for a modern man. In the general sense of the word, all that represents the scope of mycology.

By all its aspects, biotechnological, mycotoxicological, ecological and other, mycology gather researchers of different profiles with the intention of obtaining more comprehensive and exact data, primarily for the sake of human health.

The first scientific meeting MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES with international participation is organized with the aim of gathering as many as possible researchers from our country and other countries as well, dealing with the same problematics.

In this Collection of *Papers for Sciences of Matica srpska* the majority of papers to be presented at the scientific meeting have been published. The remaining papers will be published in the next *Collection* volume or another adequate journal.

The members of the Organizing Committee of The first scientific meeting MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES with international participation express profound gratitude to all those who financially supported organization and holding of this scientific meeting.

The first scientific meeting MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES with international participation is organized within Department of Sciences of Matica srpska, and held from 20—22 April 2005 in Matica srpska.

President of the Scientific Committee
Professor Maria Skrinjar, Ph.D.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 9—15, 2005

UDC 613.27/.28:615.9

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FUNGI AND OCHRATOXIN A — FREQUENCY IN FOOD AND RAW MATERIALS FOR THEIR PRODUCTION IN SERBIA

ABSTRACT: Contamination of various types of foods (cereals and their products, raisins, dry sausages, cheese) with moulds and ochratoxin A (OTA) was examined. All samples were contaminated with moulds to a different degree. About 25% of wheat samples from the localities Niš and Leskovac, 70% from the locality Kikinda, graham flour (1 sample), barley flakes (1 sample), graham bread, barley bread, 60% of "healthy food" product samples, 20% of dry sausage samples and 20% of melted cheese samples were contaminated with OTA.

KEY WORDS: fungi, ochratoxin A, food, raw materials

INTRODUCTION

Ochratoxin A (OTA) in a mycotoxin very often detected in our country in feeds and foods, as well as in raw materials for their production (Škrinjar et al., 1992, 2003, 2004; Stojanović, 1999; Škrinjar and Kocić-Tanackov, 2005).

OTA is a secondary metabolite of some fungal species from the genera *Aspergillus* and *Penicillium*. According to Pohland et al. (1992), a fungal species, first found to be able to produce a toxic metabolite, later named OTA, is *A. ochraceus*. Our few-years investigations indicate that in mycopopulations isolated from feeds and foods ochratoxin A — producing *Penicillium* species are predominant (Škrinjar, 1984, 1992; Škrinjar et al., 1989, 1991, 2002; Škrinjar and Danev, 1994; Stojanović, 1999; Stojanović et al., 2002). Having this in mind, it is necessary to point out high frequency of *P. aurantiogriseum* and *P. chrysogenum*. These species are known for the production of various toxic metabolites, including OTA (Samson and van Reenen-Hoekstra, 1988; Škrinjar, 1984; Škrinjar et al., 1992).

OTA can induce nephrotoxicity in various animal species. It is known as an immunosuppressor, a teratogen, a genotoxin and a carcinogen metabolite. Also, it is possibly involved in the Balkan endemic nephropathy in humans (Petkova-Bocharova and Castegnaro, 1985; Petkova-Bocharova et al., 1988).

In this paper the results of the presence of OTA in some agricultural products and various kinds of food will be presented.

MATERIALS AND METHODS

Contamination of various agricultural products and some kinds of foods with OTA and moulds was investigated as follows: wheat (25 samples), flour (3 samples — graham, rye, barley) bran (1 sample), "kernels of health" (1 sample), bread (4 samples), bio goods (1 sample), "healthy food" products (corn flakes — 3 samples, wheat flakes — 3 samples, oat flakes — 3 samples, whole grain rice — 3 samples, raisins — 3 samples), dry sausages (15 samples) and cheeses (145 samples).

In all samples mycological (determination of total viable counts of moulds per 1 g, isolation and identification of fungal species) and mycotoxicological analyses were carried out.

Mycological analyses. The determination of the total number of fungi per 1 g of sample and their isolation were performed using standard Koch's method and/or direct plating method. Sabouraud maltose agar with streptomycin (0.01—0.02%) was used as an isolation medium. Incubation was carried out at 25°C for 5 to 7 days. Identification of fungal species was done according to Samson and van Reenen-Hoekstra (1988).

OTA analyses. Qualitative and quantitative determination of OTA was performed by TLC (Balzer et al., 1978) and fluorometric methods (Fluorometer, VICAM, Series 4, USA).

RESULTS AND DISCUSSION

All wheat samples originating from localities Niš and Leskovac were contaminated with moulds (0.8 to 2.5 per kernel). OTA was detected in five samples (Table 1) in concentrations from traces to 40.0 μg/kg.

Table 1. Occurrence of ochratoxin A in wheat originated from Niš and Leskovac

Sample no.	Wheat/Sort	Locality	Conc. of OTA $\mu g \cdot kg^{-1}$
1	Rodna	Niš	—[a]
2	Pobeda	Niš	—
3	Evropa	Niš	—
4	Danica	Niš	8.50
5	Novosadska rana	Niš	traces
6	Proteinska	Niš	—

7	Milica	Niš	40.0
8	Slavija	Niš	—
9	Dična	Niš	16.0
10	Studenica	Niš	40.0
11	Kragujevačka	Niš	—
12	Proteinska	Leskovac	—
13	Milica	Leskovac	—
14	Slavija	Leskovac	—
15	Studenica	Leskovac	—
16	PKB-Mlinarska	Leskovac	—
17	Sunce	Leskovac	—
18	Kragujevačka 56	Leskovac	—
19	Beograđanka	Leskovac	—
20	Srbijanka	Leskovac	—

[a] toxin was not detected

Wheat kernels originated from the locality Kikinda were infested with moulds to the same degree (0.9 to 3.2 fungy/kernel). It was found that even 70% of these samples were contaminated with OTA in concentrations between 8.0 (Nevesinjka — very fusarious fraction) and 48.0 μg/kg (Nora — very fusarious fraction) (Table 2).

Table 2. Occurrence of ochratoxin A in different wheat fractions obtained from the region of Kikinda

Sort	Wheat Fraction	Conc. of OTA $\mu g \cdot kg^{-1}$
Nevesinjka	healthy	—[a]
	blackpoint	—
	little fusarious	—
	very fusarious	8.0
Jarebica	healthy	16.0
	blackpoint	32.0
	little fusarious	32.0
	very fusarious	32.0
Levčanka	healthy	32.0
	blackpoint	—
	little fusarious	—
	very fusarious	32.0
Kg 56 S	healthy	—
	blackpoint	34.0
	little fusarious	11.5
	very fusarious	34.0
Nora	healthy	11.0
	blackpoint	11.0
	little fusarious	16.0
	very fusarious	48.0

[a] toxin was not detected

By investigating the fungal contamination of some types of flour and related products it was established that the total viable counts of moulds varied from 10.0 (graham flour) to $8.5x10^3$ (rye flour) per 1g. Fungi isolated from these samples were classified into 10 genera and 21 species. The most frequent were fungi belonging to the genera *Aspergillus*, *Eurotium* and *Penicillium*. OTA was found in samples of graham flour and barley flakes (traces) (Table 3).

Table 3. Ochratoxin A in some milling products intended for special kinds of bread and goods

Sample	Conc. of OTA μg · kg^{-1}
Graham flour	traces
Rye flour	—[a]
Barley flakes	traces
Bran	—
"Kernels of health"	—

[a] toxin was not detected

It was interesting to point out that bread prepared from OTA — contaminated graham flour and barley flakes consisted significant amounts of OTA (360.0 and 80.0 μg/kg) (Table 4). An inadequate sampling of graham flour and barley flakes was probably the reason of low concentrations of OTA in them.

Table 4. Ochratoxin A in different types of bread and goods

Sample	Conc. of OTA μg · kg^{-1}
Graham bread	360.0
Rye bread	—[a]
Barley bread	80.0
Diet bread	—
Bio goods	—

[a] toxin was not detected

All "healthy food" products were contaminated with fungi. Their number ranged from 2.0 (oat flakes) to $4.0x10^2$ per 1 g (corn flakes). About 20 different fungal species were isolated from the products. *Aspergillus flavus*, *Eurotium herbariourum* and *Penicillium aurantiogriseum* were the most frequent fungi. OTA was found to contaminate even 60% of these samples (Table 5).

Table 5. "Healthy food" products contaminated with ochratoxin A

Sample	No. of sample	Conc. of OTA μg · kg^{-1}
Corn flakes	1	40.0
	2	—[a]
	3	—

Wheat flakes	1	—
	2	160.0
	3	80.0
Oat flakes	1	80.0
	2	160.0
	3	traces
Whole grain rice	1	—
	2	80.0
	3	—
Raisins	1	56.0
	2	40.0
	3	—

[a] toxin was not detected

Two tea sausage samples were also contaminated with OTA (12.0 μg/kg) as well as one samples of "kulen" (traces) (Table 6).

Table 6. Ochratoxin A in some dry sausages

Sausage	Sample no.	Conc. of OTA μg · kg^{-1}
Tea sausage	1	—[a]
	2	12.0
	3	12.0
	4	—
	5	—
	6	—
Budim sausage	1	—
	2	—
	3	—
	4	—
	5	—
	6	—
"Kulen"	1	traces
	2	—
	3	—

[a] toxin was not detected

OTA was not detected in Trappist, Edam and Junior cheese samples (Table 7). But, 17 samples of melted cheese were contaminated with the toxin in concentrations from 5.5 to 16.5 μg/kg. Further, 6 samples of melted cheese were suspected on the presence of OTA.

Table 7. Contamination of cheeses with ochratoxin A

Product	No. of investigated	No. of OTA — positive samples	Conc. of OTA $\mu g \cdot kg^{-1}$
Trappist cheese	17	0	—[a]
Edam cheese	32	0	—
Junior cheese	12	0	—
Melted cheese	84	17	5.5—16.5
		6	suspected

[a] toxin was not detected

CONCLUSIONS

A high incidence of ochratoxin A contamination of various types of agricultural products and foods produced in our country was observed during this few-years investigations.

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ГЉИВИЦЕ И OCHRATOXIN A — ФРЕКВЕНЦИЈА РАСПРОСТРАЊЕЊА У ЖИВОТНИМ НАМИРНИЦАМА И СИРОВИНАМА ЗА ЊИХОВУ ПРОИЗВОДЊУ У СРБИЈИ

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Резиме

Извршена су испитивања контаминације различитих врста животних намирница (житарице и њихови производи, суво грожђе, трајне кобасице, сир) плеснима и охратоксином А (ОТА). Сви узорци били су контаминирани плеснима у различитом степену. Око 25% узорка пшенице са локалитета Ниш и Лесковац, 70% са локалитета Кикинда, грахам брашно (1 узорак), јечмене пахуљице (1 узорак), грахам хлеб и јечмени хлеб, 60% производа „здраве хране", 20% узорка трајних кобасица и 20% узорка топљеног сира било је контаминирано са ОТА.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 17—23, 2005

UDC 613.2:615.9(497.113)

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EVALUATION OF FEED COMPONENTS CONTAMINATION WITH OCHRATOXIN IN VOJVODINA

ABSTRACT: Ochratoxin A is cancerogenic, teratogenetic, immunotoxic and nephrotoxic. The mentioned order stresses the importance of this toxin concerning its harm to human health. The harmful effects of ochratoxin A include the effects at molecular level, such as DNA fragmentation, protein synthesis inhibition, gluconeogenesis, lipid peroxidation, disorder of oxydative phosphorization in mitochondria, inhibition of blood coagulation and apoptosis. The presence of ochratoxin A in a great number of food samples, both of plant and animal origin, is the obvious risk to human health, which is confirmed by the high incidence of this toxin in samples of human serum and milk. It could be stated, with certainty, that the above — mentioned facts are the reason for which the EU has paid great attention to this mycotoxin in recent years.

This paper deals with the results of the analysis of the animal feed component samples for the period 2000—2003 concerning the ochratoxin A content. The analysed feed components were taken from the farms with significant health problems of animals (not monitoring). The samples were analysed by chromatography on a thin layer and with a limited detection method for ochratoxin A of 40 ppb. The analysis was carried out on 108 maize samples, 11 barley samples, 21 wheat samples, 42 sunflower pellets samples and 47 soybean pellets samples (Table 1). The samples of sunflower pellets were contaminated in the greatest percentage, which indicates the inadequate storage of this feed component.

KEY WORDS: barley, maize, ochratoxin A, soybean pellets, sunflower pellets

INTRODUCTION

Ochratoxin A (OTA) is a product of several moulds of the genuses: *Aspergillus* and *Penicillium*. In the temperate climate zone this toxin is mostly the product of mould *P. verrucosum*. A great number of countries, in which *P. verrucosum* has been indentified as toxin — producing, have registered a significant increase in its production during 2001. At present the toxin — producing *P. verrucosum* is not only present in Holland, Sweden, Norway, England, Germany and Austria but also in Italy, Spain, France and Portugal. Still *P.*

verrucosum is the only ochratoxin producer found. More than 100 isolates of *P. verrucosum* from these environments have been fingerprinted phenotypically and examined for the production of ochratoxin A, and many different clones have been found (O l s e n, 2002).

According to the other authors *P. verrucosum* sometimes produces, besides ochratoxin A, also citrinin which is concidered to be its major synergist (L a w i o r, 2001). The most frequent contaminated feed components are maize, wheat, barley and rye. And according to our research a significant contamination of sunflower and soybean pellets has been observed. The infection occurs before harvest and after harvest in storehouses, but the prevalent formation of ochratoxin is during storage. The problem lies in an inadequate drying of feed components prior to storage or the poor storage conditions leading to "hot-spots" of contamination. *P. verrucosum* requires a high water activity (a_w = 0.995), while its other characteristic is the tolerance to high concentration of CO_2 (even 50%).

A good storage practice comprises:

— Design of the top ventilation
— Control of the silo conditions before harvest — to avoid leakage
— Inspection of the upper layer before unloading
— Aeration system in silo
— Grain temperature system

Model describing the risk for condensation of water in the headspace.

In the grinding process, concerning wheat, a part of ochratoxin remains in bran, a part in grinding waste, whereas 50% of ochratoxin A remains in white flour.

In extruded products the quantity of ochratoxin is slightly decreased depending on the temperature, moisture content and screw speed.

Ochratoxin destribution in feed components is rather heterogenic thus making the sampling a critical point in its detection. The sample is taken from different places, its size depending on the amount of the stored food or the size of the means of transportation which makes it possible to avoid taking only contaminated food.

After oral ingestion with foods and feeds, ochratoxin A is slowly absorbed from the upper small intestine. Reaching the systemic circulation, it binds extensively to serum proteins, and translocates to and accumulates in the kidney resulting in measurable residues, whereas lower residual concentrations are found in the liver, muscle and fat. Transfer to milk has been demonstrated in rats, rabbits and humans, but the percentage of ochratoxin A excreted with milk of ruminants is limited, owing to the degradation of ochratoxin A by the rumen microflora (Kwaliteitsreeks nr 89, 2003).

The Scientific Committee on Food expressed the opinion that exposure should be below 5 ng/kg b. w./day (EC, 1998). The Joint Expert Committee on Food Additives, in 2001, retained its previouonal Tolerable Weekly Intake of 100 ng/kg b. w. Per week, pending the results of on-going studies on the mechanisms of nephrotoxicity and carcinogenicity (WHO/FAO, 2001). The International Agency for Research on Cancer (IARC) had evaluated ochratoxin A in 1993 (IARC, 1993), and classified it as possibly carcinogenic to humans

(group 2B), based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans.

Approximately 40 countries around the world set regulatory or guideline levels for ochratoxin A in food and animal feed in 2003 (FAO, 2004). In the EU, harmonised regulations exist for ochratoxin A in raw cereal grains including rice and buckwheat, all products derived from cereals, dried vine fruit and baby food (EC, 2002). EU-harmonised specific limits for ochratoxin A in animal feedstuffs have not been proposed yet, but limits have been established at the national level in 8 countries. These countries include Estonia, Lithuania, Slovenia and Sweden, reporting the existence of limits for ochratoxin A in various feedstuffs (including feeds for cattle, pigs, poultry ond other farm animals). In addition to legislation on ochratoxin A in animal feed, national legislation exists on ochratoxin A in products of animal origin in three countries: Denmark (for pig kidney), Estonia (for pig liver) and Italy (for pig and derived products) (EFSA, 2004). In our country the maximum quantity of ochratoxin A is regulated by the Act "on maximum quantity of harmful substances and ingredients in animal feed" (1990). The Act "on the amounts of pesticides, metals and metalloids and other poisonous substances, chemotherapeutics, anabolics and other substances which can be found in foodstuffs" (1992) regulates the maximum amount of ochratoxin A allowed in wheat, flour, cereals, coffee, spices, meat and meat products, milk and milk products.

MATERIAL AND METHODS

The analysis of 108 maize samples, 11 barley samples, 21 wheat samples, 42 sunflower pellets samples and 47 soybean pellets samples was carrid out. All the analysed feed components were delivered to our laboratory between 2000 and 2003 from the farms with various health problems in animals and from the animal feed plants. The content of ochratoxin in the mentioned feed components was determined by the method according to Balzer et al. (1978). The procedure itself consists of the extraction of ochratoxin A from the sample using acetonitrile, as well as by the extraction and purification of mycotoxin using the chloroform in acid and base media. Qualitative and semi-qualitative determination of ochratoxin A was carried out by chromatography on a thin layer, with a limited detection method for ochratoxin A of 40 ppb.

RESULTS AND DICUSSION

Ochratoxin A occurrence in animal feed and feed components is predominantly a problem of poor or inadequate drying of cereals prior to storage, or poor storage conditions leading to "hot-spots" of contamination.

The results of our research are shown in Table 1. In the period of three years 229 samples of feed components were analysed, almost half of which were maize samples, which is quite understandable for our region where it is the basic component of a feeding ratio for almost all the animal species, thus

making the maize the most obvious cause of the health problems on a farm. This was not without grounds in this case either as 50% of samples, according to our analyses, were contaminated with ochratoxin A, and 6.5% of the total number of the maize samples were contaminated with the highest amount of ochratoxin of 0.5—1.0 mg/kg. What makes our analyses distinctive compared with the majority of others is a high contamination of sunflower pellets with ochratoxin A both in the previous period (J u r i ć, 1999) and today. The results of these analyses show a 100% contamination of sunflower pellets sample with 42.8% of the analysed samples of this feed component was contaminated with the maximum amount of ochratoxin A (0.5—1.0 mg/kg).

Table 1. Ochratoxin A content in animal feed components obtained in Vojvodina region from 2000—2003 (mg/kg).

Feeds	Total number of samples	Number of positive samples			
		< 0.04	0.04—0.1	0.1—0,5	0,5—1,0
Maize	108	45	25	21	7
Barley	11	5	4	2	0
Wheat	21	7	11	3	0
Sunflower pellets	42	0	6	8	18
Soybean pellets	47	24	14	7	2

The high prevalence and high levels of OTA contamination in feed grains can be explained by the unfavourable storage conditions, and this finding suggests that OA-related health problems may arise in animals, and that foods of animal origin may be contaminated with this mycotoxin.

According to the EFSA data (2004) there is a significant correlation between ochratoxin A in grains, moisture content, storage period and geographic location. According to the EU data (EC, 2002) for 1500 samples of wheat, oats, rye, barley and maize obtained from 1995—1999 in 11 European countries only 61 samples (4%) contained over 1 μg/kg of ochratoxin A. The other data obtained by EFSA (2004) are 11% of samples with ochratoxin content higher than 1 μg/kg according to the researcher MacDonald or 28% of samples with the ochratoxin A content 1 μg/kg according to Jonsson and Pettersson. Particularly high levels of ochratoxin A contamination (1000 μg/kg) were reported in samples of maize, wheat, rye, oats and barley from Austria, Bulgaria, Poland and the Czech Republic. Data from Hungary (F a z e k a s, 2002) reported results from 30 feeding wheat, 32 feeding maize and 20 feeding barley samples. OTA contamination was found in 26.7% of the feeding wheat, 15.6% of the feeding maize and 35% of the feeding barley samples. The average values and the range of OTA levels found in the above samples were 12.2 and 0.3—62.8 ng/g, 4.9 and 1.9—8.3 ng/g, and 72 and 0.14—212 ng/g, respectively.

Ochratoxin A is lethal in five to six days at 1 mg/kg body weight. OTA fed at 2.5 ppm in the diets of growing pigs was found to reduce growth rates. Levels as low as 0.2 ppm for several weeks can induce detectable renal lesions. Additional clinical signs are diarrhoea, anorexia and dehydration. Someti-

mes clinical signs are not observed, the only gross evidence being the appearance of pale firm kidneys at slaughter (L a w i o r, 2001). OTA is a carcinogen and nephrotoxin which can enter the food chain resulting in human exposure. As pig herds are exposed to OTA through their feed, their kidneys, liver and pork meat are considered as a possible route of exposure for humans. The programme randomly sampled 300 health and 100 nephropathic pig kidneys in 1997 and 710 healthy pig kidneys in 1998. Less than 10% of samples were significantly contaminated by OTA: in the 1997 survey, 1% of the samples contained 0.40—1.40 μg/kg of OTA and in the 1998 survey 7.6% exhibited OTA levels in the range 0.5—5 μg/kg. In the case of nephropathic kidneys, only traces of OTA (0.16—0.48 μg/kg) were detected in six samples out of 100. Even if not a major route of exposure for humans, pigs are clearly exposed to this mycotoxin and monitoring of pork products and of feed for swine is necessary (D r a g a c c i, 2000). Blood serum, kidney, liver and muscle sample per animal were collected from slaughtered pigs (n = 52) in Romania. A total of 98% serum samples were OTA positive in the range of 0.05—13.4 ng/ml. The incidences of OTA in kidney and liver were very similar (79%, 75%) with mean levels of 0.54 ng/g and 0.16 ng/g, respectively. The lowest incidence (17%) and the lowest mean level contamination (0.15 ng/g) were in muscle samples. The mean distribution in tissues followed the pattern serum kidney liver muscle (100%; 26%, 8.5%; 2.57%) (C u r t u i, 2001).

Multiple source exposure assessment indicates that the overall contribution of animal products to human exposure does generally not exceed 3—10% (EFSA, 2004).

CONCLUSION

The conclusion of this paper could be the recommendation of EFSA (2004) to all EU member states and future member states.

1. There is a need to establish measures to reduce the formation of ochratoxin A in feed commodities during transport and storage, including on-farm storage, and to implement adequate control of moisture (water activity) and temperature changes during storage.

2. Analytical methods with appropriate limits of quantification for feeding stuff need to be validated by collaborative studies.

3. The efficacy of feed control programmes should be assessed by surveys of blood levels of ochratoxin A in pigs at slaughter.

4. More data are needed in order to establish a NOEL for pigs and poultry.

5. In order to assess the significance of residue levels in animal tissues, both with respect to animal health and to human exposure, more extensive occurrence data on ochratoxin A in animal tissues and products thereof and from other foods, covering all member states, are required.

The paper has been financed by the Ministry for Science, Technology and Development of the Republic of Serbia (Project: The development of the new

and the improvement of the existing analytical methods and procedures for monitoring the quality of the environment. Project No. 1622)

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ЕВАЛУАЦИЈА КОНТАМИНАЦИЈЕ ХРАНИВА ЗА ЖИВОТИЊЕ ОХРАТОКСИНОМ А У ВОЈВОДИНИ

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Резиме

Охратоксин А је канцероген, теретоген, имунотоксичан и нефротоксичан. Набројани редослед даје важност овом токсину у погледу штетности људском здрављу. Штетности охратоксина А укључују ефекте на молекуларном нивоу, као што су оштећења ДНА, инхибиција синтезе протеина, глугонеогенеза, липидне пероксидације, поремећај оксидативне фосфорилације у митохондријама, инхибиција згрушавања крви и апоптоза. Присуство охратоксина А у великом броју узорака хране, како биљног тако и животињског порекла, евидентан је ризик за људско здравље, што потврђује висока фреквенција присуства овог токсина у узорцима хуманог серума и млека. Могло би се са сигурношћу рећи да су управо напред наведене чињенице разлог што EU последњих неколико година највећу пажњу поклања овом микотоксину.

У овом раду приказани су резултати анализе узорака хранива за животиње за период 2000/03. године на садржај охратоксина А. Анализирана хранива потицала су са фарми на којима су били изражени здравствени проблеми код животиња (није мониторинг). Узорци су анализирани хроматографијом на танком слоју и са лимитом детекције методе за охратоксин А од 40 ppb. Анализирано је 108 узорака кукуруза, 11 узорака јечма, 21 узорак пшенице, 42 узорка сунцокретове сачме и 47 узорака сојине сачме (Табела 1). Узорци сунцокретове сачме контаминирани су у највећем проценту, што указује на неадекватно складиштење овог хранива.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 25—30, 2005

UDC 633.13:632.4

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SURFACE MYCOFLORA IN STORED GRAINS OF NAKED OATS

ABSTRACT: In the framework of small-site experiments we examined the biopreparation Supresivit S 2 containing the propagules of the antagonistic fungus *Trichoderma harzianum*.

The small-site experiments, which were carried out in the experimental plot of the Department of Crop Production (University of South Bohemia in České Budějovice, Faculty of Agriculture), were aimed at impairing the biotic fungistasis by biological means applied to the grains of naked oats, variety Adam. Our objective was to accomplish surface microbial analysis of stored naked oats grains, variety Adam, after the application of the biofungicide Supresivit S 2 in combination with surface treatment of the grains and biological screen applied during vegetation period.

In particular variants of the experiment we estimated the values of the levels of bacteria and fungi representation in the surface parts of stored grains and analysed the contents of some mycotoxins produced by the fungi of the genus *Fusarium*.

The highest numbers of bacteria and fungi on the surface of the investigated grains were found in the variant treated by the bioagent (Supresivit S 2), namely 82.000.000 spores of bacteria and 110.000 spores of fungi (*Cladosporium, Verticillium, Coelomycetum*) per 1 g of grain. Also the fungi of the genus *Fusarium* were most numerous in the variant oats — bioagent, namely 250 spores per 1 g of grain.

In all particular variants the amounts of mycotoxins were considerably below the known effective doses for animals and plants. The largest amount of vomitoxin was found in the variant chemical standard (Rovral TS, effective substance Carbendazim 17.5% and Iprodion 35%) — 18 ug. Zearalenon was found in all variants below 5 ug.

Lower doses of ergosterin were found in the variant treated by the bioagent 4.7 mg/kg.

KEY WORDS: grains, mycotoxins, naked oats

INTRODUCTION

The aim of small-plot experiments carried out in university experimental field belonging to the Department of Plant Production, University of South Bohemia, was to disturb biotic fungistasis by means of a biological agent (Supresivit S 2-antagonistic fungus *Trichoderma harzianum*), applied to the grains

of naked oats, variety Adam. We respected the fact that the most important component of the environment influencing the survival of pathogene development stages seems to consist in biological factors. Authors Sivan & Chet (1989) carried out the planting of *T. harzianum* fungus in the roots of the grown plants and thus reduced the amount of fungi *Fusarium* in the rhizosphere.

In our operational experiments we applied the bioagent in the form of solution during vegetation period with the aim to influence the microflora of phyloplan. Also the authors Okrouhlá (1993) & Michalíková (1994) investigated the protection of plants by antagonistic fungus *T. harzianum* against pathogenes attacking the above-ground parts of plants — phyloplan protection.

Surface microbial analyses of grains after naked oats harvest were carried out in cooperation with the Microbiological Institute in Linz (Lew 1995).

MATERIAL AND METHODS

Naked oats, variety Adam, was sown in the operational area in the plot of the department of plant production by means of small-plot sowing machine. The experiment took place in 12 small plots, each with the area of 6 square meters. In variant no. 2 (oats, chemical standard) the grains were soaked with Rovral TS (effective substance was Carbendazim 17.5% and Iprodione 35%).

In variant no. 3, oats were dusted with Supresivit S 2 and, during vegetation period at the beginning of wax ripeness in phase 83 DC, it underwent a single treatment with Supresivit S 2. The amount of sprinkling solution was about 2 litres per variant and was provided by hand pressure sprinkler Pilmet (see Table 1).

Table 1: Organization of the small-plot experiment

Variant no	Dose of Supresivit per 2 kg of grains	Dose of Rovral per 1 kg of grains	Supresivit application (phase DC)
1. oats control	—	—	—
2. oats chemical standard	—	10 g	—
3. oats bioagent	20 g	—	83

During vegetation period we evaluated the health state of the observed cereals. The development of diseases was assessed according to subjective scale 1—9 (9-healthy plants with no symptoms of disease).

The harvest of naked oats, variety Adam, took place at the beginning of full ripeness in phase 91 DC, during sunny, warm weather. The harvest itself was done in the individual small plots. Particular variants were hand-mown and individual yields were thrashed by the stationary thrasher Veb Fortschritt K-119. In the presence of Dr. H. Lew & Dr. A. Adler (Bundesanstalt für Agrarbiologie, Linz), 1 kg oats specimens of each observed variety were taken and transported to Linz for microbial analysis of fungi and bacteria colonizing

grain surfaces in stored variants, as well as for the assessment of the occurrence of Fusaria secondary metabolites. Bacteria were investigated by cultivation on IAG — bacterial agar; the representation of *Verticillium, Cladosporium, Coelomycetum* fungi was tested on IAG — fungal agar, and fungi of the genus *Fusarium* by cultivation on modified nutritional substance according to Papavizas (1985). Under laboratory conditions the average yield characteristics were investigated (weight of one thousand grains and bulk weight).

RESULTS AND DISCUSSION

Macroscopic evaluation of the growth from the viewpoint of the attack of serious pathogenes (*Fusarium* sp. div., *B. graminis, P. graminis, U. avenae*) took place at the end of tasseling in phase 59 DC. The development of fungal disease was assessed according to the scale of 1 to 9 points. In oats we observed moderate development of the fungus *P. graminis* in variant no. 2, chemical standard (8 points); in variants no. 1 (control) and no. 3 (bioagent) the intensity of the disease was classified with 7 points (as shown in Table 2). Further attacks of infection were stopped by high temperatures due to intense sunshine in summer months. Fungi of *Fusarium* genus, powdery mildew of cereals *B. graminis* and loose smut of oats *U. avenae* were not observed in our small-plot experiments.

Table 2: Evaluation of naked oats plants (variety Adam) attacked by particular pathogenes

Variant no	Fusarium sp. div.	Blumeria graminis	Puccinia graminis	Ustilago avenae
1. oats control	—	—	7	—
2. oats chemical standard	—	—	8	—
3. oats bioagent	—	—	7	—

The highest numbers of bacteria and fungi on the surface of the observed grains were found in variant no. 3, oats, bioagent, namely 82.000.000 spores of bacteria and 110.000 spores of fungi (*Cladosporium, Verticillium, Coelomycetum*) in 1 g of oats. Also *Fusarium* fungi were most numerous in variant no. 3, oats, bioagent, namely 250 spores in 1 g of grains (Table 3).

Table 3: Quantities of fungi and bacteria on the surface of naked oats

Variant no	Bacteria	Fungi (total)	Fusarium sp. div.
1. oats control	65.000.000	40.000	150
2. oats chemical standard	60.000.000	85.000	150
3. oats bioagent	82.000.000	110.000	250

The table gives numbers of spores per 1 g of grains

The values of mycotoxin levels (Table 4) in particular variants were in all cases deep below the known effective levels for animals and plants. The lar-

gest amount of Vomitoxin was found in variant no. 2, oats, chemical standard — 18 ug. Zearalenon was detected in all variants at levels below 5 ug.

Higher amounts of Ergosterin were found in variants no. 1, oats, control — 5.3 mg, and oats, chem. standard — 5.2 mg, compared to variant no. 3, oats, bioagent — 4.7 mg/kg of grains where the measured values were lower (Table 4).

Table 4: Results of mycotoxins and ergosterin analyses in naked oats

Variant no	Vomitoxin mg/kg	Zearalenon mg/kg	Ergosterin mg/kg
1. oats control	11	< 5	5.3
1. oats chemical standard	18	< 5	5.2
3. oats bioagent	7	< 5	4.7

In laboratory conditions we examined basic yield characteristics, namely the weight of 1.000 grains and bulk weight (Table 5). The highest values were found in the variant treated with the bioagent (Supresivit S 2).

According to Hausvater & Trnková (1993), Voženíl ková (1993) and Dušková (1994), the stimulating effects of the fungi of *Trichoderma* genus in plants manifest themselves by better and faster germination and sprouting, faster and entirely bigger growth, increased formation of leaves, the root system is more powerful, and the total yield is increasing.

In our experiments the stimulating effects of the used biopreparation were apparent in variant no. 3, oats, bioagent, the weight of 1.000 grains 33.0 g, and bulk weight 703.3 g $.1^{-1}$ in comparison with the control untreated variant where the values were considerably lower (var. no. 1, oats, control, HTZ 30.6 g, OH 672,0 g $.1^{-1}$). The variant treated with Rovral TS showed lower weight of 1.000 grains, and also bulk weight was lower compared to the variant with bioagent treatment (var. no. 2, oats, chemical standard, HTZ 31.2 g, OH 688.5 g $.1^{-1}$), as shown in Table 5.

Table 5: Bulk weight (OH) and weight of 1.000 grains (HTZ)

Variant no	Oats-variety Adam	
	OH	HTZ
1. Control	672.0	30.6
2. Chemical standard (Rovral TS)	688.5	31.2
3. Supresivit S (T. harzianum)	703.3	33.0

CONCLUSION

In small-plot experiments we tested the biopreparation Supresivit S 2 containing the propagules of fungus *T. harzianum* utilized against selected diseases in cereals (*Fusarium* sp. div., *B. graminis, P. graminis, U. avenae*). The aim consisted in surface microbial analysis of the grains of naked oats var. Adam after the application of the biofungicide Supresivit S 2 in combination

with surface modification of the grains and biological screen applied during vegetation period. Owing to high temperatures and intense sunshine during summer months the infection causing the observed diseases was hindered and thus it was difficult to precisely assess the efficiency of the applied bioagent against selected pathogenes.

We examined the basic yield characteristics, the weight of 1.000 grains and bulk weight. The highest values within the experiment were found in the variant treated by the bioagent (Supresivit S 2-naked oats, variety Adam).

Very hot weather during maturing period and harvest influenced the quality of harvested grains of naked oats (var. Adam). In all observed variants, the levels of mycotoxins were considerably lower than the known effective doses for animals and plants.

The contribution was supported by project MSM: J06/98: 122200002

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ПОВРШИНСКА МИКОФЛОРА У УСКЛАДИШТЕНИМ ЗРНИМА ГОЛОСЕМЕНОГ ОВСА

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Резиме

У оквиру експеримената на малим огледним пољима проучавали смо биопрепарат Supersivit S 2 који садржи честице антагонистичке гљивице *Trichoderma harzianum*.

Експерименти на малим огледним пољима који су спроведени на огледној парцели Департмана за ратарство (Универзитет Јужне Бохемије у Чешким Буђејовицама, Пољопривредни факултет) били су усмерени ка спречавању биотичке фунгистазе применом биолошких средстава на зрна голосеменог овса, варијетета Адам. Наш циљ је био површинска микробиолошка анализа ускладиштених зрна голосеменог овса, варијетет Адам, након примене биофунгицида Supersivit S 2 у комбинацији са површинским третманом зрна и биолошким заслоном примењеним током вегетацијског периода.

Код посебних варијаната овог експеримента одредили смо вредности нивоа појаве бактерија и гљивица на површинским деловима ускладиштених зрна и анализирали садржај неких микотоксина узрокованих гљивицама из рода *Fusarium*.

Највећи број бактерија и гљивица на површини испитиваних зрна констатован је код варијанте третиране биоагентом (Supersivit S 2), и то 82.000.000 спора бактерија и 110.000 спора гљивица (*Cladosporium, Verticillium, Coelomycetum*) по једном граму зрна. Такође, гљивице из рода *Fusarium* биле су најмногобројније у варијанти овас — биоагент, и то 250 спора по 1 граму зрна.

У свим посебним варијантама количине микотоксина биле су знатно ниже од познатих ефективних доза за животиње и биљке. Највећа количина вомитоксина констатована је код варијанте хемијског стандарда (Rovral TS, ефективна супстанца Carbendazim 17,5%, и Iprodion 35%) — 18 μg. Зеарленон је констатован код свих варијаната количини мањој од 5 μg.

Ниже дозе ергостерина констатоване су код варијаната третираних биоагентом 4.7 mg/kg.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 31—35, 2005

UDC 614.31:616-78

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DETERMINATION OF AFLATOXINS IN FOODSTUFFS EMPLOYING FLUOROMETRIC METHOD

ABSTRACT: Results from the examination of total aflatoxins in foodstuffs are presented. Determination of the aflatoxins was done employing rapid and reliable method, based on immunnosorbent columns clean-up and measurement of the aflatoxins by fluorometer (Vicam, USA). Quantification and detection limits of the method were 1.0 and 0.1 ppb respectively. This method is especially suitable when laboratories are dealing with big number of samples. 20% of the samples were found to content total aflatoxins over MRL (5 μg/kg). The positive samples were further analysed with TLC as a confirmatory method, whereby over 95% of positive samples where confirmed by TLC technique. Application of this rapid aflatoxin method significantly simplifies their analysis, especially when laboratories are dealing with big number of samples.

KEY WORDS: aflatoxins, fluorometric determination, immunoaffinity columns

INTRODUCTION

Aflatoxins are highly toxic metabolites produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. They can be found in a wide range of food and feeding stuff, and are potentially hazardous to human and animals. Aflatoxins B_1, B_2, G_1 and G_2 have found to be naturally prevalent and also their potent carcinogenic and mutagenic characteristics in a number of animals and humans (B u l l e r m a n 1986). The Europen Commision has set limits for the maximum levels of total aflatoxins (EU regulation 194/97) at 4 ng mL^{-1}. According to our national legislation (Official paper of RM), the maximum permitted level for total aflatoxins is 5 μg kg^{-1}. The monitoring of aflatoxins depends on precise and reliable analytical methods. Food products being commonly contaminated with aflatoxins are nuts (peanuts and pistachios), dried fruits (figs), tee (black tee), grains (wheat and corn) and spices (paprika). Mo-

nitoring of aflatoxins in these products is not only of importance for consumer protection, but also for producers of raw products prior to intensive processing or transport.

Current mycotoxin analysis are done by various methods including thin-layer chromatography (TLC) (Lin et al., 1998, Stroka et al., 2000), liquid chromatography (LC) and high-performance liquid chromatography (HPLC) (Jaimez et al., 2000, Akiyama et al., 2000), and microtitre plate enzyme-linked immunosorbent assay (ELISA) (Turner et al., 1998). All of these methods are long time consuming and delicate analysis, and thus require specialist equipment operated by skilled personnel.

In resent years solid-phase clean-up methods especially with immunosorbent columns have been widely applied for aflatoxins in foodstuffs (Dimitrieska-Stojkovic et al., 2002). Rapid solid-phase immunosorbent test are very useful for screening the total aflatoxin level, yet these tests are not able to perform quantitative analysis. In this paper a results from the determination of aflatoxins in our laboratory in the past year will be presented. Analysis was done by fluorometric method with previous clean-up with aflatoxin immunosorbent column. The two-dimensional TLC followed by the same sample preparation was employed as a confirmatory method. We selected it as a second method of choice, since in the analysis of mycotoxins TLC applications are still used in the great majority of samples qualitatively and/or quantitatively. One-dimensional TLC has been widely used, and two-dimensional and bidirectional developments are also frequently employed.

MATERIALS AND METHODS

235 different foodstuffs were analyzed on the presence of aflatoxins, mostly delivered from border health inspectors. Samples were ground and homogenized with 60% methanol (HPLC grade) followed by addition of sodium chloride. An aliquot of the filtered solution was diluted with water (HPLC grade) and applied to the immunosorbent column (AflaTest P, Vicam, USA) through glass microfibre filter (Whatman). Column is washed with water, and analytes were eluted with 1 mL methanol in cuvette. Eluate was diluted with 1 mL aflatest Developer solution (Vicam, USA). Aflatoxin concentration was measured on fluorometer (Vicam, USA) after 60 seconds. Quantification limit (LOC) of the method is 1 μg/kg, and the detection limit (LOD) is 0.1 μg/kg.

When applying this method on certain samples (black tea, cocoa) false positive results occur. To eliminate this problem, instead of pure water, the dilution of the filtrate was done with 10% solution of Polyoxyethylene-Sorbitan Monolaurate (Vicam, USA). When this clean-up procedure is employed the LOD and LOQ are 2 μg/kg 0.5 μg/kg, respectively.

Eluted samples after the clean up are gently evaporated under stream of nitrogen at 40°C. The samples are dissolved in 1 mL benzene:acetonitrile (98:2 v/v). A series of standards with concentration 0.5, 1.0, 2.5 and 5.0 μg mL^{-1}, together with samples are applied on Kiselgel 60 F 254 plate (20 x 20 cm) using LINOMAT IV device (Camag). The volume of the applied stan-

dards and samples was 100 μL, while the start line was 20 mm from the plate edge. Plates were developed two-dimensionally using mixture benzol : methanol : acetic acid (9 : 0.5 : 0.5 v/v) as solvent A in the first direction, and consequently with chloroform : acetone (9 : 1 v/v) in the other direction for about 60 minutes each, or when solvent front reaches about 15—17 cm height. After drying, plate spots were detected by illuminating in UV chamber (Camag) at 254 nm. The R_f-values for aflatoxins B_1, B_2, G_1 and G_2 are 0.42, 0.36, 0.30 and 0.26 respectively, with quantification limit of 1.0 μg mL^{-1}.

RESULTS AND DISCUSSION

The results from the analyzed 235 samples are presented in Table 1. The results for samples that were found to be under the LOD, under the MRL (maximum residue level), over the MRL, are presented separately to express the food safety situation regarding aflatoxin content. According to our national legislation, MRL's for total aflatoxins (B_1, B_2, G_1, G_2) are set at 5 μg/kg.

Quality control of the method is performed by calibration of the instrument on daily basis with two levels of concentration 1 l and 22 μg/kg, and by determining the recovery of the each set of 20 samples. The recovery value in average was over 90%. Total method can be completed in 30 minutes, which is significantly shortening the analysis time, especially when a laboratory is dealing with many samples that have to be analyzed in short time.

From the results presented in the table it can be concluded that 60.2% (142 out of 235) of the samples contain aflatoxins under the LOD. The number of samples with total aflatoxin concentration over MRL is 47 (20.0%). Positive results for these samples have to be confirmed with other method, and in our practice as a confirmatory method we are employing TLC.

Table 1. Results form the analysis of total aflatoxins in with fluorometric and TLC method

Sample type	Number of samples under LOQ	Number of samples under MRL	Number of samples over MRL	γ/μg/kg over MRL	Confirmed with TLC
Green coffee beans	7	—	1	7.0	1
Nuts	14	12	6	6.7—16.0	5
Cereals	46	4	3	9.6—13.0	3
Dried vegetables	23	4	5	5.2—21.0	4
Dried fruits	13	6	1	27.0	1
Spices	15	4	9	6.1—42	8
Corn maize	4	—	1	26.0	1
Egg powder	3	4	—	—	—
Black Tea	5	1	17	5.1—22.0	16
Cocoa powder	12	3	4	5.1—23.0	4

We decided to use two-dimensional TLC for its improved performances and reliability. The two-dimensional TLC excludes the possibility of appearan-

ce an interfering spot with similar R_f-values like targeted aflatoxins. If a spot coming from interfering compound appears with the development in the first direction, the second development (when the plate is rotated for 90°) will very probably separate it. Obtained detection limit for previously described experimental conditions was satisfactory and in a good agreement with some published papers for mycotoxin analysis with TLC (Lin et al., 1998).

CONCLUSIONS

In this paper a results from employing fast, easy to use and reliable method for total aflatoxin analysis were presented. 60.2% (142 out of 235) of the samples contain aflatoxins under the LOD, whereby the number of samples with total aflatoxin concentration over MRL is 47 (20.0%). As a confirmatory method for positive samples, two-dimensional TLC with previous immunoaffinity column sample preparation was performed. The percentage of confirmed positive samples with the second method (over 90%) proves the method reliability.

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ОДРЕЂИВАЊЕ АФЛАТОКСИНА У НАМИРНИЦАМА ПРИМЕНОМ ФЛУОРОМЕТРИЈЕ

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Резиме

Представљени су резултати испитивања укупних афлатоксина у намирницама. Одређивање афлатоксина је извршено применом брзог и поузданог поступка заснованог на чишћењу имуносорбентних колона и мерењу афлатоксина помоћу флуорометра (Vicam, USA). Квантификациони и детекциони лимити овог метода били су 1.0 и 0.1 ppb. Овај метод је посебно погодан када лабораторије имају велик број узорака. Констатовано је да 20% узорака садрже укупне афлатоксине преко MRL (5 μg/kg). Позитивни узорци су даље анализирани применом TLC методе за потврђивање, где је преко 95% позитивних узорака потврђено TLC техником. Примена ове брзе методе за афлатоксине значајно поједностављује њихову анализу, нарочито када лабораторије имају велик број узорака.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 37—42, 2005

UDC 633.11:582.28
664.641.12:582.28

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MYCOLOGICAL AND MYCOTOXICOLOGICAL QUALITY OF WHEAT AND FLOUR FRACTIONS

ABSTRACT: The seed infection is a result of complex factors influence: weather conditions, health conditions of used seed, quantity of infective potentila in soil, etc. By visual evaluation, initial wheat sample has been divided in four fractions: healthy, dark germed, slightly and very fusarious. Three varietes from two localities 1 and 2 have been included in analyses. Beside the wheat, the mycotoxicological contamination of flour produced by grounding of given samples was monitored, too.

The representatives of genera *Fusarium* were dominating, and the most frequent was *F. oxysporum*.

The wheat and flour samples have also been analysed on presence of aflatoxin B_1 "AB_1" and G_1 "AG_1", ochratoxin A "OA" and zearalenone "F-2" toxin. AG_1 had the lowest representation (2,3 μg/kg) and the highest representation was of F-2 toxin (even 500 μg/kg).

KEY WORDS: flour, mycotoxins, processing quality, wheat

INTRODUCTION

A mycological analysis of each batch of wheat is a prerequisite for the production of sanitarily correct nutriments produced from wheat grains. Thanks to its chemical composition the wheat represents a very suitable substrate for various phytopathogenic and saprophytic microorganisms. Many fungi of the genera *Fusarium, Alternaria, Penicillium, Aspergillus, Cladosporium* and others are well known as producers of a great number of various toxic metabolites. The produced mycotoxins are thermo resistant and lose none of their toxicity during thermal processing.

The most important mycotoxins produced by fungi of the genus *Fusarium*, synthesized in the cereals before the harvest, are zearalenone and ochratoxin A.

Fusarioses have a very negative effect on the technological quality of the grain. The infested grains have a higher content of ash and protein (but the

quality of proteins is poor), and a lower content of moist gluten (Šarić et. al., 2004).

The aim of this investigation was monitoring of micosys and mycotoxicosys infection degree, to observe the contamination level influence of technological quality of wheat.

MATERIALS AND METHODS

Depending on the degree and type of mycological infection, the wheat grains were manually divided into the following categories: healthy, dark germ (with typically noticeable dark germ), slightly fusarious contaminated (grains infested with fungi of the genus *Fusarium* in the late mature phase), and very fusarious contaminated grains (grains infested with fungi of the genus *Fusarium* in the early growth phase). Twenty four samples of each fraction were tested.

The identification of the fungi to the species was made by comparison with the corresponding reference books (Raper and Fennell, 1963; Ames, 1969; Ellis, 1971, Nelson et. al., 1983). The flour from all the wheat samples was tested for mycotoxin presence in accordance with the Official Methods of Analysis of the A. O. A. C. (1990).

RESULTS

Mycological Analysis

The results presented in Figure 1 were obtained by the mycological analysis of the wheat samples from the localities 1 and 2.

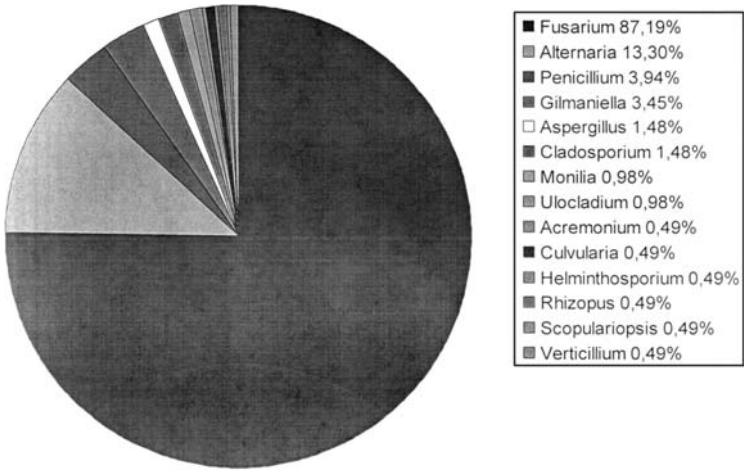

Figure 1. Distribution of the determined fungial genera in the wheat samples from the localities 1 and 2.

Mycotoxicological Investigations

Table 1 and 2 present the level of contamination by aflatoxin B_1 nad G_1, ochratoxin A and zearalenone in the examined wheat samples at localities 1 and 2.

Table 1. Number of wheat samples contaminated by AB_1, AG_1, OA and F-2 toxin at locality 1

Variety	Category of wheat	N° of wheat samples	N° of wheat samples contaminated by moulds	Concentration of			
				AB_1	AG_1	OA	F-2 toxin
				(μg kg^{-1})			
1	H	4 x 12	52	0	0	0	230
	DG	4 x 12	75	0	0	0	240
	SF	4 x 12	89	0	0	0	170
	VF	4 x 12	91	0	0	48	300
2	H	4 x 12	53	0	0	0	0
	DG	4 x 12	68	0	0	0	200
	SF	4 x 12	74	0	0	32	200
	VF	4 x 12	82	0	0	48	250
3	H	4 x 12	55	0	0	0	0
	DG	4 x 12	66	0	0	0	200
	SF	4 x 12	68	0	0	0	160
	VF	4 x 12	83	0	2,3	48	400

Legend: H — healthy; DG — dark germ; SF — slightly fusarious; VF — very fusariuos

Table 2. Number of wheat samples contaminated by AB_1, AG_1, OA and F-2 toxin at locality 2

Variety	Category of wheat	N° of wheat samples	N° of wheat samples contaminated by moulds	Concentration of			
				AB_1	AG_1	OA	F-2 toxin
				(μg kg^{-1})			
1	H	4 x 12	35	0	0	16	0
	DG	4 x 12	69	0	0	32	0
	SF	4 x 12	82	0	0	32	0
	VF	4 x 12	89	0	0	32	0
2	H	4 x 12	37	0	0	32	0
	DG	4 x 12	67	0	0	0	0
	SF	4 x 12	83	0	0	0	0
	VF	4 x 12	83	0	0	32	400
3	H	4 x 12	41	0	0	0	0
	DG	4 x 12	46	0	0	34	200
	SF	4 x 12	58	0	0	11.5	0
	VF	4 x 12	64	0	0	34	500

Samples of flour obtained by milling the above listed categories of the wheat grains were also subjected to the mycotoxicological analysis. During the milling, the outer part of the grain, i. e. the coat, where the greatest quantity of mycotoxins is concentrated, was separated as bran. Therefore, a considerably lower mycotoxin content could be expected in flour samples obtained from these grains. In Table 3 are shown the results of the mycotoxicological analysis of the flour from native grain from the localities 1 and 2.

Table 3. Flour contamination by mycotoxins

Variety	Locality	Mycotoxin (μg kg^{-1})			
		AB_1	AG_1	OA	F-2 toxin
1	1	0	0	0	66.67
2	1	0	0	26.67	0
3	1	0	1.4	5.71	0
1	2	0	0	16	0
2	2	0	0	0	0
3	2	0	0	16	200

DISCUSSION

Fungi of the genus *Fusarium* were the most frequent contaminants of the wheat grains from both localities.

From the 12 identified species, *F. oxysporum* was found to be present in the greatest number of tested samples (40.88%). *F. oxysporum* is very frequent in the micro population attacking the cereals and it is mentioned in reference literature as the producer of zearalenone. (T u t e l y a n and K r a v č e n k o, 1985).

As expected, very fusarious contaminated grains were most often infested by this fungus. Also, species *F. subglutinans*, a representative of the *Liseola* section, was detected with considerable frequency (17.61%). This species infected in greater degree the categories of healthy, dark germ and slightly fusarious contaminated grains, while its content in very fusarious contaminated grains was low. The presence of species *F. poae*, a representative of *Sporotrichiella* section, amounted to 8.80%. This fungus is generally a weak pathogen for cereals, and many other plants. In our investigations, it had mostly infected the dark germ category of wheat grain. This grain category is, however, most often infected by fungi of the family *Dematiaceae*. Apart from *F. poae*, the most frequently detected *Fusarium* species was *F. avenaceum*, a representative of the *Roseum* section (6.92%).

One of the most frequent contaminants of the wheat is certainly *F. graminearum*. In our investigations, *F. graminearum* was found to be present in the amount of 5.03%, compared to other species of the genus *Fusarium*, as a consequence of disappearance of this typical field mycopopulation during storage.

Other fungi of the genus *Fusarium* were represented in a less amount: *F. chlamydosporum* and *F. sambucinum* (4.40%), *F. lateritium* var. *cerealis* (3.14%), *F. graminum* (0.62%), *F. solani* (1.88%), *F. dimerum* and *F. orthosporoides* (0.62%).

The presence of the representatives of the genus *Alternaria* was less prominent and amounted to 13.30%.

Other fungi genera were distributed with low frequency and, therefore, it can be assumed that they did not significantly influence the mycotoxicological picture of the wheat.

MYCOTOXICOLOGICAL ANALYSIS OF THE WHEAT

In the analyzed samples of certain wheat grain categories AB_1 was detected in none of the samples from both localities. The presence of AG_1 was found in very low concentrations (2.3 μg kg^{-1}) and only in one sample of the third variety from the locality 1.

OA distribution was much higher. The highest infection with this mycotoxin had very fusarious contaminated grains from locality 1 (OA concentration in all varieties was 48 μg kg^{-1}). OA frequency in wheat samples from the other locality was much higher (75%), and even the healthy grains were infected with this toxin (varieties 1 and 2, where the concentrations were 16 and 32 μg kg^{-1}, respectively).

The greatest OA contamination of the wheat samples from locality 2 had the dark germ and very fusarious contaminated grains categories, and the concentration was 34 μg kg^{-1}. The fungi of the genus *Penicillium* detected in these samples were potential producers of OA, so the presence of this secondary metabolite *Penicillium* spp. was expected.

Zearalenone was in our investigations detected in extremely high concentrations in 54% of the tested wheat samples. The concentrations varied between 160 and 500 μg kg^{-1}. The highest contamination had the very fusarious contaminated grains, as was to be expected.

MYCOTOXICOLOGICAL ANALYSIS OF FLOUR

AB_1 was not detected in any of the flour samples from both localities, which was expected since it had not been detected in any of the wheat samples from which the flour was obtained. The presence of AG_1 was detected in only one of the flour samples (variety 3 from locality 1) in concentration of 0.14 μg kg^{-1}. The OA contamination was considerably higher. As many as 66% of the flour samples were infected with this toxin. The concentration varied between 5.71 and 26.67 μg kg^{-1}.

It can be supposed that the grain infection with store fungi as well as its storage under inappropriate conditions favored the diffusion of the produced toxins through the grain coat into the endosperm, and the so produced toxins remained in the flour after milling.

It is interesting to note that the zearalenone contamination of the native flour samples was much lower in comparison to the corresponding grain samples. The contaminated samples were 33.33% and the concentration varied between 66.67 and 200 μg kg^{-1}.

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МИКОЛОШКИ И МИКОТОКСИКОЛОШКИ КВАЛИТЕТ ФРАКЦИЈА ПШЕНИЦЕ И БРАШНА

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Резиме

Зараженост семена резултат је деловања комплекса фактора: временских прилика, здравствене кондиције употребљеног семена, количине инфекционог потенцијала у земљишту итд. Визуелним прегледом полазни узорци пшенице подељени су у неколико категорија. Анализама су биле обухваћене три сорте пшенице које су потицале са два локалитета. Циљ рада био је праћење степена миколошке инфекције и микотоксиколошке контаминације појединих категорија пшеничног зрна да би се јасније сагледао утицај степена ове контаминације на њен технолошки квалитет. Осим пшенице, праћена је и микотоксиколошка инфекција брашна, добијеног млевењем ових узорака пшенице.

Узорци пшенице и брашна анализирани су на присуство афлатоксина B_1 (AB_1) и G_1 (AG_1), охратоксина А (ОА) и зеараленона F-2 токсина. Концентрације детерминисаних токсина варирале су у широким границама. Најмању заступљеност показивао је aflatoxin G_1 (2,3 μgkg^{-1}), а највећу зеараленон (чак 500 μgkg^{-1}).

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 43—50, 2005

UDC 633.11:582.28

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FUSARIOSES OF CERTAIN WHEAT GRAIN CATEGORIES AND THEIR MYCOTOXICOLOGICAL INFECTION

ABSTRACT: Certain climatological conditions favor the infection of wheat by field moulds, and influence very much the yield, the overall quality and the hygienic properties of the wheat. The investigations included 6 wheat varieties from the first locality and 5 varieties from the second locality. The investigated samples were most often contaminated by fungi of the genus *Fusarium*. Each wheat sample was classified with respect to the degree of contamination by these fungi as slightly fusarious contaminated and very fusarious contaminated grain.

The mycotoxins, their metabolites, were isolated from the greatest number of contaminated grains.

KEY WORDS: field moulds, mycotoxins, wheat

INTRODUCTION

The wheat grain, with its adequate chemical composition, abundance of nutritional and biologically valuable substances, makes a very suitable substrate for settling and development of various groups of microorganisms. The most important contaminants are moulds, and the most dangerous ones are the mycotoxicogenic forms (Stojanović et al., 2002).

Certainly, among the most frequent contaminants of the wheat are the species of the genus *Fusarium*. Most of the representatives of this genus are able to synthesize a wide variety of toxic metabolites (zearalenone and its derivatives, tri hotellens, moniliformin and others) (Nelson et al., 1983).

The scope of this work was to study the mycological and mycotoxicological infections of the wheat grain attacked to a greater or less extent by the fungi of the genus *Fusarium*, and the influence of this infection on the hygienic properties of the grain.

MATERIAL AND METHODS

Mycological and mycotoxicological analysis was carried out on 22 samples of slightly fusarious and very fusarious contaminated wheat grains.

Mycological investigations

To determine the fungi count in the wheat grain, samples of 10 g were measured and treated with 100 ml of sodium hypochlorite to eliminate all the saprophytic fungi concentrated on the grain surface. The 300 ml Erlenmeyer flasks containing the sample and sodium hypochlorite were shaken on a rotary shaker for 2 minutes. Then, each sample was rinsed with distilled water (2 x 100 ml). Under sterile conditions, 12 grains of each sample were placed on dextrose Sabouraud agar with antibiotic added (1 ml of 2% streptomycin solution/100 ml of medium). The culture dispersed in Petri dishes was left to incubate for seven days at 26°C. Each test was repeated two times. After that the results were recorded and presented as the average fungi count per wheat grain.

Further cultivation of pure fungi isolates was carried out on potato dextrose agar (PDA) and carnation leaves agar (CLA) purifying the cultures until obtaining the pure monospore isolate. The determination of the fungi of the genus *Fusarium* was made according to Burgess et al., 1988.

Mycotoxicological analyses

The qualitative and quantitative determination of mycotoxins was carried out by multimycotoxic method proposed by Balzer et al., 1978.

RESULTS

In Table 1 are given the results of the investigation of the wheat grains contamination by moulds.

Table 1. Average number of moulds found per kernel in wheat fractions

Fraction of wheat	No of wheat samples	Variety	Locality	Number of moulds per kernel
LF	2	1	1	2.59
VF	2	1	1	2.37
LF	2	2	1	3.24
VF	2	2	1	3.30
LF	2	3	1	2.51
VF	2	3	1	2.76
LF	2	4	1	2.53

VF	2	4	1	2.88
LF	2	5	1	2.62
VF	2	5	1	3.21
LF	2	6	1	2.63
VF	2	6	1	2.76
LF	2	1	2	2.87
VF	2	1	2	3.21
LF	2	2	2	3.12
VF	2	2	2	3.25
LF	2	3	2	3.21
VF	2	3	2	3.21
LF	2	4	2	2.12
VF	2	4	2	2.20
LF	2	5	2	2.94
VF	2	5	2	3.14

Legend: LF — little fusarious; VF — very fusarious

The contamination degree of slightly fusarious and very fusarious contaminated wheat grains by species of the genus *Fusarium* is given in Figures 1 and 2.

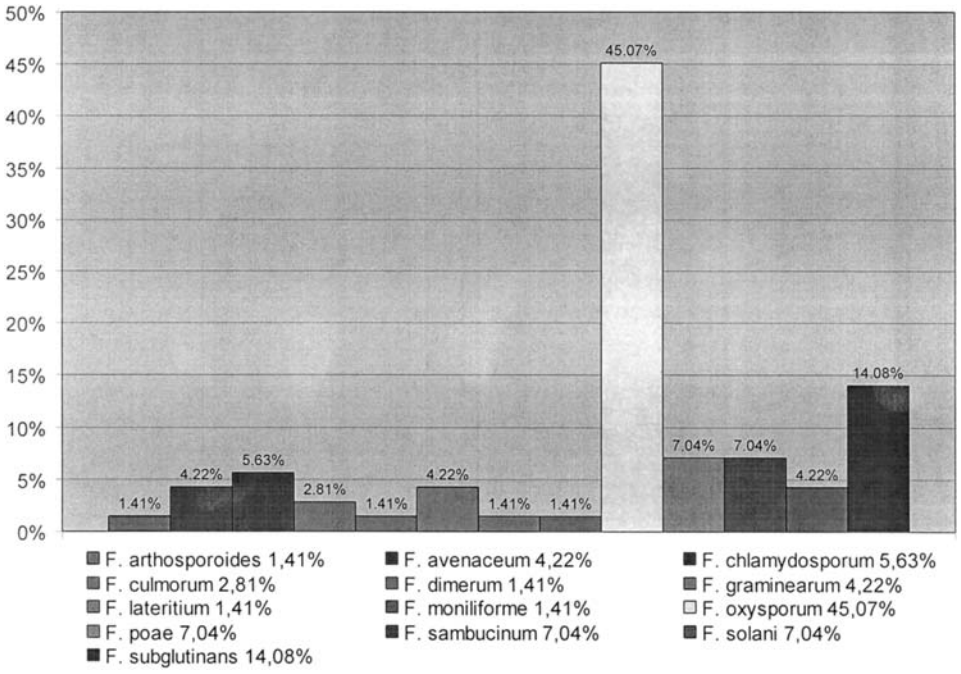

Figure 1. Contamination of little fusarious wheat kernel by *Fusarium* species at locality 1 and 2

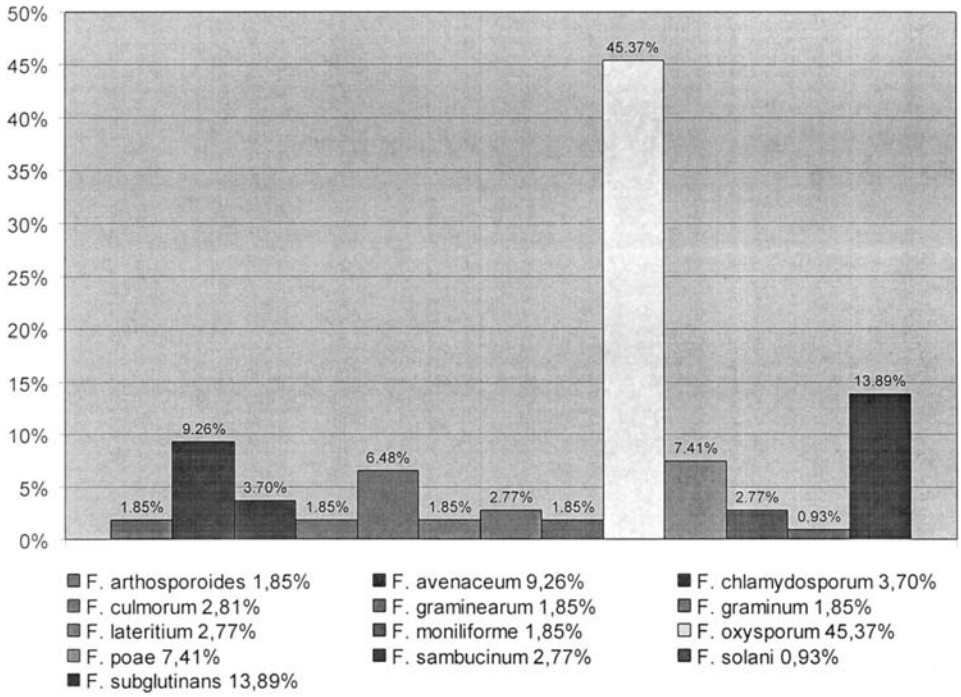

Figure 2. Contamination of very fusarious wheat kernel by *Fusarium* species at locality 1 and 2

The results of the mycotoxical analyses of slightly fusarious and very fusarious contaminated wheat grains are given in Table 2.

Table 2. Number of wheat samples contaminated by zearalenone at locality 1 and 2

Variety	Locality	Fraction of wheat	Zearalenone μgkg^{-1}
1	1	LF	240
1	1	VF	1600
2	1	LF	170
2	1	VF	360
3	1	LF	200
3	1	VF	250
4	1	LF	160
4	1	VF	400
5	1	LF	280
5	1	VF	360
6	1	LF	160
6	1	VF	360
1	2	LF	0
1	2	VF	280

2	2	LF	0
2	2	VF	0
3	2	LF	0
3	2	VF	400
4	2	LF	0
4	2	VF	500
5	2	LF	250
5	2	VF	350

DISCUSSION

The mycological analyses showed that all the wheat samples had been infected by moulds, as was supposed, since many of the grains were visibly damaged and shriveled. This was especially true of the very fusarious contaminated grains. The mould count per wheat grain did not vary much, since the samples were of similar grain categories, but it certainly was very high, amounting to 2.12 in slightly fusarious contaminated grains, and 3.30 in very fusarious contaminated wheat.

The most frequent contaminants of all wheat varieties from localities 1 and 2 were species of the genus *Fusarium*. The fungus *F. oxysporum* was the most frequent type with both slightly fusarious contaminated and very fusarious contaminated wheat grains. *F. oxysporum* is genetically very variable species with a high degree of variability of both morphological and physiological properties (B u r g e s s et al., 1989). This fungus is a very strong soil sapro-

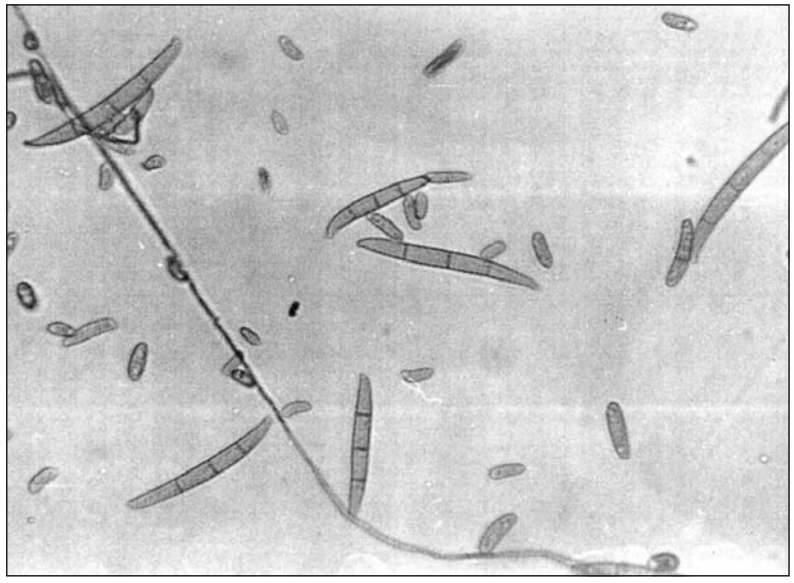

Figure 3. Micro and macro conidia
Microscopis view of *E. oxysporum* (PDA, 26°C, 7 days)

phyte, and beside cereals it often attacks and causes wilting of pepper, tomato, watermelon, melon, and other plants (N e l s o n et al., 1981). Many forms of *F. oxysporum* are toxicogenic, as mentioned in investigations by other authors. The microscopic view of *F. oxysporum* is given in Figure 3.

The species *F. subglutinans, F. avenaceum, F. chlamydosporum* and others were significantly less frequent.

In very contaminated grains, the content of *F. avenaceum* fungus was 9.26%. This fungus is characterized by growth on PDA with thick aerial pink mycelium. This representative of the section *Roseum* has thin, needle-like macro conidia, with missing chlamydospores (Figure 4).

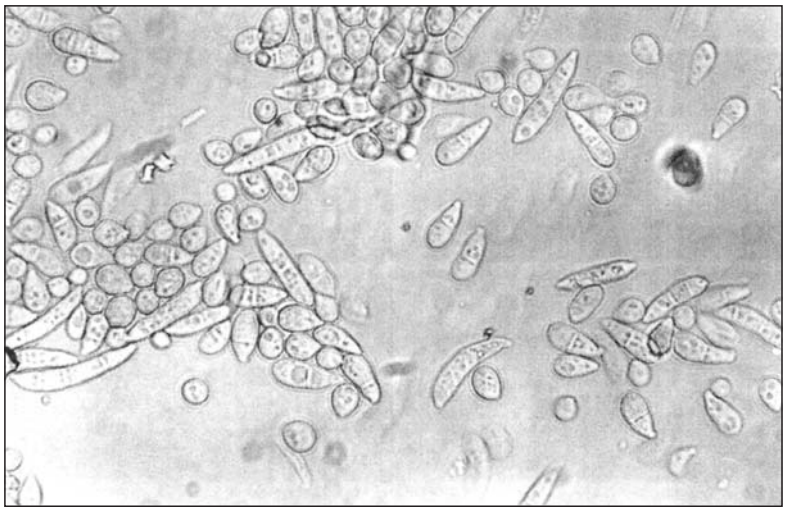

Figure 4. Micro and macro conidia
Microscopis view of *F. poae* (PDA, 26°C, 7 days)

Considerable contamination by fungus *F. poae* was found in both slightly and very fusarious contaminated wheat grains (7.04% and 7.41%, respectively). This fungus often produces micro conidia. Macro conidia are of crescent shape and they are typical of this genus (Figure 5).

F. chlamydosporum infection of slightly fusarious wheat grains was 5.63%, while its count in very fusarious contaminated grains was lower and amounted to 3.70%. Also, this fungus is known as a frequent producer of various toxic metabolites (moniliformin, neosolanio and others) (M a r a s a s et al., 1984), although this author does not list it as the producer of zearalenone.

The fungus *F. graminearium* was found to be present in very fusarious contaminated grains (6.48%). This species is considered the most virulent one, and very often it biosynthesizes zearalenone, fusarine, monoacetoxyscirpenol, nivalenol and other toxins. Although the slightly fusarious contaminated and very fusarious contaminated grains were also infected by other fungi of the genus *Fusarium* (13 species contained in each grain category), their occurrence was considerably lower compared to the species listed above.

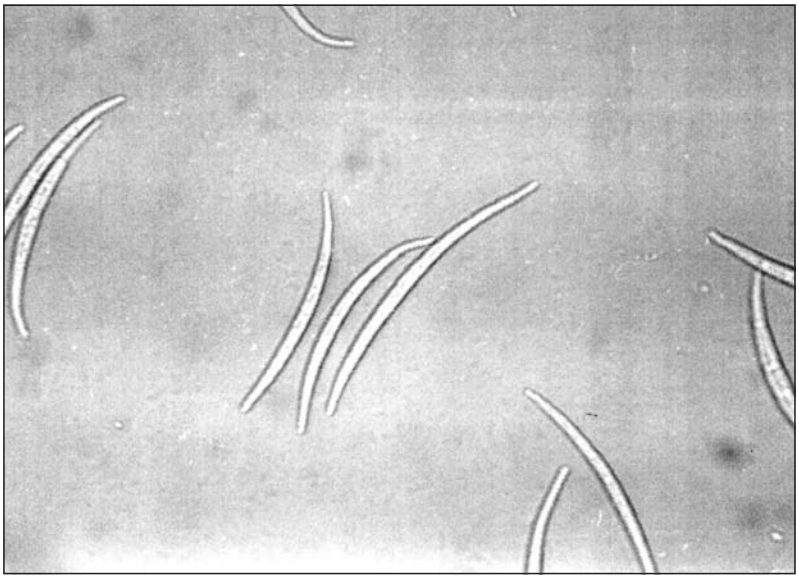

Figure 5. Macro conidia
Microscopic view of *F. avanaceum* (PDA, 26°C, 7 days)

Mycotoxicological analyses showed extremely high contamination by zearalenone (to the extent that 77% of the samples were infected). The concentrations varied between 160 and 1600 μg kg^{-1}. Zearalenone is a toxic metabolite of the field mycopopulation synthesized in the grain before the harvest. Having in mind that the samples investigated were visibly damaged grains (especially, the very fusarious contaminated fraction), these results were expected.

Unfortunately, it is very hard to separate the fusarious contaminated grains from the bulk of grains, so that, apart from drastic impairment of technological quality, they influence, to great extent, the sanitary properties of the wheat.

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ФУЗАРИОЗЕ ПОЈЕДИНИХ КАТЕГОРИЈА ПШЕНИЧНИХ ЗРНА И ЊИХОВА МИКОТОКСИКОЛОШКА ИНФЕКЦИЈА

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Резиме

Одређени климатски услови фаворизују инфекцију пшенице пољским плеснима и јако утичу на приносе, квалитет у целини и хигијенску исправност пшенице. Истраживањима је било обухваћено 6 сорти пшенице пореклом са првог локалитета и 5 сорти пшенице са другог локалитета. Гљивице рода *Fusarium* биле су најчешћи контаминенти испитиваних узорака. Сваки узорак пшенице је према степену оштећености гљивицама овог рода био подељен на мало фузариозна и јако фузариозна зрна.

Код највише контаминираних зрна изоловани су њихови метаболити — микотоксини.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 51—57, 2005

UDC 631.563:582.28

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MYCOTOXINS IN THE SILAGE — CAUSES OF CREATING, AFTERMATH AND PROTECTION FROM ACTING

ABSTRACT: The causes of appearance of mold in the silage, genus and species of fungi which biosynthesize mycotoxins, acting consequences of micotoxins, prevention of fungi growth and possibilities to prevent their negative effects are shown in this paper. Also, the results of mycotoxins presence in the silage (corn and lucerne) in Vojvodina in the period 2000—2004 are presented. The most commonly found mycotoxins were zearalenone in 60.6% of analyzed samples and DAS in 30.3% of samples. Silage contamination with ochratoxin A, aflatoxin B1 and T-2 toxin was between 15.2 and 21.2%. The content of mycotoxins DAS and T2 toxin was above the values allowed by regulative. The solutions which contribute to the prevention of development of the molds and elimination of negative effects of mycotoxins in silage were analyzed and suggested.

KEY WORDS: adsorbents, helath, mycotoxins, silage

INTRODUCTION

Silaging of animal feed is a possibility for keeping the quality of feed in fresh stage for a long time. That is happening because of the presence and activity of lactic acid, acetic acid and butyric acid bacteria. Besides them, in the ensilig material the molds and yeasts which produce numerous metabolites are present. The main bacteria activity product in the silage is lactic acid which is at the same time most favourable one, too. In the silage material, some minor quantities of acetic acid, butyric acid and propionic acid, ethyl alcohol, carbon dioxide and ammonium appear. Acetic acid, butiric and propionic acid are weak conservators and, at the same time, indicators of undesirable processes in the silage, like missing in ensiling. These acids, especially propionic acid, act as fungicides, in order to prevent molds growth, compared to a

lactic acid, so in some cases they are favourable in the silage. Because of that, propionic acid is used during the storage of animal feed.

Silage is very favourable environment for molds development. Humidity in the silage, depending on the type of plant material, is from 30 to 80%, temperature from 5 to 40°C, while concentration and availability of the nutritive materials is high. Molds may already biosynthetize mycotoxins in the growth phase of plants which during the silage preparation are carried into the silo. Molds are mostly active in the environment with oxygen, so they grow on the surface of silage or in the parts where the air is not pressed out enough. Compared with lactic acid bacteria, molds endure much higher acidity and may be active in the environments which have pH between 2 and 3 or lower. The most common genus/species of molds and their mycotoxins in silage (C h a d d, 2004) are shown in Table 1.

Table 1. Common molds in European silage and their mycotoxins (C h a d d, 2004)

Silage	Genus	Species	Toxin
Grass Silage	*Penicillium*	*roqueforti*	Roquefortin
	Aspergillus	*fumigatus*	Aflatoxin
	Aspergillus	*flavus*	Aflatoxin
	Fusarium	*moniliforme*	Fusaric acid
Maize silage	*Fusarium*	*graminearum*	Zearalenone
	Fusarium	*roseum*	Fumonisins B_1, B_2
	Fusarium	*verticillioides*	Fumonisins B_1, B_2
	Fusarium	*moniliforme*	Ochratoxin A
	Penicillium	*verrucosum*	Ochratoxin A

DISORDERS CAUSED BY MOLDS AND MYCOTOXINS

Harmful effects of molds on animal health may be seen in a form of illness-mycosis, while the influence of their mycotoxins manifests in a form of intoxications — mycotoxicoses. The most frequent mycosis is aspergillosis caused by mold from genius *Aspergillus* (*A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus*), as well as by molds form genuses *Mucor* i *Absidia* which are commonly found in hay.

According to their activity, mycotoxins may be: hepatotoxins, nephrotoxins, neurotoxins, citotoxins, estrogen toxins, immunosupresive toxins and photosensibile toxins (S i n o v e c et al., 2003). In this work more attention was payed to the influence of mycotoxins on cuds, because silage in their feed is the biggest portion of diet.

In beef and sheep mycotoxins cause lower consumption and feed uptake, decreasing of milk yield, weaker growth of socket in fat and rejuvenate, high number of abortuses, not regular and not enough expressed estrus, decreasing the rate of pregnancy, retaining of the endometrium, uterus inflammation, dislocation of abomasus, ketosis, appearance of the oily degeneration of liver, weaker immunity, higher number of somatic cells in milk and accumulation of residua of mycotoxins in milk and meat. The cattle is less sensitive because microorganisms of rumen with their enzymes degradate the mycotoxins and

thus decrease their negative effects (Whitlow et al., 2004) The most common molds are from genus *Penicillium, Aspergillus* and *Fusarium* (Fink-Gremmels, 2004; Auerbach, 2003; Mašić et al., 2003 b).

Penicillium. From the kinds of this genus the most frequent is *P. roquerforti*. It biosynthetizes toxins rokvefortin C, patulin, mycofenol acid, PR toxin and penicillin acid. Ensiling material may be naturally contaminated by some of these mycotoxins (Fink-Gremmels, 2004). Penicillin acid is the first metabolite which appears after ensiling (13 days), followed by patulin (22—27 days), mycofenol acid (36 days) and PR toxin (49 days).

Rokvefortin C and patulin are neurotoxins which in the cattle cause muscle weakness and disarrangement of coordination. Symptoms are not specific in the cattle because they are more resistant to these mycotoxins. Cattle may be more sensitive to mycofenol acid, penicillin acid and PR toxin. They may have negative influence on development of the microorganisms population in the rumen and cause local inflammatory processes. Toxins which are biosynthetized by *P. rubrum* may cause hemorrhagic disfunctions in blood capillaries in the cattle. *P. citrinum* biosynthetizes citrine and the symptoms of this toxin poisoning are laxity renal insufficiency and uremia which in some cases finish by death. The autopsy usually show changes in the mouth, oesophagus and omasum, especially in the form of perirenal edem. Molds from genus *Penicillium* produce the ochratoxin A which does not cause severe consequences in grown cattle, because in the large amount (more the 50%) this toxin is degraded by microorganisms of rumen.

Aspergillus. Molds of this genus are very often found in the silage. The most common is *A. fumigatus* which biosynthetizes micotoxins: verukologen, fumitremorgen and peniterm. These toxins cause tremor and other kinds of neurotoxicology in sheep. Symptoms of intoxication are difficult to notice in the farm conditions, in contrast to intoxications with lolitrems and ergovalins which causes *Neotyphodium* mold. Disposal of animals to the spores of *A. fumigatus* cause bronchitis, mastitis and abortus of cattle. *A. flavus, A. parasiticus* and *A. nomius* biosynthetize extremely toxic mycotoxins (aflatoksin B_1, B_2, G_1, G_2) which cause the symptoms characteristic for aflatoxicoses — weak growing up, leaning and decreasing the milk yield and lead to the harder kind of indigestion disturbed work of browels, neuro disarrangements, damaging the capillaries and dysentery. If the poisoning is heavy, the death comes in 1—3 days. Compared to cattle, sheep are more resistant to aflatoxins. Between 0.3 and 6.2% of dietary aflatoxins B1 and B2 were found in the milk of cows in a form of aflatoxins M1 and M2 exuding within 4—5 days after consumption of contaminated feed. Also, some special types of molds from the genus *Aspergillus*, biosynthetize the ochratoxin A, which, thanks to the activity of microorganisms in rumen, does not cause severe damages.

Fusarium. Molds of this genus biosynthetize mycotoxins zearalenone and trichothecenes from which the most common are: deoxynivalenol or vomitoxin (DON), diacetoxiscirpenol (DAS), T_2-toxin and fumonisins B_1 i B_2. Zearalenone is biosynthetized by species *F. graminearum, F. culmorum, F. oxysporum* and others. This toxin may bind estrogen receptors, cause hyper estrogenism and lower fertility. Trichotecenes mycotoxins are produced by *F. graminea-*

rum, F. culmorum, F. tricinctum, F. sporotrichioides, F. poae, F. semitectum, F. nivale and other species of the same genus. Also, trichotecenes are biosynthetized by other genus of fungi: *Trichothecium*, *Trichoderma, Cephalosporium* etc. These toxins have influence on weak feed consumption, damage the mucous membranes of rumen and rennet, appearence of laxity, hemorrhagic changes on the heart, kidneys, spleen, and urinal bubble, and act as the immune suppressives. Till now, not enough investigated toxins fumonizins B_1 and B_2 are produced by species *F. verticillioides and F. proliferatum;* these toxins may damage the liver and decrease the milk production in cows.

PRESENCE OF MYCOTOXINS IN SILAGES IN OUR COUNTRY

The most common producers of mycotoxins in Serbia are molds of genus *Fusarium*, particularly species *F. graminearum, F. verticillioides, F. subglutinans, F. oxysporum* i *F. proliferatum*. They are isolated on the corn and grain. Besides these species, on lucerne there are isolated other species of molds from the same genus, like *F. avenaceum, F. tricinctum, F. sporotrichioides, F. solani* (K r n j a j a et al., 2004). The most common toxin which they biosynthesize is zearalenone. Besides zearalenone, the most often isolated is T-2 toxin, as well as the other trichotecenes. In the first place it is DAS which follows T-2 toxin and DON which follows zearalenone. M a š i c et al. (2003a), in investigated corn samples in Serbia, fortified the presence of zearalenone in 36,88% of samples (average concentration 750 μg/kg), trichotecenes in 31,82% of samples (240 μg/kg), ochratoxin A in 27,05% of samples (69 μg/kg) and aflatoxin B_1 in 19,67% of samples (36 μg/kg). The quantity of mycotoxines in the silage originated from part of Vojvodina, fortified by B o č a r o v - S t a n č i ć, 2005 are shown in Tables 2 and 3.

An official tolerance level of mycotoxins in feed and mixture are allowed by Regulations on maximal permitted quantities of harmful materials and components in cattle feed (Official Gazette SRJ, br.2/90). According to these regulations, however, no tolerance level of mycotoxins in silage is given.

Table 2. Mycotoxins in silage samples in Vojvodina region, for the period 2000—2004 (B o č a r o v - S t a n č i ć, 2005)

Silage	*Aflatoxin*		*Ochratoxin A*		*Zearalenone*	
	Contamination sample, %	Average, μg/kg	Contamination sample, %	Average, μg/kg	Contamination sample, %	Average, μg/kg
Corn plant	25	3	37,5	75	43,8	1450
Corn dent	0	0	9,1	130	63,6	1640
Lucerne	16,7	3	0	0	100,0	730

Table 3. Mycotoxins (trihotecens) in silage samples in Vojvodina region for the period 2000—2004 (Bočarov-Stančić, 2005)

Silage	*T-2 toksin*		*DAS*	
	Contamination sample, %	Average, μg/kg	Contamination sample, %	Average, μg/kg
Corn plant	25	310	43,8	620
Corn dent	27,3	500	18,2	1380
Lucerne	0	0	16,7	250

POSSIBILITIES OF PROTECTION FROM MOLD AND MYCOTOXINS ACTION

Protection from mold and mycotoxins may be done in three basic phases: 1) on the arable — regular crop rotation, optimal terms of works, plant protection by fungicides and insecticides, using healthy seed, making more resistant genotypes of plants on development the molds, destroying the damagers etc. 2) during the ensiling — conveying adequate technology of silaging, using bacterial-enzyme inoculants, agents against developing of the mold, protection against progressing the air, water, rodents and other kinds of protection 3) during the usage of silage — nutritive intervention in the diet like increasing the content of energy and proteins additive to probiotics, enzymes, vitamins, amino acids and anti oxidants, adsorbents of mycotoxins from organic or mineral origins. Very efficient and rational way to prevent the negative effects of mycotoxins is focused on the application of inorganic mineral adsorbents based on natural zeolites of domestic origin. They can be used in the phase of ensiling, apropos using the silage if there is fortified presence of mycotoxins in it. The new mineral adsorbent of mycotoxins obtained by cation exchange of inorganic cations on the zeolite surface with organic cations (“Min-a-Zel Plus”) is produced in the Institute for the Technology of Nuclear and Other Mineral Raw Materials, Belgrade. The addition of “Min-a-Zel Plus” to the green mass of plant of corn during the ensiling (0.2% from the mass of plant) has influence on increased of production of lactic acid, decreasment of the number of molds and mycotoxins in the silage (zearalenon, T-2 toxin and DAS), as well as the ammonia nitrogen (Table 4).

Table 4. Efficiency of “Min-a-Zel Plus” in silaging of corn (Adamović et al., 2001)

Parametar	Without “Min-a-Zel Plus”	With “Min-a-Zel Plus”
Dry mater, %	22,34	24,28
Lactic acid, %	49,52	75,40
Acetic acidd, %	50,48	24,61
pH	3,36	3,40
NH_3-N	0,03	0,02
Total mold in 1 g	1000	100
Zearalenone, mg/kg	1,02	0,90
T-2 toxin, mg/kg	0,50	0,25
DAS, mg/kg	0,50	0,00

Đorđević et al. (2003) and Đorđević et al. (2004) reported the similar results for application of products based on zeolite during the ensiling of sugar beet pulp. Additional regulations and procedures of protection from mycotoxins in the silage may be: regular organoleptic and laboratory valuation of the silage, bringing law regulations about limited quantities of mycotoxins in the silage, harmonizing the methods and the procedures for determination of mycotoxins, equiping the laboratories, education of laboratory personnel and development of the efficient procedures and instruments for protecting the animals from mycotoxins.

CONCLUSION

Protecting against molds and mycotoxines in the silage needs to start just on arable and to continue in the process of silage, apropos using the silage. Regular control supported by law regulations may have important contribution to the success in the fight against mycotoxins.

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МИКОТОКСИНИ У СИЛАЖИ — УЗРОЦИ СТВАРАЊА, ПОСЛЕДИЦЕ И ЗАШТИТА ОД ДЕЛОВАЊА

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Резиме

У раду се указује на узроке појаве плесни у силажи, родове/врсте плесни које биосинтетишу микотоксине, последице њиховог деловања, мере спречавања развоја и могућности отклањања штетног утицаја. Истакнуто је да услед пропуста у технологији силирања (недовољно гажење, присуство ваздуха) при влажности масе за силирање, постоје повољни услови за развој плесни које биосинтетишу токсине штетне по здравље животиња и људи. Они могу да изазову здравствене поремећаје укључујући и патохистолошке промене на органима и ткивима, депонују се у производима (млеку, месу и јајима) и на тај начин угрозе здравље људи. У раду су изложени резултати присуства микотоксина у силажи (кукуруз и луцерка) у Војводини у периоду 2000—2004. Најчешће присутан микотоксин био је зеараленон у 60,6% анализираних узорака, а потом ДАС у 30,3% узорака. Контаминираност силаже охратоксином А, афлатоксином Б1 и Т-2 токсином била је између 15,2 и 21,2%. Садржај микотоксина ДАС и Т-2 токсина био је на граници или изнад вредности које дозвољава *Правилник* (Службени лист СРЈ, бр. 2/90). Вредности за остале микотоксине биле су испод максимално дозвољених количина. Анализирана су и предложена решења која доприносе сузбијању развоја плесни и микотоксина.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 59—67, 2005

UDC 633.31:631.563]:582.28

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MYCOPOPULATIONS OF ALFALFA SILAGE WITH PARTICULAR REWIEV ON TOXIGENIC *FUSARIUM* spp.

ABSTRACT: Mycological and mycotoxicological investigations of alfalfa samples (initial not fermented plant material, as well as silage obtained from unfaded and faded state of the same) were performed during the year 2003. Total of 14 fungal species, included in 11 genera, were identified during the present study. The most frequent moulds were *Chaetomium piluliferum* and their anamorph *Botryotrichum piluliferum* (83.3% and 66.7% respectively). Potentially toxigenic *Fusarium* spp. (*F. culmorum, F. semitectum* and *F. sporotrichioides*) were observed less frequently (from 16.7% to 33.0%) and only on initial not fermented alfalfa.

From the sample of alfalfa faded state contaminated with 0.25 mg/kg of diacetoxyscirpenol (DAS) and 1.28 mg/kg of zearalenone (ZEA) *F. semitectum* SL-B was isolated. The production of fusariotoxins by this strain was later on tested *in vitro* conditions. Different aeration treatments in semisynthetic medium with glucose (GPK) or sucrose (SPK), as well as cultivation on sterilized corn kernels (moisture content 47%) were used. The highest yield od DAS (64.0 mg/l) was obtained during submerged cultivation of *F. semitectum* SL-B in GPK (210 rpms, 3 days, room temperature). Production of T-2 toxin, but rather poor (0.08 mg/1), was detected only in SPK (150 rpms, 3 days, room temperature). ZEA was found exclusively after 25 days of cultivation on corn kernels at room temperature (21—25°C).

KEY WORDS: alfalfa, *Fusarium*, moulds, silage, toxicity

INTRODUCTION

Silage moulds can occur because of existence of pockets containing residual oxygen, or subsequent introduction of air into ensilaged material. Although this problem can be overcome by good pressurizing of the mass and its covering, superficial parts of silage, then those which are in contact with the walls of silo structures, as well as silage composed of more mature material which is harder to pressurize, represent potential places for contamination with

fungal species (Đ o r đ e v i ć et al., 2004). According to S e l g a r (2004), some silage moulds can also grow in conditions of low oxygen level and moderately low pH value. However, their survival is limited by competition with anaerobic bacteria. The reason of occurrence of fungi in silage is usually an increase in pH value which is due to consumption of lactic acid by yeasts which become active after the introduction of oxygen into a silo. *Candida* and *Hansenula* are microorganisms which consume lactic acid, and when their count exceeds 10^5 cells per gram of stock-cattle feed, increased mould activity occurs.

Two types of mycopopulation can cause deterioration of ensilaged material. The so-called "field fungi" which develop during the vegetation period in crops, infect either the grain or the forage, generally in conditions of high humidity (> 70%) and temperature variations (warm days and cold nights). According to S e l g a r ' s assertion (2004), most frequently they do not develop on stored silage because of low pH and lack of oxygen. However, this does not exclude presence of their toxic metabolites which biosynthesis can be initiated already in field conditions. In this respect, the most interesting ones are *Fusarium* species which are also connected with production of mycotoxins, or human and animal diseases, unlike other field mycopopulations, which cause only plant diseases (*Diplodia, Anthrachnose, Helminthosporium, Ustilago*).

Moulds which develop on stored feed — "storage fungi", usually do not attack crops before harvest. Spores of these moulds which originate in soil are brought into the silo together with stock-cattle feed. Dominant moulds in North America isolated from silage are: *Mucor, Penicillium, Aspergillus*, and *Monilla* (S e l g a r, 2004). Most of about twenty fungal species of this type, identified on ensilaged material in the USA, are thought not to have the ability of mycotoxins' biosynthesis. Even though *A. flavus* has been classified as a storage microorganism, in its case production of aflatoxins can take place already in field conditions.

MATERIALS AND METHODS

Fungal cultures. Isolation of fungal species has been carried out by standard mycological methods (M u n t a ñ o l a - C v e t k o v i ć, 1987). Culture identifications were carried out according to N e l s o n et al. (1983) for *Fusarium* species, whereas other moulds have been determined according to D o m s h et al. (1980) and E l l i s (1971). *Fusarium semitectum* SL-B has been isolated from a sample of unfaded alfalfa which contained 0.25 mg/kg of diacetoxiscirpenol (DAS), and 1.28 mg/kg of zearalenone (ZEA). The cultures have been kept on potato-dextrose agar (PDA) at 6°C. Prior to preparation of medium for testing the ability of biosynthesis of fusariotoxins, the isolates were subcultivated on PDA for the period of seven days at 27°C.

Cultivation types. A. GPK liquid medium (5% glucose + 0.1% yeast extract + 0.1% peptone, pH 5.3) 250/500 ml, rotatory laboratory shaker (210 rpm) during the period of three days at room temperature (21—25°C); **B**. GPK liquid medium 250/500 ml, rotatory laboratory shaker (150 rpm) during the

period of three days at room temperature (21—25°C); **C**. SPK liquid medium (5% saccharose + 0.1% yeast extract + 0.1% peptone, pH 5.3) 250/500 ml, rotatory laboratory shaker during the period of three days at room temperature (21—25°C); **D**. SPK liquid medium, 100/500 ml, stationary cultivation during 25 days at room temperature (21—25°C); **E**. sterile corn kernels (uncontaminated by mycotoxins, water content 47%) stationary cultivation during 25 days at room temperature (21—25°C).

Determination of fusariotoxins. Qualitative and quantitative ZEA determination in liquid media (**A—D**) was carried out by applying modified method of Pepeljnjak and Babić (1991). The modification consisted in adding 20% anhyd. Na_2SO_4 and silica gel to fungal culture filtrate during initial extraction of toxin with acetonitrile. The rest of the analysis has been carried out according to the given procedure. Identification of ZEA in corn kernels (**E**) was performed according to the *Regulations on sampling methods and methods of physical, chemical and microbiological analyses of fodder* (Official Gazette of SFRJ, No. 15/87).

Extraction of A type trihotecenes (T-2 toxin and DAS) was carried out in all cases with ethyl acetate, according to Romer et al. (1978), and TLC determination by Pepeljnjak and Babić (1991) method.

RESULTS AND DISCUSSION

The results of mycological study of alfalfa and silage are shown in Table 1.

During the present study, a total of 14 species were identified, namely, 11 fungal genera, of which the most frequent one was the species *Chaetomium piluliferum* and its anamorph *Bolriotrichum piluliferum* (83.3% and 66.7% respectively). *Bolriolrichum piluliferum*, according to the data provided by Domsh et al. (1980), is widely spread all around the world (soil, plant rhizosphere, animal excrement etc.). Optimal growth temperature for this mould is 25—30°C, and pH 5.5. In addition to these features, it is distinguished by the ability to dissolve starch, pectin, xylan, and carboxymethyl cellulose. Bearing in mind these features, as well as the fact that when *Bolriotrichum piluliferum* grows on straw it dissolves well cellulose and lignin, producing humus substances in the course, the presence of this anamorph and its teleomorph on faded alfalfa and alfalfa silage is only logical.

The highest number of fungal species (6) has been identified on unfaded alfalfa and silage obtained from it (**SL2** and **SL4**) independently of the type of treading. The majority of moulds identified on these three types of samples belonged to typical field mycopopulation, such as the genera *Alternaria, Cladosporium* and *Fusarium*. (Table 1).

In addition to other species of fungi identified during the present study, in Serbia and Montenegro there are listed: on ensilaged corn kernels — *Penicillium aurantiogriseum* (Bočarov-Stančić, 2003), on the silage of the whole corn plant *Aspergillus versicolor* (Djordjević et al., 2004), and on ensilaged soy grain *A. versicolor, F. oxysporum, F. subglutinanas*, and *Rhisopus nigricans* (Bočarov-Stančić, 2003).

Table 1. Identified fungal species on alfalfa and alfalfa silage

No.	Species	A	B	SL2	SL4	SL6	SL8
1.	*Acremoniella atra* (Fr.) Keissl.	—	—	+	+	—	+
2.	*Alternaria alternata* (Corda) Sacc.	+	—	—	—	—	—
3.	*Bolryolrichum piluliferum* Sacc. & March.	—	—	+	+	+	+
4.	*Chaetomium globosum* Kunze ex Steud.	—	—	+	—	—	—
5.	*C. piluliferum* J. Daniels	—	+	+	+	+	+
6.	*Cladosporium herbarum* (Pers.) Link ex Gray	+	—	+	—	—	—
7.	*Epicoccum purpurascens* Ehrenb. ex Sclecht.	+	—	—	—	—	—
8.	*Fusarium culmorum* (W. G. Sm.) Sacc.	—	+	—	—	—	—
9.	*F. semitectum* Berck & Rav.	+	+	—	—	—	—
10.	*F. sporolrichioides* Shreb.	+	—	—	—	—	—
11.	*Geotrichum candidum* Link ex Leman	—	—	—	+	—	—
12.	*Mucor racemosus* Fres.	—	—	+	+	+	+
13.	*Penicillium sp.*	—	—	+	—	—	—
14.	*Trichocladium opacum Ellis.* (Corda) Hughes	—	—	—	+	—	—
	TOTAL	6	3	6	6	3	4

Legenda: **A** — initial unfaded material;
B — initial faded material;
SL2 — silage from **A**, better treading;
SL4 — silage from **A**, weaker treading;
SL6 — silage from **B**, better treading;
SL8 — silage from **B**, weaker treading.

Potentially toxygenic *Fusarium* species (*F. culmorum, F. semitectum, F. sporolrichioides*) were determined only on initial, not ensilaged material, with 16.7 to 33.0%. Although K r n j a j a et al. (2004) did not find these species on the diseased alfalfa plants, except for *F. sporotrichioides*, but primarily *F. oxysporum, F. solani, F. equiseli*, and *F. proliferatum*, it is necessary to specially pay attention to them, considering the fact that these *Fusarium* species are potential producers of a larger number of mycotoxins and that they are connected not only to the occurrence of plant diseases, but also diseases in humans and animals (Table 2). In other words, it is known that mycotoxins: aflatoxin, deoxynivalenol, zearelenone, and fumonysins are usually diagnosed in silage (D j o r d j e v i ć et al., 2004; S e l g a r, 2004).

Table 2. Toxigenic potential of *Fusarium* spp. identified on alfalfa (literature data)

Species	*F. culmorum*	*F. semitectum*	*F. sporotrichioides*
Type A trichotecenes			
1. 4-Acetoxyscirpendiol	—	a	—
2. Diacetoxyscirpenol	a, b	a	a
3. Monoacetoxyscirpenol	—	a	—
4. Neosolaniol	a, b	a	a
5. Scirpentriol	—	a	—
6. HT-2 toxin	—	—	a

7. T-2 tetraol	—	—	a, b
8. T-2 toxin	a	a	a
Type B trichotecenes			
9. 3-Acetyldeoxynivalenol	a	—	—
10. Deoxynivalenol	a, b	a	a
11. Diacetylnivalenol	a	a	a
12. Nivalenol	—	a	a
13. Nivalenol diacetate	—	a	—
14. Nivalenol monoacetat	—	a	a
Zearalcnone and derivatives			
15. Zearalenol	—	a	—
16. Zearalenone	a	a	a
Other fusariotoxins			
17. Butenolide	—	a	a

Legend: **a** — according to Marasas et al. (1984),
b — according to Ožegović and Pepeljnjak (1995).

Taking into account the data presented in Table 2, the culture *F. semitectum* SL-B, isolated from the sample of faded alfalfa which contained 0.25 mg/kg of DAS and 1.28 mg/kg of ZEA, was subjected to toxicological *in vitro* study. *F. semitectum* is a cosmopolite fungus, which is according to Domsh et al. (1980), most frequently isolated from tropical and subtropical regions of the world. This saprogenic soil microorganism can also be found on decaying vegetal material in the countries of temperate zone of Europe and North America. In our country, the species *F. semitectum* is quoted as causing agent of soy and aubergine (eggplant) seed diseases (Jovičević and Milošević, 1990), sunflower seed (Noory, 1983), wheat and barley seed (Bočarov-Stančić et al., 2000), and corn in field conditions (Lević et al., 2004). This mould is, according to Marasas et al., (1984), the only toxic species from the Arthrosporiela section, which is related to the following human and animal diseases: a) **degnala disease** in water buffalo and cattle in India and Pakistan, which is characterized by edematous swelling of legs, necrosis, and appearance of necrosed skin on extremities, and b) **human esophageal cancer** in the Chinese province Henan.

In the course of the present study, significant impact of cultivation condition on the biosynthesis of fusariotoxins through *F. semitectum* SL-B, that is, on the type of toxin and its yield was established (Table 3).

Table 3. The yield of fusariotoxins and basic cultivation parameters of *F. semilectum* SL-B *in vitro* conditions

Med.	Cult. type	pH	Microscopic characteristics	Toxin yield (mg/l od mg/kg)		
				DAS	T-2	ZEA
GPY	A	4.2	Exceptionally fatt, segmented and vacuolized hyphae with outstanding spherical deformations.	64.0	0	0
	B	4.0	Medium fatt, segmented and vacuolized hyphae, with periodical spherical deformations.	31.8	0	0
SPY	C	3.5	Loose mycelium. Fatt, segmented and vacuolized hyphae.	9.6	0.08	0
	D	3.0	Fatt, segmented and vacuolized hyphae with pearl like deformations.	0	0	0
C. K.	E	—	—	4.0	0	6.4

Legend: GPY — 5% glucose + 0,1% yeast extract + 0,1% peptone, pH 5.3;
SPY — 5% saccharose + 0.1% yeast extract + 0.1% peptone, pH 5.3;
C. K. — corn kernels (47% water content).

Stationary cultivation in semisynthetic liquid medium with saccharose (**D**) did not produce a positive result in the case of any of tested mycotoxins, whereas at submersed cultivation in the same medium (**C**), biosynthesis of only trichotecen of the A type (0.08 mg/1 T-2 of toxin, and 9.6 mg/l of DAS) was achieved. The results obtained by the use of nutritive medium with glucose (**A** and **B**) also speak of the positive impact of aeration on DAS production. In both cases (Table 3) there are detected not only considerably higher yields of the same trichotecen than in SPK, but also two times higher when cultivating *F. semitectum* SL-B fungus in conditions of increased aeration (64.0 mg/l with respect to 31.8 mg/l). Although Marasas et al. (1984) most frequently propose PSC medium and Chapek's broth as media for studying toxigenicity of *Fusarium* spp. and the temperature of 25°C, we decided to use the liquid media mentioned above (SPK and GPK) in which we achieved satisfactory results, taking into account our previous studies (Bočarov-Stančić et al., 2004).

According to the data provided by literature (Ožegović and Pepeljnjak, 1995), *Fusarium* species begin to decay at the substrate humidity of 12—13%, whereas at 22—23% they multiply intensively. Lević et al. (2004) quote slightly higher values — minimum water contents which enable development of the representatives of the same genus are 18—19%. At the same time, this represents a limiting value for biosynthesis of mycotoxins in cereals, although better yields were, as a rule, obtained on natural substrates with a higher water content. In accordance with this, in the present study sterile, uncontaminated corn kernels with initial of 47% water content were used. Although literature quotes temperature stress as optimal condition for ZEA biosynthesis (a higher number of weeks at 25°C, which are followed by a couple of weeks at 10°C), Marasas et al. (1984), show that in the case of some *Fusarium* isolates higher yields can be obtained by cultivation at constant temperature. For all these reasons, in the present study of toxigenic potential

of *F. semitectum* SL-B was used for cultivation at room temperature (21—25°C) during four weeks. As Table 3 shows, in the given conditions (**E**), production of ZEA in the quantity of 6.4 mg/kg was verified. Relatively low yield of this mycotoxin obtained can be explained by weak potential for biosynthesis of *F. semitectum* strains in comparison with the main ZEA producers: *F. graminearum*, of which, according to Ožegović and Pepeljnjak (1995), even 93% of the strains are toxigenic, or *F. culmorum* with 63% of isolates as producers of the same fusariotoxin. DAS yields on the same substrate were also rather low (4.0 mg/kg), although according to Marasas et al. (1984), larger quantities of the same mycotoxin (up to 23.49 mg/kg) can be produced on cereals. Even though the same authors quote examples of biosynthesis of T-2 toxin which can develop on rice (30°C, seven days), or on ground white corn kernels (21 days, 15°C), during the present study it was not possible to detect this type A trichotecene in corn kernels used for cultivation (E).

CONCLUSIONS

On ensilaged material, despite the low pH value and lack of oxygen, certain types of field moulds and fungi characteristic of feed storing may develop, some of which are potential producers of mycotoxins.

On alfalfa and various types of its silage, the dominant types were pectinolitic, chemicellulolitic, and cellulolitic fungi — teleomorph *Chaetomium piluliferum* and its anamorph *Bolryotrichum piluliferum* (83.3% and 66.7%, respectively).

Typical field mycopopulation (*Alternaria, Cladosporium*, and *Fusarium*) were found mainly on the initial poorly areated material and the silage obtained from it.

From three potentially toxigenic *Fusarium* species isolated from alfalfa, with culture *F. semitectum* SL-B it was established that the biosynthesis of fusariotoxins *in vitro* conditions was favoured by: 1) DAS — increased aeration (210 rpm) and use of glucose as the C atom source; 2) T-2 toxin — saccharose as the source of carbon, and 3) ZEA — cultivation on the corn kernels and the temperature of 21—25°C.

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ACKNOWLEDGEMENTS

The paper is part of the investigations realized in the scope of Project No. BTR. 5.05.4335 financially supportet by the Ministry for Science, Technology and Development of SR Srbija.

МИКОПОПУЛАЦИЈЕ СИЛАЖЕ ОД ЛУЦЕРКЕ, С ПОСЕБНИМ ОСВРТОМ НА ПРИСУСТВО ТОКСИГЕНИХ *FUSARIUM* ВРСТА

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Резиме

Током 2003. год. извршена су миколошка и микотоксиколошка истраживања узорака луцерке (непровенули, провенули полазни материјал и силажа од истих). Идентификовано је укупно 14 врста, односно 11 родова гљива од којих су са највећом учесталошћу забележени *Chaetomium piluliferum* и његов анаморф *Bolryotrichum piluliferum* (83.3 односно 66.7%). Потенцијално токсигене *Fusarium* spp. (*F. culmorum, F. semilectum* и *F. sporotrichioides*) уочене су знатно ређе (од 16.7 до 33.0%). Из узорка провенуле луцерке контаминиране са зеараленоном (ЗЕА) и диацетоксисцирпенолом (ДАС) изолован је сој *F. semitectum* SL-B чија је способност за биосинтезу фузариотоксина испитана у *in vitro* условима. Највећи принос ДАС-а (64.0 mg/l) је постигнут при субверзној култивацији у течној подлози са глукозом. Слаба производња Т-2 токсина (0.08 mg/kg) је добијена само у течној подлози са сахарозом. ЗЕА је нађен искључиво при култивацији на влажном стерилном зрну кукуруза (6.4 mg/kg).

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 69—76, 2005

UDC 633.83:582.28
633.83:615.9

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MYCOLOGICAL AND MYCO-TOXICOLOGICAL ANALYSIS OF A HERB (OREGANO) USED IN THE MEAT INDUSTRY

ABSTRACT: Raw materials used in meat industry can be contaminated with toxigenic moulds and mycotoxins. Such raw materials present a source of contamination of final products with these compounds. This paper gives a review of the presence of aflatoxin B1, ochratoxin A and zearalenon and moulds in five different samples of oregano.

KEY WORDS: aflatoxins, ELISA, moulds, mycotoxins, ochratoxins, zearalenon

INTRODUCTION

The technology of food production is constantly evolving; new products are developed in accordance with very exact health protection and advancement requirements.

Control of food products for the presence of moulds and their toxic metabolites — mycotoxins — is exceptionally important in the drive to manufacture products which are safe and good for human health.

It is known that over 100 different genera of moulds are capable of producing mycotoxins. Several species can produce the same toxin; it has also been established that some moulds can produce two or more different toxic metabolites.

Contemporary meat-seasoning technology is based on the use of as many spices as possible, some basic and others supplementary. Pepper, paprika, pimento, garlic and onion are regarded as primary herbs (Savić and Bem, 1983). Additional seasonings (clove, cardamom, ginger, mace and rosemary) are used to supplement the taste and aroma of the finished product. Cumin and thyme belong to both categories. Oregano is widespread in Europe, Asia and North America. Oregano contains 0.15—3% of ethereous oils, primarily carvacrol. Its scent varies from finely to powerfully aromatic, and its taste is bur-

ning and bitter. Oregano is a seasoning much favoured in Italy and some other Mediterranean countries.

Microorganisms can contaminate herbs, cereal grains, flour and other products of vegetable origin, leading to undesirable consequences. Irrespective of the level of contamination, the presence of moulds in seasonings can besides changing colours under certain conditions also lead to the secretion of toxic metabolites. Mycotoxic-producing moulds can be classified into three groups by the time of development and contamination on plants and their products: field moulds (*Alternaria, Helminthosporium, Cladosporium, Fusarium*), barn and storage moulds (*Penicillium, Aspergillus*) and advanced decay moulds (*Papulospora, Sordaria, Mucor, Rhizopus*) (Ožegović and Pepeljnjak, 1995).

Mycotoxins are toxic secondary metabolites of moulds. They get into food destined for humans and animals through spores and mycelium fragments; they have a wide range of action dependent on their diversity. It has been established that over 100 different genera of moulds are capable of producing mycotoxins. Many species can produce the same mycotoxin, while others can generate two or more different toxic metabolites.

Mycotoxicoses are abnormal conditions caused by the alimentary intake of mould toxins into the human and animal organism. They are neither infectious nor transmittable by contact. They cause various powerful biological effects. Some are especially attracted to liver, kidney, heart or circulatory system tissues. Mycotoxicoses provoke no immunological response and cannot be treated by traditional medicament-based methods (Mašić et al., 2000). Aflatoxins are products of the secondary metabolism of *Aspergillus flavus* and *A. parasticus* (Krogh, 1987). Almost all genera of *A. parasiticus* are toxicogenic, while the synthesis of aflatoxins in *A. flavus* varies considerablly from one genus to another. The most important are aflatoxin B_1 (AB_1), G (AG_1) and their dihydro derivatives AB_2 and AG_2. Most often found in food and animal fodder is AB_1, which is grouped among highly toxic compounds. Given that it is one of the most powerful mutagens and carcinogens, on the basis of all-round scientific knowledge the IARC has classified it in Group 1 of human carcinogens (Scimeca, 1995). It is followed by AG1, AM1, AB2 (Duraković et al., 1989). Hydroxil derivatives of the toxins AB2 and AG2, AB2a and AG2a, were isolated later.

Ochratoxins are toxic products of storage moulds of the genera *Aspergillus* and *Penicillium*. They were first isolated from the species *A. ochraceus*, after which they were named. Probably the most important producers of this group of mycotoxins in tropical and sub-tropical regions are believed to be representatives of the groups *A. ochraceus* (*A. sclerotiorum, A. sulphureus*, *A. ostianus A. melleus*) (Muntanola-Cvetković, 1987).

However, some *Penicillium* species also synthesise ochratoxins (*P. viridicatum, P. aurantiogriseum, P. chrysogenum, P. commune* etc.) (Wyllie, Morehouse, 1977). Varga et al. (1996) have detected this mycotoxin in *A. albertensis, A. auricomus* and *A. wentii*.

Ochratoxins appear in several forms, the most widespread and also most toxic being ochratoxin A (OA). It has been established that OA causes serious kidney and liver damage in humans and animals, urinary tract cancer and da-

mage to the immunological system. It has been hypothesized that it is responsible for the Balkan endemic nephropathy which affects humans in Yugoslavia, Romania and Bulgaria, as well as human chronic nephropathy in Tunisia (Mašić et al., 2000).

Zearalenon is a toxic product of species in the genus *Fusarium*. It is synthesized primarily by *F. graminearum*, and also *F. culmorum*, *F. roseum*, *F. moniliforme*, *F. solani* and other species of the genus (Bočarov-Stančić, 1996a). Zearalenon belongs to the phytoestrogen group; a total of 15 different derivatives with varying biological activity have been identified so far.

MATERIAL AND METHODS OF WORK

Mycological research

The mycological research encompassed determination of the total number of moulds in 1 g of the tested seasoning (oregano) and their identification. Five different samples were tested.

The determination of the total number of moulds in 1 g of spice was conducted according to the standard laboratory procedure (Škrinjar, 2000), repeated twice.

Two types of selective culture media were used: Sabouraud-maltose agar (SMA) with the addition of antibiotics (1 ml chloramphenicol per 100ml of medium) and maltose yeast extract agar with 50% glucose. The species isolated were identified on the basis of investigation of the macromorphological properties of colonies and micromorphological properties of conidial and other structures by key: (Raper, Fenel, 1965), (Samson and van Reenen-Hoekstra, 1988), (Ainsworth and assoc., 1973), (Ellis, 1971), (Gerlach and Nirenberg, 1982) and (Ramirez, 1982).

Mycotoxicological research

The presence of Aflatoxin B_1, ochratoxin A and zearalenon were determined by the ELISA testing method.

RESULTS AND DISCUSSION

Table 1. Total number of moulds in 1g of oregano

Sample	Total No. of moulds / g	
	SMA	MY 50 G
1	40	$1.6 \cdot 10^2$
2	15	$1.2 \cdot 10^3$
3	10	$4.5 \cdot 10^2$
4	40	$1.0 \cdot 10^2$
5	15	$1.0 \cdot 10^3$

In oregano (Table 1) on an SMA medium, sample no. 3 had the smallest mould contamination, and samples 1 and 4 (40.0) the highest. On a MY50G medium, sample no. 1 ($1.6 \cdot 10^2$) had the smallest contamination level, while sample no. 2 ($1.2 \cdot 10^3$) had the highest. Comparison of the results points to drastic differences between the numbers of moulds grown on the two culture mediums. The highest number of moulds were generated on the MY50G medium.

Mycopopulations in oregano

Table 2 shows the results of mycological testing of oregano. Most of the species found were from the genus *Aspergillus* (4); they made up 57.14% of all the isolated species. The remaining moulds were from the genera *Alternaria, Rhizopus* and *Penicillium*, 14.28% of the total each.

A total of seven species were identified.

Table 2. Types of moulds isolated in the oregano sample

genus	species	percentage of total (%)
Aspergillus	*A. flavus* *A. niger* *A. rubrum* *A. candidus*	57.14
Alternaria	*A. alternata*	14.28
Rhizopus	*R. stolonifer*	14.28
Penicillium	*P. sp.*	14.28

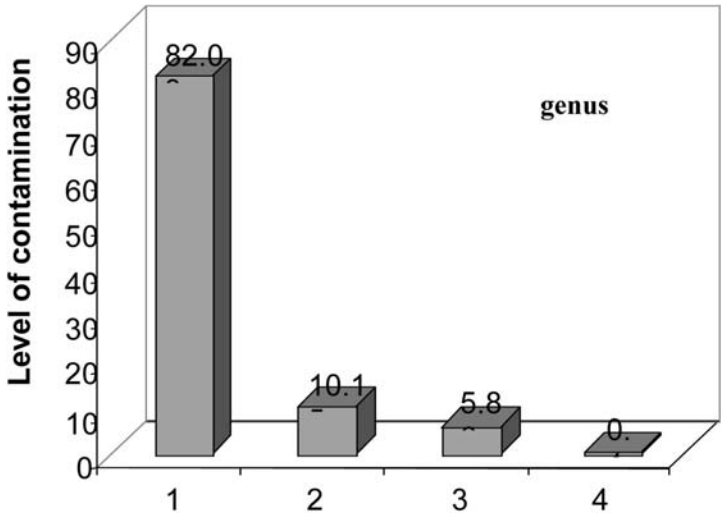

Figure 1. Frequency of genera in the oregano myco-population (1 — *Aspergillus*, 2 — *Rhizopus*, 3 — *Penicillium*, 4 — *Alternaria*)

As shown in Figure 1, storage moulds, most of them toxicogenic, were dominant in the samples. *Aspergillus* accounted for fully 82.0% of the overall oregano contamination myco-population, *Rhizopus* made up 10.15%, *Alternaria* 5.6% and *Penicillium* just 0.4%.

Figure 2. shows that *A. flavus* (19.75%) i *A. rubrum* (50.30%) were predominant in the oregano mycopopulations.

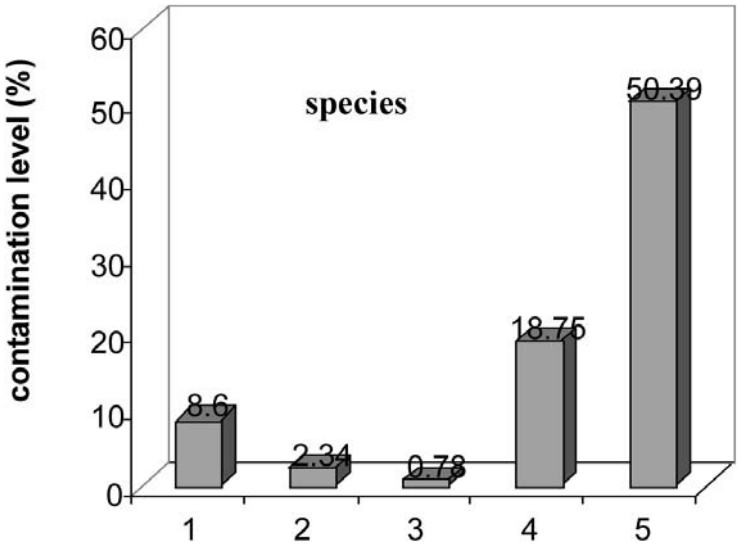

Figure 2. Frequency of *Aspergillus* species in oregano myco-populations
(1 — *A. niger*, 2 — *A. fumigatus*, 3 — *A. candidus*, 4 — *A. flavus*, 5 — *A. rubrum*)

The presence of toxic metabolites in seasonings

The research showed the presence of toxic mould metabolites in the tested seasoning (Table 3)

Table 3. Mycotoxins in five different samples

Sample	Aflatoxin B1 ($\mu g \cdot kg^{-1}$)	Ochratoxin A ($\mu g \cdot kg^{-1}$)	Zearalenon ($\mu g \cdot kg^{-1}$)
Oregano 1	22	75	17.5
2	17.5	93	8.5
3	27.5	85.5	16.5
4	17	79.5	10
5	15	82.5	8

AB1 was found in all spice samples. Ožegović and Pepeljnjak (1995) noted that aflatoxin was not isolated just in animal feed, but also in very diverse human food samples (soy, peanuts, meal, cheese, meat, eggs, coffee, even mother's milk).

Finoli and Ferrari (1994) analysed 40 samples of various spices, 30 of which were found to contain aflatoxins. The biggest concentration of AB1 (234,3 μg/kg) was found in Cayenne pepper.

In the U.S., Cook et al. (1989) detected aflatoxicoses in swine (400 animals found diseased) which had been fed with corn. The quantity of aflatoxin found was from 1200 to 2000 mg/kg.

OA was found in all spice samples. Jurić et al. (2001) tested animal fodder for OA in the 1994—99 period, a total of 269 samples. All tested samples of sunflower seed contained OA, some even as high as 10 ppm. OA has been found in blood plasma and mother's milk. The effect of mother's milk contaminated with OA on children was researched by Skaug (1999) in Norway, where it was found in 3.3% of all milk samples in a quantity of 40 ng/l.

Scientists studied this phenomenon in ochratoxins, which are particularly noxious elements with a special affinity for coffee and cereals. They estimated that the grains lost between 2% and 3% of their contamination during washing, between 3% and 44% during defatting, up to 60% during grinding and between 5% and 10% during boiling Naresh (2004).

The presence of zearelenon was established in all samples. Bočarov-Stančić et al. (1998) conducted myco-toxicological research of maize in the 1990—1995 period. ZEA was identified in just 13—19% of all samples of naturally dried maize (max. concentration 10 000 μg/kg), but in 25—27% of the samples of artificially-dried corn (max. concentration 960 μg/kg).

According to published reports, fusariotoxins represent one of the most important groups of toxic contaminants of cereal grains, their products and animal feeds.

CONCLUSION

The objective of regular mycological and mycotoxicological control of spices before the start of the technological processing of food is to reduce the risks to human health and also economic damage to the food industry.

What else can be done?

The strategy involved identifying the critical points in the food chains and defining the procedures to avoid these situations — from the pre-harvest stage right up to marketing and including storage, and all the other stages along the way. Known as HACCP (Hazard Analysis by Critical Control Points), this approach is now traditional practice at the industrial processing stage of food production, although less common upstream.

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МИКОЛОШКА И МИКОТОКСИКОЛОШКА АНАЛИЗА БИЉКЕ ОРИГАНО КОЈА СЕ ПРИМЕЊУЈЕ У ИНДУСТРИЈИ МЕСА

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Резиме

Сировине које се примењују у месној индустрији могу бити контаминиране токсичким гљивицама и микотоксинима. Такве сировине представљају извор контаминације финалног производа који их садржи. Овај рад пружа преглед присуства афлатоксина $Б_1$, охратоксина А и зеараленона и гљивица у пет различитих узорака оригана.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 77—84, 2005

UDC 633.8:582.28
633.8:615.9

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PRESENCE OF MOULDS AND MYCOTOXINS IN SPICES

ABSTRACT: In this paper there are presented the results of mycologic and mycotoxicologic analysis of seven spices which are being used for production of meat products. Using standard mycologic methods, in all the tested samples, we noticed a presence of moulds. By quality and quantity, most represented are genera: *Aspergillus* and *Penicillium*. With smaller occurrence, there are presented genera: *Rhizopus*, *Mucor*, *Paecylomyces* and *Absydia*. Mycotoxins — ochratoxin, aflatoxins and zearalenon, are detected in samples of ground white pepper, ginger, cloves and ground caraway.

KEY WORDS: *Aspergillus spp*, fungi, mycotoxins, *Penicillium spp*, spices

INTRODUCTION

Foodstuffs of plant origin, to which species belong, are favourable substrate for development of moulds. Besides changes which challenge, like discoloration, some genera of moulds, in some definite condition, can produce toxic extracellular metabolites-mycotoxins.

A great number of moulds have the ability to produce mycotoxins. More species can produce the same mycotoxin, or the same species can produce more different mycotoxins. With food, moulds and mycotoxins are carried into human and animal organisms. Most often, they challenge toxigenic effect for all the cells in the organism, and some have specific affinity for cells liver, kidney, heart, nerve and hematopoetic tissue (Ožegović, Pepeljnjak, 1995). Mycotoxins have cumulative ability, and they are eliminated hard from the organism, which is proved on experimental animals. Also, for mycotoxicoses it is characteristic, that they are not treated with classic medical treatment (Mašić et al., 2000).

Spices are often contaminated with moulds, which origin can be from ground, feces (bird, insect, rodent). Number of moulds in spices grows up because of irregular treatment at drying, and reduces when activity of water is (a_w value) under 0,60. Special problem are xerophil moulds, which can develop on or in a medium with lower free water content (under a_w 0,80), and mostly belong to genus *Aspergillus* and *Penicillium*, which most often contaminate spices (Horie, 1971; Stefanović et al., 1973; Takatori, 1977; Ayres, 1980; Leistner, 1985, Frisvad, 1988). Apart from the mentioned ones, in literature there are quoted the following species of genera: *Cladosporium*, *Fusarium*, *Rhizopus*, *Mucor*, *Eurotium*, *Alternaria* (Misra, 1981; Roy et al., 1988; Dimić and Škrinjar, 1996; Aziz et al., 1998).

In spices, most often detected are the following mycotoxins: ochratoxin A, aflatoxins B_1, G_1 and zearalenon (Misra, 1981; Roy et al., 1988). Mycotoxins fall into the group of heat stable compounds and have long toxigenic effect during process of production of food products. It is advised, when using spices in meat technology for meat products, that they are not thermically treated (fermented drying products), they should be already sterilized (Hadlok, 1969; Leistner, 1985).

The aim of this paper is to isolate and determinate moulds from some spices, which are being used for production of meat products and to determinate mycotoxins.

MATERIALS AND METHODS

Using mycologic and mycotoxicologic analysis we analysed seven spices mostly used in meat processing (black and white pepper granule and ground, garlic ground, onion ground and cutting, ginger, ground cloves, caraway granule and ground, and tree samples of ground red paprika) (Table 2).

Total count of moulds in 1 g of spices is determined with standard laboratory procedure (Škrinjar, 2000), with two repetitions. For the isolation of moulds, there are used two selected media: Sabouraud-malt agar (SMA), with addition of antibiotic and malt yeast extract agar with 50% glucose (MY50G). Petri plate are incubated for seven days at 25°C. Growth of moulds was controlled every day, and results were obtained after seven days. By visual control it was determined percentage of presence of colony. Isolation was done on standard mycologic medium (Malt agar, Czapek agar) with the aim of determination of isolate.

Thin layer chromatography (TLC) was applied for quantitative and qualitative determination of aflatoxins B_1+G_1 (AFT B_1 + G_1), ohratoxins A (OTA) and zearalenon ZEA, Balzer et al. (1978).

Spices are stored properly in dry and dark place, at 16—18°C, in adequate package. Contents of moisture, ash, essential oils, capsantin and piperin in spices, are determined in accreditation laboratory at import (Table 2).

RESULTS AND DISSCUSION

In all the tested a presence of moulds is detected. Spices of genera *Aspergillus* are isolated from all samples with different in presence — by quality and quantity. Spices from genera *Penicillium* are isolated from white pepper-granule and ground, ground garlic, ground onion, onion-cutting, ground cloves, caraway-granule and ground and from one samples ground red paprika. Species of moulds from this two genera are most represented. In much less number of samples, a presence spices from genera *Rhizopus* is detected — isolated from black pepper-granule, caraway-granule, onion-cutting and from two samples of ground red paprika. We sporadically isolated species of genera: *Mucor* — (ground caraway and two samples of ground red paprika), *Paecylomyces* — (ground black and white pepper) and *Apsydia* — (ground caraway) (Table 1).

Table 1. Review of genera of moulds, isolated from spicesa

N°	Name of spice	Genera of moulds
1.	Black pepper — granule	*Aspergillus* spp., *Rhizopus* spp.
2.	Black pepper — ground	*Aspergillus* spp., *Paecylomyces* spp.
3.	White pepper — granule	*Aspergillus* spp., *Penicillium* spp.
4.	White pepper — ground	*Aspergillus* spp., *Penicillium* spp., *Paecylomyces* spp.
5.	Garlic — ground	*Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp.
6.	Onion — ground	*Aspergillus* spp., *Penicillium* spp.
7.	Onion — cutting	*Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp.
8.	Ginger	*Aspergillus* spp.
9.	Cloves — ground	*Aspergillus* spp., *Penicillium* spp.
10.	Caraway — granule	*Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp.
11.	Caraway — ground	*Aspergillus* spp., *Penicillium* spp., *Mucor* spp., *Absydia* spp.
12.	Red paprika I — ground	*Aspergillus* spp., *Mucor* spp., *Rhizopus* spp., *Penicillium* spp.
13.	Red paprika II — ground	*Aspergillus* spp., *Rhizopus* spp., *Mucor* spp.
14.	Red paprika III — ground	*Aspergillus* spp., *Rhizopus* spp.

Our results are in accordance with the results of investigation of many authors. So, in spices and medicinal plant samples, there are isolated species of moulds, which belong in most cases, to genera *Aspergillus* and *Penicillium*, while in substantialy less number, to genera: *Fusarium*, *Absydia*, *Cladosporium*, *Rhizopus* and *Scopulariopsis* (Aziz et al., 1998).

Also, Takatori (1977) and Ayres (1980) found a high degree of contamination in spices (cardamon, cinnamon, fennel, coriander, cumin, black cumin and white pepper), by species from genera *Aspergillus* and *Penicillium*. Misra (1981) and Roy et al. (1988) isolated from coriander, cumin, black pepper and cinnamon, most number of species of genus *Aspergillus*, and one species from genera: *Chaetomium*, *Fusarium*, *Penicillium*, *Alternaria*, *Curvula-*

ria and *Rhizopus*. From ground red paprika by mycologic method most species from genera *Aspergillus* and *Mucor* are isolated, but the most frequent were genera *Rhizopus* and *Eurotium* (Dimić and Škrinjar, 1996).

There is obvious identity of our results with the research results of many authors, who indicate to dominate existence of moulds in spices from genera: *Aspergillus* and *Penicillium.*

Quantity analysis of the tested spices are made on the base of number of colony in 1 g of spices (Figure 1). The most represented on both media was in sample number 6 (onion-ground), than in sample number 4 (ground white pepper) and number 7 (onion-cutting). All the other samples were significant by less contaminated, and differences between them were small.

Providing that a presence and growth of moulds are in positive correlation with quality of spices, were made certain chemical investigation. From the results showed in Table 2, it is clearly seen that percent ages of moisture in tasted spices were from 2,37% to 13,68%, and ash content was from 1,08% to 6,98%, which is in accordance with to the "Book of Regulations (Official paper SFRJ, number 4/85 and 84/87)". The contents of ether oils, capsantin and piperin, are in accordance with the literature results (Savić and Danon, 1982) and "Book of Regulations (Official paper SFRJ, number 4/85 and 84/87)".

Table 2: List of analysed species of spices and representation of moisture, ash, essential oils, capsantin and piperin

N°	Name of spice — botanic name	Moisture %	Ash %	Essential oil %	Capsantin mg %	Piperin mg %
1.	Black pepper — granule (*Piper nigrum* L.)	13,09	4,14	2,20	—	5,10
2.	Black pepper — ground (*Piper nigrum* L.)	10,94	4,72	1,10	—	—
3.	White pepper — granule (*Piper nigrum* L.)	8,76	1,75	2,00	—	—
4.	White pepper — ground (*Piper nigrum* L.)	13,68	1,08	1,70	—	—
5.	Garlic — ground (*Allium sativum* L.)	5,09	6,98	—	—	—
6.	Onion — ground (*Allium cepa* L.)	2,37	6,30	—	—	—
7.	Onion — cutting (*Allium cepa* L.)	9,81	5,85	—	—	—
8.	Ginger (*Zingiber officinale* Rosc)	10,18	6,47	2,50	—	—
9.	Cloves-ground (*Eugenia caryophyllata*)	9,93	4,98	10,00	—	—
10.	Caraway — granule (*Carum carvi* L.)	10,15	6,80	2,70	—	—
11.	Caraway — ground (*Carum carvi* L.)	10,78	6,29	1,60	—	—
12.	Red paprika I (*Capsicum annum* L.)	10,02	6,48	—	2,16	—
13.	Red paprika II (*Capsicum annum* L.)	10,93	6,01	—	2,47	—
14.	Red paprika III (*Capsicum annum* L.)	9,81	6,53	—	2,32	—

The fact, that the smallest number of colonies was isolated from ground cloves could be explained with the finding that eugenol extracted from cloves

completely inhibited growth of *A. flavus* and *A. versicolor* (H i t o k o t o et al., 1980). On the basis of previous fact, we may suppose that essential oils from other spices could also influence the number and composition of moulds (Figure 1).

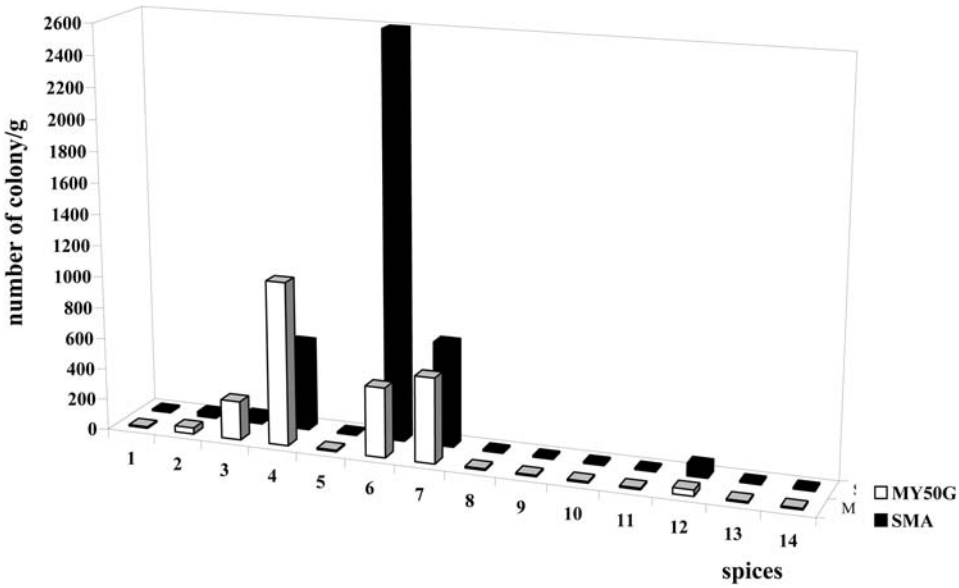

Figure 1. Presence of moulds of media MY50G i SMA

Mycotoxins are detected in four samples, of total fourteen analyses samples of spices. In ground white pepper and ground caraway OTA is detected, in ginger ZEA and in ground cloves $AFTB_1+G_1$. The detected count OTA and ZEA is not in accordance with the "Book of Regulations (Official paper SFRJ, number 5/92 and 11/92)", while the count of AFT B_1 + G_1 is in accordance with the "Book of Regulations" (Table 3).

Table 3. Results of mycotoxicologic analysis of spices

Spice	Ohratoksin A (OTA) *(max. 10 μg/kg)	Aflatoksin B_1 + G_1 (AFT B_1 + G_1) *(max. 30 μg/kg)	Zearalenon (ZEA) *(max. 1 mg/kg)
1. White pepper — ground	30 μg/kg		
2. Ginger			2 mg/kg
3. Cloves — ground		15 μg/kg)	
4. Caraway — ground	28 μg/kg		

* maximal allowed count in accordance with the "Book of Regulations, (Official paper SFRJ, number 5/92 and 11/92)"

It is known that, potential producers of aflatoxins are species of genera *Aspergillus* (*A. flavus* and *A. parasiticus*) (K r o g h, 1987), while production

of toxins depends on the interaction of genotip and conditions of medium in which a fungus grows up (Moreau and Moss, 1979). In our investigation aflatoxin is isolated from ground cloves, where colonis in small number os detected.

A. niger, which is a potential producer. According Pantović and Adamović (1974) of moulds of genera *Mucor* and *Penicillium*, can also synthesize AFT. Earlier investigation approved the presence of this toxin in samples of food: soya, peanut, flour, cheese, meat, eggs, etc. (Ožegović and Pepeljnjak, 1995).

In synthetic medium *A. flavus* produced high concentrations of aflatoxins (mostly alfatoxin B1, less B1 and B2, B1 and G2 and B1 and G1), while in natural medium (medicinal plants and spices) it produced low concentrations of alfatoxins (Aziz et al., 1998).

It is known that species of genera *Aspergillus* and *Penicillium* (in the first place *A. ochraceus* and *P. viridictum*) are OTA — producers. In our investigation we detected a presence OTA as a species of genera *Aspergillus* and *Penicillium*, at ground white pepper and ground caraway.

From ginger small number of colonis of genera *Aspergillus* is isolated, while it is registered a presence of zearalenon which producers are species of genera *Fusarium*. On the basis of presence of toxins, we can conclude that species of genera were presented in ginger in some phase of growth or during production.

CONCLUSION

At all the tested samples of spices, presence of moulds is detected. Species of genera *Aspergillus and Aspergillus* are mostly presented, and a greater number of colony from 1 g of spice is isolated from ground white pepper, ground onion and onion-cutting.

Of fourteen tested samples of spices, mycotoxins are detected in four samples. Concentration of OTA at ground white pepper and ground caraway, as like ZEA at ginger, was higher than limited, while concentration of $AFTB_1$ + G_1 at ground cloves, was in recommended limits.

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ПРИСУСТВО ПЛЕСНИ И МИКОТОКСИНА У ЗАЧИНИМА

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Резиме

У овом раду су приказани резултати миколошке и микотоксиколошке анализе седам зачина који се најчешће користе у преради меса (црни и бели бибер у зрну и млевени, бели лук у праху, црни лук млевени и резанци, ингвер, млевени каранфилић, ким у зрну и млевени, као и три узорка млевене слатке паприке).

Укупан број плесни у 1 g зачина, одређен је по стандардном лабораторијском поступку (Шкрињар, 2000) у два понављања. За изолацију плесни коришћене су две селективне подлоге: Сабоурауд-малтозни агар (SMA) уз додатак антибиотика и сладни квашчев екстракт агар са 50% глукозе (MY50G).

Квалитативно и квантитативно одређивање афлатоксина B_1 + G_1 (AFT B_1 + G_1), охратоксина А (OTA) и зеараленона ZEA), извршено је применом танкослојне хроматографије (TLC методологије) Balzer et al. (1978).

У свим испитиваним узорцима зачина утврђено је присуство плесни. Врсте рода *Aspergillus* су изоловане из свих узорака са разликама у квалитативној и квантитативној заступљености. Врсте из рода *Penicillium* су изоловане из белог бибера у зрну и млевеног, белог лука у праху, млевеног црног лука, црног лука у резанцима, млевеног каранфилића, кима у зрну и млевеног и из једног узорка млевене слатке паприке. Врсте плесни из ова два рода су најзаступљеније. У много мањем броју узорака утврђено је присуство врста из рода *Rhizopus* — изоловане су из црног бибера у зрну, црног лука у резанцима, кима у зрну и из два узорка слатке паприке. Спорадично су изоловане врсте родова: *Mucor* — (млевени ким и два узорка млевене слатке паприке), *Paecylomyces* — (млевени црни и бели бибер) и *Apsydia* — (млевени ким).

С обзиром да су присуство и развој плесни у позитивној корелацији са квалитетом зачина, обављена су одређена хемијска испитивања: садржај влаге, пепела, етарских уља, капсантина и пиперина. Проценат влаге код испитиваних зачина износио од 2,37% до 13,68%, а садржај пепела кретао се од 1,08% до 6,98%, што је у складу са важећим *Правилником* (Сл. лист СФРЈ, бр. 4/85 и 84/87). Садржај етарских уља, капсантина и пиперина је у складу са литературним подацима (Савић и Данон, 1982) и *Правилником* (Сл. лист СФРЈ, бр. 4/85 и 84/87).

Чињеница да је најмањи број колонија изолован из млевеног каранфилића можда може да се објасни налазом да еугенол екстрахован из каранфилића потпуно инхибира раст *A. flavus* и *A. versicolor* (Хитокото и сар., 1980). На основу претходне констатације може се претпоставити да су етарска уља и код осталих зачина могла утицати на бројност и састав плесни.

Од укупно четрнаест анализираних узорака зачина, микотоксини су утврђени код четири узорка. Садржај OTA код млевеног белог бибера и млевеног кима, као и ZEA код ингвера, био је изнад прописаног, док је садржај AFTB_1 + G_1 код млевеног каранфилића био у границама дефинисаним важећим *Правилником*, који регулише ову област.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 85—93, 2005

UDC 632.4:636.085

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OCCURENCE OF OCHRATOXIN A IN FEED AND RESIDUE IN PORCINE LIVER AND KIDNEY

APSTRACT: The effect of ochratoxin A in feed for pigs, and the incidence of its residue in liver and kidney were investigated. The samples were taken from farms and corresponding slaughterhouse in different areas of Serbia. The criteria for OTA residue examination in the mentioned tissues were macroscopic alterations in kidneys, that is a marked kidney ishemia. 14 feed samples, 12 kidney samples and 12 liver samples in total were examined. The average OTA concentration in feed was 25,24 μg/kg (0,0—85 μg/kg). The presence of the OTA residue was found in all of examined tissues samples. The average OTA concentration in kidneys was 2,37 μg/kg (1,0—8,2 μg/kg), in liver was 2,66 μg/kg (1,2—5,5 μg/kg). The experiment showed that the average OTA concentration in feed of farm A in contrast to farm B was significantly low ($p < 0,05$), in liver was significantly lower ($p < 0,01$), while in kidneys was not significantly low ($p < 0,05$). The correlation between these three findings was postulated and discussed.

KEY WORDS: feed, ochratoxin A, pigs, residue

INTRODUCTION

Information about fungi associated with food and feeds is important in assessing risk of mycotoxin contamination. Therefore, the presence of certain fungi implicates a potential risk for animal health (5). Feeding mouldly feeds contaminated by mycotoxins is able to cause adverse effects on animal health and productivity and large economical losses. The carry over of mycotoxins or their metabolites into foodstuffs of animal origin (9) are also essential information elements. The role of carry-over of a certain mycotoxins has especially to be seen in the light of possible consequences for humans health (13) consuming contaminated foodstuffs of animal origin. Meat of this animals has poor production features (20). Ochratoxin A (OTA) is a mycotoxin produced by se-

veral fungi of the genera *Aspergillus* and *Penicillium*, (12) principaly *P. verrucosum* in temperate climates, and *A. ochraceus* in varm region, (24) and other moulds of ochraceus group (12, 26). Importance of OTA is usualy evaluated by nutritive-health impact, but real and final opinion of OTA importance is possible to acquire only on the basis of data that residue of OTA is appears in tissues of animals, feeded feeds contaminated by mycotoxin. The number of data on OTA in meat and meat products was reported (11, 12, 19, 28, 35). Fact that is in our area pigmeat most present in human consumption make a possibility to human ochratoxicosis. Ochratoxin A is a nephrotoxic mycotoxin with carcinogenic, (24) teratogenic, immunotoxic, (6, 18, 33) genotoxic effects in many species, and possibly neurotoxic properties (4, 22). Ochratoxin A has been implicated in the ethiology of Balkan endemic nephropathy, (17) and urinary tract tumors in humans (24, 29). Therefore, from a toxicological (7, 23, 31) and economical point of view, OTA appears to takes place in many scientific investigation.

The aim of this study was to determine the presence of OTA residue in liver and kidneys of fattening swine, slaughtered regularly, depending on feed levels of OTA in samples of complete feedmixes intended for fattening swine. On the slaughterhouse line, during meat inspections, the samples of kidneys where macroscopic alteration noticeably expressed that is a kidney ishemia were taken. The samples of liver from the same carcasses were taken. The correlation between the three findings was postulated and discussed.

MATERIALS AND METHODS

The investigations were carried out in Central (Niški) and North region of Serbia, on the farms and corresponding slaghterhouses. With the aim to determine the content of OTA in samples of complete feedmixes intended for fattening swine, samples were taken every 30 days. The investigations included kidney and liver samples from normally slaughtered pigs. The criteria for OTA residue examination in the stated tissues were characteristic colour and size renal changes, such as “mottled or pale enlarged kidneys”. The samples of liver were taken from the same carcasses. Thin layer chromatography was applied for sample analysis according to the method described by Balzer et al. (1978), and for tissues samples we used the same method modified by Pepeljnjak et al. (1982).

RESULTS AND DISSCUSION

The obtained results are summarized in Table 1, 2, 3, 4, 5, 6 and in a Picture 1.

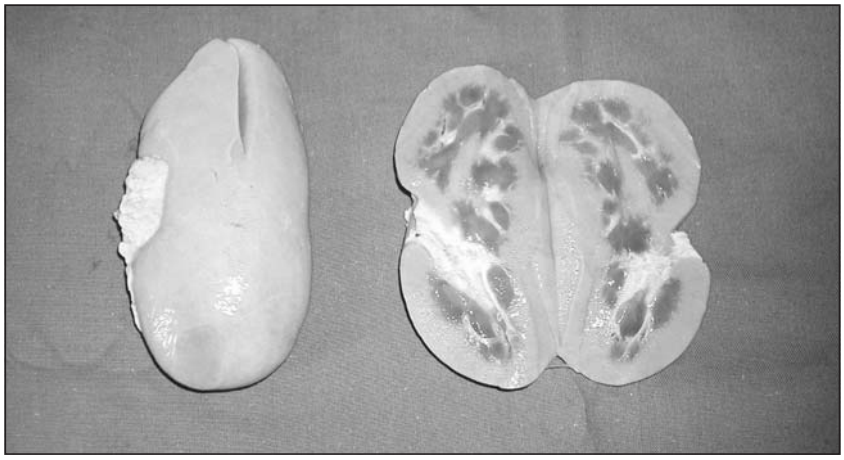

Figure 1. Macroscopic changes of the type "mottled or pale enlarged kidneys" during meat inspection

Table 1. The contents of OTA in samples of complete feedmixes for fattening swine [μg/kg]

Farm	N	Statistical parameters					
		$\bar{x}$	±	S_x	σ	Cv	Iv
A	7	31,40[a]	±	10,28	27,20	86,62	0,0—85,0
B	7	19,00[b]	±	3,48	9,22	48,52	8,0—36,0
Total	14	25,21	±	7,76	20,54	81,47	0,0—85,0

[a:b] $p < 0,05$

The OTA results in samples of complete feedmixes intended for fattening swine collected from farm A and farm B, are shown in Table 1. The obtained results indicate that the average OTA concentration (31,4 μg/kg) in samples of complete feedmixes intended for fattening swine from farm A, varied between 0,0—85,0 μg/kg, and the higest concentration (85,0 μg/kg) was found in December, while in feed samples from farm B, the average OTA concentration (19,0 μg/kg), varied between 8,0—36,0 μg/kg, and the higest concentration (36,0 μg/kg) was found in March. On the basis of the results in mycotoxicological surveys, it can be concluded that contamination of animal feeds by muolds caused OTA productions, under storage condition.

The obtained results are in accordance with tose of domestic and foreign authors (3, 8, 21, 34). Also, the obtained results support the opinion that in our areas OTA and moulds are producers of OTA prevalent that aflatoxin (14, 36). The experiment showed that the average OTA concentration in samples of complete feedmixes intended for swine of farm A in contrast to farm B was significantly low ($p < 0,05$). However, the presence of OTA was accordance with the values stated in the Book of regulations on the maximum tolerable levels of harmul substances and compounds in animal feed, it can be concluded that the pressence of OTA in the obtained values is able to cause disturbance

of health and production results in swine breeding production. In conclusion, the natural occurrence (frequency and concentration) in relation to toxicological relevance of OTA, play an important role, especially in subclinical toxicosis.

Table 2. The occurrence of characteristic macroscopic changes of the type "mottled or pale enlarged kidneys" during meat inspection. (%)

Farm	I	II	III	IV	V	VI	VII	Total
A	70	78	85	80	65	61	66	72,14
B	40	50	40	47	55	80	60	53,14
Total	55	64	62,5	63,5	60	70,5	63	62,64

The occurrence of characteristic macroscopic changes of the type "mottled or pale enlarged kidneys" during meat inspection are shown in Table 2. A kidney in pigs with characteristic macroscopic changes of the type "mottled or pale enlarged kidneys" has been frequently identified at meat inspection of slaughtered pigs.

The analysis of this trial shows that the frequency of observed kidney with characteristic macroscopic changes of type "mottled or pale enlarged kidneys" varied significantly in the examined farms. From 61—85% in farm A, to 40—60% of slaughtered pigs from farm B, had kidney lesions of the type "enlarged mottled or pale kidneys" established during meat inspection. The investigations of kidney samples of pigs from farms A and B, showed that the frequency of observed kidney were very similar compared to those from other countries (19, 32, 37).

Table 3. Residue of ochratoxin A in tissues of slaugtered pigs [μg/kg]

Farm	N	Statistical parameters					
		$\bar{x}$	±	S_x	σ	Cv	Iv
Liver							
A	6	3,62[a]	±	0.50	1,24	34,25	2,2—5,5
B	6	1,70[b]	±	0.20	0,49	28.82	1,2—2,5
Total	12	2,66	±	0.55	1.35	50.76	1.2—5.5
Kidneys							
A	6	3,15	±	1.07	2,62	83,17	1,0—8,2
B	6	1,58	±	0.29	0,72	45,56	1,0—3,0
Total	12	2,37	±	0.82	2,01	84,81	1,0—8,2

[a:b] $p < 0,01$

The presence of OTA residue in kidneys and liver of slaughtered pigs from farm A and farm B, are shown in Table 3. The toxicological investigations of kidneys and liver samples of pigs from various farms, showed that the residue of OTA in examined tissues were presented. The obtained results showed that the contamination levels of OTA in the kidneys and liver samples are very similar compared to those from other authors (2, 11, 35, 37).

The results of these analysis made it possible for us to compare the average concentration of OTA in liver and kidney samples between farm A and B. The average concentration of OTA in liver samples from farm A was 3,62 μg/kg in comparison to 1,70 μg/kg in liver samples from farm B. The difference was statistically significantly lower ($p < 0,01$). The average concentration of OTA in kidney samples from farm A was 3,15 μg/kg in comparison to 1,58 μg/kg in kidney samples from farm B, and this difference was not significantly low ($p < 0,05$). The average OTA concentration in kidneys in contrast to liver was not significantly low ($p < 0,05$). These findings confirm that after resorption, higher amount of OTA occur in liver then in kidney and muscle, (10) because of its first-passing through the liver. The effects of OTA appeared to be longer-lasting than those of other mycotoxins, and possessed cumulative feature.

Table 4. Correlation between feed level and tissue levels (residues) of ochratoxin A in pigs [R]

Farm A	Liver	Kidney	Feedmixes
Feedmixes	0,011	0,933	—
Kidney	0,07	—	
Liver	—		

Table 5. Correlation between feed level and tissue levels (residues) of ochratoxin A in pigs [R]

Farm B	Liver	Kidney	Feedmixes
Feedmixes	0,608	0,727	—
Kidney	0,864	—	
Liver	—		

Table 6. Correlation between feed level and tissue levels (residues) of ochratoxin A in pigs [R]

Total	Liver	Kidney	Feedmixes
Feedmixes	0,345	0,913	—
Kidney	0,313	—	
Liver	—		

The carry-over of OTA from feed to tissues was elucidated in this study. The correlation between feed level and tissue levels (residue) of OTA in pigs is shown in Table 4, 5 and 6. It has been established that there is a statistical association between the correlation of OTA in feed samples and tissues samples. The results obtained indicate that between feed level and tissue levels of OTA there exist positive correlation, and regression line caracterizes polynomial functional dependance. There was a high correlation between the feed level of OTA and the residue levels in the kidneys (R 0,933), while a low correlation was established between the feed levels of OTA and the residue level in the liver (R 0,011), as well as between the residue level in the liver and the residue level in the kidneys from farm A (R 0,07).

The surveys conducted in farm B showed that there was a middle correlation between the feed level of OTA and the residue level in the liver (R 0,608), as well as between the feed level of OTA and the residue level in the kidneys (R 0,727). There was a high correlation between the feed level of OTA and the residue level in kidney (R 0,864).

The results from these surveys indicated that there was a low correlation between the feed level of OTA and the residue level in liver (R 0,345), as well as between the residue level in kidneys and the residue level of OTA in liver (R 0,313). There was a high correlation between the feed level of OTA and the residue levels in the kidneys (R 0,913). These results are in accordance with those of other studies (15, 16).

CONCLUSION

On the basis of the results in toxicological surveys, it can be concluded that:

1. Ochratoxin A was detected in over 70% of examined samples of complete feedmixes intended for swine. The presence of OTA was according with the values stated in the Book of regulations of the maximum tolerable levels of harmful substances and compounds in animal feed. The experiment showed that the average concentration of OTA in samples of complete feedmixes intended for swine of farm A in contrast to farm B was significantly low ($p < 0,05$).

2. The analysis of this trial shows that the frequency of the observed kidney with characteristic macroscopic changes of type "mottled or pale enlarged kidneys" varied significantly in the examined farms. From 61—85% in farm A, to 40—60% of slaughtered pigs from farm B had kidney lesions of the type "enlarged mottled or pale kidneys" established during meat inspection.

3. The toxicological investigations of kidney and liver samples of pigs from various farms, showed that the residue of OTA in the mentioned tissues were presented. The average concentration of OTA in liver samples from farm A was 3,62 μg/kg in comparison to 1,70 μg/kg in liver samples from farm B. The difference was statistically significantly lower ($p < 0,01$). The average concentration of OTA in kidney samples from farm A was 3,15 μg/kg in comparison to 1,58 μg/kg in kidney samples from farm B, and this difference was not significantly low ($p < 0,05$). The average OTA concentration in kidneys in contrast to liver was not significantly low ($p < 0,05$).

4. The results obtained indicate that the between feed level and tissue levels of OTA there exist a positive correlation, and regression line caracterizes polynomial functional dependance. The results from these surveys indicated that there was a low correlation between the feed level of OTA and the residue level in liver (R 0,345), as well as between the residue level in kidneys and the residue level of OTA in liver (R 0,313). There was a high correlation between the feed level of OTA and the residue levels in the kidneys (R 0,913).

5. The contamination of animal feed with moulds causing many deleterious effect. The main importance fungal contamination of feeds is because of its potential for mycotoxin production. Feeding mouldly feeds contaminated by mycotoxins is able to cause adverse effects on animal health and productivity and large economical losses. Besides protecting pigs from deleterious OTA effects, the main goal in swine breeding production is to provide foodstuffs of high value for human consumption.

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ЗАСТУПЉЕНОСТ ОХРАТОКСИНА А У ХРАНИ И РЕЗИДУА У ЈЕТРИ И БУБРЕЗИМА СВИЊА

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Резиме

У овом раду испитиван је утицај охратоксина А у храни за исхрану свиња на појаву резидуа у јетри и бубрезима. За испитивање степена контаминације смеша за завршни тов свиња охратоксином А узорци су узимани на сваких 30 дана. Поред смеша, за испитивање садржаја резидуа у унутрашњим органима клинички здравих свиња на линији клања истог дана су узимани унутрашњи органи свиња пореклом са праћених фарми. Током прегледа закланих свиња на линији клања посебна пажња била је посвећена морфолошким променама бубрега типа бледих (исхемичних), „као куваних" бубрега. Промењени бубрези су узимани, а затим и припадајућа јетра. Присуство ОТА је утврђено у преко 70% испитиваних узорака смеша и то у дозвољеним границама, а разлике у посматраном обележју између појединих група биле су статистички значајне ($p < 0,05$). Макроскопске промене боје, облика и величине бубрега, уочене на линији клања код клинички здравих свиња, заступљене су у 40—85% случајева у зависности од периода. У свим узорцима морфолошки промењених бубрега, као и у узорцима јетара животиња са уоченим морфолошким променама на бубрезима, утврђено је присуство резидуа ОТА. Утврђена разлика у садржају ОТА у јетрама са одговарајућих фарми показала се статистички врло значајном ($p < 0,01$) док се садржај ОТА у бубрезима са одговарајућих фарми и поред изражених нумеричких разлика није статистички значајно разликовао ($p > 0,05$). Садржај ОТА у бубрезима и јетри са одговарајућих фарми није се статистички значајно разликовао ($p > 0,05$). Испитивањем међусобне зависности садржаја ОТА у смешама и органима редовно закланих, клинички здравих свиња, збирно за цео оглед, утврђен је низак степен позитивне корелације између садржаја ОТА у храни и садржаја у јетри, као и између садржаја ОТА у јетри и бубрезима (R 0,345 и R 0,313), док је између садржаја ОТА у храни и садржаја у бубрезима утврђен врло висок степен корелације (R 0,913).

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 95—102, 2005

UDC 633.11:632.4

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EFFICIENCY OF CRUDE CORN EXTRACT CLEAN-UP ON DIFFERENT COLUMNS IN FUMONISINS DETERMINATION

ABSTRACT: The efficiencies of different clean-up procedures for crude corn extract from corn samples naturally contaminated by fumonisins B_1 and B_2 were compared. These procedures precede liquid chromatography determination with fluorescence detection. The efficiencies of immunoaffinity columns (IMA), strong anion exchange columns (SAX), as well as columns with reversed-phase C_{18} (RP C_{18}) were investigated. No significant differences in the obtained results were found, regardless of the crude extract clean-up procedure. However, the use of IMA columns for clean-up provided better chromatographic resolution, with the clean-up procedure being the simplest and the fastest. Also, because of the possibility of IMA column regeneration, it is possible to prepare ten samples on one column, so all in all, the lower price of SAX and RP C_{18} columns is of no great significance.

KEY WORDS: Clean-up extract (IMA, SAX, RP C_{18} columns), fumonisins, liquid chromatography, maize

INTRODUCTION

Fumonisins are a group of compounds isolated for the first time from a fungus culture *Fusarium moniliforme* in 1988 (Gelderblom et al., 1988). They are produced by *Fusarium moniliforme* Sheldon (also known as *Fusarium verticillioides* (Sacc.) Nirenberg) and *Fusarium proliferatum*, which are widely spread in nature, and most frequently contaminate corn and corn products (WHO, 2000).

All quantitative techniques used for fumonisins determination, except enzyme-linked immunosorbent assay (ELISA) and in some cases liquid chromatography (LC) with mass detector, require clean-up of crude extract of the analyzed sample (Abramović et al., 2002; Jakšić, 2004). Cartridges with different sorbents are used for that purpose: columns filled with C_{18} re-

verse phase (RP C_{18}), columns with strong anion exchange (SAX), as well as immunoaffinity (IMA) columns.

Comparison of efficiencies of the above said three types of columns for crude extract clean-up from different samples was the subject of several papers. For example, Müller and Gustavsson (2000) determined fumonisins B_1 (FB_1) and B_2 (FB_2) in different maize products by two different methods based on clean-up steps using an immunoaffinity column and a combination of SAX and C_{18} columns, respectively. Recovery, repeatability, and results from the survey showed comparable results among these methods. Furthermore, De Girolamo et al. (2001) compared the efficiency of clean-up of extracts from different maize products on SAX and IMA columns. They found that the use of SAX clean-up column for maize flour, muffins and infant formula gave better fumonisins recoveries than IMA clean-up, while this was not the case with corn flakes and extruded maize. However, a peak interfering with FB_1 appears in the chromatograms of some extracts when a SAX column is used for clean-up, which, according to the authors makes the use of IMA columns obligatory.

Continuing our previously begun research (Abramović et al., 2005), the aim of this work was to compare the efficiencies of all three types of columns in clean-up of spiked corn extract, as well as to investigate the possibility of multiple use of SAX and RP C_{18} columns, *i.e.* their regeneration. Finally, the investigations were performed using crude extract of corn samples naturally contaminated with fumonisins B_1 and B_2 in respect to recovery of fumonisins and chromatography resolution.

MATERIAL AND METHODS

All solvents used for fumonisins extraction from corn samples, as well as those for the mobile phase preparation were of LC grade. All chemicals used in the investigation were of reagent grade. Solutions were prepared in deionized water except when stated otherwise.

Fumonisins calibrant solutions. Fumonisins B_1 (Sigma, from *Fusarium moniliforme*, approx. 98% TLC) and B_2 (Sigma, from *Fusarium moniliforme*) were purchased as analytical standards. Calibrant solutions were prepared in acetonitrile-water (50:50, v/v) at concentration of 100 μg/ml for FB_1 and FB_2. Fumonisin calibrant solutions are stable up to 6 months when stored at 4°C (Visconti et al., 2001). Stock solution containing FB_1 at 10 ng/μl and FB_2 at 5 ng/μl was prepared by measuring 500 μl calibrant solution FB_1 and 250 μl calibrant solution FB_2 into 5 ml volumetric flasks and diluting to volume with acetonitrile-water (50:50, v/v). Working calibrant solutions were prepared by appropriate dilution of stock solutions with acetonitrile-water (50:50, v/v). Standard solutions were stored at 4°C.

Preparation of phosphate-buffered saline (PBS). 8.0 g NaCl, 1.2 g anhydrous Na_2HPO_4, 0.2 g KH_2PO_4, and 0.2 g KCl were dissolved in about 990 ml water, pH was adjusted to 7.0 and the solution was diluted to 1 l.

Preparation of o-phthaldialdehyde-2-mercaptoethanol reagent. 40 mg o-phthaldialdehyde (OPA, Sigma, min. 99%) were dissolved in 1 ml methanol, diluted with 5 ml 0.1 mol/l $Na_2B_4O_7$, and 50 ml 2-mercaptoethanol (MCE, Serva) were added. This reagent is stable up to 8 days in a capped, aluminum foil-covered vial in the dark.

LC mobile phase. Methanol-0.1 mol/l NaH_2PO_4 (Merck, extra pure) (78: 22, v/v), with pH adjustment to 3.3 with *o*-phosphoric acid. The mobile phase was filtered through a 0.45 mm membrane (ISO-DISC™ Filters PTFE 25-4, Supelco).

Sample preparation. Corn samples were collected during autumn of 2002 from four localities in Vojvodina. Immediately after sampling, 1000 g of each sample were prepared by grinding in a laboratory mill in such a way that 93% passes through a sieve with pore diameter of 0.8 mm. After that, the sample was homogenized by mixing. Samples prepared in such a way were packed in plastic bags and stored in a freezer at –20°C until analysis. Prior to each analysis, the samples were allowed to reach room temperature.

Principle of determination

Fumonisins were extracted from corn with acetonitrile-methanol-water. After filtration (and dilution), the crude extract was cleaned-up on one of the columns. The final sample extract was derivatized with o-phthaldialdehyde and 2-mercaptoethanol and analyzed by reversed-phase liquid chromatography with fluorescence detector.

Extraction. 20.0 g samples were extracted twice with 50 ml of acetonitrile-methanol-water (25:25:50, v/v/v) in a laboratory blender for two minutes. The combined extracts were centrifuged at 3000 x g for 10 min, with subsequent supernatant filtration through filter paper (Macherey-Nagel, Type 751, Düren, Germany).

Clean-up of raw extract

Immunoaffinity column clean-up. 10 ml of the filtered extract was diluted with 40 ml of PBS. The diluted extract was then filtered through a 1.0-μm microfiber filter (Vicam, Watertown, MA, USA). 10 ml of the filtrate were then applied to an IMA column (FumoniTest™, Vicam, Watertown, MA, USA). After rinsing of the column with 10 ml of PBS, fumonisins were eluted using 3 ml of methanol (MeOH), at a rate of 1 drop per 10—15 seconds. The eluate was evaporated just to dryness at 60°C. Purified residue was redissolved in 200 μl acetonitrile-water (50:50, v/v).

Regeneration of the IMA columns. One IMA column was used five times in a row. Each time, after the elution of toxins, the diluted extract was applied to the same column. After that, the column was washed with 10 ml of PBS solution, leaving a part of the solution on the column. The column was regenerated at 4°C for 24 h.

SAX column clean-up. 2.5 ml of the filtered extract were applied to a SAX column (LC-SAX SPE Supelclean™, Supelco, Bellefonte, USA, capacity 3 ml, with a 500 mg filling) which was previously conditioned with 3 ml MeOH and 3 ml of mixture MeOH-water (3:1, v/v). The column was rinsed with 3 ml of mixture MeOH-water (3:1, v/v) and 3 ml MeOH. After that, fumonisins were eluted from the column with 3 ml of mixture MeOH-acetic acid (99:1, v/v). The rate of elution was 1 drop per 10—15 seconds. The eluate was evaporated just to dryness at 80°C, 1—2 ml of MeOH were added and once again evaporated to dryness. Purified residue was redissolved in 200 μl acetonitrile-water (50:50, v/v).

Regeneration of the SAX columns. One SAX column was used five times in a row. Each time, after the elution of toxins, the raw extract was applied to the same column. After that, the column was regenerated by washing with 5 ml of 0.1 mol/l water solution of HCl and 8 ml water, filled with MeOH and left at room temperature for 24 h.

RP C_{18} column clean-up. 5 ml of the filtered extract were diluted with 12 ml of 1% KCl solution and filtered through 1.0-μm microfiber filter paper. After that, 8.5 ml of the diluted extract were applied to a RP C_{18} column (LC-18 SPE Tubes Supelclean™, Supelco, Bellefonte, USA, ~10% C, endcapped, capacity 6 ml, with a 1 g filling), previously conditioned with 5 ml MeOH and 5 ml 1% KCl solution. The column was eluted with 10 ml of acetonitrile-1% KCl (2:8, v/v) mixture. Fumonisins were subsequently eluted with 12 ml of mixture acetonitrile-water (7:3, v/v), the rate of elution being 1 drop per 10—15 seconds. The eluate was evaporated just to dryness at 60°C. Purified residue was redissolved in 200 μl acetonitrile-water (50:50, v/v).

Derivatization and liquid chromatography

A 50 μl aliquot of the extract was mixed with 50 μl of the OPA-MCE reagent at room temperature with the reaction time of one minute with stirring. 20 μl of derivatized solution were injected into the LC system. The equipment consisted of an LC system — BioRad 2800 with Supelcosil™ LC-18-DB column (250 x 4.6 mm id, particle size 5 mm) with a fluorescence detector Hewlett Packard 1046A, response time 4 s, flash frequency 220 Hz. LC pump delivered 1 ml/min constant flow rate. Wavelength of excitation radiation was 220 nm and emission 440 nm.

RESULTS AND DISSCUSION

In our previous work, optimal conditions were established for separation and determination of fumonisins by the LC method, as well as the clean-up efficiency of IMA columns for spiked corn sample crude extract (Abramović et al., 2005). The possibility of multiple use and regeneration of IMA columns was investigated as well. It was found that the efficiency of successive clean-up on one IMA column for FB_1 is 89.0±7.1%, and for FB_2 88.5±1.3%,

i.e. after column regeneration 88.5±11.6% for FB_1 and 92.5±7.5% for FB_2 (the average of 5 measurements in both cases).

The study of efficiency of crude spiked corn sample extract clean-up on SAX and RP C_{18} columns, as well as the possibility of their multiple use and regeneration was continued in this work. It was found that SAX columns can be used five times in a row and at least once more after regeneration. The achieved efficiency of SAX columns is higher and has a value of 109.4±6.9% for FB_1 and 92.1±6.8% for FB_2 (the average of 5 measurements), and after regeneration 109.7% and 95.9%. However, the clean-up procedure is significantly more complicated and long-lasting in comparison to IMA columns.

When the possibility of multiple use and regeneration of RP C_{18} columns was investigated, it was found that they can be used only once, as well as that they can't be regenerated. Also, the efficiency of FB_1 and FB_2 determination in the spiked corn sample after crude extract clean-up is the lowest on RP C_{18} columns, its value being 82.4±9.2% for FB_1 and 74.5±5.2% for FB_2.

At the end, all three clean-up procedures for crude extract of corn samples naturally contaminated with FB_1 and FB_2 used for LC determination were compared in this work. Chromatograms for corn sample with the highest fumonisins content after crude extract clean-up by use of all three types of columns are presented in Fig. 1, and the results of fumonisins determination are given in Table 1. As can be seen, chromatographic separation of FB_1 peak from the matrix peaks is poorer in the case of eluate from SAX and RP C_{18} columns.

Tab. 1 — The influence of crude extract clean-up procedure on the results of fumonisins determination in corn

Locality	Crop	Clean-up procedure	FB1 content (mg/g)	FB2 content (mg/g)
		IMA	< 0.02	< 0.06
Kikinda	2002	SAX	< 0.02	< 0.05
		RP C18	0.06	< 0.05
		IMA	0.13	< 0.06
Bačka Topola	2001	SAX	0.12	< 0.05
		RP C18	0.07	< 0.05
		IMA	0.33	0.12
Subotica	2002	SAX	0.34	0.15
		RP C18	0.30	0.09
		IMA	0.79	0.28
Bač	2002	SAX	0.61	0.18
		RP C18	0.70	0.18

In chromatograms presented in Fig. 1 a peak appearing immediately before FB_2 can be noted. From the results of authors who also determined FB_3 (Sydenham et al., 1992; Fazekas et al., 2000; VICAM, 1997), because of the similarity in the appearance of chromatograms, its presence in the sample analyzed in this work can be presumed. Unfortunately, due to the lack of the standard we weren't able to confirm this presumption.

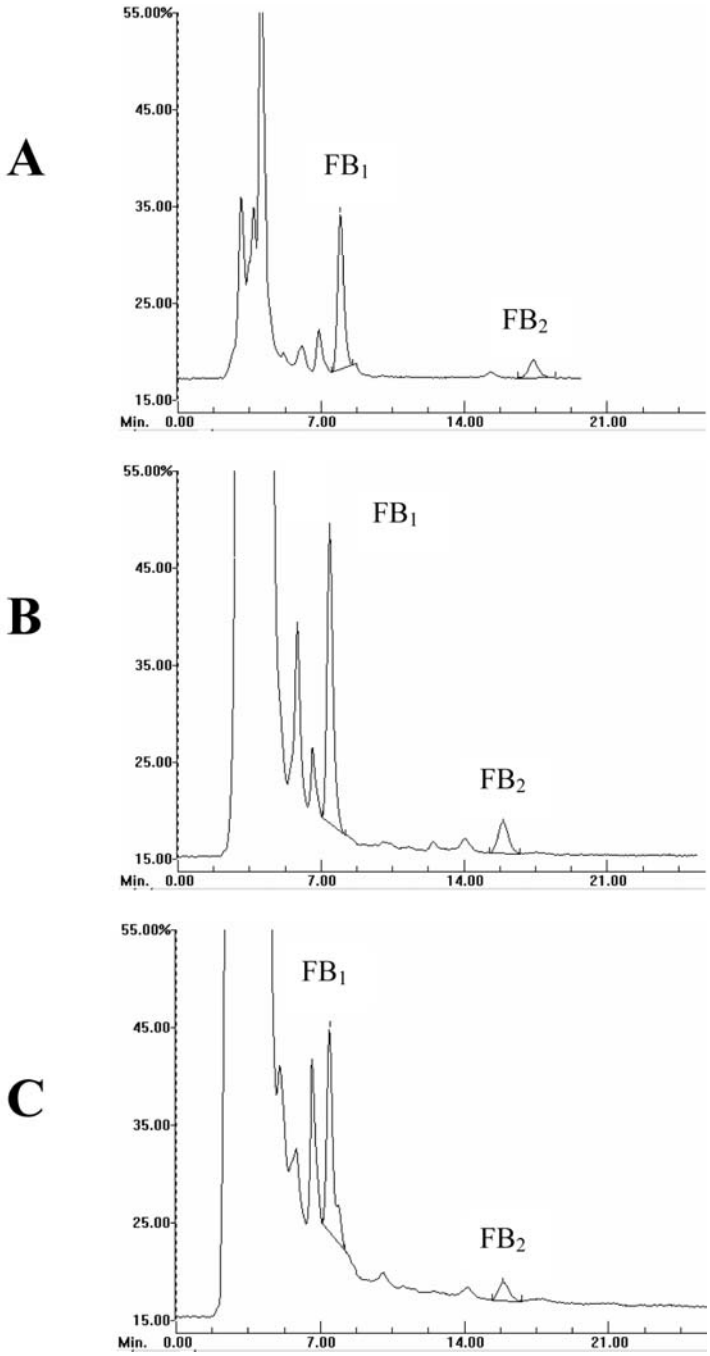

Fig. 1. Chromatograms of naturally contaminated corn sample (Bač 2002) obtained by application of different extract clean-up procedures: (A) IMA column; (B) SAX column and (C) RP C_{18} column

As can be seen in Table 1, there is no significant difference in the obtained results for fumonisins content, regardless of the crude extract clean-up procedure.

From the above results, it can be concluded that the use of IMA columns for clean-up of crude extract of naturally contaminated corn samples and LC with fluorescence detection provide the best results. Aside from the fact that the purest extract was obtained by clean-up of crude corn extract on IMA columns, the clean-up procedure is the simplest and the fastest. Also, because of the possibility of regeneration of IMA columns (Abramović et al., 2005), it is possible to prepare more samples using one column. Therefore, having all said in mind, lower price of SAX and RP C_{18} columns is not of great significance.

ACKNOWLEDGEMENTS

The work was financed by the Ministry of Science and Environment Protection of the Republic of Serbia (Project: "Development of New and Improvement of the Existing Analytical Methods and Techniques for Monitoring Quality of the Environment", No 1622).

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ЕФИКАСНОСТ ПРЕЧИШЋАВАЊА СИРОВОГ ЕКСТРАКТА КУКУРУЗА ПРИМЕНОМ РАЗЛИЧИТИХ КОЛОНА ПРИ ОДРЕЂИВАЊУ ФУМОНИЗИНА

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Резиме

Фумонизини су група једињења сврстана у фузаријумске микотоксине. Чести су контаминенти кукуруза и производа од кукуруза. Поред тога што изазивају токсичне ефекте код различитих врста животиња, сматра се да изазивају канцер једњака код људи. У литератури су описане различите технике за одређивање фумонизина, као и различите методе одређивања применом исте технике. С обзиром на чињеницу да су фумонизини присутни у веома малим концентрацијама а у веома комплексним медијумима, један од најзначајнијих корака у анализи је припрема, односно пречишћавање сировог екстракта узорка.

Примењујући претходно утврђене оптималне услове за одређивање фумонизина $Б_1$ и $Б_2$ течном хроматографијом са флуоресцентним детектором, у овом раду је упоређена ефикасност различитих начина пречишћавања сировог екстракта из узорка кукуруза које претходи њиховом одређивању. Испитана је ефикасност одређивања фумонизина у природно контаминираним узорцима кукуруза након пречишћавања сировог екстракта на имуноафинитетним (IMA) колонама, јаким анјонским измењивачким (SAX) колонама, као и на колонама са C_{18} реверзном фазом (RP C_{18}).

На основу постигнутих резултата испитивања може се констатовати да нема сигнификантне разлике у добијеним резултатима садржаја фумонизина независно од начина пречишћавања сировог екстракта. Међутим, примена IMA колона за пречишћавање даје најчистији екстракт, а сам поступак пречишћавања је најједноставнији и најбржи. Такође, због могућности регенерације IMA колона, могуће је припремити десет узорака на једној колони, те узимајући све наведено у обзир, нижа цена SAX и RP C_{18} колона нема већег значаја.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 103—108, 2005

UDC 636.5:632.4

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PATHOHISTOLOGICAL CHANGES IN KIDNEY AND LDH ACTIVITY IN BROILER TREATED WITH DIFFERENT DOSES OF OCHRATOXIN A

ABSTRACT: The three-week long trial was performed on day-old Hybro broilers divided into four groups. After 14 days long preexperimental period, the experimental groups were offered feed contaminated with 0.5, 1.0 and 1.5 ppm OA during 7, respectively. At the end of the trial blood and kidney samples were taken for investigations.

In broilers feed with 1.5 ppm of OA histopathological examination of the kidney tissue revealed changes located in proximal tubules. Some cells were dim and swollen. These changes produced particular or total reduction in tubular lumen of kidney. Acute tubular necrosis existed in some of tubulocites in form of small foci. Fragmentation of necrotic mass and presence of fresh red blood cells were also detected.

The LDH activity was significantly greater in broilers of experimental groups compared with control group.

All presented data indicated that intensity of pathohistological alterations and LDH activity depends upon dietary OTA level. Positive correlation between pathohistological changes and increased LDH activity caused by OTA was noticed. Thus, LDH activity measure could be used as early diagnostic tool in measuring changes caused by OTA.

KEY WORDS: ochratoxin, broiler, kidney, LDH

INTRODUCTION

Ochratoxins are highly toxic compounds commonly produced as secondary metabolites by two species of fungi: *Penicillium verrucosum* Dierckx and *Aspergillus ochraceus* Wilhelm *(alutaceus)* (Frisyud and Samson, 1991). In recent years, ochratoxin A (OA) has received considerable attention because it can not only seriously affect animal performance and health, but it may also have deleterious effects on humans. Of greatest concern in humans (Marquardt and Frohlich, 1992) is its implicated role in an irreversible and fatal kidney disease (Balkan endemic nephropathy).

Microscopic lesions in ochratoxicosis are most prominent in the kidney. On light microscopy, severe distension, enlargement and hypertrophy of the renal proximal convoluted tubules and thickening of the glomerular basement membrane are seen in kidney sections of broilers receiving 2—4 ppm dietary OA for 20 days. (Dwivedi and Burns, 1984). The same pathohistological changes were reported by Mraz and Kosutzky (1992), after feeding broilers with 0.85 ppm OA during 42 days. Pathohistological examination revealed epithelial dystrophy of proximal tubules, presence of eosinophilic granulocites in tubular lumen, glomerular dystrophy and cell infiltrate of intertubular space.

The broilers' kidneys have high activity of LDH comparing to the other animals (Cubena, 1974). Increased serum LDH was reported only in broilers with kidney disorders. Kubena and Harvey (1994) described a significantly increased LDH activity in broilers treated with 2 ppm OTA for 21 days. Ayed (1991) also described an increased LDH activity in broilers treated with low doses of OTA (0.5 ppm/7 days).

The present study was, therefore, designed to assess the effect of short--term treatment with graded levels of dietary OA on the pathohistological changes in kidney tissue of broilers, as well as correlation between pathohistological changes and increased LDH activity caused by OTA.

MATERIAL AND METHODS

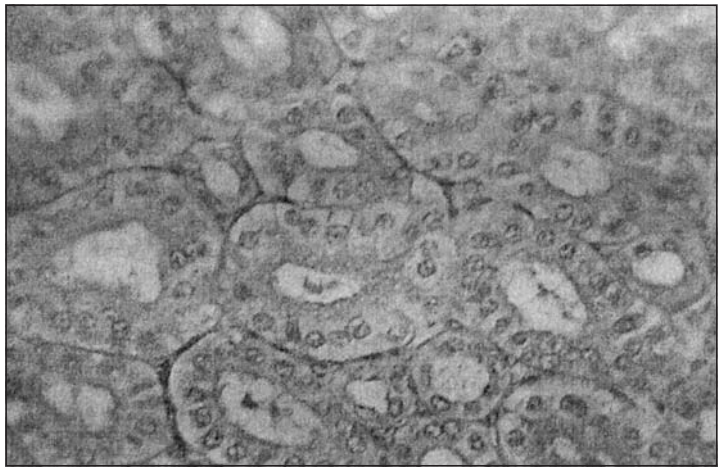
Experimental design. After 14 days long preexperimental period, a total of 48 broilers were submitted to the trial. Birds were divided into three experimental groups (A, B, C), and one control group (K). Experimental groups were fed with contaminated feed.

Diet. All groups of broilers were fed with commercial mash, which consisted of standard feedstuffs and contained enough nutrients to meet all requirements. In the mixture for A, B and C experimental groups the 99% pure ochratoxin A (Sigma, O — 1877), obtained from *Aspergillus ochraceus* culture (303-47-9), was added in an amount enough to provide 0.5, 1.0 and 1.5 mg OTA/kg of feed, respectively.

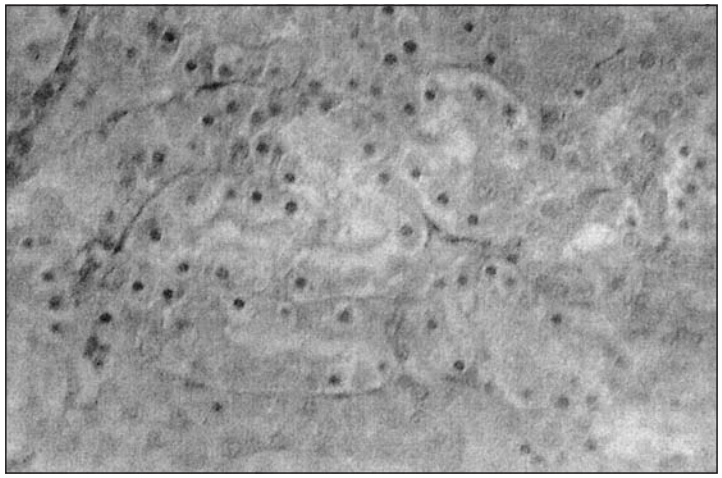
Sample collection. Kidney and blood samples were taken after the period of toxin administration (21st day). In the shortest possible period the samples of kidney were taken for histological investigation. Kidney samples were fixed in 10% neutral formalin and absolute alcohol and were formed in paraffin. Thickness of the cut was 5—8 μm and they were stained (Scheur and Chalk, 1986) using standard methods (HE). Also, using an automated, clinical-chemistry analyzer (SMAC Technicum 3000) the determination of the serum activities of LDH was done.

RESULTS

Histopathological changes in kidney were not detected in broilers of control group and broilers of experimental groups fed with 0.5 and 1.0 ppm of OA.

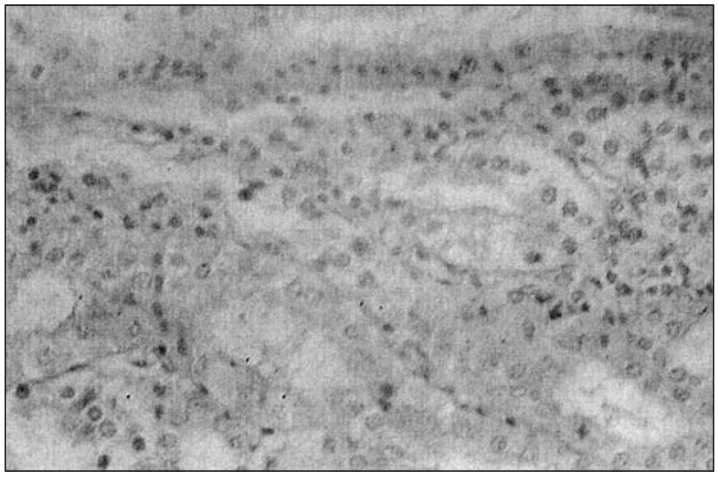


In broilers fed with high doses of OA (1.5 ppm) during 7 days period histopathological examination of the kidney tissue revealed changes located in proximal tubules. Some cells were dim and swollen. These changes produced particular or total reduction in tubular lumen of kidney.

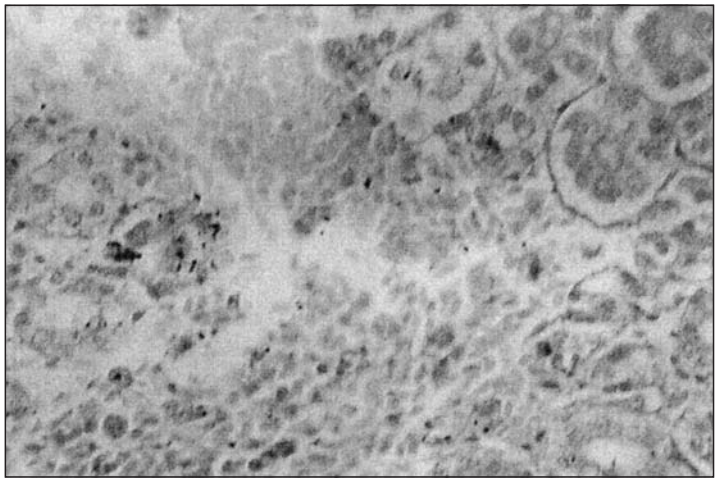


Basement membrane of epithelial cells was intact. Cytoplasm of tubulocites was filled with fine granules and nucleus was masked. Also, reduction in volume and hyperchromatosis of nucleus in epithelial cells of proximal tubules was detected. In some of epithelial cells in changed tubules light vacuolization was expressed and transparent cytoplasm was detected.

Acute tubular necrosis existed in some of tubulocites in form of small foci. Fragmentation of necrotic mass and presence of fresh red blood cells were also detected. In two of six sacrificed broilers intensive extravasation near necrotic center was found. The structure of glomeruli was better preserved than tubules; thus their normal formation is more easily observed.



The LDH activity was significantly greater in broilers of experimental groups compared with the control group.



DISCUSSION

In our trial pathohistological changes were detected in tissue samples of animals receiving 1.5 mg OA/kg feed during 7 days. According to our findings, Kubena et al. (1989) detected in broilers fed 2 mg OA/kg feed enlargement of kidney epithelial cells with dark nuclei, which indicate early degenerative changes in proximal tubules of intoxicated animals. Similar changes, although with almost double dose of toxin, were described by Harvey et al. (1987), who fed broilers with 3.5 mg OA/kg feed during 28 days. Pathohistological changes included dilatation and necrosis of tubules.

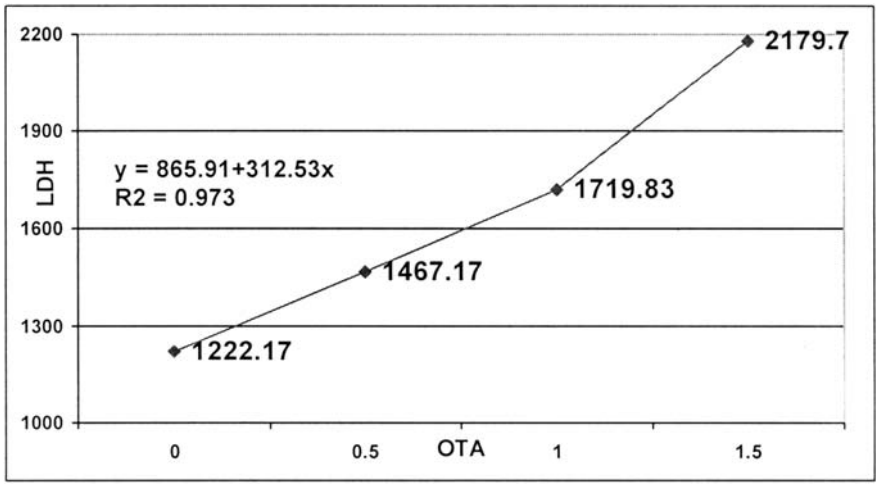


Figure 1. The enzyme LDH concentrations in broilers 21-days of experiments

Stojković et al. (1984) presented persistence of a protein fraction with small molecular weight in blood (20.000 Da) which binds OA more specifically than plasma albumins. The authors concluded that the binding of OA to this protein might be relevant to its predominant nephrotoxic effect, because such molecules can easily pass through the normal glomerular membrane, enabling the accumulation of OA in the kidney.

All described pathological changes induced after OA treatment had a primary localization in proximal kidney tubules and could be connected with toxin metabolism. Increasing LDH activity could indicate alteration of tubulocites related with OTA effect.

All the presented data indicated that intensity of pathohistological alterations and LDH activity depends upon dietary OTA level. A positive correlation between pathohistological changes and increased LDH activity caused by OTA was noticed. Thus, LDH activity measure could be used as an early diagnostic tool in measuring changes caused by OTA.

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ПАТОХИСТОЛОШКЕ ПРОМЕНЕ У БУБРЕЗИМА БРОЈЛЕРА И АКТИВНОСТ ЛАКТАТ ДЕХИДРОГЕНАЗЕ ТРЕТИРАНИХ РАЗЛИЧИТИМ КОЛИЧИНАМА ОХРАТОКСИНА А

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Резиме

Оглед је изведен на Hybro-бројлерима подељеним у четири групе и трајао је 21 дан. Након четрнаестодневног припремног периода огледне групе су храњене храном контаминираном охратоксином А у количини од 0.5; 1.0 и 1.5 ppm током 7 дана. На крају огледа узети су узорци крви и ткива за испитивања.

Код животиња које су 7 дана добијале количину од 1.5 ppm ОА патохистолошким прегледом бубрега уочава се да су променама углавном захваћени проксимални бубрежни тубули. Поједине епителне ћелије су мутне и набубреле, што је довело до делимичног или потпуног сужења лумена бубрежних тубула. Код мањег броја бубрежних тубула запажа се акутна тубуларна некроза у виду ситних огњишта. Честа је појава фрагментације некротичне масе, као и налаз свежих еритроцита.

Активност ензима LDH на крају огледа била је сигнификантно виша код бројлера свих огледних група у односу на контролну групу.

Све наведене чињенице указују да интензитет патохистолошких промена у бубрегу зависи од концентрације охратоксина А у храни, при чему активност ензима LDH може да послужи као користан параметар за процењивање степена алтерација бубрега.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 109—119, 2005

UDC 633.15:632.4

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EXPERT SYSTEM FOR DETERMINATION OF FUMONISINS IN CORN SAMPLES

ABSTRACT: An expert system (ES) to solve the problem of choosing an optimal procedure for the determination of fumonisins in corn samples was developed, having in mind that these toxins most frequently contaminate this particular cereal. In constructing the ES use was made of the deterministic approach starting from the assumption that the experts in the field have a profound knowledge about the problem in question. The ES knowledge base contains the solutions that have been published in the pertinent literature, as well as some solutions and recommendations, which we have developed and introduced. On the basis of this information, as well as on the basis of the demanded method detection limit, available equipment, chemicals, as well as the time the experimenter has at his disposal for the determination, the ES proposes a procedure for solving the given analytical problem, starting from sampling, preparation of all the necessary solutions, the appropriate apparatus, probe preparation, the mode of determining results, calculation of the results, and provides a survey of all the relevant literature references. The base of ES is a shell, which can work under a variety of Microsoft Windows operating systems. In the development of ES and its adaptation for users who are not familiar with computer techniques, different tools, which operate with Microsoft Windows operating systems, as well as the tools within Microsoft Office are used.

KEY WORDS: Corn analysis, expert system, fumonisins, mycotoxins

INTRODUCTION

Expert systems (ES) are a branch of applied artificial intelligence (AI) which began to develop by the AI community in the mid-1960s (L i a o, 2005). The basic idea behind ES is simply that expertise, which is the vast body of task-specific knowledge, is transferred from a human to a computer. This knowledge is then stored in the computer as a software package and users call

upon the computer for specific advice as needed. In the field of analytical chemistry a larger number of ESs have already been developed (Abramović et al., 1996; Namiesnik, 2000; Bonastre et al., 2001; Peris, 2002).

Although, the replacement of the human expert by advanced computer-aided systems in chemical analysis laboratories in the food industry has been perhaps slower than in other fields of analytical chemistry (Peris, 2002), ESs are straightforward with important advantages, namely:

1. ESs can work 24 hours over the year without stopping, but chemists cannot.
2. ESs can work in difficult working conditions, but chemists cannot.
3. Copies of an ES can easily be made for as many locations as needed, while training new analysts is expensive and time-consuming.
4. ESs do not forget even the large number of parameters, which often have to be taken in account during the analyses, but chemists may.
5. ESs make comparable recommendations for like situations, similar cases being handled in the same way, whereas, humans are influenced by primacy effects (early information dominates the judgment) and contemporary effects (most recent information having a disproportionate impact on judgment).
6. Mistakes and errors can be prevented.
7. In the real-time systems information needed for decision-making is available sooner than from humans and even from different and sometimes very distant locations.
8. The knowledge of multiple human experts and even from different fields of science can be combined to give a system more breadth than a single person is likely to achieve.

However, ESs are still no match for chemists in food analysis in terms of:

1. Sensory experience: Human experts have available to them a wide range of sensory experience, whilst ESs are currently very often dependent only on symbolic input.
2. Degradation: ESs are very often not good at recognizing when no answer exists or when the problem is outside their area of expertise.
3. Learning: Human experts automatically adapt to changing environments and unlike them, ESs must be explicitly updated. ES combined with database, case-based reasoning and neural networks are methods that can incorporate learning.
4. Creativity: Analysts can respond creatively to unusual situations; ESs cannot.

Up to now, no one ES was developed in the field of mycotoxin analysis. In view of this, the aim of the present work was to develop an ES for solving the problem of choosing an analytical procedure for the determination of fumonisins in corn.

BASIC PRINCIPLES IN THE DEVELOPMENT OF THE EXPERT SYSTEM

In developing the ES the guiding idea was that it has to satisfy several goals, namely:

1) To be modular and easy to expand;

2) That new information and knowledge can be easily updated in the ES, without, or with a minimal change in the formal logic system;

3) That the acquisition and preparation of data and procedures can be conveniently carried out by experts to their final implementation into the ES, so that the communication between the expert and system engineer is made easier, as well as that a fast implementation is ensured;

4) That the once developed ES can be easily translated into other languages, with minimum change of the inference engine and that its updating in that language should be as simple as possible;

5) That the developed ES and its upgrades easily can be downloaded over the Internet.

With the aim of fulfilling these requirements we designed the ES with often use of graphical parameters. The system of formal logic has mostly been designed to operate with symbolic parameters, which are independent of the precise content of its graphical presentation. In this way, we were able to develop a system of formal logic, which is intelligible and easy for checking, modification, or expansion. In the graphical presentation of the parameters a full freedom is ensured in choosing the text, language, tables, mathematical or chemical formulas, pictures or drawings.

In designing the knowledge base it was extremely important to carry out formalization, organization and parameterization of the knowledge base in a way, which will be user-friendly, and its usage utmost freed of possible ambiguities. The inference engine was made so that it ensures the shortest way in obtaining an answer, which ought to be clear and unambiguous, and provides the user full information about the procedure to be applied. Also in the developing of the present ES we have chosen the certain criteria, which then were used for designing the hierarchical structure and in the making the branching choice.

In developing of the ES and adapting it to wide variety of the users even to those, which are not familiar with the computer technique, beside ES shell, some of Microsoft Office tools and some of the software tools operating with the Microsoft Windows operating systems were used. The ES was constructed having constantly in mind that its application should be maximally simplified and easy to use for specialists in chemistry but also with those who are not.

BASIC CARACTERISTICS OF FUMONISINS

Fumonisins, secondary metabolites of fungi from the genera *Fusarium*, are mycotoxins, of importance to human and animal health (WHO, 2000). They are the most frequently found as natural contaminants in corn and

corn-based products worldwide (S h e p h a r d et al., 1996). Fumonisins have experimentally been shown to be a causative agent of equine leukoencephalomalacia, porcine pulmonary edema syndrome and to produce liver cancer in rats (WHO, 2000). Acute fumonisin toxicity in humans was not confirmed, but the presence of fumonisins in corn was statistically associated with high incidence of esophageal cancer in people from South Africa (S y d e n h a m et al., 1990) and China (C h u and L i, 1994). International Agency of Cancer Research has classified *Fusarium moniliforme* toxins as potential carcinogens for humans (class 2B carcinogens), similar to ochratoxin A (IARC, 1993). An official tolerance value for dry corn products (1 μg/g) has been issued only in Switzerland, while only recommendations have been issued in the United States and France (S o l f r i z z o et al., 2001). To date, European Commission has recommended, but not legislated, maximum levels for combinations of fumonisins B_1 and B_2 which range from 2000 μg/kg for unprocessed corn to 100 μg/kg for infant food (EMAN, 2000). The tolerance level for fumonisins in feed and groceries has not yet been set in Serbia and Montenegro.

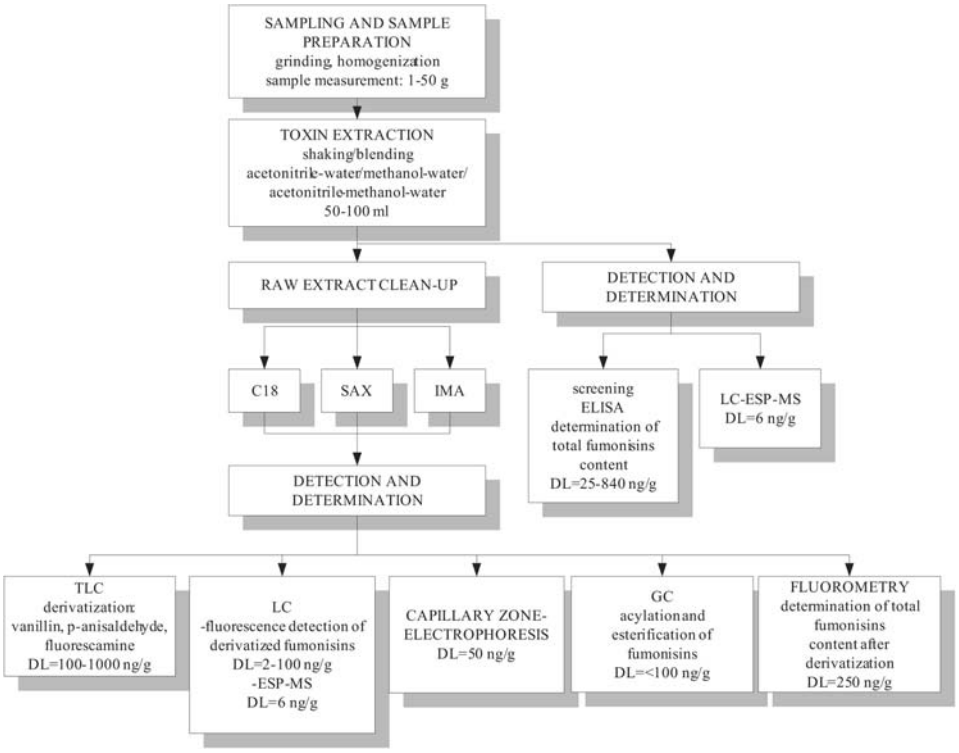


Fig. 1. Presentation of methods for determination of fumonisins. (IMA — immunoaffinity column; SAX — column with strong anion exchange; C18 — column with C_{18} reverse phase; ELISA — enzyme-linked immunosorbent assay; LC-ESP-MS — liquid chromatography with electrospray mass spectrometry; TLC — thin-layer chromatography; LC — liquid chromatography; GC — gas chromatography; DL — detection limit)

By their chemical structure, fumonisins are polar organic compounds with a long hydrocarbon chain. According to their structure, there are four series: A, B, C and P. The most attention is devoted to the toxins from the B series, since they are the most toxic. These toxins are diesters of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethylpolyhydroxyeicosanes. Many analytical procedures have been developed for determining fumonisins in corn and corn-based foods and feeds (J a k š i ć, 2004). Besides the fact that methods for fumonisins B series determination differ in detection limit (Fig. 1), there is a significant difference in the price of analysis, as well as in duration, which was also considered in creating the ES.

BASIC HIERARCHICAL ORGANIZATION OF THE EXPERT SYSTEM

To get an impression about the system as a whole and of its parts let's have a look at the following basic flow-chart. (Fig. 2). First of all, the user obtains some basic information about the ES (Fig. 2, Q2 and Q3) and then, if the user so desires, basic information on fumonisins (production and presence in nature, chemical structure of fumonisins, toxic effects of fumonisins, carcinogeneity, legislative regulations, as well as the detection limits for various methods, Fig. 2, Q5—Q10). This information on fumonisins can be later omitted. Parameter Q11 is introduced to enable simpler spreading, *i.e.* branching of ES parts, as well as for easier control. After that, the user has to choose with what sensitivity, *i.e.* detection limit (DL) of the method he wants to determine fumonisins (Fig. 2, Q12). In this ES, methods are divided into four groups according to DL. Liquid chromatography has the lowest DL (0.01 μg/g), followed by capillary zone electrophoresis (0.05 μg/g), gas chromatography and thin-layer chromatography (0.1 μg/g), and enzyme-linked immunosorbent assay (ELISA) and fluorometry (0.25 μg/g). If the user wishes to apply the method with the lowest DL, *i.e.* liquid chromatography (LC), he should answer the question about the availability of the equipment. If the answer is affirmative (Fig. 2, Q13, 1), the next question is whether cartridges are available (immunoaffinity column, column with strong anion exchange, and/or C_{18} reverse phase), or not (Fig. 2, Q15). If the answer is affirmative, seven options are available, depending on whether the user has only one column at disposal (Fig. 2, Q16, 1—3), two columns (any two) (Fig. 2, Q16, 4—6) or all three columns (Fig. 2, Q16, 7). If only one column is at disposal, ES gives him an appropriate procedure (choices 2—4). However, if the ES user has more than one cartridge at his disposal, a more appropriate procedure is suggested, providing a chromatogram with fewer interfering peaks. If, however, the user does not have cartridges at his disposal (Fig. 2, Q15, 2), individual cartridges are then suggested within the HELP, along with additional information about their advantages, *i.e.* disadvantages (Fig. 2, Q22—Q24).

As a result of the consultation, the user gets basic information about the technique (in this case about LC), along with a detailed determination procedure, starting from sampling, necessary chemicals as well as preparation of all

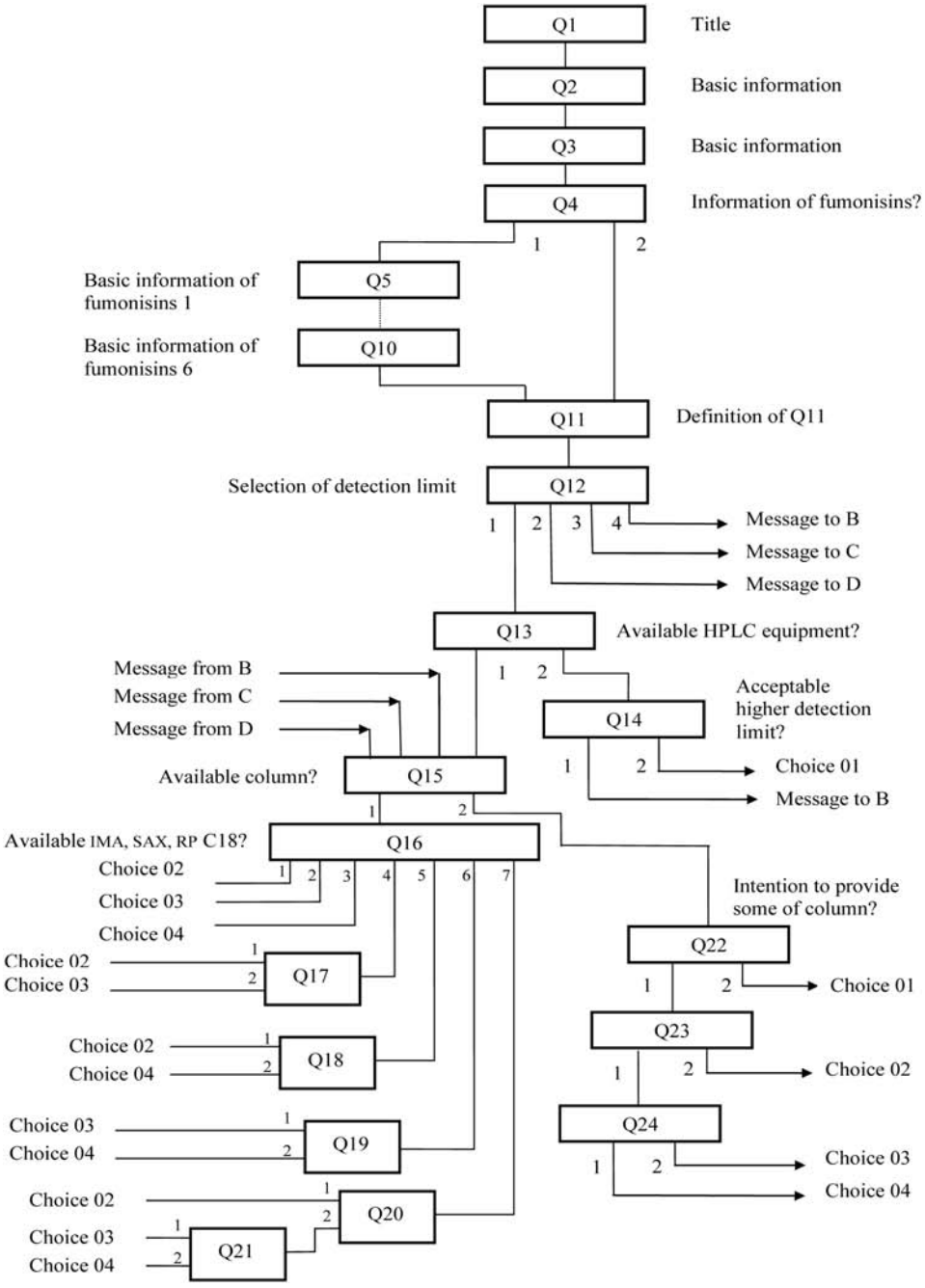
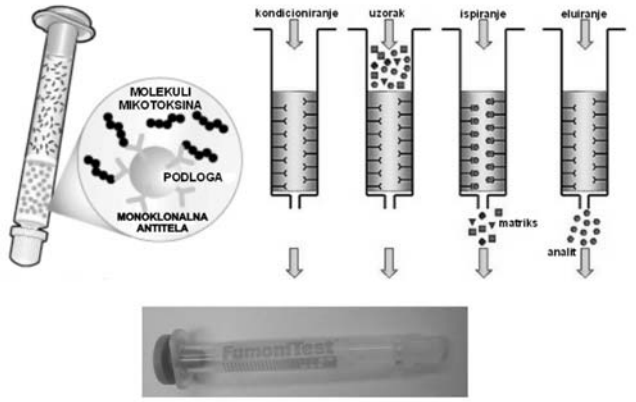


Fig. 2. Flow chart of one part of ES for determination of fumonisins

APARATURA

Imunoafinitetna kolona (FumoniTest™, Vicam, Watertown, MA, USA). Po deklaraciji kolona je za jednokratnu upotrebu, a kapacitet kolone je ≥ 10 μg fumonizina B_1 i B_2. Međutim, prema našim istraživanjima kolona se može primeniti 10 puta nakon regeneracije kako je opisano kasnije.

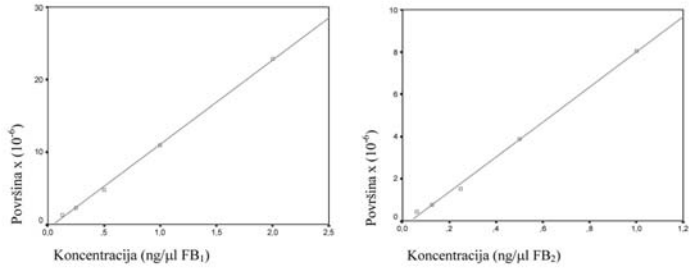


Izgled FumoniTest ™kolone i faze prečišćavanja ekstrakta pomoću IMA-kolona.

ANALITIČKI POSTUPAK *(nastavak)*

Kalibraciona kriva

Pripremi se kalibraciona kriva pomoću serije standardnih rastvora. Ovi rastvori obuhvataju opseg koncentracija od 0,125 – 2,000 μg/g FB_1 i 0,0625 – 1,000 μg/g FB_2. Kalibracionu krivu treba snimiti pre LC analize i proveriti linearnost.



Kalibracione krive za određivanje FB_1 i FB_2.

Kalibracione krive, koeficijenti korelacije i rezidualne standardne devijacije za određivanje FB_1 i FB_2

Mikotoksin	Opseg koncentracija (ng/μl)	Kalibraciona kriva	Koeficijent korelacije	Rezidualna standardna devijacija
FB_1	0,125 – 2,000	$y = 11{,}64 \cdot 10^6\, c - 0{,}57 \cdot 10^6$	0,999	0,37
FB_2	0,0625 – 1,000	$y = 8{,}30 \cdot 10^6\, c - 0{,}29 \cdot 10^6$	0,999	0,18

Linearnost je data prema jednačini: $y = mx + b$ (m–nagib, b–odsečak na y-osi).
Linearnost za FB_1 i FB_2 je određena kalibracijom pomoću pet tačaka.

Fig. 3. Graphic presentation of a few parts of the results of consultation

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RULES:
----------------------------------------

RULE NUMBER: 1
IF:
       Ekran 2 OK
  AND  Ekran 3 OK
  AND  Da li zelite dodatne informacije o fumonizinima? da
  AND  info01 OK
  AND  info02 OK
  AND  info03 OK
  AND  info04 OK
  AND  info05 OK
  AND  info06 OK

THEN:
       A1 1
----------------------------------------

RULE NUMBER: 5
IF:
       A1 1
AND    Odaberite granicu detekcije metode kojom zelite da detektujete i
       odredite sadrzaj fumonizina?
       = 0,01
AND    Za ovo odredjivanje Vam je potreban tecni hromatograf sa
       fluorescentnim detektorom. Da li raspolazete sa potrebnom opremom?
       DA

THEN:
       A2 1

ELSE:
       A2 0
----------------------------------------

RULE NUMBER: 8
IF:
       A2 1
OR:    A16 1
AND    Za preciscavanje sirovog ekstrakta kukuruza mogu se primeniti IMA,
       SAX i RP C18 kolone. Da li raspolazete nekom od njih?
       Da
AND    Kojom od navedenih kolona raspolazete? IMA AND SAX AND RP C18
AND    Da li zelite da primenite kolonu IMA?
       Da

THEN:
       Postupak Choice02 - Confidence=10/10
  and  REPORT(IMA01.out)
  and  REPORT(IMA02.out)
  and  REPORT(IMA03.out)
  and  REPORT(IMA04.out)
  and  REPORT(IMA05.out)
  and  REPORT(IMA06.out)
  and  REPORT(IMA07.out)
  and  REPORT(IMA08.out)
  and  REPORT(IMA09.out)
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Fig. 4. Few rules for defining one of the consultation results

the necessary solutions, the appropriate apparatus, probe preparation, the mode of determining results, calculation of the results, and provides a survey of all the relevant literature references. Some of the screens are presented in Fig. 3 for the sake of illustration, and the way of coming to one such solution, *i.e.* rules which ensure it are presented in Fig. 4.

If, however, an LC is not available to the user, he should define whether a higher DL is acceptable for his determination of fumonisins in corn (Fig. 2, Q14). If the answer is YES (number 1), then branching is provided toward branch B. If, however, the answer is NO (number 2), the user gets the information that by using the other technique the desired DL cannot be achieved and that the consultation is ending (Fig. 2, choice 1).

If the user defined a higher DL at the beginning of the job, the choice of methods is certainly higher (Fig. 2, branching toward branches B—D). For example, if the DL of 0.25 μg/g is acceptable, the user can apply, aside from LC, capillary zone electrophoresis, gas chromatography, thin-layer chromatography, as well as ELISA and fluorometry. To narrow down the choice, a possibility of choosing a method is offered to the user on the basis of analysis cost, as well as its duration (not shown in Fig. 2).

ACKNOWLEDGEMENTS

The work was partially financed by the Ministry of Science and Environment Protection of the Republic of Serbia (Project: "Development of New and Improvement of the Existing Analytical Methods and Techniques for Monitoring Quality of the Environment". No 1622).

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ЕКСПЕРТНИ СИСТЕМ ЗА ОДРЕЂИВАЊЕ ФУМОНИЗИНА У КУКУРУЗУ

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Резиме

Експертни системи се последњих петнаестак година примењују у све већем обиму у различитим областима људске делатности као изванредно средство за решавање комплексних проблема који захтевају знање и искуство одговарајућих експерата. Једна од тих области је и хемијска анализа у којој је већ развијен значајан број експертних система.

Циљ овог рада је био да се реализује експертни систем за решавање проблема избора оптималног поступка одређивања фумонизина. У првој фази развоја експертног система определили смо се за одређивање фумонизина у кукурузу имајући у виду да ови токсини најчешће контаминирају управо ову житарицу. При изради експертног система прихваћен је детерминистички приступ који полази од претпоставке доброг познавања наведене проблематике од стране експерата из дате области. Системом су обухваћена решења која су досада објављена у литератури за наведену област, као и наша искуства до којих смо дошли током испитивања појединих метода. На основу тих информација, као и на основу захтеване границе детекције методе, расположиве опреме, хемикалија, као и времена које експериментатор има на располагању за одређивање, експертни систем

даје предлог оптималног поступка одређивања фумонизина у кукурузу почевши од узимања узорка за анализу, припремања свих потребних раствора, поступка одређивања, израчунавања резултата, па до прегледа релевантне литературе. Основа експертног система је љуска која може да ради под разним верзијама Windows оперативног система. У његовом потпуном уобличавању и прилагођавању корисницима који рачунарску технику не познају довољно коришћен је алат обухваћен унутар Microsoft Windows и Microsoft Office. При изради експертног система водило се рачуна да његова примена буде максимално поједностављена за кориснике.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 121—127, 2005

UDC 579.6:612.6

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ZEARALENONE ENDOCRINE SYSTEM CATCH

ABSTRACT: This paper deals with the contamination of our environment with thousands of both natural and man-made chemicals which affect the endocrine system of humans and animals. These so-called endocrine disrupting chemicals (EDCs) are thought to mimic or block the action of hormones and therefore disrupt sexual development *in utero*. EDCs are organochlorine pesticides, dioxin compounds, polychlorinated biphenyls, alkylpolyethoxylates, plastic additives and phytoestrogens (occurring naturally in foods: isoflavones, coumenestans and zearalenone). The structure of zearalenone is similar to the structure of estrogens and it enables binding to the estrogenic receptors. DNA laddering on gel electrophoresis was present 12 h after dosing thus indicating a conclusion that there was apoptosis. Apoptosis is the principal mechanism contributing to germ cell depletion and testicular atrophy following zearalenone exposure.

KEY WORDS: apoptosis, EDCs, testis, zearalenone

INTRODUCTION

Our environment is contaminated with thousands of man-made chemicals that can interact with the endocrine system of humans and animals. More recently animal models have shown that maternal EDC exposure induces reproductive disfunction in male offspring. Environmental chemicals known to do this do so most often with receptors derived from the steroid/ thyroid/ retinoid gene family (M c L a c h l a n, 2001). They include ubiquitous and persistent organochlorines, as well as pesticides, dioxin compounds, polychlorinated biphenyls, alkylpolyethoxylates, plastic additives and phytoestrogens (occurring naturally in foods) have known endocrine disrupting effects. Many plants and fungi contain naturally occurring phytoestrogens and humans are probably exposed to milligram quantities every day. Active substances are isoflavones, coumenestans and the fungal metabolite zearalenone.

In order to understand the mechanisms by which EDCs affect the development of the human fetal gonad, it is important to determine the key processes regulating human gonad development. Testis development is initiated in the embryo as a response to the expression of the sex-determining gene, SRY. In addition to SRY, there are several downstream effectors and autosomal ge-

nes that are required for normal differentiation of the male primary gonad. The appearance of Sertoli cells at 6—7 weeks is one of the first morphological signs of testis development. Between 10 and 15 weeks, Sertoli cells in the newly differentiated testis produce Mullerian inhibiting substance (MIS). This causes the regression of the Mullerian ducts, which would otherwise differentiate into the female genitalia. Persistence of these ducts is associated with failed testis descent, hence MIS is thought to be critical for masculinisation and thus a likely target for EDCs (Murray et al., 2001).

Sertoli cell multiplication occurs during fetal, neonatal and prepubertal life and is dependant on FSH stimulation. Circulating FSH levels have been shown to rise accordingly as gestation progresses from 17 to 40 weeks. Inhibin production, which is reported to predominantly occur in the adult Sertoli cells, suppresses FSH production by the pituitary. In the adult testis each Sertoli cell "nurses" a fixed number of germ cells into spermatozoa. It follows therefore that the establishment of a normal adult sperm count is dependant on the generation of Sertoli cells. Hence, adequate Sertoli cell proliferation during fetal, postnatal and prepubertal windows is vital to establish a normal sperm count in adulthood.

The interstitial fetal Leydig cells begin differentiation during the eighth week of gestation and secrete testosterone to promote differentiation of the Wolffian ducts into the seminal vesicles. The Leydig cells undergo rapid proliferation until week 18 when a plateau is reached and Leydig cell number remains at this level until the third trimester. The period of maximum Leydig cell number is also coincidental with a peak in fetal testosterone synthesis between 14 and 18 weeks of gestation equivalent to levels observed in the adult male. Luteinizing hormone (LH) is the main hormone stimulus on Leydig cells for the intrauterine production of testosterone (Radunović et al. 1993).

Zearalenone has several cell types to target, but the function of these cells is not fully understood; it is thought that zearalenone is involved in Sertoli/Leydig cell interactions.

Zearalenone is a non-steroid estrogenic complex produced by several species of *Fusarium* which grow on cereals. Zearalenone and its metabolites bind to human estrogenic receptors thus demonstrating estrogenic and anabolic properties similar to FSH (Abid-Essefi, 2003). After ingestion zearalenone decreases the level of FSH and LH.

Zearalenone can be genotoxic *in vivo* causing damage to DNA and causing the chromosome aberration. The elevated concentration of this toxin causes the DNA fragmentation resulting in the "ladder-like" appearance on the agar gel during the electrophoresis. Such an appearance indicates the apoptosis (Hwan et al., 2003).

Apoptosis or the programmed cell death is a normal component of the development and health of the multicellular organisms. The number of cells can be regulated by apoptosis in the normal state of health; for exampe, the number of neurons during development which are "siucidal" so as to keep their number at a controlled level. Apoptosis can also occur in diseases, AIDS e.g., where there is an increased apoptosis of immune cells, consequently a

weaker immunity. It occurs due to the cell death as a response to the variety of stimuluses (radiation, tissue lesion, various genotoxic agents, EDC etc.) and during the apoptosis it happens in a controlled and regulated way in contrast to the necrosis, in which the cell death leads to the destruction of cells, inflammations and serious health problems (T e r z i ć, 2004). The most obvious example for understanding the apoptotic changes is with neurons. At the place of the primary impact the neurons undergo necrosis and instantly die but those neurons further from the primary impact are partly damaged and can survive so that they can be saved by a certain treatment since their death is delayed, thus enabling the treatment. But, if the neurons realize that death is inevitable (that they are too damaged) they employ the self-destruction mechanisms (D e m j e n, 2004).

After receiving certain signals a cell undergoes the apoptosis i.e. a certain number of characteristic morphological changes. A group of proteins known as caspases are activated in the early stages of apoptosis. They divide into parts which are essential for normal cell functions, including structural proteins in cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can activate the other degradative enzymes (DNases which begin to cleave the DNA in the nucleus). The result of these biochemical changes is the appearance of morphological changes in a cell. To summarize, when a cell wants to kill itself different ways of signal transduction are employed (transmission of a suicidal signal) which lead to the activation of certain enzymes — caspases which cut (disintegrate) their substrates (proteins essential for the life of a cell — structural, nuclear proteins) and a cell dies (S h i g e k a z u, 1999).

THE PROCESS OF APOPTOSIS

The cytoplasm begins to shrink following the cleavage of lamins and actin filaments (structural proteins) (A). The nuclear condensation can be observed through the destruction of chromatins and nuclear-structural proteins, and in many cases the nucleus of apoptotic cells takes on the shape of a horseshoe (B). The cells continue to shrink (C) packaging in a shape which allows for easy clearance by macrophages (a macrophage "eats" them and there are no inflammatory reactions in contrast to necrosis). The phagocytic cells are responsible for the elimination of apoptotic cells from tissues in a clean and tidy way which avoids many of the problems associated with the necrotic death of a cell. In order to promote their phagocytosis through macrophages, apoptotic cells often undergo the change of the plasm membrane which causes the macrophagic reaction. One of such changes is the transmission of the phosphatidylserine from the inner leaflet of the cell towards the outer surface. The changes in the membrane can often be observed morphologically through the occurence of membrane blebs or blisters (D), which often appear at the end of the apoptotic processes. Namely, the dying cells develop phosphatidylserine on the outer membrane which is a sign to macrophages to "eat" them. Small vesicles called apoptotic bodies can sometimes be observed (D?) and are the sign that cells die during apoptosis (Fig. 1).

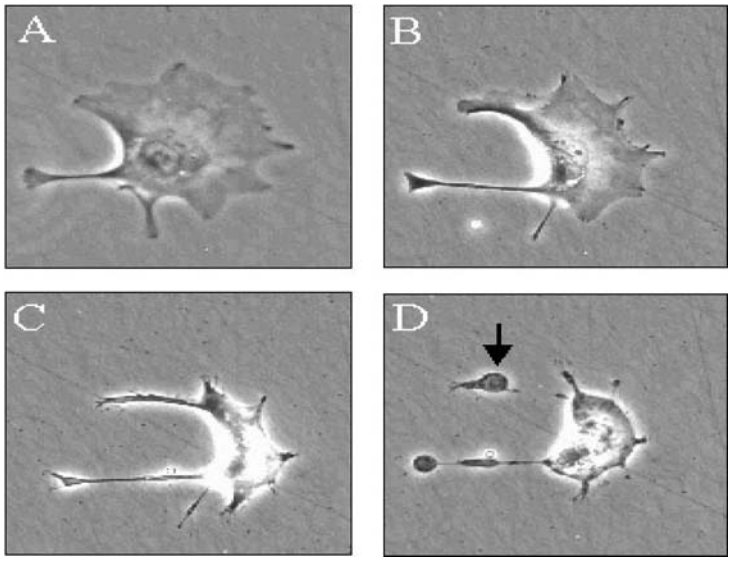

Figure 1. Apoptosis process (Ashton et al., 2004)

APOPTOSIS AND EDCs

Apoptosis is an important feature during testis development and this is partially controlled through the expression of a large family of related apoptotic regulatory genes. The prototype apoptosis inhibitor is the protein product of the proto-oncogene *bcl-2*. The gene product forms homo- or hetero-dimers with apoptosis inducing factors such as bax. Over-expression of bax negates the death repressor effects of bcl-2 and promotes apoptotic cell death, hence the bcl-2/bax ratio is an important component of cell survival.

The expression of Fas ligand (FasL) and Fas receptor (FasR) is also important in the regulation of apoptosis. When FasL crosses links with its receptor, the apoptosis is induced in FasR-positive cells. Since FasR is localised to adult rodent germ cells and FasL to the Sertoli cells the role of this system in adult testis function remains to be clarified. In addition, an estrogen response element (ERE) motif has been identified in the promoter region of the FasL gene. These findings suggest that EDCs recognised by the estrogen receptor could disrupt FasL expression in the testis, hence affecting testis development and function. However, human studies have failed to detect FasR or FasL mRNA in fetal testis at 20—22 weeks of gestation hence EDC action through this mechanism may be limited to certain gestational windows.

Zearalenone is estrogenic and therefore can bind to the ERα/β-positive Leydig cells in the interstitium. This could reduce testosterone secretion and thus availability for binding to the AR expressed on the PMCs. Since testosterone is reported to protect against testicular apoptosis, this may be associated with reduced levels of bcl-2 in the PMCs resulting in apoptosis.

The ways of the assumed mechanisms by which EDCs may induce disregulation of apoptosis in the human fetal testis are schematically shown (Fig. 2). A. Normal function: Testosterone (T), produced by Leydig cells [1], targets the peritubular myoid cell (PMC) where most androgen receptor (AR) is located [2]. Consequently, this cell functions normally and interacts with Sertoli cells [3]. B. EDC action on estrogen receptor: After crossing the placental 'barrier', dieldrin (Diel) reaches the fetal testis [4] and docks with the ER expressed by the Leydig cell [5]. This causes estrogen mediated effects, such as decreasing T output by the cell [6] and hence, the AR on the PMC does not receive adequate T [7]. This causes bcl-2 levels to fall resulting in apoptosis of the PMC [8]. Finally, PMC-Sertoli cell interaction is affected [9]. C. EDC action on androgen receptor: Diel docks with the AR expressed by the PMC [10] thus blocking T binding to the AR on the PMC [11]. Inadequate binding of T causes bcl-2 levels to fall resulting in apoptosis of the PMC [12]. Finally,

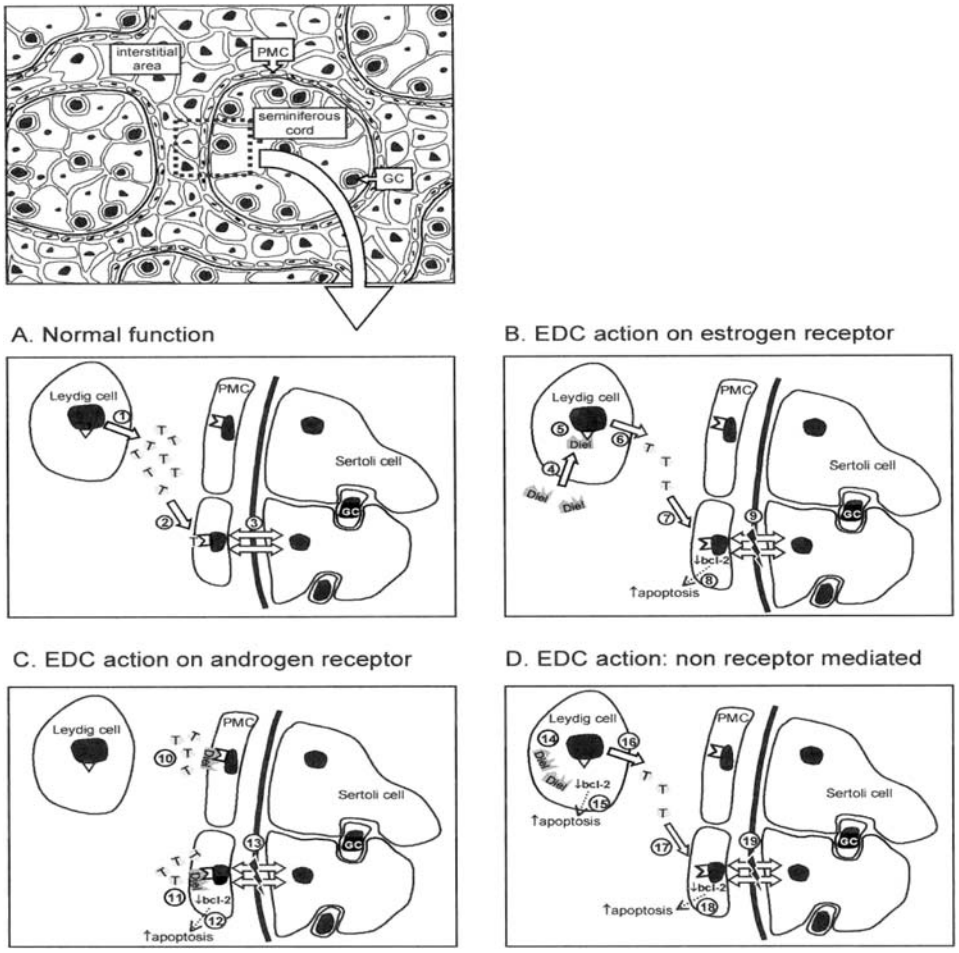

Figure 2. Schematic presentation of EDCs action (M u r r a y, 2001)

PMC-Sertoli cell interaction is affected [13]. D. EDC action: non-receptor mediated: Unbound Diel present within the Leydig cell [14] may cause bcl-2 levels to fall resulting in apoptosis of the Leydig cell [15]. Consequently, less T is produced by the Leydig population. 17. Hence, the AR on the PMC does not receive adequate T. 18. This causes bcl-2 levels to fall resulting in apoptosis of the PMC. 19. Finally, PMC-Sertoli cell interaction is affected [19].

CONCLUSION

Until recently moulds were concidered to be harmless, although it was proved that some moulds were harmful or toxic to people, plants and animals. Zearalenone belongs to the group of phytoestrogens and is a secondary metabolite of *Fusarium* spp. This mycotoxin, present in this region, has proven endocrine — disrupting effects and negatively affects the reproductive development in male population. So far the studies have given rise to the significant concern about the EDCs effect from the environment on the development of male fetus, followed by the effect on the reproductive health in adults. Scientists have succeeded in identifying EDC sensitive genes but there is still a lot to be explained regarding the meshanisms i.e. the disrupted apoptosis of the testis.

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ЗЕАРАЛЕНОН КЛОПКА ЕНДОКРИНОГ СИСТЕМА

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Резиме

Наше окружење контаминирано је са 1000 хемикалија које је човек створио и које могу ступити у интеракцију са ендокриним системом људи и животиња. Сматра се да ове тзв. супстанце које разарају ендокрини систем (ЕДЦ) блокирају рад хормона и тако ометају сексуални развој *in utero*. Недавно су животињски модели показали да материнска изложеност ЕДЦ супстанцама индукује репродуктивне дисфункције код мушких потомака. Ове студије су довеле до значајне забринутости у продору ЕДЦ из окружења на развој мушког фетуса, а потом и репродуктивног здравља одраслог.

У хемикалије са ендокрино-разарајућим ефектима убрајају се: органохлорни пестициди, диоксини, полихролни бифенили, алкилополиетоксилати, пластични адитиви и фитоестрогени који се јављају природни у храни. Активне супстанце су изофлавони, комествроли и фунгални метаболит зеараленон (ЗЕР). Зеараленон као естрогенска супстанца изазива прерани сексуални развој код незрелих женки, док код мужјака инхибира нормалан развој тестиса. ДНК фрагментација уочена „мердевинастим" изгледом ДНК на агарозном гелу током електрофорезе била је очигледна након 12 сати деловања ЗЕР и упућивала је на настанак апоптозе. Апоптоза је главни механизам који доприноси пражњењу семених ћелија и атрофији тестиса.

Постоје докази да се мушка репродуктивна функција смањује код људи и популацији у дивљини. Докази су подударни са повећаном употребом и већим утицајем хемикалија као и фитоестрогена у окружењу током последњих 50 година. Сходно томе, хопотезе су усмерене на механизам деловања ЕДЦ у индукцији дисрегулисања апоптозе у људском феталном тестису.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 129—137, 2005

UDC 633.11:632.4

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CORRELATION OF CONCENTRATION OF FUMONISINS AND YIELD GRAIN OF WHEAT

ABSTRACT: Wheat from different locations was served for the isolation of *Fusarium* spp. Isolates were precisely identified and multiplied for artificial inoculation. Three sorts of winter wheat were chosen: PKB Lepoklasa, Jugoslavija and Francuska. To these sorts three different treatments were applied: artificial inoculation with the isolates of *Fusarium* spp., protection of wheat with fungicide Impact-C and follow-up of a spontaneous infection in different phenophasis of wheat development. The control was done with the same sort, of the same location, not covered by an experiment. The research lasted for three years.

Phytopathological evaluation was done twice during vegetation. The sort of Jugoslavija had an average of 85% of infected plants, Francuska 65%, but PKB Lepoklasa during each of three years had 100% of infection by *Fusarium* spp. fungi.

Presence of fungi *Fusarium* spp. causes production of numerous mycotoxins and we determined presence of fumonisins. The mentioned mycotoxin was found only in the treatment of artificial inoculation for each sort. Presence of fumonisins was proved by fluorometric method and concentration was by sorts as follows: Jugoslavija 0.30 mg/kg, Francuska 0.62 mg/kg, and PKB Lepoklasa 0.56mg/kg. In grains 100% infected by fungus, the concentration of mentioned toxins is of a greater quantity.

KEY WORDS: Fusariosis, mycotoxin, wheat, yield grain

PREFACE

The increase of population brings the problem of increased food production and food keeping in better storing conditions, by the help of which healthy satisfactory food could be provided. According to the data of FAO, it is estimated that one fifth of all losses of produced food is due to insects, microorganism and other pests. The reducing of these losses induces the reducing of hunger problem in the world.

Mycotoxins are secondary metabolites produced by fungi which may cause harm to health of people and animals. Wheat may be infected with fungus *Fusarium* spp. in the field, as well as during the storage. The most significant

Fusarium toxins, frequently appearing as cereals are fumonisins, zearalenone and trichothecenes. Working group for cancerogenic risk put toxins from *F. moniliforne* (including fumonisins) in group 2B, as possibly carcinogen for people.

For our territory, concerning geographical position and climatic characteristic, the most significant mycotoxins, originating from fungus *Fusarium* spp. are zearalenone and deoxynivalenol, and for fumonisins, occurring in wheat, there are no data for out country, but in the world these mycotoxins are combined mainly for corn.

MATERIAL AND METHODS OF WORK

To examine the presence of fumonisins in wheat grain three varieties of winter wheat were chosen: PKB Lepoklasa, Jugoslavija and Francuska. To these varieties three different treatments were applied and the first was the protection of wheat with fungicide Impact-C. The second treatment was the artificial inoculation with the isolates of *Fusarium* spp. at the beginnig if wheat flowering. Wheat, grown in various regions, was used for isolation of *Fusarium* spp. Isolation was done according to the method of Nelson et al. (1979) and there was done suspension for artificial inoculation with fungi *Fusarium* spp. The third treatment was the monitoring of a spontaneous infection in different phenophases of wheat development. The control was done with the same sort, of the same location, not covered by an experiment. The research lasted for three years.

This experiment in the field was implemented. For this test, it was used the block system repeated four times. The size of the field was $5m^2$ (1x5).

The spikes (50 for treatments) were taken in different phenophases of wheat growing (milk ripeness, waxy ripeness and full ripeness). Germinating ability was tested by spikes analysis and the identification of *Fusarium* spp. was done according to the method of Nelson et al. (1979).

After the harvest, the presence and concentration of the mycotoxins in the wheat grain were determined. The degree of infection with fungi *Fusarium* spp. is variable, with the aim to compare obtained results, grains 100% infected with fungus were isolated from the wheat samples artificially inoculated. Fumonisins were determined by a modified HPLC method, by fluorometric method, that is, imunoafinitive column chromatography on fluorometer Vicam, Watertown, MA (Scott et al., 1995, Ware et al., 1994).

There were done statistic data processing, analysis of variations, simple correlation and regression. The analysis covered different years, sorts and treatments. The results are shown as a three year average for all examined characteristics.

RESULTS OF RESEARCH

Variety Jugoslavija, on allotments where artificial infection by fungus, resulted with 85% of sick plants. Protection by fungicide was effective for Fran-

cuska variety as during the course of three years the symptoms of fusiriosis were not observed. During the first and the second year an artificial infection occurred on 60% infected plants and during the third year on 75%. Plants of PKB Lepoklasa variety were 100% infected by fungus in a treatment of an artificial inoculation, 1% was naturally infected and there were no damages with protected plants (Table 1).

Table 1. Percentage of infection by fungus *Fusarium* spp. of different genotypes of winter wheat by treatments

VARIETY(S)	TREATMENT (T)			$S\bar{x}$
	Protection with Impact C	Artificial inoculation with *Fusarium* spp.	Natural infection	
Jugoslavija	0.2	85	0.7	28.6
Francuska	0	65	0.3	21.8
PKB Lepoklasa	0	100	1	33.7
$T\bar{x}$	0.07	83.3	0.7	28.0

The spikes were taken in different phenophases of wheat growing (milk ripeness, waxy ripeness and full ripeness). Germinating ability was tested by spike analysis and there was done identification of *Fusarium* spp. Dominant fungus at selected varieties of winter wheat was *F. graminearum*, *F. moniliphorme* and *F. proliteratum* found in minor percentage. Results of regression analysis show that there is a high negative correlation between the germinating ability and the percentage of spike infection by fungi (r = –0,90).

During the course of three years study the grain yield of selected varieties of winter wheat was as follows: Francuska 7,77 t/ha, PKB Lepoklasa 7,76 t/ha and Jugoslavija 7,71 t/ha.

Regression analysis between the mass of 1.000 grains and concentration of fumonisins at wheat there was deduced grain a high negative correlation coefficient r = –0,99. For seed germination and concentration fumonisins at wheat grain, there was confirmed string correlation coefficient r = –0,19.

Small detected difference between analysed varieties is not statistically significant. Wheat protected with an fungicide Impact-C had 0,74 t/ha more grain yield than grain from spontaneous infection, by which this scientific farming method has been already justified in wide wheat production for twenty years (Table 2).

Table 2. Accomplished yield in wheat grain of different varieties of winter wheat within different treatments(t/ha)

VARIETY (S)	TREATMENT (T)			$S\bar{x}$
	Protection with Impact C	Artificial inoculation with *Fusarium* spp.	Natural infection	
Jugoslavija	9,06	6,08	7,98	7,71
Francuska	8,74	6,28	8,31	7,77
PKB-Lepoklasa	8,80	6,37	8,10	7,76
$T\bar{x}$	8,87	6,24	8,13	7,75

Mass of thousand grains is an attribute that depends on sort, climatic and edaphic factors, as well as on the level of applied farming method. Within the analysed varieties mass of thousand grains, irrespective of treatment, moved from 38,1 g (Francuska) up to 44,2 g (Jugoslavija). Variety PKB-Lepoklasa kept mass of thousand grains 41,0 g. Regression analysis between the mass of thousand grains and the degree of the percentage of infection of wheat spikes by fungi *Fusarium* spp. shows a mild correlation r = 0,12.

Absolute mass of grains (mass of thousand grains) is a significant indicator of external quality of grains. Increased number and mass of grains per spikes does not always results in increasing yield of grains.

Number mass of grains per spikes, in the face of treatment, was utmost in variety Francuska (35,5), something lower in variety PKB-Lepoklasa, and the least for wheat variety Jugoslavija (32,4).

Overall fumonisins are found in wheat grains from the treatment of artificial inoculation (Table 3). The lowest concentration of fumonisins is determined with grain of variety Jugoslavija. With grain of variety Francuska utmost concentration of fumonisins is measured, one with another 0,62 mg/kg and most at third years of research 0,64 mg/kg, when there was a high relative humidity of air folowed by high temperature. String negative correlation r = –0,11 was found in concentration of fumonisins at wheat grains and degree of infection wheat spikes. Regression analysis between mass of thousand grains and concentration of fumonisins in wheat grains found a high negative correlation coefficient r = –0,99. For germination ability of wheat grains and concentration of fumonisins in wheat grains, there was found the string correlation coefficient r = –0,19

Table 3. Contents of Fumonisins mg/kg in wheat grain of different varieties of winter wheat within different treatments

VARIETY	TREATEMENT	FUMONISINS mg/kg
Jugoslavija	*Fusarium* spp.	0,30
	Fusarium spp. 100%	0,40
Francuska	*Fusarium* spp.	0,62
	Fusarium spp. 100%	0,80
PKB-Lepoklasa	*Fusarium* spp.	0,56
	Fusarium spp. 100%	0,60

By comparison average of concentration of fumonisins per varieties in wheat grains originated from the treatment of artificial inoculation with grains from the same treatment with 100% infection, it can be proved that in the latter case, the concentration of fumonisins per varieties is increased (Table 3).

DEBATE

Fusariosis of wheat is a disease that irregularly appears in the areas occupied by this culture, and it results in reduction of crops and technological

quality. This disease is provoked by many kinds of Fusarium types and the most frequent are: *F. graminearum, F. culmorum, F. avenaceum.* The damage is the consequence of class sickness which leads to the considerable reduction of grain germination, bad grain quality with defected starch granulation and stored proteins (Bechtel et al., 1985). Infected grain proved damages on seeds (Manka, 1989).

Symptoms of mycotoxicosis is the result of the interaction of mycotoxins with functional molecule and subcells organellas at mamalias cells. Per Kiessling et al. (1986) many mycotoxins affect DNK level, by disturbing process of transcription and inhibiting relayed broadcast by synthesis of protein. During germination of wheat grains the activity from the proteinase enzyme is accelerated, mycotoxins block protein synthesis, due to which wheat grains infected with fungi have reduced germinating ability.

Weinert and Wolf (1995) found that after infection the fungus settles the infected spikelet and starts to grow downwards in the rachis. It invades successively the spikelets mainly in basipetal direction. The vessels in the rachis may get obstructed or destroyed, it is not provided with water and nutritient anymore and start to wit. Yield reduction and infection of kernels are mostly directly correlated with disorders of ripeness.

In a three-year-study during 1994—1996. Golinski et al. (1997) observed 39,2—84,7% infection of wheat grains with fungi *Fusarium* spp. and reduction of the mass of 1.000 grains was 80,5—92,5% in relation to control.

According to Balaž (1989) a high degree efficiency at patronage of spike from causing fusariosis were evienced by preparates with two active matters (Impact-C) and mixtures of two fungicides, applied in the beginning of wheat flowering. Increasing of yield and mass of 1000 grains are at correlation with effect with patronage wheat from disease, so that relatively increassing yield at relation at control is about 2,1—14,5%.

Mycotoxins synthetised by fungi *Fusarium* spp. are stabile in production unit process and there is a tendency to accumulate in products being mainly used as food for people and cattle. During increased moisture, in spring and summer, zearalenone and deoxynivalenol are dominant, and during drought — fumonisins. Mycotoxins do not represent enough great molecul mass so that animal and human organic structure could create related antibody.

After their disclosure, fumonisins (Gelderblom et al., 1988) turned to be an object of many researches because of their potential adverse toxic effects to the health of humans and animals. Out of seven fumonisins identified — A_1, A_2, B_1, B_2, B_3, B_4 and C_1, B_1 (FB_1) is the most abundant and commonly found in corn (Truckessetal, 1995).

Fumonisins were isolated in South Africa from culture material of *F. moniliforme* MRC 826, which had been proved to cause leucoencephalomalacia in horses and was hepatocarcinogenic in rats (Gelderblom et al., 1988) *F. moniliforme* contaminated corn has been associated with human esophageal cancer (Marasas et al., 1988) and was implicated as a cause of pulmonary edema in pigs (Kriek et al., 1981).

F. moniliforme and *F. proliferatum* are producers of fumonisins. *F. moniliforme* is cardinal pathogen in corn at tropics and moist temperate regions and

main abiot stocks and bonds for toxigenesis are temperature and humidity. *F. proliferatum* is mostly identified as *F. moniliforme* J. Sheld. and there are quite few data on its geographical distribution. It is settled in corn and causes sickness of class, stalk and seminal. Along with *F. moniliforme*, it provokes animal toxicosis. Fumonisin B_1 is the most significant mycotoxin originated in both species. Risk from metabolise fumonisins is in postharvest period, before drying. During drying and processing conditions of cereals the level of fumonisins is not reduced (B a r s et al., 1994).

During the experiment, cows were fed with addition of pure FB_1 oraly 1,5—5 mg/kg per body weight/day and intravenosly with 0,05 and 0,2 mg/kg per body weight/day, it was proved the presence of FB_1 in milk stable in cooking and freezing (B e n e t t et al., 1996).

During 1993—1995. it was confirmed in Hungary that 70—73% of visibly mouldy samples and 30% visibly mould-free samples were contaminated with FB_1 (F a s e k a s et al., 1998). Two weaned piglets were fed with a diet containing 330 mg/kg FB_1 of feed.The experimental animals developed hydrotorax and pulmonary oedema and died in 5—6 days. Kidneys are sensitive in FB_1 because of shingolipid metabolism, and urine is liquor for detection of free sphingoid base which results in influence of FB_1 in kidney destruction (G e l d e r b l o m et al., 1988).

R i c h a r d et al. (1996) nurtured cows with FB_1, FB_2 and FB_3 those being mixed with meal during 14 days and consummation had been 3 mg/kg per body weight/day for FB_1. To absence of detectable fumonisins in milk of cow there were detected. Except passing diarrhoea and increased serum blood-fat, helpmate hematological and clinical changes were not observed.

A l i et al. (1998) examined 16 corn samples collected from Indonesia for aflatoksines, fumonisins, trihotecenes and zearalenone. Fumonisins were detected in all of the samples at a mean level of 895 mg/kg, deoxynivalenol, nivalenol and zearalenone in 2 samples and in 11 samples aflatoksins were detected.

In Germany 38 samples of sweet corn were tested during 1993. and 1994, 29% contained fumonisins with the highest concentration of 193 mg/kg. During 1994/95. out of 66 samples corn product 14 samples were contaminated with FB_1 in concentration varied from 127 — 9.818 μg/kg and the rest from 6 samples — 453 μg/kg. In 1996. 17 samples were contaminated one with another 43 μg/kg (B r e s c h et al., 1998).

Corn products obtained from retail store in the Netherlands were analysed for FB_1 contamination. Out of 78 samples, 36% were contaminated with FB_1. About 45% sample corn products had FB_1 over 380 μg/kg and 25% processed foods (maize bread, maize chips, maize starch and cornflakes) were contaminated with FB_1 at up to 1430 μg/kg (D e n i j s et al., 1998).

Forty-one samples of beer were analyzed for Fumonisins FB_1 and FB_2, recoveries ranged from 0,4—1 μg/mL (S c o t t et al., 1996).

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КОРЕЛАЦИЈА ИЗМЕЂУ КОНЦЕНТРАЦИЈЕ ФУМОНИЗИНА И ПРИНОСА ЗРНА ПШЕНИЦЕ

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Резиме

На три сорте озиме пшенице ПКБ-Лепокласа, Југославија и Француска примењена су три третмана: вештачка инокулација изолатима *Fusarium* spp., заштита пшенице фунгицидом Impact-C и праћење спонтане инфекције у различитим фенофазама развоја пшенице.

Фитопатолошка оцена извршена је два пута у току вегетације. Узорци класова су испитивани на клијавост зрна и извршена је идентификација *Fusarium* spp.

Сорта Југославија имала је у просеку 85% инфицираних биљака, Француска 65% а ПКБ-Лепокласа је у све три године имала 100% инфекцију гљивама *Fusarium* spp.

Доминантна гљива на одабраним генотиповима озиме пшенице била је *F. graminearum*, а *F. moniliforme* и *F. proliferatum* су нађене у мањем проценту.

Присуство гљива изазвало је смањену клијавост, јер микотоксини инхибирају синтезу протеина. Развој гљива на биљкама пшенице довео је до увенућа зр-

на због оштећења спроводног система а самим тим и до смањења приноса, који је статистички значајан.

Гљиве из рода *Fusarium* spp. изазивају продукцију многих микотоксина. За подручје наше земље најзначајнији микотоксини, пореклом од гљива *Fusarium* spp., су зеараленон и деоксиниваленол, а за фумонизине, присутне на пшеници, у нашој земљи нема података, а у свету су ови микотоксини везани углавном за кукуруз.

Фумонизини су одређени модификованом HPLC флуориметријском методом.

Најниже концентрације фумонизина утврђене су у зрнима сорте Југославија. У зрнима сорте Француска измерене су највеће концентрације фумонизина, у просеку 0,62 mg/kg. Ниска негативна корелација r = –0,11 утврђена је за концентрације фумонизина у зрнима пшенице и степен инфекције класова пшенице гљивама. Регресионом анализом између масе 1.000 зрна и концентрације фумонизина у зрнима пшенице утврђен је висок негативни корелациони коефицијент r = –0,99. За клијавост семена пшенице и концентрацију фумонизина у зрнима пшенице утврђен је ниски корелациони коефицијент r = –0,19.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 139—146, 2005

UDC 633.15:632.25

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DETERMINATION OF DEOXYNIVALENOL IN CORN

ABSTRACT: By applying previously established optimal conditions for determination of deoxynivalenol by liquid chromatography with DAD detector, its content was determined in this work in corn samples artificially inoculated with *Fusarium graminearum.* The samples presented material of different genetic origin, and are frequently used as parent components of corn hybrids in the moderate belt. The obtained values for deoxynivalenol content were not higher than the maximum permitted level (1 μg/g), regardless of the fact that the fungus was artificially inoculated. This is probably a consequence of the absence of humid weather, as well as extremely high temperatures during the sampling period (year 2003).

KEY WORDS: Corn analysis, deoxynivalenol, liquid chromatography, mycotoxins, solid-phase extraction columns

INTRODUCTION

Deoxynivalenol (DON) belongs to trichotecenes, a group of mycotoxins, which, beside aflatoxin, zearalenone, ochratoxin and fumonisins, is most frequently found in foodstuffs and feed. DON is the most widely spread mycotoxin from the trichotecene group, produced by fungi from the genera *Fusarium*, most frequently *Fusarium graminearum* and *Fusarium culmorum.* The presence of these fungi is pronounced in cereals, wheat and corn being especially sensitive, causing their rotting. In foreign literature, a disease of wheat caused by this fungus is known as *Fusarium* head blight, while in corn it is known as *Gibberella* ear rot (JECFA, 2001). Infection of these cereals has for a consequence a decrease in grain size and the amount of protein in the grain, as well as a harmful effect on germination. The end result is a decrease in yield and feed quality.

Regardless of the fact that DON belongs to the group of the least toxic trichotecenes, knowledge of its content is of great importance for food quality control, because of its exceptional prevalence and its use as a kind of an indicator of possible presence of other, significantly more toxic trichotecenes (Lombaert, 2002). Its presence in feed is manifested by rejection of feed, vomiting, diarrhea and finally, the weight loss in livestock (Kuiper-Goodman, 2002).

The most sensitive animal species to the presence of deoxynivalenol is the pig, hence already at concentrations of 1 mg/kg in feed a certain percentage of these animals refuse food. That is exactly why this deoxynivalenol amount is the maximum permitted in feed intended for this animal species in most countries worldwide. Maximum permitted level of deoxynivalenol in feed for milch cows is also 1 mg/kg, while the level considered to be permitted for cattle and sheep is 5 mg/kg, although these species can tolerate levels above 10 mg/kg. The limiting level of deoxynivalenol in feed for horses is 1 mg/kg, although there are no thorough data about its effect on this species, except that the animals become depressed and excrete a larger amount of saliva. The least sensitive species to the presence of this mycotoxin in feed is poultry, although the maximum permitted level of deoxynivalenol in this case is 5 mg/kg (Canadian Grain Commission, 1999).

The maximum permitted levels for this mycotoxin, however, haven't yet been legally regulated in our country, neither in foodstuffs nor in feed.

Most frequently used for quantitative determination of DON are chromatographic methods, *i.e.* liquid chromatography (LC) with and without derivatization of DON, as well as gas chromatography with almost compulsory derivatization, and somewhat less commonly used thin-layer chromatography (TLC), immunochemical method and others (JECFA, 2001; Krska, 2001; Lombaert, 2002; Jajić, 2004).

All quantitative methods for deoxynivalenol determination (except immunochemical) require clean-up of crude sample extract by solid phase extraction (SPE). To that purpose, columns with different sorbents are used: activated charcoal, alumina and celite (Eppley et al., 1986), fluorisil (Sano et al., 1987), silica-gel, ion exchange resins, as well as different combinations of the above sorbents (Lauren and Greenhalgh, 1987). Lately, the most frequently used are multifunctional, so called MycoSep columns (Weingaertner et al., 1997; Mateo et al., 2001) and to some extent less often immunoaffinity columns filled with antibodies specific for an individual mycotoxin (Cahill et al., 1999).

The aim of this work is to determine DON content in corn samples used for artificial inoculation of *Fusarium graminearum* by applying previously established optimal conditions for DON determination by liquid chromatography with DAD detector (Abramović et al., 2005). Samples represented material of different genetic origin, and are frequently used as parent components of corn hybrids in the moderate belt.

MATERIAL AND METHODS

Materials

All solvents used for DON extraction from corn samples, as well as for the mobile phase preparation were of LC grade. All chemicals used in the investigation were of reagent grade. Solutions were prepared in deionized water except when stated otherwise.

Deoxynivalenol calibrant solutions. Deoxynivalenol (Biopure, Tulln, Austria) was purchased as an analytical standard. Calibrant solution was prepared in ethyl acetate-methanol (19:1, v/v) at the concentration of 144.0 μg/cm^3 from crystalline substance according to AOAC method 986.17. Stock solution containing DON at 14.4 μg/cm^3 was prepared by measuring 1.00 cm^3 calibrant solution of DON into a 10 cm^3 volumetric flask and diluting to volume with ethyl acetate-methanol (19:1, v/v). Working calibrant solutions were prepared by evaporation of the appropriate volume of the stock solution and dilution with the appropriate volume of methanol. Standard solutions were stored at 4°C.

Sample and preparation. Three types of corn samples, presenting material of different genetic origin, frequently used as parent components of corn hybrids in the moderate belt, were obtained from the Department for Maize of the Institute of Field and Vegetable Crops Novi Sad. These samples were used, ten days after fertilization, to artificially inoculate fungus *Fusarium graminearum*, known to be one of the most frequent deoxynivalenol producers. Immediately after sampling, each of the samples was prepared by grinding in a laboratory mill. After that, the sample was homogenized by mixing. Sample prepared in such a way were packed in plastic bags and stored in a freezer at –20°C until analysis. Prior to each analysis, the samples were allowed to reach room temperature.

Apparatus

The equipment consisted of an LC system — HP1090 Liquid Chromatograph (Hewlett Packard, Palo Alto, CA, USA) with a DAD detector (Hewlett Packard, Palo Alto, CA, USA) and a column Hypersil ODS (100 x 4.6 mm i.d., particle size 5 mm, Agilent Technologies, USA).

For thin-layer chromatography plate preparation, silica gel 60 H was used (Merck, Darmstadt), as well as commercially available plates Alugram Sil G/UV$_{254}$ (Macherey-Nagel, Düren, Germany).

Activated charcoal-alumina-Celite-cation exchange resin (CACC) column. The column was prepared in the following way: a plug of glass wool was inserted into the tapered end of a glass tube (9 cm x 1.5 cm i.d.); then 0.1 g of Celite (545, Merck, Darmstadt), 1.5 g of activated charcoal (Darco G-60, Sigma-Aldrich, Steinheim), alumina (70—230 mesh, Merck, Darmstadt), and Celite mixture (7:5:3) were added, loosely packed, and tapped to level. 2 g of prewashed with 10 cm^3 of methanol cation exchange resin (0.3—0.9 mm, Ke-

mika, Zagreb) were added and lightly compacted above activated charcoal-alumina-Celite by pushing down a second glass wool plug.

The following equipment was used to perform the analyses: magnetic stirrer (MM-530, Tehtnica Železniki, Yugoslavia), sample evaporator (Rotavapor-R, Buchl, Switzerland), 1.5 μm microfiber filters (110 mm i.d., Vicam, Watertown, MA, USA), pipettes of different volumes (Eppendorf, Hamburg, Germany), 5B Advantec filter paper (0.13 mg/circle, 125 mm i.d., Toyo Roshi Kaisha, Ltd., Japan), and single position pump stand (Vicam, Watertown, MA, USA).

Procedure

Principle. DON was extracted from corn with acetonitrile (ACN)-water. After filtration, the crude extract was cleaned-up on CACC column. Cleaned-up extract was evaporated just to dryness, residue redissolved in methanol in case of analysis by reversed-phase LC with DAD detection or in a mixture chloroform-ACN when analyzed by TLC.

Extraction and clean-up. 25.0 g of the sample were extracted with 100 cm^3 of ACN-water (84:16, v/v) and shaken on a magnetic stirrer for 60 minutes. After filtration through Advantec filter paper, 3.0 cm^3 of the extract were applied to the prepared column. The column was then washed with 5 cm^3 of the solvent mixture comprising of ACN-water (84:16, v/v) at about 0.6 cm^3/min. The cleaned-up extract was evaporated to dryness, dissolved in 3 cm^3 of ethyl acetate and quantitatively transferred to an evaporation vessel by triple washing with 1.5 cm^3 ethyl acetate. The eluate was evaporated just to dryness.

Liquid chromatography. The purified, evaporated residue was redissolved in 300 μl methanol, and a 15 μl aliquot of the solution was injected into the LC system at following chromatographic conditions: mobile phase, a mixture of solvents ACN-water (16:84, v/v), λ = 220 nm, flowrate 0.6 cm^3/min. Calibration curves used for quantitative determination were constructed on the basis of the area under the DON chromatographic peaks, using working standard solutions.

Thin-layer chromatography. Evaporated residue was redissolved in 100 μl of the mixture chloroform-ACN (4:1, v/v). 25 and 50 μl of corn extract were applied to the plate using a micropipette, along with 5, 10, 15, 20 and 25 μl of diluted standard DON solution. Chromatographic plates were developed by a mixture of solvents chloroform-acetone-isopropanol (8:1:1, v/v/v). After developing, the plates were left to air-dry for at least 10 minutes, subsequently sprayed with a 20% solution of $AlCl_3$ and dried in a drying oven for 7 minutes at 120°C. After cooling, blue fluorescent spots, resulting from DON presence, appear on the plates in the presence of UV light. Quantitative determination was based on comparison of fluorescence intensity of sample spots with standard solution spots. Fluorescence intensity of individual spots was determined after photographing chromatographic plates by a digital camera, using *Scion Image* program.

RESULTS AND DISSCUSION

Samples of three types of corn, material of different genetic origin, frequently used as parent components of corn hybrids in the moderate belt, were analyzed. Fungus *Fusarium graminearum*, known to be one of the most frequent producers of deoxynivalenol, was artificially inoculated by these samples ten days after fertilization. A chromatogram for one of the analyzed corn samples is presented in Fig. 1, while the results of deoxynivalenol determination in these corn samples are presented in Table 1.

Table 1. DON content in corn samples used for artificial inoculation of *Fusarium graminearum*

Description of lines	Sowing number	DON (μg/g)	
		LC	TLC
Lines derived from a synthetic population Iowa Corn Borer Synthetic (BSCB1)	31	ND	ND
	56	0.57	0.54
Lines of BSSS type (Iowa Stiff Stalk Synthetic)	86	0.33	0.20
	93	0.36	0.20
	157	0.08	ND
Lines derived from Lancaster based material	137	ND	ND
	193	ND	ND
	203	0.08	ND
	209	0.51	0.41

LC — liquid chromatography; TLC — thin-layer chromatography; ND — not detected

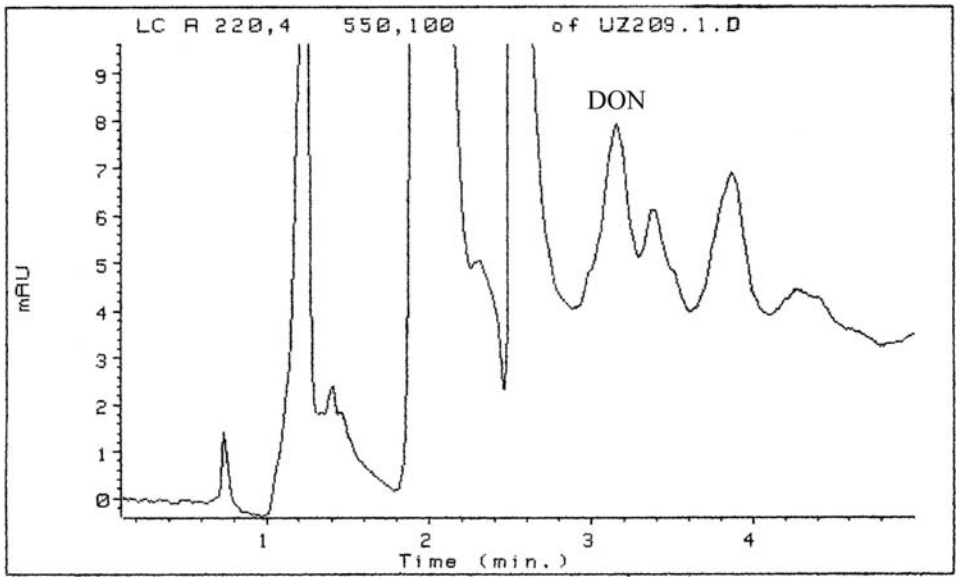

Fig. 1. Chromatogram of a corn sample used for artificial inoculation of *Fusarium graminearum*

As can be seen, in none of the corn samples did DON content surpass the value of 1 μg/g, which is the maximum permitted level for DON legislated in most countries, while in 3 samples the content was below the detection limit of liquid chromatography (0.06 μg/g DON). Besides that, no significant difference is observed in tolerance to fungus *Fusarium graminearum*, as at least one sample appears in each parent line in which the presence of DON is detected. By comparing the results acquired by use of liquid chromatography and thin-layer chromatography, it can be stated that somewhat higher results are obtained when liquid chromatography is used. Such low concentrations of DON in these samples, however, are not surprising, having in mind that all analyzed corn samples belong to the 2003 crop. This year was exceptionally unfavorable for fungus development, *i.e.* mycotoxin development, because of a long-lasting drought period. Consequently, even in these samples by which fungus *Fusarium graminearum*, proven to produce deoxynivalenol, was artificially inoculated, no significant production of this mycotoxin occurred. The absence of humid weather as well as extremely high temperatures, which occurred during the entire plant growth period, caused a poorer development of the inoculated fungus, having as a logical consequence lower DON content than expected. This research presented only a part of long-lasting and detailed investigations undertaken at Department for Maize of the Institute of Field and Vegetable Crops Novi Sad, with a purpose to generate a hybrid of the best quality possible, resistant to fungi and different climatic conditions.

ACKNOWLEDGEMENTS

The work was financed by the Ministry of Science and Environment Protection of the Republic of Serbia (Project: "Development of New and Improvement of the Existing Analytical Methods and Techniques for Monitoring Quality of the Environment", No 1622).

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ОДРЕЂИВАЊЕ ДЕОКСИНИВАЛЕНОЛА У КУКУРУЗУ

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Резиме

Деоксиниваленол, који је по својој хемијској структури 12,13-епокси-3α, 7α,15-трихидрокситрихотец-9-ен-8-он, је микотоксин који припада групи трихотецена. Већина плесни које га продукују припадају роду *Fusarium*, конкретно *Fusarium graminearum* и *Fusarium culmorum*. Без обзира на чињеницу да деоксиниваленол припада групи најмање токсичних трихотецена, због његове изузетно велике распрострањености у усевима широм света, као и због чињенице да је он нека врста индикатора за могуће присуство других, знатно токсичнијих трихотецена, познавање садржаја овог токсина је од велике важности за безбедност хра-

не. Највећа осетљивост на присуство деоксиниваленола у храни је изражена код свиња, а његова акутна токсичност на животињама манифестује се одбијањем хране, повраћањем, дијарејом и коначно губитком тежине.

Примењујући претходно утврђене оптималне услове за одређивање деоксиниваленола течном хроматографијом са DAD детектором, у овом раду је одређен његов садржај у узорцима кукуруза којима је вештачки инокулисана *Fusarium graminearum*. Узорци су представљали материјал различите генетске основе који се користи за синтезу хибрида кукуруза умереног појаса. Добијене вредности садржаја деоксиниваленола нису биле више од максимално дозвољених вредности без обзира на чињеницу да је плесан била вештачки инокулисана. Ово је вероватно последица одсуства влажног времена и изузетно високих температура у периоду када су узорци узимани (2003. година).

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 147—155, 2005

UDC 632.25:636.085.3
615.9:636.085.3

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PREVENTION AND CONTROL OF MYCOTOXICOSES USING MODIFIED CLINOPTILOLITE

INTRODUCTION

Fungi may develop in stored grains following field inoculation. During the process of host colonization fungi utilize plant nutrients, and may also produce toxins that are harmful for humans and animals (Viera, 2003). Fluctuations in animal performance are relatively common, and sudden losses in feed conversion, as well as in other performances, are in general attributed to mycotoxin contaminated feeds.

It is assumed that at least 25% of grains are contaminated with known mycotoxins and probably more with still unidentified toxins (Leibetseder, 1989). Regarding those facts, presence of mycotoxins in animal feed represents a great problem for animal production in our country. During 1999—2000 presence of zearalenone, ochratoxin A, aflatoxin B_1 and T-2 toxin was faund out in 72.3—74.5, 41.2—63.6, 20.1—21.65 and 29.7—45.1% of scrutinized feed samples (Bočarov-Stančić et al., 2000). Moreover, laboratory data demonstrate that over 70% of feed samples are contaminated with two or more mycotoxins in amounts above maximal tolerable levels (Šefer et al., 1994; Mašić et al., 2002).

Based on a risk assessment, the prevention and control of mould development and mycotoxin in the food chain can be undertaken at consecutive levels. Basically, the best way to minimize the risk for a mycotoxin to come into food chain would be to prevent its formation in crop production and or during storage of feedstuffs. The use of contaminated feeds requires either the dilution with non-contaminated grains to an acceptable level or uses some of known methods to alleviate detrimental effects of mycotoxins, but financial, nutritional and toxicological considerations will temper this decision (Danicke, 2002).

PREVENTION AND CONTROL OF MYCOTOXINS

From a practical standpoint, the best means of restricting mycotoxin contamination is by prevention, in particular, by excluding or reducing toxigenic mould growth in the raw and processed material. When mycotoxin contaminated material is suspected or identified, it may be salvaged by the removal of contaminated material by mechanical separation techniques, by chemical extraction or by decontamination/detoxification of the material by physical, chemical or biological techniques (Smith and Moss, 1985).

Prevention. Mycotoxins can be produced by toxigenic moulds growing on (1) living plants, (2) decaying plant material and (3) stored products. Thus, mycotoxin prevention is based on making unfavourable conditions for moulds growing (Sinovec et al., 2000).

Pre-harvest treatment is based on methods of cultivating to improve plant vigor, the judicious use of insecticides and fungicides to reduce insect and fungal infestation, irrigation to avoid drought condition and, more recently, genetically methods to develop commercially acceptable varieties of crops that will be more resistant to toxigenic moulds or will inhibit toxin production.

Post-harvest treatment is mainly based on physical and chemical methods (Frisvad and Samson, 1991). *Physical methods*, in order to prevent or to delay mould spoilage of feeds, include heating, pasteurization and sterilization, cooling, vacuum packing, canning, drying and irradiation. Chemical methods are based on using preservatives (substances which inhibit or kill microorganisms in feeds) and, in practice, organic acids (propionic, sorbic, formic, etc.) are the most common group of chemical used.

Elimination. Once a product is contaminated with mycotoxins there are only two options if it is to be used for animal nutrition: (1) the toxin can be removed and (2) the toxin can be degraded into less toxic or non-toxic compounds (Smith and Moss, 1985).

Physical separation is connected with taking apart loose-shelled kernels, shriveled kernels and discolored or off-colored kernels. Such kernels can be separated either by hand or by color sorters.

Chemical separation includes numerous processes that have been developed to remove toxin from contaminated materials by various extraction techniques. Several procedures can be used to remove toxin and include extraction of toxin with appropriate solvents, simultaneous solvent extraction of oil and toxin and selective extraction of toxins from oil.

Degradation or detoxification are the methods of choice when mycotoxin contaminated material is suspected or identified. Making toxin less toxic or non-toxic (degradation) or extracting from contaminated materials (detoxification) seems to be the most promising way in a combat with mycotoxins. Many physical, chemical and biological methods have been investigated to eliminate (degrade or detoxify) *in situ* mycotoxin in raw material.

Physical methods include mainly heating and irradiation, but the adverse effects of heat treatment on the appearance and nutritive value of the products

makes the practical application of these methods highly doubtful. Better results could be achieved by combination of heating and increasing moisture content (e. g. hydro-thermic treatment).

Chemical methods include a wide range of chemicals that have been tested as reagents for the destruction of toxins such as acids, alkalis, aldehydes, oxidizing agents and several gases. Although technical treatments of contaminated feedstuffs have been proven to successfully degrade several mycotoxins, most of the procedures are expensive or were only labtested. Besides, limitations associated with decontamination process (changing nutritive value) and insufficient degradation makes this process untenable.

Biological methods are based on the natural ability of some microorganisms (bacteria, yeasts) to degrade most molecules of mycotoxins. Some investigations clearly show that where marked reduction in toxin presence in feedstuffs after inoculation of some microorganisms (P a s t e i n e r, 1998). Whether this is due to inhibition of production or breakdown of the toxin has not yet been determinated. The possibility of producing mycotoxin-degrading enzymes would have considerable commercial interest and application.

Nutritive approach. As the ingestion of contaminated feed is the main way of input mycotoxins in to animal body, a nutritional approach is the logical way to reduce impact of mycotoxins. Nutritive treatment is based primarily on raising quality of feed (increasing nutrients) in order to avoid or alleviate negative effects after mycotoxin ingestion (P i v a and G a l v a n o, 1999).

Due to protein synthesis inhibition as prime mode of action, increasing the protein content by 1—2% in the ratio could prevent mycotoxin effects. Besides, adding methionine (glutathione detoxification), phenylalanine (ochratoxin A) or neutral amino acids (competition with triptophane) is recommended in some cases. Increasing antioxidating nutrients (selenium, vitamin E, A, C) is the approach to reduce cell wall altering by action of free radicals.

However, most of these procedures are expensive, but more elegant and inexpensive solution would be a feed additive, which would detoxify the contaminated feeds *in situ*, i. e. during passage through the digestive tract. For this purpose, several adsorbents are used in practice.

Adsorbents are the substances, which are not absorbed from intestines, and they have the ability to physically bind some chemical structures decreasing their absorption. Many adsorbents were tested for binding capacity, and the degree of adsorption depends on the chemical structure of the mycotoxin in relation to the surface properties, and geometry of the adsorbent, as well as their polarity.

Several commercially available adsorbents claimed to bind mycotoxins were tested for adsorption of some mycotoxins. Most studies have used aluminosilicates such as hydrated sodium calcium aluminosilicates and zeolites, or even natural clays containing them. Similarly, the same effects could be achieved by addition of cell wall mannanoligosaccharide obtained from *S. cerevisaea* (D e v e g o w d a et al., 1998).

ZEOLITES

Zeolites are natural hydrated aluminosilicates with unlimited three-dimensional crystalline structure with large surface and negative polarity. Utilization of natural zeolites depends more or less upon their physical and chemical characteristics, like cation exchange, adsorption, hidratation/dehidratation, size and shape of mineral granules, porosity and hardness. Specific adsorption (chemisorptions), as well as other physical and chemical properties of zeolites (gas and fumes adsorption, hydratation, ion exchange, catalytic ability, etc.) is basically determined by crystal structure with pores system, acting as a sieve of molecular range. Natural zeolites, unlike other aluminosilicates, exhibit high adsorption ability even when low concentration of adsorbing material is present. According to the research data, zeolites can adsorb and inactivate about 35% of ochratoxin (Dumić et al., 1998).

The modified mineral adsorbents acquire wider role in human and veterinary medicine (Rodrigues et al., 1997). Changeable cations in zeolite minerals are bound by weak forces into tethraedric structure, with the possibility to be relatively easy removed or replaced by other cations from the solution. Changeable cations are positioned into zeolite canals of clinoptilolite, thus by ion exchange minerals with new characteristics could be produced, without disturbing original clinoptilolite structure (Tomašević-Čanović et al., 1997, 1998). Mineral adsorbent based upon clinoptilolite structure, with NH_4^+ changeable cation was created by technological manufacture of natural zeolites. Roentgen diffraction of natural (Ca-dominant) and modified (NH_4^+ — dominant) clinoptilolite were identical, while results of thermal analysis were different because the part NH_4^+ — ions were liberated upon heating.

In vitro trial. Two studies were conducted under *in vitro* conditions, particularly the feasibility of utilizing a clinoptilolite, aimed at alleviating and/or preventing harmful effects of AFB_1 and OTA. For experimental purposes MC was obtained by technological processing of natural zeolites, bearing a dominant Ca ion in exchangeable position. In order to contain a reversible NH_4^+ cation mineral adsorbent was grinding on particles under 63 μ, mixing with 10% solution of 1M NH_4Cl and keeping at 20°C during 3 subsequent days.

First study of MC binding capacity for AFB_1 was done in electrolyte made by the addition of 0.1M HCl/dm^3 and 0.05M NaCl/dm^3 in water solution, as well as 0.1M NaOH for achieving 2 or 7 pH of solution (Resanović, 2000). The 99% pure crystallyne AFB_1 (Sigma, 1162-62-8) was diluted in methanol in concentration of 0.1 mg AFB_1/mL and after that water was adjoined up to 100 mL. Experimental solutions were carried at 37°C and gentle shaking after modified clinoptilolite addition in the amount of 10 mg/cm^3. Index of absorption was calculated relative to the determined amount of pure AFB_1 in solution, followed the adding modified clinoptilolite after 5 and 30 min, as well as after 6, 24 and 48 hours.

On both pH values adsorbtion of AFB_1 by MC began with fast reaction and almost all AFB_1 were adsorbed in a few first minutes of contact. Later,

between 1—48 h, adsorbtion is slower and significantly less amounts of AFB_1 were binding to active center of mineral adsorbent, which is in agreement with other findings (P h i l i p s et al., 1995). An obtained result clearly shows that saturation of all MC binding center were not achieved by used AFB_1 concentration. AFB_1 adsorption index upon modified clinoptilolite was satisfying, ranging between 90 and 95% on both pH values (2 and 7) in the concentration range of 50—300 μg/g.

Second study of MC binding capacity for ochratoxin A was done in electrolyte made by the addition of 0.1 M HCl/dm^3 and 0.05M NaCl/dm^3 in water solution as well as 0.1 M NaOH for achieving 3.8 pH of solution (Z u - r o v a c - K u z m a n, 2002). The 99% pure crystalline OTA (Sigma, O-1877) was diluted in methanol in the concentration of 0.2 mg OTA/mL and after that water was adjoined up to 100 mL. Experimental solutions were carried at 37°C and gentle shaking after addition modified clinoptilolite in the amount of 10 mg/cm^3. Index of absorption was calculated relative to the determined amount of pure OTA in solution followed the adding modified clinoptilolite after 120 min.

MC exhibits high adsorption ability even when low concentration of adsorbing material is present. According to the research data, zeolites can adsorb and inactivate about 35% of ochratoxin (D u m i c et al., 1998).

The achieved results show that MC could very efficiently remove AFB_1 or OTA from the water solution. The obtained data are comparable with similar results done by P h i l l i p s et al. (1995), K u b e n a et al. (1990, 1993) and H a r v e y at al. (1993) that there was reached high level mycotoxin adsorption by different adsorbents (zeolites, silicates, phylosilicates) on different pH values, as well as different temperature. It has to be pointed that it is not possible to totally compare achieved results with other findings (P h i l l i p s et al., 1995), because accurate physical and chemical characteristics of used adsorbents were not cited.

In vivo trials. Many adsorbents in *in vitro* conditions show the ability to bind and adsorb different mycotoxins, but there are only a few references describing *in vivo* results. Thus, after preliminary study, further investigations were performed in an *in vivo* trial.

The aim of the first study was to examine the protective effects of modified clinoptilolite (MC) on the adverse effects of aflatoxin B_1 (AFB_1) on poultry (R e s a n o v i ć et al., 2001). The three-week long trial was performed on 21-day-old Hybro broilers divided into three groups. The control, as well as experimental groups was fed with mashes without AFB_1, while 0.5% MC was added to the feed for the second experimental group. The AFB_1 was administrated per os to both experimental groups in dose of 0.1 mg/kg BW daily. At the end of the trial all broilers were sacrificed and samples of livers were taken for pathohistological examination, as well as for determination of AFB_1 residues using TLC.

The liver of the treated broilers was enlarged, dark yellow colored and tender in consistence. In some cases punctiformes and maculoses extravasation could be seen. Varied amounts of fatty droplets could be detected in hepatocytes. Progressive fatty vacuolization, i. e. a different degree of fatty meta-

morphosis were spread centrolobularly or panlobularly. In altered areas focuses of extensive necrosis could be seen. Hyperplasia of the intrahepatic bile ducts was also prominent. Residues of AFB_1 were detected in all liver samples.

In the liver samples of the control group, as well as the group offered feed with added MC, no histopathological alteration or presence of AFB_1 residues was detected. The obtained results suggest that modified clinoptilolite represent a strong binding agent for AFB_1 and could prevent the adverse effects of AFB_1.

The aim of the second study was to examine the protective effects of MC on the adverse effects of OTA in poultry (Nedeljković-Trailović et al., 2001). The 42-day long trial was performed on 36 day-old Hybro broilers divided into three groups. After a 14-day long preexperimental period, the experimental groups were offered feed contaminated with OTA in the amount of 1.0 mg/kg, while 0.5% MC was added to the feed for the second experimental group. At the end of the trial all broilers were sacrificed and samples of kidneys were taken for pathohistological examination, as well as for determination of OTA residues using TLC.

Proximal tubules were predominantly affected, while glomerules were chiefly preserved. Cytoplasm of tubulocytes was microgranulated and the nuclei were masked. Vacuolization was noticed in a certain number of altered cells. Foci of acute tubular necrosis were noticed in a few tubules. In some cases weak hemorrhage could be seen in affected areas. Residues of OTA in the amount of 3.23±0.80 ppm were detected in all kidney samples.

In the kidney samples of the group offered contaminated feed with added MC morphological alterations were expressed in the form of intracellular edema. Epithelial cells of proximal tubules were enlarged with opaque cytoplasm, which caused tubule lumen stenosis. Apoptotic body could be noticed between some tubulocytes. Residues of OTA in the amount of 1.43±0.39 ppm were detected in all kidney samples.

CONCLUSIONS

During the process of host colonization fungi utilize plant nutrients, and also may produce toxins that are harmful for humans and animals. The extent of mould growth determines the degree of depletion in the nutrient content of the feedstuff. Mould mainly uses its host as a source of energy, and besides carbohydrates, fat utilization during mould development may be extensive. Reduction in performance of animals is usually credited only to mycotoxin effects, but it would be more accurate to accept an interaction between the reduction in nutrient content and the mycotoxin effects, especially when low levels of mycotoxin are present.

Based on a risk assessment, the prevention and control of mould development and mycotoxin in the food chain can be undertaken at consecutive levels. The use of contaminated feeds requires either the dilution with non-contaminated grains to an acceptable level or uses some of known methods to alleviate

detrimental effects of mycotoxins, but financial, nutritional and toxicological considerations will temper these decisions.

A new approach to mycotoxin control is the use of selective chemisorbents which added to feed are capable of forming irreversible complexes with mycotoxin molecules in the intestinal lumen. Such complexes are not digestible, pass down the digestive tract and are excreted in the feces. The net effect is to reduce the dose of adsorbed toxin to the point that it is below biological threshold. This allows contaminated feed to be fed with minimal losses in performance. The presented data clearly indicated that using modified clinoptilolite as adsorbent might reduce the negative impact of mycotoxins. Adsorptive capacity of modified clinoptilolite, obtained by technological processing of natural zeolites, was significantly increased by changing molecular arrangement and polarity.

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ПРЕВЕНЦИЈА И КОНТРОЛА МИКОТОКСИКОЗА КОРИШЋЕЊЕМ МОДИФИКОВАНОГ КЛИНОПТИЛОЛИТА

Златан Ј. Синовец, Радмила Д. Ресановић

Резиме

Микотоксини представљају сталну опасност у узгоју животиња јер су присутни у хранивима и негативно утичу на здравствено стање и производне резултате чак и у малим количинама. Поремећаји производних резултата животиња су врло чести, а слабија конверзија хране, као и слабији параметри производње, генерално се везују за коришћење хране контаминиране микотоксинима.

С обзиром да је главни пут уношења микотоксина ингестија контаминиране хране, оптимално решење је превенција контаминације спречавањем или редукцијом раста токсин-продукујућих плесни на хранивима. Са друге стране, у

случајевима када се посумња или идентификује присуство микотоксина у храниивима и/или храни елиминација се може извршити механичком сепарацијом, хемијском екстракцијом и деконтаминацијом или детоксификацијом коришћењем физичке, хемијске или биолошке методе.

Ефикасни методи за деконтаминацију хране веома су скупи и непрактични, посебно ако се ради о великој количини хране. Зато могућност избора представља коришћење адсорбената у циљу ублажавања негативних ефеката микотоксина на производне резултате животиња. Адсорбенти имају могућност да вежу микотоксине током пасаже хране кроз дигестивни тракт, односно да смање ресорпцију микотоксина присутних у храни.

Истраживања изведена последњих година, као и резултати сопствене лабораторије, указују да је степен контаминације хране веома висок са тенденцијом раста, што указује на чињеницу да ће микотоксини још дуго бити један од примарних проблема у исхрани животиња. Досадашња искуства, експериментална и практична, указују да коришћење адсорбената може да ублажи и/или превенира негативне ефекте микотоксина. Адсорпциони капацитет модификованог клиноптилолита, добијеног технолошком прерадом зеолита, значајно расте модификацијом молекулске структуре и поларности.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 157—165, 2005

UDC 633.16:632.4

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CAPACITY OF *FUSARIUM* SPECIES ISOLATED FROM BREWER'S BARLEY TO SYNTHESISE ZEARALENONE

ABSTRACT: Fungi of the genus *Fusarium*, known as toxigenic species, are very often parasites and contaminants of brewer's barley. In this paper, the composition of the genus *Fusarium* species in brewer's barley samples and their potential in the zearalenone synthesis were investigated.

The tests were done on different brewer's barley varieties, crop 2003, samples (SSK1, SSK2, SSK3, SSK4, SSK5, SSK6, SSK7, SSK8, SSK9, SSK10 and SSK12) from Kragujevac locality. The isolation and identification of the *Fusarium* species were done according to the methods described by Nelson et al. (1983). The identified *Fusarium* species (6) were tested for their capacity to synthesise zearalenone. The isolates were cultivated on sterilised barley grains at the temperature of 25°C for 14 days, and then the zearalenone concentration was determined by the fluorometric method on the fluorometer "VICAM" series 4.

The following seven *Fusarium* species were isolated from barley samples: *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. poae*, *F. sporotrichioides* and *F. tricinctum. F. poae* was the most distributed species (10.26%). The zearalenone concentration within the range of 12.0 to 430.0 $\mu g \cdot kg^{-1}$ was determined in cultures of barley grain inoculated with *F. avenacuem* (SSK6 and SSK12), *F. culmorum* (SSK8), *F. tricinctum* (SSK1), *F. sporotrichioides* (SSK7 and SSK12) and *F. poae* (SSK5, SSK9 and SSK10). Isolates of *F. equiseti* (SSK2) and *F. poae* (SSK6) did not express capacity to synthesise this toxic metabolite.

KEY WORDS: barley, *Fusarium* species, zearalenone

INTRODUCTION

Barley grains similar to other cereal grains are a good substrate for the development of numerous microorganisms, especially fungi (Škrinjar, 2001; Kocić-Tanackov and Škrinjar, 2004). The process of contamination and fungi development actually starts in the field (Schollenberger et al., 2005), during harvest, transport and storage or is activated in the certain stages of the grain technological processing. Fungi of the genus *Fusarium*, a

common contaminant of cereal grains (Duraković and Duraković, 2003), can cause the loss of colour, change of flavour or essence, loss in the nutritive value, quality of milling, cooking and roasting, contamination with mycotoxins, etc. (Šarić i sar., 1980; Bočarov-Stančić, 2001). Infection with *Fusarium* species is very critical for barley that is malted and which malt is used in brewing beer.

Species of the genus *Fusarium* can deteriorate brewer's barley quality in several ways:

— they decrease the average size, weight and nutritive value of the grain (Salas et al., 1999; Vanne and Haikara, 2001);

— they inhibit barley grain germination during the process of malting, decrease the α-amylase activity and cause beer gushing (Noots et al., 1998; Kleemola et al., 2001; Schwarz et al., 2001);

— they synthesise alkaline proteases that hydrolyse brewer's barley proteins (Pekkarinen et al., 2003);

— they produce mycotoxins (zearalenone, DON, DAS, fumonisine, moniliformin, etc.) (Schollenberger et al., 2005) that are thermostable and are not destroyed during the malting process, wort production, beer pasteurisation and therefore can be transmitted to beer (Scott, 1996).

Due to all above mentioned and especially due to their capacity to produce mycotoxins that are harmful to human health, the objective of the present study was to analyse both, the composition of the genus *Fusarium* species in brewer's barley samples that is used as a raw material in the beer production and the potential of isolated species to synthesise zearalenone.

MATERIAL AND METHODS

Samples of brewing barley. Two-rowed winter barley samples, crop 2003, were collected in storage rooms at Kragujevac locality. A total of 3 kg each was sampled from the following 11 different varieties of brewer's barley: SSK1, SSK2, SSK3, SSK4, SSK5, SSK6, SSK7, SSK8, SSK9, SSK10 and SSK12.

Isolation and identification of fungi. A total of 100 barley kernels was taken from each sample and treated by shaking with 100 ml of 4% NaOCl solution for 2 minutes. Then, the sample was rinsed twice with 100 ml of sterile distilled water. Filter papers soaked with 10 ml of sterile distilled water were placed in four 130 mm Petri dishes. Twenty five barley kernels were placed in each of four Petri dishes. The incubation of cultivated samples was proceeded at the temperature of 25°C during the 14-day period. An additional wetting of the filter paper with 7 ml of sterile distilled water was done on the 7th day. In order to obtain a pure culture and identification fungi, on the basis of the macro-morphological properties of colonies, fungi were re-cultivated to the Sabouraud maltose agar (SMA) or to the Capek medium. According to macro-morphological properties, all colonies, which were assumed to be representatives of the genus *Aspergillus* or *Penicillium*, were transferred to the Capek medium,

while all other colonies remained on the Sabouraud maltose agar (SMA). Cultivated Petri dishes were incubated at 25°C for 7 days. Subsequent to the incubation, isolates, determined to belong to the genus *Fusarium*, were used for producing monosporous cultures on the potato dextrose agar (PDA) and 2% carnation leaf agar (CLA) according the procedure described by Nelson et al. (1983). In order to stimulate the formation of conidiogenic structures, cultivated media were incubated in the 12 h UV light/dark cyclic regime. Monosporous cultures were incubated under stated conditions at the temperature of 25°C for 10 to 14 days.

The determination of isolated pure fungal cultures was done in accordance with taxonomic properties described by Ellis (1971, 1976), Nelson et al. (1983) and Samson and van Reenen-Hoekstra (1988).

In vitro zearalenone production. Six species of the genus *Fusarium* were investigated for their potential in zearalenone synthesis. Zearalenone-free barley sample were used for this investigation. Eleven sub-samples of 20 g, taken from the primary sample, were coarsely milled and autoclaved at 121°C for 30 minutes. Afterwards they were inoculated with a suspension of *Fusarium* spp. Number *Fusarium* spp. spores was determined according to the dilution method. The inoculum was incubated at 25°C for 14 days, with the periodic addition of sterile distilled water and daily shaking of the sample.

Zearalenone analysis. The zearalenone isolation was done in barley samples naturally contaminated with species of the genus *Fusarium* and in barley samples that had been inoculated with cultures of *Fusarium* spp. The zearalenone extraction from the barley samples was done by mixing 20 g of milled sample with 2 g NaCl and 50 mL of the mixture of 90:10 acetonitrile-water in the high-speed glass blender for 2 minutes. The obtained extract was filtrated through the filter paper, and then 10 mL of the filtrate was mixed with 40 mL of washing buffer (PBS/0.1% Tween-20, VICAM). The homogenised mixture was filtrated through the 1.0 μg microfibre filter. The obtained filtrate (10 mL) was passed through a Zearalenone Test immunoaffinity column (1—2 drops/second). 10 mL of washing buffer and 10 mL of distilled water (1—2 drops/second) were passed through the column. Zearalenone was rinsed out from the column with 1.0 mL of methanol (HPLC purity) into the glass test tube by speed of approximately 1 drop/second. Then, 1 mL of the Zearala Test developer (VICAM) was added to the tube and the content was well homogenised. The test tube was placed into the graduated fluorometer (VICAM series 4), in which the zearalenone concentration was read in PPB (VICAM, 1997).

RESULTS AND DISCUSSION

Isolated fungal species. Fourteen different fungal species were isolated from two-rowed winter barley samples. On the basis of their taxonomic properties these species were classified into the following genera: *Alternaria, Cladosporium, Fusarium, Mycelia sterilia, Rhizopus, Scopulariopsis* and *Ulocladium* (Figure 1).

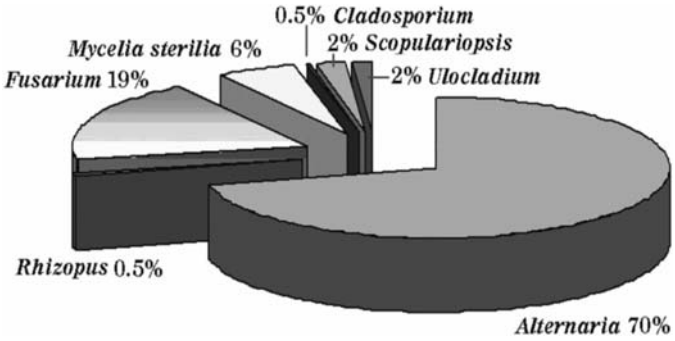

Figure 1. Distribution (%) of certain fungal genera in two-rowed winter barley samples

Out of totally isolated mycopopulations, the highest distribution (70%) in all samples was determined for the species of the genus *Alternaria* (Fig. 1): *A. alternata* (54%), *A. brassicicola* (14%) and *A. tenuissima* (2%). *A. brassicicola* was distributed with 19% within the genus *Alternaria*.

The genus *Fusarium* in the studied barley samples ranked second by its distribution (19%), but it was represented by a greater number of different species (7) than the genus *Alternaria*. The species *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. poae*, *F. sporotrichioides* and *F. tricinctum* were isolated from the genus *Fusarium*. *F. poae* dominated in all barley samples (Figure 2).

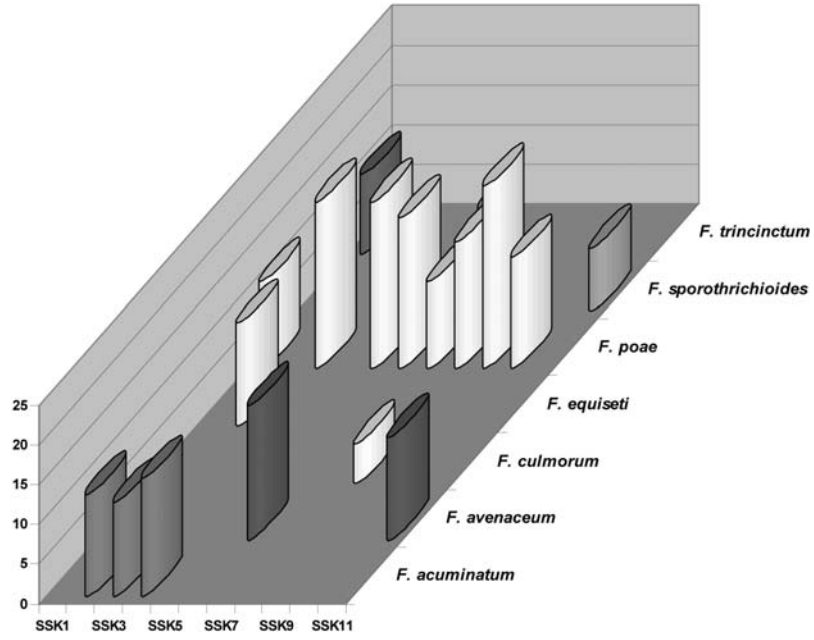

Figure 2. Distribution (%) of certain species of the genus *Fusarium* in two-rowed winter barley samples

One species each was isolated from genera *Cladosporium*, *Rhizopus*, *Scopulariopsis* and *Ulocladium* as follows: *C. herbarum*, *R. stolonifer*, *S. fusca* and *U. charatum*.

The obtained data on the distribution of genera *Alternaria* and *Fusarium* are in accordance with the literature data obtained by international and national researchers. Mycological studies on 260 samples of wheat, barley and oat showed similar results in relation to the frequencies of these two genera in Norway (K o s i a k et al., 2004). A high distribution of *F. poae* (approximately 20%) was determined in barley grain in the U.S.A. (S a l a s et al., 1999) and Czech Republic (H y s e k et al., 2000), as well as, in oat grain in Canada (T e k a u z, 2002). The dominance of *Alternaria* spp. (up to 72%) and *Fusarium* spp., especially *F. poae* (53%), in barley, i.e. wheat grain, was determined by B o č a r o v - S t a n č i ć et al. (2001), i.e. B a l a ž et al. (2003) and D o p u đ a and L e v i ć (2004), respectively.

Zearalenone content. The determined zearalenone content in barley grain samples naturally contaminated with species of the genus *Fusarium* ranged from 5.2 to 52.0 $\mu g \cdot kg^{-1}$ (Table 1). These levels were higher than the maximum tolerable levels for zearalenone in cereals according to the Regulations on critical amounts of pesticides, metal, metalloids and other toxic substances, homotherapeutics, antibiotics and other substances included into food commodities (Official Gazette of FRY, Issue 5, 1992, Article 15).

Table 1. Natural occurrence ($\mu g \cdot kg^{-1}$) of zearalenone in barley samples

Sample number	Barley sample	Zearalenone content ($\mu g \cdot kg^{-1}$)
1.	SSK1	22.0
2.	SSK2	5.2
3.	SSK3	8.6
4.	SSK4	5.8
5.	SSK5	7.1
6.	SSK6	8.2
7.	SSK7	52.0
8.	SSK8	24.0
9.	SSK9	37.0
10.	SSK10	19.0
11.	SSK12	16.0

The zearalenone content in barley samples inoculated with cultures of *Fusarium* spp. ranged from 0.0 to 430 $\mu g \cdot kg^{-1}$ (Table 2). After the 14-day incubation on sterilised barley grains, *F. avenaceum* isolates, originating from barley samples SSK6 and SSK12, synthesised 430—330 $\mu g \cdot kg^{-1}$ of zearalenone.

Table 2. Zearalenone content (μg · kg^{-1}) in barley samples incubated with isolated cultures of *Fusarium* spp.

Sample number	*Fusarium* species	Origin of isolate of *Fusarium spp.* (barley variety)	Concentration of spores in suspension for barley grain inoculation (spore ml^{-1})	Zearalenone (μg · kg^{-1})
1.	*F. avenaceum*	SSK6	8x10^5	430,00
2.	*F. avenaceum*	SSK12	1x10^5	330,00
3.	*F. culmorum*	SSK8	1x10^5	220,00
4.	*F. equiseti*	SSK2	1x10^5	0,00
5.	*F. tricinctum*	SSK1	4x10^5	220,00
6.	*F. sporotrichioides*	SSK7	1x10^6	290,00
7.	*F. sporotrichioides*	SSK12	8x10^6	340,00
8.	*F. poae*	SSK5	2—10^5	120,00
9.	*F. poae*	SSK9	2—10^5	120,00
10.	*F. poae*	SSK10	16—10^6	140,00
11.	*F. poae*	SSK6	1—10^5	0,00

An isolate of *F. sporotrichioides*, originating from the barley sample SSK7, synthesised 290.0 μg · kg^{-1} of zearalenone while another, originating from the sample SSK12, synthesised 340.00 μg · kg^{-1} of zearalenone. *F. poae* isolates, originating from three different barley samples (SSK5, SSK9 and SSK10), synthesised 120.00—140.00 μg · kg^{-1} of zearalenone. *F. culmorum* and *F. tricinctum* synthesised equal content of zearalenone (220.00 μg · kg^{-1}), while a *F. equiseti* isolate and a *F. poae* isolate did not synthesise zearalenone.

In cases when a greater initial number of spores for the same species of the genus *Fusarium* was used for the inoculation of sterilised barley grain, the fungi produced a higher zearalenone content (Table 2): two isolates of *F. avenaceum* (samples No. 1 and 2) and *F. sporotrichioides* (samples No. 6 and 7), and especially three isolates of *F. poae* (samples No. 8, 9, and 10).

In comparison with other species, the lowest zearalenone content in three isolates of *F. poae* (samples No. 8, 9 and 10) or even non detectable contents of this mycotoxin in cultures of *F. equiseti* (sample No. 4) and *F. poae* (sample No. 11) most probably point out to the fact that these species or particular isolates had no genetic background for the zearalenone synthesis.

CONCLUSIONS

According to the obtained results on the composition of mycopopulations and zearalenone content in naturally and artificially inoculated brewer's barley grains with species of the genus *Fusarium* the following conclusions can be drawn:

— fungi of the genera *Alternaria* (70%) and *Fusarium* (19%) dominate in samples of brewer's barley, while percentage of remaining fungal genera

ranged from 6—2% (*Mycelia sterilia*, *Ulocladium*, *Scopularipsis*) to 0.5% (*Rhizopus*, *Cladosporium*);

— *A. alternata* (54,6%) and *F. poae* (10,26%), were the most distributed within the genus *Alternaria*, i.e. *Fusarium*, respectively;

— several species of the genus *Fusarium* (*F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. poae*, *F. sporotrichioides* and *F. tricinctum*) were isolated from brewer's barley grain;

— natural occurrence of zearalenone varied from 5.2 to 52.0 $\mu g \cdot kg^{-1}$, i.e. it was higher than the maximum tolerable levels prescribed by legal regulations for content in cereals;

— the zearalenone content varied from 120.0 to 430.0 $\mu g \cdot kg^{-1}$ in cultures of *F. avenacuem* (2), *F. culmorum* (1), *F. tricinctum* (1), *F. sporotrichioides* (2) and *F. poae* (3);

— the highest, i.e. lowest potential in zearalenone synthesis was expressed by the isolates of *F. avenaceum* and *F. sporotrichioides*, i.e. *F. equiseti* and *F. poae*, respectively;

— only one isolate of *F. equiseti* (sample No. 4) and of *F. poae* (sample No. 11) did not synthesised zearalenone;

— the same species of the genus *Fusarium* synthesise a higher zearalenone content if a higher concentration of spore suspension is used for the inoculation of sterilised barley grain.

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СПОСОБНОСТ СИНТЕЗЕ ЗЕАРАЛЕНОНА *FUSARIUM* ВРСТА ИЗОЛОВАНИХ ИЗ ПИВСКОГ ЈЕЧМА

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Резиме

Гљиве из рода *Fusarium*, познате као токсигене врсте, чести су паразити и контаминанти пивског јечма. У овом раду испитиван је састав врста рода *Fusarium*, пореклом из пивског јечма, као и њихов потенцијал у синтези зеараленона.

У раду су испитивања вршена на узорцима различитих сорти пивског јечма (SSK1, SSK2, SSK3, SSK4, SSK5, SSK6, SSK7, SSK8, SSK9, SSK10 и SSK12), рода 2003. године, који су прикупљени у локалитету Крагујевац. Изоловање и идентификација *Fusarium* врста извршено је према методама које су описали Нелсон и сар. (1983). Идентификоване *Fusarium* врсте (6) тестиране су на способност синтезе зеараленона. Изолати су гајени на стерилисаним зрнима јечма, инкубирани на 25°C у трајању 14 дана, након чега је одређена концентрација зеараленона флуорометријском методом на флуорометру „VICAM" серије 4.

Из узорака јечма изоловано је 7 *Fusarium* врста, и то: *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. poae*, *F. sporotrichioides* и *F. tricinctum.* Најзаступљенија је била врста *F. poae* (10,26%). У културама инокулисаног зрна јечма са *F. avenacuem* (SSK6 и SSK12), *F. culmorum* (SSK8), *F. tricinctum* (SSK1), *F. sporotrichioides* (SSK7 і SSK12) и *F. poae* (SSK5, SSK9 и SSK10) утврђена је концентрација зеараленона од 120,0 до 430,0 $\mu g \cdot kg^{-1}$. *F. equiseti* (SSK2) и *F. poae* (SSK6) нису показале способност синтезе овог токсичног метаболита.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 167—171, 2005

UDC 633.31:632.4

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VIRULENCE OF FUSARIUM SPECIES TO ALFALFA SEEDLINGS

ABSTRACT: In *in vitro* conditions, virulence of 91 isolates of species *Fusarium* genus (*F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* and *F. graminearum*) towards alfalfa seedlings was investigated. Isolates of investigated species originated from diseased alfalfa plants collected at four locations in Serbia based on symptoms of wilting caused by fusarium and root rotting. Pathogenicity and virulence of investigated isolates of *Fusarium* spp. were determined by visual evaluation of inoculated seedlings of cultivar K28 in laboratory conditions. All isolated of investigated species had pathogenic effect on alfalfa seedlings, which expressed symptoms such as necrosis of root, moist rotting and "melting of seedlings". Colour of necrotic root tissue varied from light brown, brown, lipstick red to explicit black, depending on the *Fusarium* species. Strong virulence was established in 48 isolates, medium virulence in 31 and weak virulence in 12 isolates.

KEY WORDS: alfalfa (*Medicago sativa* L.), *Fusarium* spp., seedlings, virulence

INTRODUCTION

Longevity of alfalfa crops is conditioned by condition of root system, primarily in root neck and small roots in the most active zone of the root. Therefore, rotting of root and root neck, since frequent in case of alfalfa, is one of the most important factors which reduces the longevity of alfalfa crops, yield and quality of alfalfa. *Fusarium* species isolated from diseased alfalfa roots, especially root neck, were more frequent than any other type of fungus. Except type of rot, symptoms of disease are manifested in form of chlorisis of leaves and lower plants.

Fusarium oxysporum Schlect., *F. solani* (Mart.) Appel & Wollenw. Emend. Snyder & Hansen and *F. roseum* Lk. ex Fr. Emend. Snyd. & Hans. are species constantly isolated from alfalfa root (O'Rourke and Millar, 1966; Graham et al., 1979). According to the data from literature regarding the

etiology of root rot, other *Fusarium* species are also important, such as *F. avenaceum* (Fr.) Sacc., *F. arthrosporioides* Sherb., *F. culmorum* (W. G. Smith) Sacc., *F. scirpi* Lamb, et Fautr. var. *acuminatum, F. poae* (Peck) Wr., *F. sambucinum* Fuckel and *F. tricinctum* (Corda) Sacc. (Erwin, 1954; Chi et al., 1964; Nedelnik, 1988; Hwang et al., 1989).

On the territory of Serbia, from alfalfa plants demonstrating symptoms of wilting and rotting of root and root neck, most frequently isolated were numerous *Fusarium* species (Milijić et al., 1984, 1986, Vico et al., 1996; Krnjaja and Ivanović, 2001; Krnjaja et al., 2002). Damage caused by nematodes (*Pratylenchus penetrans* Cobb) enables more intensive development of *Fusarium* wilt (*Fusarium oxysporum* var. *medicaginis*) on alfalfa root (Grujičić et al., 1984). *Fusarium* species were isolated also from alfalfa seed (Krnjaja et al., 2003), which could be a source of further spreading of pathogens on vegetative parts of the plant and cause problem in establishing of alfalfa crops.

Considering how frequent incidences of fuzariozing wilting and root rotting are in alfalfa crops on the territory of Serbia as well as great number of isolated *Fusarium* species, the objective of this research was to investigate virulence of different types of *Fusarium* species to alfalfa seedlings.

MATERIAL AND METHODS

Applying standard phyto-pathological methods, isolates of *Fusarium* spp. were separated from alfalfa plants, collected in the vicinity of Belgrade (Zemun, Padinska Skela), Novi Sad (Rimski Šančevi) and Kruševac, with symptoms of *Fusarium* wilt and root rot. According to morphological characteristics described by Nelson et al. (1983) and Burgess et al. (1994), investigated isolates belong to the following species: *F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* and *F. graminearum*.

In *in vitro* conditions, inoculation of seedlings of cultivar K28 was carried out according to the method described by Chi et al. (1964). Alfalfa seed was disinfected in 95% ethanol for 10 seconds, subsequently in 7% sodium hypo chlorite (NaOCl) for 10 minutes, rinsed in sterile water and dried at room temperature. Section of the colony of investigated isolates 4—5 mm^2 in diameter and five days old was placed in the centre of Petri dish with 1.7% potato dextrose agar. Around the section of colony, at distance of 2 cm in diameter, 15 seeds of alfalfa were placed. Petri dishes were incubated at room temperature. After two days, primary roots were placed so that their tips were touching the rim of the fungus colony in the centre of Petri dish.

After 10 day incubation, degree of pathogenicity (virulence) of isolates was evaluated by visual inspection of necrotic areas according to the following scale: 0 = no virulence (no necrotic areas on the root), 1 = weak virulence (necrosis on the tip of the root), 2 = medium virulence (root and low part of the stem — stem butt, but necrosis or fungus mycelium didn't spread on leaves and upper section of stem) and 3 = strong virulence (necrosis or fungus

mycelium have spread entirely over root, stems and leaves, and in some cases even "melting" of seedlings occurred).

RESULTS OF INVESTIGATION

By inoculation of alfalfa seedlings in laboratory conditions it was established that all 11 isolates of *Fusarium* species were pathogenic. Two days subsequent to contact between root and fungus colony necrosis appeared in all investigated isolates. Necrosis spread vertically and after 10 days of incubation isolates of strong virulence were completely spread over root, stems and leaves of seedlings, causing in some cases so called "melting" of seedlings (Fig. 1).

Necrotic tissue of the root was rotten and decayed. In case of isolates which haven't caused spreading of necrosis further from the root, herbaceous parts of seedlings which weren't necrotic tore easily when pulled from disintegrated and softened root tissue. Colour of necrotic root parts was light brown, brown, red brown, and lipstick red to black (Fig. 1). Roots of control seedlings were without necrosis, healthy and with stabile structure.

Among investigated isolates of *Fusarium* spp., 48 demonstrated strong virulence, 31 isolates medium virulence, and weak virulence 12 isolates (Tab. 1). Among investigated isolates of *F. oxysporum*, 16 isolates demonstrated strong virulence (grade 3), 14 isolates demonstrated medium virulence (grade 2), and 4 isolates demonstrated weak virulence (grade 1). Among investigated isolates of *F. solani*, 16 isolates demonstrated strong virulence, and one isolate demonstrated medium virulence. Seven isolates of *F. acuminatum* demonstrated strong, and two weak virulence. All investigated isolates of *F. equiseti* demonstrated weak virulence. Six isolates of *F. arthrosporioides* demonstrated medium virulence and one isolate strong virulence. All investigated isolates of *F. proliferatum* demonstrated strong virulence. One isolate of *F. avenaceum* demonstrated strong, and two isolates medium virulence. All investigated isolates of species *F. semitectum* and *F. sporotrichioides* demonstrated medium virulence. One isolate of *F. tricinctum* demonstrated strong, and one medium virulence. One investigated isolate of *F. graminearum* demonstrated strong virulence (Tab. 1).

DISCUSSION

In the test for control of pathogenicity of *Fusarium* species to alfalfa seedlings pathogenicity of isolates of all investigated *Fusarium* spp. was established, as well as high sensitivity of alfalfa in pheno — stage of seedlings. Similar results were confirmed in previous investigations (Weimer, 1927, 1928, loc. cit. Schmittenner, 1964; Chi et al., 1964; Hancock, 1983, 1985), when it was proved that *Fusarium* species can infect alfalfa seedlings. Weimer (1927, loc. cit. Schmittenner, 1964) has established that *Fusarium* spp. and *Rhizoctonia* spp., isolated from rotten root neck and root of alfalfa cause moist rotting of seedlings. *F. oxysporum* f. sp. *medicaginis* is also

pathogenic to alfalfa seedlings (W e i m e r, 1928 loc. cit. S c h m i t t e n n e r, 1964). Isloates of *Rhizoctonia* spp. and *F. oxysporum* f. sp. *medicaginis* originating from alfalfa have demonstrated strong pathogenicity not only to alfalfa seedlings, but also to seedlings of bird's foot trefoil, red and white clover (V i c o, 1997). Histological researches have shown that penetration and further development of *F. avenaceum, F. oxysporum* and *F. solani* are similar in case of alfalfa and red clover seedlings (C h i et al., 1964). The results obtained by these authors indicate that all three *Fusarium* species have penetrated into uninjured epidermal root cells, seed coat and cotyledons by direct penetration without formation of apresoria. The penetration was intercellular and intracellular. Most frequently, pathogens penetrated the meristematic tissue, although regions of cell magnification and differentiation were also affected. Pathogens colonize completely the cortex of the alfalfa root. All three species colonize xylem, and *F. solani* is most limited when developing in epidermal and cortical tissues. Tips of roots are affected by pathogens in two-day old seedlings. Seed coat was colonized quickly by all three fungis species. Lots of hyphae were found in cotyledons, leaf primordia and young stems. No difference was established between plants which became diseased naturally and artificially inoculated plants in regard to development of fungus (C h i et al., 1964).

CONCLUSION

Investigations of pathogenicity and virulence of *Fusarium* species *in vitro* have lead to the following conclusions:

— *F. oxysporum, F. solani, F. acuminatum, F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum, F. tricinctum, F. sporotrichioides* and *F. graminearum* are pathogen to seedlings of K28 alfalfa;

— Main symptoms of disease are change of colour from brown to black depending on the investigated species, necrosis of root, moist rotting and "melting of seedlings";

— Most of the isolates demonstrated strong virulence (48) to medium virulence (31), and only 12 weak virulence, none of the isolates were of no virulence;

— *F. solani* and *F. acuminatum* demonstrated mostly strong virulence, *F. arthrosporioides* medium virulence, whereas virulence of *F. oxysporum* varied from weak to strong;

— All isolated Species of *F. proliferatum* demonstrated strong virulence, and of *F. equiseti* weak virulence;

— Less present species on alfalfa root, such as *F. avenaceum, F. tricinctum* and *F. graminearum*, demonstrated medium to strong virulence, and *F. semitectum* and *F. sporotrichioides* medium virulence.

ВИРУЛЕНТНОСТ ВРСТА РОДА *FUSARIUM* ПРЕМА КЛИЈАНЦИМА ЛУЦЕРКЕ

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Резиме

У *in vitro* условима проучена је вирулентност 91-ог изолата врста рода *Fusarium* (*F. oxysporum, F. solani, F. acuminatum. F. equiseti, F. arthrosporioides, F. proliferatum, F. avenaceum, F. semitectum. F. tricinctum, F. sporotrichioides* и *F. graminearum*) према клијанцима луцерке. Изолати испитиваних врста пореклом су из оболелих биљака луцерке које су прикупљене из четири локалитета у Србији на основу симптома фузариозног увенућа и трулежи корена. Патогеност и вирулентност испитиваних изолата *Fusarium* spp. утврђени су визуелним оцењивањем инокулисаних клијанаца сорте К28 у лабораторијским условима. Сви изолати испитиваних врста патогени су према клијанцима луцерке, који су испољили симптоме у виду некрозе корена, влажне трулежи и „топљења клијанаца". Боја некротираног ткива корена варира од светло смеђе, смеђе, црвено-смеђе, кармин-црвене до изразито црне, зависно од врсте рода *Fusarium*. Јаку вирулентност испољило је 48 изолата, средњу 31 изолат, а слабу 12 изолата.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 173—179, 2005

UDC 636.4.085.3:[615.9:582.28

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EFFICACY OF DIFFERENT ADSORBENTS IN ALLEVIATING ZEARALENONE EFFECTS ON PERFORMANCE OF PIGS

ABSTRACT: The investigation of the influence of zearalenone effects on performance of pigs and the possibility of the application of modified clinoptilolite (MC) and esterified glucomanane (EGM) in alleviating and/or prevention of harmful effects was performed by the experiment of pigs nutrition. The experiment lasted for 31 days, and the group of pigs was fed with uncontaminated feed, while experimental groups were fed with feed containing 3.84 ppm of zearalenone. In pig feed of the second and third experimental group MC and EGM in the amount of 0,2 and 0,1% were added.

The pigs of the control group accomplished daily growth of 0.569 kg with feed conversion of 1.793 kg. The presence of F-2 toxin in feed affected adversely production results. The addition of MC or EGM to contaminated feed improved production results, but not to the level of the control group. The obtained results point at the fact that adsorbents can only partially prevent harmful effects of F-2 toxin.

KEY WORDS: adsorbents, pigs, production results, zearalenone

INTRODUCTION

Mycotoxin zearalenone is one of the most important secondary metabolites of *Fusarium* fungi. It is a phytoestrogen, lacton 6-b-resorcilic acid (6-[10--hydroxy-6-oxy-trans-1-undecenil]-b-resorcilic acid). Biological activity of this toxin can be explained as competition with 17-b-oestradiol for the specific binding points at oestrogenic receptors.

Pigs are more sensitive (SCF 2000) than other animal species, like ruminants and poultry, especially fowl. Oestrogenic syndrome (K r o g h, 1987) is dominant phenomenon in clinical picture. Hyperemia and oedema of vulva with lightly clouded mucilaginous vaginal discharge are symptoms of intoxication. In severe cases vaginal and rectal prolapse can be seen (S y d e n h a m

et al., 1988). Clinical signs are more expressed in immature than in mature animals (James and Smith, 1982; Kuiper-Goodman et al., 1987).

Corn is usually affected, especially hybrid sorts with long vegetation and a lot of humidity in the moment of ingathering, but the fungi growth and toxin synthesis prolong further during storage (EFSA 2004). Mycotoxin content in feedstuffs and/or feed is usually lower (Šefer et al., 1994; Mašić et al., 2001) and it doesn't provoke significant health disturbance, so it is manifested as a chronic mycotoxicosis (Humphreys, 1988). Chronic primary mycotoxicosis is manifested by different grade of decrease of performances: retarded growth, lower feed conversion, feed refusal, vomiting and diarrhea (Šefer, 2000).

Numerous strategies for the detoxification of mycotoxin-contaminated crops have been reported in the scientific literature and recently reported (Phillips et al., 1994). These include methods of mycotoxin extraction, separation, decontamination, thermal inactivation, irradiation, microbial degradation, and treatment with variety of chemicals (Piva and Galvano, 1999). Many of these techniques are impractical, ineffective, and/or potentially unsafe (Sinovec et al., 2000). A new approach to mycotoxin control is the use of selective chemisorbents which added to feed is capable of forming irreversible complexes with mycotoxin molecules in the intestinal lumen. Such complexes are not digestible, pass down the digestive tract and are excreted in the feces. The net effect is to reduce the dose of adsorbed toxin to the point that it is below the biological threshold. This allows contaminated feed to be fed with minimal losses in performance.

Previous investigations have shown quite satisfactory effects in binding F-2 toxin by zeolite, especially after organic modification, i.e. after the change of superficial charge and hydrophobia when better adsorption (over 90%) of less polar molecules has been obtained (Tomašević-Čanović et al., 2001). Biotechnology also offers application of organic adsorbents, especially glucans (Devegowda et al., 1998) which have been obtained from cell wall of yeast *Saccharomices cerevisiae* and which possess significant capability of adsorption of a wide spectrum of mycotoxins based on bipolar molecular charge.

The primary objective of this study was not only to investigate the harmful effects of dietary F-2 toxin on piglet's performances, but also to assess the feasibility of utilizing a modified clinoptilolite and esterified glucomannane aimed at alleviating and/or preventing its harmful effects.

MATERIALS AND METHODS

Animals. Experiment was performed on a total of 32 pigs of Swedish and Dutch Landrace cross-breed. Pigs were of both sexes with 14.40 kg average body weight, 60 days old.

Diets. All groups of pigs were fed with a commercial feed mixture which consisted of standard feedstuffs and contained enough nutrients to satisfy the piglet requirements. Corn naturally contaminated with zearalenone (12.8 ppm)

was used in the amount of 30% in experimental groups. Zearalenone free corn was given to pigs in control group. Modified clinoptilolite (MC), as mineral adsorbent was introduced in contaminated feed of the second experimental group and esterified glucomannane (EGM) as organic adsorbent in contaminated feed of the third experimental group.

Experimental design. A trial 31 days long was conducted on a total of 32 pigs, divided into four groups, each containing 8 pigs. Control group (K) received feed without toxin, while experimental groups offered F2-toxin contaminated feed in an amount of 3.84 ppm. Pigs in the first experimental group (O-I) were given feed with toxin only. At the same time, 0,2% MC (Minazel Plus) and 0,1% EGM (Mycosorb) was added to the feed for the second and the third experimental group, respectively.

Data and sample collection. The following data were monitored during the trial: body weight (BW), weight gain (AWG), feed intake (FI) and feed consumption ratio (FCR).

Statistical analysis. All data were statistically processed (S n e d e c o r and C o c h r a n, 1971) and an appraisal was made of the significance of differences in mean values between the groups of pigs.

RESULTS

From Table 1 it can be seen that pigs were of adequate body weight for their breed and age at the beginning of the trial without significant differences between groups (p 0.05). Obtained performances differed at the end of the trial between control and experimental groups. By comparing experimental group of pigs fed with contaminated feed (O-I) with control group (K) it could be seen that zearalenone had harmful influence on body weight which was decreased for 10.84% ($p > 0.05$).

Table 1. Body weight and weight gain* of pigs, [kg]

Group	n	BW initial	BW final	AWG	
K	8	14,38±1,53	32,00±3,38	0,569±0,08	a
O-I	8	14,38±1,22	28,53±4,87	0,456±0,13	b
O-II	8	14,31±1,22	30,13±1,92	0,510±0,03	
O-III	8	14,00±1,22	30,00±2,83	0,516±0,03	

* Value expressed as x±Sd $^{a, b}$ $p < 0.05$

Although body weight is a respectable parameter, daily growth is reputed as better indicator of feed quality. It is particularly a pointer of hygiene propriety and animal health. Comparing experimental group (O-I) with control group of pigs (K) considerably lower daily growth, for 19.86%, could be seen ($p < 0.05$).

Application of adsorbents ensured significant recompense of lost body weight, i.e. gain of satisfactory daily growth. Average body weight and daily

weight gain of pigs fed with contaminated feed with addition of adsorbents were still lower than control values, but at the same time higher than average body weight and daily growth of pigs fed with contaminated feed without protectors. There wasn't any difference in results due to type of adsorbent.

Presence of F-2 toxin reduced feed consumption (Table 2) for 5.89%, but addition of adsorbents entirely eliminated harmful effects. Pigs fed with contaminated feed with application of adsorbents achieved larger consumption than pigs fed with the same feed without adsorbent, but it was still lower than in pigs in the control group. There wasn't any significant difference in results due to type of adsorbent.

Table 2. Feed intake and feed gain ratio, [kg]

	n	Group			
		K	O-I	O-II	O-III
FI	8	1,020	0,960	0,978	0,988
FCR	8	1,793	2,105	1,918	1,915

Feed conversion, as interaction of growth and consumption, differed between control and experimental groups. Experimental group fed with contaminated feed attained higher feed consumption ratio for 14.82%. Utilizing of adsorbents significantly alleviated harmful effects of zearalenone, but not entirely. Feed consumption ratio of pigs fed with contaminated feed with the addition of modified clinoptilolite was still higher than control value for 6.52%, but it was lower than feed conversion of pigs fed with contaminated feed only.

Feed consumption ratio of pigs fed with contaminated feed with the addition of esterified glucomannane also wasn't as efficient as control value (higher for 6.37%), but it was lower than feed conversion in group of pigs which offered contaminated feed without adsorbents.

DISCUSSION

In practice, F-2 poisoning is usually expressed in nonspecific form as chronic primary mycotoxicosis followed by retarded growth, decrease of body weight, lower feed consumption and consequently increases of feed consumption ratio (H u m p h r e y s, 1988). Based upon the obtained results, it could be concluded that zearalenone in amount of 3,84 ppm provokes such harmful effects and induces performances reduction.

As the ingestion of contaminated feed is the main way of mycotoxins input into animal body, a nutritional approach is the logical way of struggle with them. One of the elegant and inexpensive solutions would be a feed additive, which would detoxify the contaminated feeds during passage through the digestive tract. For this purpose, several commercially available adsorbents were tested in vitro and in vivo. In general, adsorbents are nutritionally inert, nonresorbable substances which could, due to their physical, chemical and electrostatic characteristic, reduce the bioavailability of mycotoxins by adsorption. If

a stable adsorbent-mycotoxin complex is formed, the resorption of mycotoxins in the gastrointestinal tract could be reduced, and thereby toxic effects for animal would be decreased.

Mycotoxin binders can be inorganic or organic polymers. Many types of inorganic polymers were tested for binding capacity, and the degree of adsorption depends on the chemical structure of the mycotoxin in relation to the surface properties and geometry of the adsorbent, as well as their polarity. Adsorbents, including zeolites, silicates, phylosilicates and modified phylosilicates, in vitro conditions show the ability to bind and adsorb different mycotoxins (Phillips et al., 1995, Coenen and Boyens, 2001). Modified clinoptilolite has a high binding capacity for different mycotoxins, including zearalenone in *in vitro* conditions (Tomašević-Čanović et al., 2001) which is confirmed in *in vivo* trials of Avakumović et al. (2000, 2001) who concluded that addition of mineral adsorbent is a very reasonable approach, as well as in trials with other toxins (Nedeljković-Trailović et al., 2001, Resanović et al., 2001).

Organic toxin binders are derived from plant or microorganism fibers. An innovation is the concept of organic polymers derived from yeast cell wall fractions. This material has a high surface area and enough specificity to allow effective mycotoxin binding at a low level of dietary inclusion. Binding efficacy of esterified glucomannane is confirmed in this trial, but not at the level as noted in literature (Devegowda et al., 1998; Devegowda and Aravind, 2002; Wust and Soriano, 2000).

Many adsorbents in *in vitro* conditions show the ability to bind and adsorb different mycotoxins, but there are only a few references describing *in vivo* results. Addition of modified clinoptilolite and esterified glucomannane in zearalenone contaminated feed approved to be quite successful in alleviating its harmful consequences. Thus, the use of adsorbents as toxin binders could be efficient approach to reduce negative mycotoxin effects to animals to a certain extent.

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ЕФИКАСНОСТ РАЗЛИЧИТИХ АДСОРБЕНАТА У УБЛАЖАВАЊУ ЕФЕКАТА ЗЕАРАЛЕНОНА НА ПРОИЗВОДНЕ РЕЗУЛТАТЕ ПРАСАДИ

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Резиме

Испитивање утицаја зеараленона на производне резултате и могућност коришћења модификованог клиноптилолита (МС) и естерификованог глукоманана (EGM) у ублажавању и/или превенирању штетних ефеката изведено огледом исхране прасади. Оглед је трајао 31 дан, а изведен је на укупно 32 грла у доби од 60 дана, подељених у четири групе са по 8 прасади. Контролна група прасади храњена је неконтаминираном храном, док су експерименталне групе храњене храном која је садржала 3.84 ppm зеараленона. У храну за прасад друге и треће експерименталне групе додат је МС и EGM у количини од 0,2 и 0,1%.

Прасад контролне групе остварила су дневни прираст од 0.569 kg уз конверзију хране од 1.793 kg. Присуство F-2 toxina у храни утицало је негативно на производне резултате. Додатак МС или EGM контаминираној храни побољшало је производне резултате, али не до нивоа као у контролној групи. Добијени резултати указују да адсорбенти могу само донекле да превенирају штетне ефекте F-2 toxina.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 181—188, 2005

UDC 639.2:579.67
639.2:502.51

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RISKS FROM MICROBIOLOGICAL AND CHEMICAL CONTAMINATION OF FISH MATERIALS

ABSTRACT: Fish is today one of the most important commercial material. In our market as in the world market they are present in fresh and manufactured products. Fish products always have some risk. They could be contaminated with dangerous chemicals and biological contaminants. From biological originated polutions *Aflatoxin* and other *Mycotoxins* are very dangerous. The contamination starts in fish ponds, in canals and swamps. Also, the contamination occurs at storage and prepararation of fish materials. It is very important to recognize hygienically correct fish material and control fish food and different products from fish by educated inspectors (V l a h o v i ć, 1999).

KEY WORDS: Contamination, Fish food, Inspectors, Storrage effect, Toxins

INTRODUCTION

The inspection program also encompasses the maintenance of databases on contaminants in fish and fish products and the inspection of fishing. Also, all consumers and trade complaints involving fish products are investigated to determine the cause of the complaint and the appropriate follow-up action.

Compared to European Union and many other countries, aquaculture in Serbia and Montenegro (SCG) is a relatively new industry. Until the 1980s, aquatic species were produced principally to provide seeds for restocking programs and family consumption. Although trout has been raised for food since early 1790s, only with the advent of commercial aquaculture of sardines, trout, carp, cat fish, perch, tolstolobic, strugeon, hybrid striped bass and pike.[1] Others aquaculture species grown for food are shrimp, oyster, clam, mussels and crawfish (crayfish). To support this fast growing segment of agriculture, and

[1] Controlled production according to European and world standard has begun.

maintaine its global competetivness, several institutions as universities, governmental institutions for agriculture and fishing union.[2] These institutions are actively engaded in research on nutrition, feeds and feeding of various commercially important fish species and other species of interest in aquaculture. The goals are to develop low-cost and environmentally-friendly feeds and more efficient feeding strategies, increase production efficiences, improve fish health, decrease production cost, improve product quality and minimize environmental impact. The major research areas include nutrient requirements and their effects on stress and disease resistance, nutrient metabolism and nutritional physiology, develoment evalution of nutritional value and nutrient bioavailability of alternative feed ingredients, effects of antinutritional factors and feed-borne toxins (mycotoxins) on fish growth, water quality, reproduction and fish health (W e i b e l, 1964).

Poisoning-algal toxins

Algae are important part of marine plankton serving as food for variety of aquatic animals. Algae are important factor of regeneration of water and treatment of water in fish pond. Algae may present variable morphology. Species can be unicellular as well as multicellular, some algae grow up to 20—30 meters of length. Algae serve as food for aquatic animals, they may be used as ingredients such as the production of agar agar, used in food, in drugs, as basis for bacteriological media and in many other ways. Chlorophyll is often hidden by yellow, brown, blue and red pigments. This gave the origin to a classification of algae in *Xantophyceae* or *Rodophyceae*. Algae produce starch, mannite, leucosin and oil. Classification of algaes is often not up date, but some are didactical and will still be used even when there is a new classification proposed, so this is why the present classification proposed by Strasburger is used here. The plant kingdom is divided in 7 great divisions:

1. *Bacterriophyta* containing bacteria,
2. *Cyanophyta* containing the blue-green alga,
3. *Phcophyta* containing all other alga,
4. *Mycophyta* containing mushrooms,
5. *Byrophyta* containing mosses,
6. *Pteridophyta* containing ferns,
7. *Spermatophyta* containing plants with seeds.

Algae which produce toxins are settled in division 2. *Cyanophyta* and division 3. *Phycophyta, Flagellatae*.

Division 2: *Chrooccocales*, generaly unicellular, forming jelly on rocks, *Hormogonales* (*filaments*)

Division 3: *Chrysomonodale, Heterochloridales, Cryptomnodales, Dinoflagellatae, Euglenales, Protochloridale, and Volvocales*.

[2] Start taking part in programmes and projects to solve the problems.

The *Flagellatae* have subdivision: *Chlorophytina* includes the green algae (*Chlorphyceae*), wich live mainly in fresh water as plankton and form the green coating on rocks and the bark of trees. Other *Flagellatae* are: *Pyrhophytina, Euglenophytina, Rhaeophytina, Rhodophytina* and *Cyanophyta*.

Algal toxins

Beside useful algae, there are many single cell algae which produce toxins. These species develop rapidly under favourable conditions forming algae carpets in seawater killing fish. Marine animals such as oysters, crustaceae and different types of fish may eat toxic algae storing toxins. According to the species of algae, the symptoms of poisoning are (W e l d e e, 1931):

1. Damage of the nervous system, (Paralytical Shellfish Poisoning).
2. Loss of memory (Amnesic Shelfish Poisoning)
3. Neurotoxic phenomena (Neurotoxic Shellfish Poisoning)
4. Sodium chanel blocking in nervous cells (Tetrodotoxin).

In summer the temperature of fish ponds, rivers, branches of river, lakes, chanels, swamps and seawater rises causing high growt of algae.

Damage of the nervous system: They are caused by toxins produced by *Dinoflagelata* such as *Alexandrium* spp. The Paralytical Shellfish Poisoning toxins are water soluble.

Damage of the digestive tract: Toxins of Diarrhetic Shellfish Poisoning are ocadic acid and the Dinophysis toxin which are liposoluble causing strong diarrhea.

Loss of memory: A poisoning with Amnesic Shellfish Poisoning which is caused by the dominoinic acid (DA) of the alga *Nitzscia pungens.* This algae is found also in Europe. The maximum tolerable amount of dominoinic acid in Germany is 20 mgDA/Kg in mollusc meat.

Neurotoxic phenomenans: The neurotix Shellfish Poisoning toxins are produced by *Gymnodinium breve*, also denominated as *Ptychodiscus brevis*. Amnesic Sellfish Poisoning toxins may be classified in to types: *Brevetoxin* A and *Brevetoxin* B.

Sodium channel blocking in nervous cells: *Tetradotoxin* is also called *Fugu-toxin*. It may be produced by some fish of the family of *Tetradontidae* (*Takifugu* sp.). The *Tetradotoxin* blocks the sodium channel of nervous cells acting neurotoxic. This toxin has no absorbtion of ultra violet light and is not fluorescent.

Saxitoxin: is an algal toxin of the Paralytical Shellfish Poisoning type, being water soluble.

Bacterial poisoning

Bacteria can settle on food. Due to industralisation and globalisation they can be widespread turning: endemic, epidemic and pandemic. The bacteria present in food can:

1. Spoil the food causing off-taste and off-smell.
2. Produce toxins under favourable conditions of growth, causing acute poisoning or subacute but very harmful alterations such as cancer.
3. Be infectious causing diarrhea and other serious diseases.
4. Be opportunists.

Microorganism which spoil food

Moulds, yeasts, *Escherichia coli* and *Proteus* can produce toxins when present in food and having sufficient time during storage under appropriate temperatures. In this case, the microorganisms do not necessarily need to be alive when reaching the final consumer. *Clostridium botulinum* produces exotoxins from type A, B, C, D, E and F. They are the strongest toxins which are known and act as neurotoxins. They inhibit the excretion of *acetylcholine* avoiding thus the transmission of signals from the nerve to the muscle causing paralysis comparable to the effect of *curare*. The endotoxins which are thermo unstable are formed in canned food with a pH higher than 4.5 and about 6 months of storage. This toxin is destroyed when food is cooked before serving. *Esherichia coli* produces an entrotoxin under bad hygienic conditions. *Salmonella entertides* produces a heat unstable exotoxin mainly in ground in eggs in poultry in milk powder, in chocolate, in fish meat and fish salads. *Shigella dysenteriae* and *Shigella sonnei* produce endotoxin or heat unstable exotoxins. *Shigella dysenteriae*, *Shigella sonnei* and *Staphylococcus aureus*, produce thermostable toxins. The toxins produced by *Staphylococcus aureus*, can be classified serologically as toxins A, B, C1, C2, D, E and F.

All microorganisms cited as the producers of toxins are able to cause infections. The microorganisms must be alive and in sufficient number to cause an infeciton.

Mycotoxins

Mycotoxins are poisonous metabolites of certain moulds which can cause pathological changes in humans and animals. The most important species which produce mycotoxins are *Aspergillus*, *Penicillium* and *Fusarium*. Intoxication takes place through ingestion of contaminated food more seldom by inhalation or skin resorption. Mycotoxins, unlike the bacterial or algal toxins, generally do not produce acute intoxication but they are known as strong cancerogenic, teratogenic with chronic activity. Direct contamination with mycotoxins can take place when moulds grow on food. Indirect contamination can take place when mycotoxins which contaminated feed are ingesteded by cattle and pork. Milk, eggs and fish meat are examples of indirect contamination of food caused by spoiled feed containing *Aflatoxins, Ochratoxin A* and some of the *Fusaria toxins*. The direct contamination caused by moulds growing on food is of great importance on cereals, oil seeds, coffee, fruits, vegetables, spices, some types of cheese, like roquefort cheese, and fish meat products with wheat flour, corn germ and rice (G a s z t o n y i, 1979).

Aflatoxins

Aflatoxins are mycotoxins which are exclusively produced by the mould *Aspergillus flavus* and *Aspergillus parasiticus* (R a d m i l o v i ć, 2002). Importante are *Aflatoxins* B1, G1 and G1. *Aflatoxin* B1 is the strongest cancerogenic compound known. It causes liver cancer. In food, *Aflatoxin* M1 is sometimes present and is almost as poisonous as *Aflatoxin* B1. In fish feed in fish pond, the most frequent *Aflatoxin* is B1 being often found together with B2, G1, G2 (F i s h b e i n, 1972).

Fusaria toxins

Fusaria toxins is a generic term for the so-called fade-toxin produced by *Fusaria* moulds which produce wrinkling of plant parts (B a m b u r g, 1968).

Ochratoxin A

Ochratoxin A is a mycotoxin produced by moulds of genus *Penicillium* and *Aspergillus*. It is a water soluble cumarin derivate, cereal, coffee, spices and other fish food (in fish pond). The growth of moulds and production of *Ochratoxin* A is speeded by high temperatures and high moisture during: harvest, handling, drying, storage and transport. *Ochratoxin* A is carcinogenic and genotoxic in mice and rats. Heating during cooking and backing does not inactivate *Ochratoxin* (S c o t, 1965). Stored cereals can be decontaminated with an atmosphere of 2% NH_3 at 20 degrees during 4 to 6 months.

Poison of heated foods

Heterocyclic aromatic amines are caused by heating protein rich foods as fish meat. They are carcinogenic. Polycyclic aromatic carbon and polycyclic aromatic hydrocarbon are formed when fat drips from grill fish meat and are brought back with smoke (smoked salmon, herring and tuna) and flames contaminating the foods. They are carcinogenic.

CHEMICAL AND BIOLOGICAL ANALYSIS OF TOXINS

For the analysis of different paralytic toxins it is recommended the ionic-pair chromatography with RP-C18 columm. High Pressure Liquid Chromatography (HPLC) is used as analytical method of Amnesic Shellfish Poising an RP-C18 column without derivatisation. Dominoinic acid down to 1,0 mg/kg in mollusc meat can be detected with derivisation before the column with fluorenilmetoxicarbonilchlorid. The leading world standardized methods for Neurotic Shellfich Poisoning analysis are HPLC-MC and immunoassays.

Saxitoxin is extracted from acid solution and after extraction purifed with periodic acid in alkaline medium. *Saxitoxin* is then read fluorometricaly against a standard curve. Standard solution: Saxitoxin dissolved in acetic acid 0.1 cm^3/dm^3. Further dilution are made with sulphuric acid in a way that 2 cm^3 of the dilution are added to 2 cm^3 NH_4OH and 0.1 cm^3 0.1 mol/dm^3 periodic acid. Reading made at 388 nm.

Sample with *Saxitoxin* purifed in column of 1.0 cm diameter, is charged with approximately 5 g resin which gives a lenght of 5 cm, is then washed with 100 cm^3 buffer of potassium acetate at a pH 5,2, followed with 50 cm^3 distillated water. Saxitoxin is then eluated with sulfuric acid 0.5 mol/dm^3 until 20 cm^3 are obtained in a volumetric flask. The velocity of elution should not exceed 3 cm^3/min. After 15 minutes 0.2 cm^3 of glacial acetic acid are added to the sollution and read aginst a blank containing the same components as before having periodic acid changed with water. With the same methods the mycotoxins are analysed.

The known fresh water fish species

In Novi Sad areas including National park Fruška Gora in fishing there occur the following fish species:

— Omnivora fishes: Barbel (*Barbus barbus*), ide, orfe (*Leucescus idus*), *Vimba vimba*, clamp (*Chondrostoma nasus*), Fresh water carp (*Pelecus cultratus*), robin (*Scardinius erytrophtalmus*), *Rutilus rutilus*, *Carassius auratus*, carp (*Carasius carassius*), tench (*Tinca tinca*), gudgeon (*Gobio gobio*), loach (*Misgurnus fossilis*), *Cabitis tornea*, *Cyprinus carpio*, carp (*Cyprinus carpio*), sturgeon (*Acipenser ruthenus*), bream (*Abramis brama*), *Blicca bjoerka*, *Abramis sapa*, *Abramis ballerus*, bleak (*Alburnus alburnus*), *Rhodeus sericeus amarus*, *Pseudorasbora parva*.

— Herbivora fishes: *Stenopharingodon idella*, *Hypophtamichtis molitex*, *Aristichtis nobilis*.

— Predators: Eel (*Anguila anguilla*), perch (*Stizostedion lucioperca*), *Stizostedion volgensis*, pike (*Esex lucius*), catfish (*Silurus glanis*), burbot (*Lota lota*), *Ictalurus nebulosus*, *Aspius aspius*, klena (*Leuciscus cephalus*), *Zingel zingel*, *Micropterus salmoides*, perch (*Perca flaviatilis, Lepomis gibbosus*).

DISCUSSION

This paper is for the purpose of the education of sanitary inspectors and in schools. There was included in the questionaries 337 pupils of "Prva vojvođanska brigada" elementary school in Novi Sad, 13—14 years of age. The questionaire deals with indirect poisoning with toxins in fish food. In summer, when in fish ponds the oxigen level is decreased, the bacterias liberate toxins are in dangerous quantity. Also the green algae develop toxins. The most dangerous is the poisoning with unadequate stored fish food. It has also to be no-

ted that the poising with fish has storage effect. The mentioned problems are included in five quetions. The questions given below had interesting answers.

1. When do you consume fish food? Unswer: never (15), rare (41), 1—2 times mounthly (119), one or two times weakly (141) and very often (21).

2. Where is fish purhased for your hause? Answer: In the restaurant (8), in fish market (124), in the shops (108), at private sellers (57), fishing by oneself (37) and not interested (3).

3. Which kind of fish do you purchase? Answer: Frish (89), frozen (130), various state (104), do not purchase (7) and not informed about (7).

4. How the consumed fish food is prepared? Answer: cooked (36) baked (101), on oil roasted (90), breaded (92), not preparared (7), smoked fish (4) and from can (7),

5. What do they do with fish food which remain after consuming? Answer: they eat it later cold (125), throw away (88), heat it again (76) and no rest (48).

— From the first question it is seen that many of then eat fish.

— The second question shows that 28% of fish food come from risky origins and sellers.

— The third question gave answer that people purchase mostly frozen fish which include many uncertaintles including technology and refrozen processes.

— From the answers to the fourth question it is seen that 55% of the prepared fish food was done in risky way.

— The fifth answer shows that 37% eat the rest fish food cold, which is a great risk like again heated food which is 23%.

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РИЗИЦИ ОД МИКРОБИОЛОШКИХ И ХЕМИЈСКИХ КОНТАМИНАТА У РИБЉЕМ МАТЕРИЈАЛУ

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Резиме

Рибљи производи су данас на светском тржишту веома цењени. Они у промет улазе као свежа риба или као прерађевине. Међутим, сви рибљи производи носе у себи опасност, пре свега од тровања. Саме рибе се често контаминирају хемијским и биолошким загађивачима вода и рибље хране. Контаминација је најизраженија у рибњацима, каналима и у барама, као што је у околини Новог Сада Петроварадинско-ковиљски рит. Одређене врсте алге или укварена, буђава храна за рибе су највећа опасност за контаминацију. У њиховим организмима токсини и друге опасне материје се постепено гомилају и кумулативним ефектом додатно угрожавају потрошача рибљег материјала. Грабљивице и рибе сваштоједи још више кумулирају у себе токсине и друге токсичне супстанце. Начине спремања и складиштења јела од риба исто тако треба убрајати у потенцијалну опасност од загађивања од токсина. Нарочито се то односи на поховане рибе, затим са брашном и пиринчем припремљене рибље специјалитете. Важно је, зато, првенствено едуковати тржишне инспекторе како би они преузели потребне превентивне мере.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 189—195, 2005

UDC 633.11:632.4

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CEREAL SEED MYCPOPULATIONS IN SERBIA

ABSTRACT: Mycopopulation of cereals-durum wheat (cultivars Žitka and Durumko), triticale (cultivars Goranac and NS tritikale), winter barley (cultivars NS 131, ZA 37 and Jagodinac) and oat (cultivars Slavuj and Rajac) had been studied during three years (harvest 2002, 2003 and 2004) from numerous Serbian localities. In all three investigated years and four cereal species the predominant fungal genus were *Alternaria* and *Fusarium.* On seeds there were determinated representatives of genus *Penicillium, Mucor, Bipolaris, Aspergillus, Stemphylium* and *Epicoccum*, too. The established fungi can significantly affect quality of seeds and flour products.

KEY WORDS: *Alternaria*, cereal seeds, *Fusarium*, mycopopulations, Serbia

INTRODUCTION

During past several years in Serbia a significant wheat seed and seed products quality decline was found. It is assumed that this occurrence was caused by mycopopulation that colonizes cereals in the field, as well as during storage. Cereal seeds are suitable substrate for development of great number of phytopathogenic and saprophytic fungi. The most frequently isolated fungi on cereal seeds belong to the genus *Fusarium, Alternaria, Mucor, Bipolaris, Epicoccum, Cladosporium, Penicillium, Stemphylium* and etc. (Ylimaki, 1970; Clear and Patrick, 1993; Šarić et al., 1997; Korona et al., 1995; Csosz, 2002).

As a result of phytopathogenic and saprophytic fungi colonisation the number and weight of seeds are lowered, and the germination precentage is declined (Szunics et al., 1987; Milus and Parsons, 1994). Beside quantitative losses very important are losses in quality parameters (Šarić et al., 1997). In infected seed fungus dezorganisates cell walls, starch granulates and proteins. Infected seeds have higher ash, and lower protein percentage. Flour quality is significantly reduced (Berova and Mladenova, 1974; Snijders, 1990; Boyacioglu and Hettiarachy, 1995; Dexter et al., 1996, 1997). Numerous *Fusarium* species are considered toxigenic, be-

cause of producing many thermostabil toxins, that are dangerous for human and animal health (Snijders, 1990; Langseth et al., 1997; Bagi et al., 2000). It is known, that among frequently isolated fungi from wheat seeds toxin producing there are also species which belong to genus *Aspergillus, Penicillium, Alternaria, Mucor, Rhizopus, Streptomyces* (Bočarov-Stančić, 1996; Šarić et al., 1997).

MATERIALS AND METHODS

In vegetation seasons 2001/2002, 2002/2003 and 2003/2004 (Tables 1, 2, 3, 4) cereal seed samples were collected after harvests from macro trials performed in various Serbian localities. In macro trials the standard cultural practice was applied.

Tab. 1. Localities and years of durum wheat grain samples

Locality Cultivar	Zrenjanin	Pančevo	Senta	Bačka Topola	Subotica	Sombor	Kikinda	Valjevo	Leskovac	Požarevac	Šabac	Zaječar
Žitka	2002 2003 2004	2004	2003 2004	2002 2004	2004	2004	2004	2002 2003	2004	2004	2003 2004	2002 2003
Durumko	2002 2003		2003 2004	2002 2003 2004			2004	2002 2003 2004	2004	2004	2002 2003 2004	2002 2003

Tab. 2. Localities and years of triticale grain samples

Locality Cultivar	Zrenjanin	Pančevo	Senta	Bačka Topola	Subotica	Sombor	Kikinda	Valjevo	Leskovac	Požarevac	Šabac	Zaječar
Goranac	2002	2003	2003 2004	2002 2003	2004		2004	2002 2003 2004	2004		2002 2003 2004	2002 2003
NS- -tritikale	2002	2003	2003 2004	2002 2003				2002 2003 2004			2002 2003 2004	2002 2003

Tab. 3. Localities and years of barley grain samples

Locality Cultivar	Zrenjanin	Pančevo	Senta	Bačka Topola	Subotica	Sombor	Kikinda	Valjevo	Leskovac	Požarevac	Šabac	Zaječar	Jagodina
NS-313	2002	2003 2004	2003 2004	2002 2003 2004	2004	2004	2004	2002 2003	2003 2004	2004	2002 2003 2004	2002 2003	2003
Jagodinac	2002			2002 2003 2004		2004		2002	2003		2002	2002 2003	2003
ZA-37	2002	2003	2003	2002 2003				2002 2003	2003		2002	2002 2003	2003

Tab. 4. Localities and years of oat grain samples

Locality Cultivar	Bačka Topola	Subotica	Kikinda
Slavuj	2002, 2003, 2004	2003, 2004	2002
Rajac	2002, 2003, 2004	2003	2002, 2004

For cereal seed mycoflora determination a method on moistured filter paper was applied (Pitt and Hocking, 1985).

Evaluation of germination and infection rate was accomplished by binoculare, while the fungi determination according to cultural and morphological characteristics using Barnett's (1969) manual. Determination of species from genus *Fusarium* were carried out according to Burgess et al. (1988). Statistical analysis of collected data were made by Duncan's multiply interval tests (Duncan, 1955).

RESULTS

Results of seed germination and infection percentage, and rate of observed fungi genuses on cereal seeds in different localities and years are presented in Tables 5, 6, 7 i 8.

Tab. 5. Percentage of germinated and diseased durum wheat grain 2002—2004

Year	germinated grain(%)	diseased grain (%)	*Fusarium* sp.	*Alternaria* sp.	*Penicillium* sp.	*Mucor* sp.	*Bipolaris* sp.	*Epicoccum* sp.
2002	84,7	36,2	7,4	25,1	3,2	0,4	—	—
2003	78,7	41,8	10,8	28,8	0,3	0,3	0,5	0,1
2004	78,1	24,16	10,7	12,3	0,2	0,8	0,05	0,11

In 2002—2004 performed studies on durum wheat mycopopulations suggest that in each year of the studies the dominant species on cereal seed belonged to genus *Alternaria*, particularly in 2002 and 2003 (Table 5). *Fusarium* infection level was between 7,4—10,8%, while species from genus *Penicillium, Mucor, Bipolaris* and *Epicoccum* were represented in much lower intensity. On the same level of infection were found mentioned fungi on trtiticale seeds (Tab. 6). In case of triticale, fungi from genus *Alternaria* and *Fusarium* were predominant, too.

Tab. 6. Percentage of germinated and diseased triticale grain 2002—2004

Year	germinated grain (%)	diseased grain (%)	*Fusarium* sp.	*Alternaria* sp.	*Penicillium* sp.	*Mucor* sp.	*Bipolaris* sp.	*Epicoccum* sp.	*Aspergillus*
2002	91,6	40,2	8,2	29,3	1,4	0,1	0,5	0,2	0,5
2003	85,5	41,4	6,2	34,0	1,2	—	—	—	—
2004	89,5	24,0	8,1	15,5	0,3	—	—	0,1	—

Tab. 7. Percentage of germinated and diseased barley grain 2002—2004

Year	germinated grain (%)	diseased grain (%)	*Fusarium* sp.	*Alternaria* sp.	*Penicillium* sp.	*Mucor* sp.	*Bipolaris* sp.	*Epicoccum* sp.	*Stemphylium*
2002	80,1	33,5	3,8	28,8	0,1	—	0,5	—	0,1
2003	71,4	61,5	4,7	55,4	0,1	0,05	1,0	0,2	
2004	36,5	39,2	3,7	34,8	0,5	—	0,1	0,1	

Analysing results of barley seed mycopopulation it is noticeble the very high *Alternaria* infection, which in 2003 reached 55,4% (Tab. 7). *Fusarium* infection level of barley seed was lower in comparison with the wheat infection level, while other fungi were determined on the same level as in cases of wheat samples.

Tab. 8. Percentage of germinated and diseased oat grain 2002—2004

Year	germinated grain (%)	diseased grain (%)	*Fusarium* sp.	*Alternaria* sp.	*Penicillium* sp.	*Mucor* sp.	*Bipolaris* sp.	*Epicoccum* sp.	*Stemphylium*
2002	22,5	83,2	10,5	72,2	0,2	—	—	—	0,2
2003	69,2	29,6	4,5	23,2	—	1,0	—	0,7	
2004	43,0	32,7	8,5	23,2	1,0	—	—	—	

During 2003 and 2004, the percentage of oat seeds infection level was the same as in wheat, barley and triticale, but in 2002 very high *Alternaria* infection level (72.2%) and seed germination level (22.5%) have been determined.

DISCUSSION

The results of three years investigation of cereal seed mycopopulations show dominatig role of species from genus *Alternaria.* This conclusion is in agreement with the data from Hungary (Csosz, 2002), according to which species from genus *Alternaria* can be found in very high percentage on cereal seeds. In 2000 Csosz (2002) found *Alternaria* infection rate of 70—100%, while in 2001 the infection level was 47—92%. On the basis of two year results this author concludes that there is no correlation between visual symptoms on seeds and infection intensity determined in moistured filter paper in laboratory conditions. For this reason, the laboratory analysis of mycoflora seed is necessary. It is well known that *Alternaria* species produce secondary metabolites, which are harmful to human and animal health (Giryn and Szteke, 1995; Andersen and Thrane, 1996; Sinha and Bhatnagar, 1998). High level of cereal seed *Alternaria* infection suggests that there exists a need for detailed studies of toxigenity of some *Alternaria* species, as well as ecological conditions that are necessary for mycotoxin production.

Species from genus *Fusarium* are also known as mycotoxin producers (Wilcoxson et al., 1988; Chelkowski, 1994). Along with species *F. culmorum* and *F. graminearum*, which cause wheat scab, on cereal seed species *F. equiseti, F. proliferatum, F. moniliforme, F. subglutinans, F. anthophilum* were determinated (Balaž et al., 2003). Phytopathogenic fungi from genus *Bipolaris* and *Stemphylium* were found in low intensity. Representatives of genus *Penicillium*, *Mucor* and *Aspergillus* do not cause visual symptoms on cereal seeds, but are considered to be saprophytic species which colonise supstrate rich in carbohydrates and produce harmful mycotoxins.

Using statistical analysis of fungal infection level according to studied localities and by comparison of investigated cultivars susceptibility, we did not find any statistical connections (Balaž et al., 2003, Bagi et al., 2004). Some statistically significant differences between localities and cultivars were found, but those differences are rather the results of microclimates and climatic specificity of year, than of characteristic of locality or cultivar.

The possible measures to good quality food obtaining are predominantly preventive ones. Beside growing resistant cultivars, some agrotechnic measures (like crop rotation, soil cultivation, fertilization, irrigation) can significantly preserve plant vitality, and prevent seed colonisation.

Beside mentioned preventive measures, during seed formation and ripening studies of influence of chemical treatment by fungicide to seed mycopopulation should be useful. Chemical measures against cereal diseases because of economic reason are hardly used in Serbia, although they it can quantitatively and qualitatively increase the growing results.

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МИКОПОПУЛАЦИЈА СЕМЕНА СТРНИХ ЖИТА У СРБИЈИ

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Резиме

Микопопулација семена стрних жита — дурум пшенице (сорте Житка и Дурумко), тритикалеа (сорте Горанац и НС тритикале), озимог јечма (сорте NS 131, ЗА 37, Јагодинац) и овса (сорте Славуј и Рајац) испитивана је током три године (жетва 2002, 2003 и 2004) са већег броја локалитета Србије. У све три године испитивања на четири испитиване врсте најзаступљеније гљиве су биле врсте из родова *Alternaria* и *Fusarium*. На семену су констатоване и гљиве из родова *Penicillium, Mucor, Bipolaris, Aspergillus, Stemphylium* и *Epicoccum*. Утврђене гљиве на семену стрних жита могу значајно утицати на квалитет зрна и производа од брашна.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 197—205, 2005

UDC 636.5.085.3:632.25

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MYCOTOXINS AND THEIR IMPACT ON POULTRY PRODUCTION

ABSTRACT: Only two years afer the great expansion of "AGROŽIV" company, it was evident that mycotoxins have great impact on all segments of poultry production. During that year we were for the first time faced up with problems in poultry fattening. It was not possible to explain the present problems only by bacterial and viral infections, so we assumed that there is another reason for the observed clinical picture. From that time we started to pay more attention on contamination of poultry feed with mycotoxins. In the four years' period, from 1988 to 2002, 57 samples were analyzed for the presence of mycotoxins. Mycotoxicological investigations revealed the presence of T-2 toxin in 19 samples at concentrations less than 0.3 mg/kg, in 18 samples at concentration of 0.5 mg/kg, and in 3 samples 1.0 mg/kg. Beside this, type A trichotecene DAS was found in 6 of tested samples, and ochratoxin A and in 1 sample. Clinical picture and damages varied depending on mycotoxins' concentrations and poutry age.

To exceede this problem in animal production we tried to use the organic and anorganic mycotoxin adsorbents as aditives of poultry feed, but the results were not satisfactory enough. So, we resumed that if we really want to resolve problem of mycotoxins we have to start from the field production of poultry feed components.

KEY WORDS: DAS, ochratoxin-A, poultry fodder, T-2 toxin

INTRODUCTION

Integral fodder mixtures used for poultry nutrition have high percentage of vegetable originated components which can be contaminated by mycotoxins, the influence of which can be more or less detrimental to animal or human organism. By this time, more than 1,100 different fungi species are known, of which more than 220 have the ability to generate over 330 different mycotoxins. Mycotoxins are respectively simple, low molecular, thermostable,

aliphatic and cyclical compounds with several carbon atoms. Depending on their chemical structure, mycotoxins taken into an organism may cause systematic effects and result in degeneration of certain organs or organic systems. Most of the mycotoxins have immunosuppressive effects due to their toxic influence over immune system, on one hand, and decreased and disordered protein synthesis on the other. Mycotoxins having been ingested enter the intestine and are discharged via faeces, often chemically modified. Mycotoxins absorption proceeds in small intestine. Certain part of absorbed mycotoxins reenters intestine along with bile, and only the remaining, usually smaller amount, enters systematic circulation, reaches predilectional organs and is consequently discharged via egg or urine after partial metabolism (G e d e k, Brigite, 1980; L e i b e t s e d e r, J., 1981). Problem of mycotoxins has been actualized for the last twenty years due to factors created as a consequents of farming policy changes (H a l a m a, A. K., 1982), due to food production technology changes, intensified poultry production, high productive hybrid usage, both in agricultural and poultry production (B r a u e r, J., 1982). In further exposition factors that actualized mycotoxins' problems, especially in poultry production, will be outlined.

Crop production. Not obeying the basic agrotechnical standards (crop rotation, inadequate mechanical land cultivation, introduction of heavy equipment in the process of land cultivation). Insisting on those cereal sorts that have high yield, and sensitive grain highly perishable while still in the ground (especially perishable are its embryonic parts-germs), leads to frequent appearance of the grains with undamaged endosperm, but with severely rotten germ. The damage of the shell and cereals' grains by means of mechanical devices is highly present with those sorts with sensitive grain. Mechanical damage can occur mainly when harvesters or pickers are used, particularly if the rollers are not properly set. The same mechanical damage is caused by cereals' elevation during the process of artificial drying. A grain can be damaged by insects, as well as with the work of unfavorable environmental factors. Usage of nitrogen fertilizers in large amounts, influences *Fusarium* to generate toxins (W o l f, Hanna, 1987).

Crops storage. Mechanical damage of grains can occur during the act of grain unloading into the elevators, particularly during the elevation into the upper strata of grain elevator. If the grain elevators are not constructed properly and if the food within is mouldy, big, heavy clots, sometimes heavier than 100kg, are created. At the same time, rise in temperature of the mixture can occur, due to the process of fermentation, pertinent to the mould growth and toxin creation. This moment is of vital importance, because when the fungi appear in normal conditions (meaning ordinary number per cm^2) they use primary metabolism. It has been proved that metabolites created during the primary metabolism of some fungi species even lower the toxic rate of certain mycotoxins. The cause of such situation is still unknown. When a fungi population reaches the certain magnitude per cm^2, that is when it has become thick enough, secondary metabolism is activated and mycotoxins synthesis begins (K r a l j, M. et al., 1988). It appears that moulds fight for nutritive substratum

in such way; in other words, it seems as if mycotoxin production were one of the biological struggle for survival.

Fodder production. Construction of fodder factories with large production capacities and stock space of their own, contributed to the process of poultry production intensification. However, the risk increased that, entering the process of contaminated raw material production, the problem caused by consumption of food with high mycotoxin concentration, may appear in larger amount and in greater area.

Fodder transportation. Constantly being on the move and being emptied daily, tank trucks used for food transportation do not represent such a frequent way of food contamination with mould, that is with mycotoxins. Nevertheless, it may happen, if a food cargo transported is mouldy and some of it remains in the tank or in discharge hose, that it will consequently contaminate healthy food. That is why it is obligatory to inspect through the cell openings, after each discharge, if there is some food leftovers stuck to the cell walls. It is understandable that hoses must explicitly be cleaned for any food remains. I, personally, find it necessary to disinfect tanks once a month by some of the chlorine preparations.

OCHRATOXICOSES

Ochratoxin-A is a derivate of *dihydroisocumarine.* Besides ochratoxin-A ochratoxines B and C are isolated. These mycotoxins were discovered in 1965 in South Africa as a result of toxicological research of a larger number of *Asperagillus ochraceus.* Later researches proved that *Penicillium verrucosum* is also a significant producer of ochratoxin and that it can be produced by some other *Aspergillus* and *Penicillium* genus. *Aspergillus* species produce ochratoxin only if humidity and temperature are high enough, while certain *Penicillium* species can produce it even at lower temperatures (even at +5°C) (G r a v i t s, R., S a l y i, G., 1988).

Consumption of ochratoxin-A rich food can cause severe dehydration with chicken, decreased growth, anorexia, deficient conversion, diarrhea, parchedness, while with laying hen further symptoms appear: lower egg production, larger number of stained eggs with thin, rubber shell and decrease in laying eggs. Higher percentage of dying embryo has been noted. The section shows hemorrhage in the glandular area of the stomach, severe renal uricosis with white urate deposits all over the body cavity and inner organs (visceral uricosis). Ochratoxin-A has cancerous, genotoxic, immunosuppressive and immunotoxic features and affects protein synthesis, and it can cause blood vessels damage along with exudative diathesis. It has been proved that it adversely affects the amount of passively acquired SN antibodies for IB virus (E l - K a r i m, S. A. et al., 1991). Ochratoxin-A can diminish amount of gamma globulin even to 38%, as a result of immune system damages, which explicates higher frequency of aerosacculitis and lower response to vaccination (M a z i j a, H. et al., 1991). Ochratoxin causes decreased bone mineralization. Value of ochratoxin-A in human medicine is significantly less studied, though

there is a widely accepted hypothesis about the role of this mycotoxin for etiology of Balkan endemic nephropathy with people from Serbia, Romania and Bulgaria, as well as the chronic intestinal nephropathy with people from Tunisia (K r a l j, M. et al., 1988). It is vital to mention that another mycotoxin belongs to the group of nephrotoxins and is called citrinin, which is often found in contaminated food along with ochratoxin-A.

TRICHOTECENOTOXICOSES

Trichotecenes are products of secondary metabolism of fungi from *Fusarium, Trichotecium, Cephalosporium, Myrothecium* and *Stachybotrys* genus. More than 150 mycotoxins, belonging to this group, are known today. *Fusarium* species belong to the group of so called 'soil moulds' the reason being that they find the arable land very propitious for their survival and growth (G r a v i t s, R., S a l y i, G., 1998).

With trichotecenic mycotoxicoses further symptoms can be observed: the loss of weight, vomiting, tachycardia, hemorrhage, edemas, skin necrosis, especially around the mouth and in it, necrosis of the tip of the tongue, hemorrhages in epithelium of the stomach lining, in small intestine, infarct in parenchymal organs, damages of hematopoietic organs, hemorrhage within meninges and, consequently, neuro disorders (M u l l e r, Th. et al., 1987).

Trichotecenes inhibit DNA and protein synthesis and because of that have a strong immunosuppressive effect. Besides, they can cause cell damages particularly of the actively dividing ones (thymus, lymph glands, testis, intestine, milt). Trichotecenes cause acute hepatic dystrophy with hemorrhages. T-2 toxin is strong irritant and causes necroses of proventriculus' mucosa, of the muscular part of the stomach, and of epithelium feather follicle. Smaller amounts of trichotecene, result, along with the loss of weight, in feathering disorder and flock unhomogeneousness. DON taken clean and in large amounts affects neither food consumption nor broiler growth, that is it affects neither egg production nor their quality when laying hen are concerned. On the other hand, according to some authors, DON produces the process of yolk retraction to run late, which precipitates decreased vitality of chickens. Some authors have published that DON concentration of 0,50 mg/kg effected wet extremities (legs) with laying hens of heavy line (G r a v i t s, R., S a l y i, G., 1998).

Humans and animals can get poisoned via ingestion of contaminated food, via skin or inhalation. When a chicken takes a larger amount of T-2 toxin, it reaches the highest concentration in liver in 2.5—3 hours, but already in 4—5 hours it is out of the organ.

In 2000, broiler poisoning was registered in Hungary. A small flock of broilers was in question where 78 out of 300 specimen died, i.e. 26%. Clinical picture said the following: bristle feather, apathy, mild diarrhea, subcutaneous edemas and heavy breathing. The section outlined muscular degeneration, necroses in oral cavity and esophagus, lymph tissue (Bursae fabricii and Thymus) atrophy, as well as heavy kidneys nephrosis. At the first stages of the disease, the cause was unfamiliar. The food having been changed, clinical picture beca-

me stable respectively and later fodder analyses demonstrated that the food contained T-2 toxin with 2.5 mg/kg concentration (B i t a y, Z. et al., 1981).

During the same year broilers in Scotland had the same clinical picture of T-2 toxicosis characterized by loud piping, strong vexation, running to and from, pecking the walls. Further on, certain number of chickens started to form groups, while the rest of them stood individually being pecked by other chickens, especially being pecked over their feet that had dry necroses and relinquished a finger, respectively. Their liver was enlarged with augmented gallbladder. Decreased feathering was noted, as well as thickening of cartilaginous discs in limbs. With those chickens that grouped themselves quaver, ataxia, uncoordinated movements, loss of getting up reflex and, consequently, exitus were observed (R o b b, J. et al., 1982). Stachybotryotoxin is a product of the secondary metabolism of *Stachybotrys alternans* mould. This mould lives as saprophyte on necrotic parts of plants, which are rich in cellulose. The most important of these toxins is satratoxin-N which is five times more toxic than T-2. Strachybotryotoxicosis is noted in almost all animals, as well as in people (in 1944 described as alimentary aleuky) (K r a l j, M. et al., 1988). Acute course is followed by depression, ailment, loss of weight, ataxia, and abrupt death. Chronic course is characterized by necrosis and appearance of pseudomembranes all over oral lining and tongue, and over digestive tract, respectively. A case of strachybotryotoxicosis has been described in Hungary, within broilers aged 3 weeks when in a flock counting 15,000 pieces 0.6% of them died (G r a v i t s, R., S a l y i, G., 1998). The skin of the scalp, crest and neck, as well as of the legs was erythematic, infiltrated with serum, while surface necroses with necrotic areas of variable areas respectively appeared. Hemorrhages and diphtheric layers with necroses occurred even on the lining of the beak and muscular part of the stomach.

MATHERIALS AND WORKING METHODS

Only two years afer the great expansion of "AGROŽIV" company, it was evident that mycotoxins have the great impact on all segments of poultry production. During that year, we were for the first time faced up with problems in poultry fattening. It was not possible to explain the present problems only by bacterial and viral infections, so we assumed that there is another reason for the observed clinical picture. From that time we started to pay more attention on contamination of poultry feed with mycotoxins. In the four years' period, from 1988 to 2002, 57 samples were analyzed for the presence of mycotoxins. Mycotoxicological investigations revealed the presence of T-2 toxin in 19 samples in concentrations less than 0.3 mg/kg, in 18 samples in concentration of 0.5 mg/kg, and in 3 samples 1.0 mg/kg. Beside this, type A trichothecene DAS was found in 6 of tested samples, and ochratoxin A and in 1 sample. Clinical picture and damages varied in dependence on mycotoxins' concentrations and poutry age.

RESULTS AND DISCUSSION

T-2 toxicosis

For broiler production mycotoxins from the group of trichotecenes were the most interesting, first of all T-2 toxin and DAS. Clinical picture and damages varied depending on mycotoxins'concentrantions and poultry age. Since the most of the specimen sent to be analyzed referred to starter, it was evident that one-day old chickens and starter chickens were the most sensitive.

Fourteen-day old chickens and 0.5 mg/kg T-2 toxin concentrations. Five-day old chickens, that is six-day old chickens clinically seem normal. The only fact that can be observed is that till the third day of their life chickens lose interest in food, start to rummage through the food and pick by beak those components of food that suit them (note: the size of food particles is appropriate to technology). After this unapparent period, the first clinical signs appear-chickens form groups around hand feeders and under the heaters. Thinking that they were cold, we tried to raise the temperature in the room for 1—2°C, but the results were not satisfying enough. Soon after that mild diarrhea appears. About 5% of chickens suffers from neuro disorders, such as the lost of rising reflex. Morbidity is present in 20% and mortality in 1—3%. Average body weight of the chickens during the test measurement is by, approximately, 40% lower than the technological one (90 g). With the chickens that died during the peracute period pathologic-anatomic test is very often negative. Catarrh enteritis can be observed sometimes. During the subacute and chronic period, with those chickens that have lost their rising reflex and the ability to walk, section displays kidney changes shaped as one strong nephrosis, which can be manifested via renal or visceral uricosis. Hemorrhages in glandular area of stomach along with necrosis of cuticula of the muscular part of stomach can occur as a consequence of thirst.

Fourteen-day old chicken and 1 mg/kg T-2 toxin concentrations. Two--day or three-day old chickens appear normal. However, already after 24 hours lower food consumption is observed. After this unapparent period very intensive chicken grouping occurs, which cannot be explained by the low temperatures in the room. Risen temperature had no effect. Consequently, profound diarrhea is noted. During 24 h the spreading becomes so wet on those places where the grouped chickens were located that damp reaches the conceit. The chickens seem as if they were scald. Down covering neck, scalp and orbital arches thins. Around 30% of the chickens gain neuro disorders such as loss of the rising effect. Certain specimens even lose pecking reflex. Other neuro disorders like one-sided, seldom both-sided, paresis and paresis of wings and legs appear. Morbidity is 60%, approximately, and mortality 3—6%. Average body weight of the chickens, during the test measurement that was undertaken for seven days, was by approximately 60% lower than the technological one (around 70 g).

The pathologic-anatomic results are similar to those when the concentration of T-2 toxin was lower than 0.5 mg/kg; only the changes were more obvious. Subcapsular hematomas in liver, fatty liver dystrophy, dilatation of gal-

lbladder and hemorrhage on kidneys are evident (peracute period). During the subacute and chronic period, kidneys demonstrate mild changes indicating fatty dystrophy, kidneys are hypertrophic, filled with urate, and if the process lasts longer severe renal or visceral uricosis occurs. Hemorrhages on Burse Fabricii plica and milt atrophy are noted.

Fourteen till fifty-six-day old chicken and 0.25—1 mg/kg T-2 toxin concentrations. Clinically, with chickens seven to fourteen days old weaker food consumption can be observed, weaker feathering, along with chicken disintegration according to average egg weight, which is some 10—20% below the technological one. The section outlines catarrh enteritis, hemorrhage at the juncture of esophagus and glandular stomach area. Sporadically, erosions are traced in muscular stomach area. Liver is still fatty dystrophic and hypertrophic with pale stripes originated as a consequence of ischemia and rib pressure over the area. Subcapsular hematomas are observable. Gallbladder is dilated and enlarged 2—3 times. Necroses of the tip of the tongue are present with older poultry that consummated T-2 toxin rich food for a longer period of time. This necroses is sometimes so strong that the tip of the tongue becomes cleft (snake's tongue). Atrophy of thymus, milt, and B. Fabricii is noted. Those chickens that had consummated contaminated food were more prone to get fibrous aerosacculitis compared to those that consummated trichotecene free food. It is noted that T-2 toxin and DAS have cumulative impact. After serological blood research on the virus of New Castle disease, via method of hemagglutination inhibition, bad immune response to vaccination is recorded. However, we have observed that the immunity of those chickens from the rooms where the food was previously healthy and their immune system good, did not deteriorate even when the chickens consumed trichotecene rich food. This leads us to conclude that trichotecenes do not affect already created immunity.

OCHRATOXICOSES

So far we had the opportunity to detect ochratoxin at the lower boarder of allowed concentration in one food sample. The chickens were eleven days old and the first clinical sign that something was arising was larger water consumption and appearance of whitish diarrhea, that is the presence of watery substance, admixed with urates over the spreading. Thinking that the reason for that was increased food salinity, we sent the sample urgently to be tested. The amount of traced NaCl was even lower than the recommended one for that chicken category. It was only then, when we demanded mycotoxic analyses to be done. The food was removed from the premises for 24 h, and was mixed with other food stocks until laboratory results appeared. In the food sample ochratoxin was traced. Its concentration was 0.25 mg/kg. Direct damages from consumption of such food were not enormous. During that year epizoologic situation was fairly unfavorable since we had the IB virus present. The chickens were vaccinated against IB on the first and seventeenth day of their life. On the premises where ochratoxin rich food was consumed, clinical manifestation of the illness appeared.

CONCLUSIONS

Based on previously stated facts, further conclusions can be drawn concerning mycotoxicoses:

1. Mycotoxicoses have been known as causes of certain illnesses, or, perhaps it would be better to say, symptoms of certain illnesses, for more than a few hundred years.

2. Diagnosis of mycotoxicoses is very complex and includes thorough anamnesis and unspecific clinical picture that characterizes all mycotoxicoses. Evident in development disorders, lower production capacities with animals, and only in those cases of timely long consumption of food with extremely high mycotoxin concentration, exitus can occur. Uncontagiousness of mycotoxicoses, abrupt betterment in health of the ill animals and return of production to technological norms based solely on food change, without treatment, are safe data for final diagnosis establishment.

3. The most frequent mycotoxins carriers are crops, especially harvest remnants, oil industry waste and warehouse food remains.

4. Mycotoxicoses, when occuring in large systems such as 'Agroživ' company, can cause major problems both by means of increased mortality and by decreased production results. Qualified personnel that lead this production sector had realized that beforehand, and there have been several years since we have started fighting this battle. We opted for the situation where mycotoxins our fodder. This is achieved via appropriate agro technical measurements applied in the fields, and certain precautions are undertaken even in the fodder factories and farms. A thorough analysis of the row materials of foreign origin is done. Quality of our raw materials is more or less known to us, so that we can decide by ourselves what to give to which animal species and category. This way of work resulted in betterment of production results that can be equally measured with the world.

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МИКОТОКСИНИ И ЊИХОВ УТИЦАЈ НА ЖИВИНАРСКУ ПРОИЗВОДЊУ

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Резиме

У компанији „Агрожив” Панчево смо две године након експанзије компаније — 1998. год. схватили значај који микотоксини могу имати у свим сегментима наше производње: почев од одгоја родитеља тешке линије, преко њихове експлоатације па до финалне бројлерске производње. Те године смо се први пут сусрели са проблемима пилића у тову чију етиологију нисмо могли да објаснимо само бактеријским и вирусним инфекцијама, него смо претпостављали да постоји још неки чинилац који је искомпликовао клиничку слику. Тада је послат и први узорак хране ради испитивања на садржај микотоксина. У периоду 1998—2002. год. послато је 57 узорака хране ради испитивања на садржај микотоксина. У 19 узорака је утврђен Т-2 токсин у концентрацији < 0,3 mg/kg. У 18 узорака је нађена концентрација Т-2 токсина од 0,5 mg/kg, а у 3 узорка је нађена концентрација од 1 mg/kg. У 6 узорака хране је осим Т-2 токсина био присутан и ДАС. У једном узорку хране био је присутан и охратоксин. Клиничка слика и штете су варирали у зависности од концентрације микотоксина и старости пилића. У борбу против микотоксина смо кренули са додавањем различитих органских и неорганских адсорбената, али смо тиме успели само да делимично решимо проблем. Схватили смо да борбу против микотоксина морамо да поведемо на широком фронту, у свим сегментима производње, а да је морамо почети у ратарској производњи — на самој њиви.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 207—215, 2005

UDC 664.642:664.15

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INFLUENCE OF ORGANIC ACIDS AND ORGANOCHLORINATED INSECTICIDES ON METABOLISM OF *SACCHAROMYCES CEREVISIAE*

ABSTRACT: *Saccharomyces cerevisiae* is exposed to different stress factors during the production: osmotic, temperature, oxidative. The response to these stresses is the adaptive mechanism of cells. The raw materials *Saccharomyces cerevisiae* is produced from, contain metabolism products of present microorganisms and protective agents used during the growth of sugar beet, for example the influence of acetic and butyric acid and organochlorinated insecticides, lindan and heptachlor, on the metabolism of *Saccharomyces cerevisiae* was investigated and presented in this work. The mentioned compounds affect negatively the specific growth rate, yield, content of proteins, phosphorus, total ribonucleic acids. These compounds influence the increase of trechalose and glycogen content in the *Saccharomyces cerevisiae* cells.

KEY WORDS: *Saccharomyces cerevisiae*, Acetic and butiryc acids, lindan, heptachlor

INTRODUCTION

The production of *Saccharomyces cerevisiae* is quantitatively and economically the most important microbiological production which is commercially exploited (Stewart, 2002). The production of *Saccharomyces cerevisiae* (baker's yeast, with 69—72% of water) is about 2,0 x 10^6 t and about 20.000 t of dry active instant yeast (with 90—94% of dry matter) (Rose, 1979). Molasses is the main raw material for the production of *Saccharomy cerevisiae* (Athnasion, 2003). The composition of molasses is very complex and depends on composition of sugar beet, year of production, applied agents for sugar beet protection during the cultivation and technological process of sugar production (Pejin, 1989). The main component of molasses is saccharose (about 50%). Molasses contains further a number of materials originating from sugar beet: betain, aminoacids, vitamins and mineral matters. As great number of microorganisms is present in molasses, their metabolism products like nitrits and lower organic acids (formic, acetic and butyric) may be found in molasses also. Fattohi (1994) reported the presence of acetic (119—260 ppm) and

butyric acid (28 to 104 ppm) in molasses. These metabolism products of microorganisms are known as inhibitors of growth and quality of baker's yeast (Olbrich, 10956; Zauner et al., 1979; Fiedler, 1981). The mentioned investigations were performed under anaerobic conditions. In our work the growth of baker's yeast in the presence of inhibitors was performed in aerobic conditions.

Many environmentally important xenobiotic chemicals introduced for industrial and agricultural use are halogenated (Deo et al., 1994). Several reviews have been published that deal with various aspects of the environmental fate of halogenated xenobiotics (Haider, 1979; Tiedje et al., 1987; Reineke and Knackmuss, 1988). Insecticides (62.7%), fungicides (6.1%) and hercbicides (32.4%) are used for the protection of sugar beet, one of the agricultural cultures (Šovljanski, 1993). 26.8 kg per ha of these agents are used for sugar beet protection, on the average. For that reason the residues of agents used for the protection of sugar beet (insecticides and herbicides) were determined in sugar beet molasses for the last three decades and their influence on *Saccharomyces cerevisiae* investigated (Schiweck and Haberl, 1973; Zauner et al., 1979). The investigators have found that the concentration of insecticides and herbicides in molasses has no inhibitory effect on yeast yield, respiration intensity and fermentation ability. Schiweck (1974) has found that insecticides, akarazides and fungicides inhibit the respiration intensity of *Saccharomyces cerevisiae*.

The aim of the work was to investigate the influence of organic acids (acetic and butyric) and organochlorinated insecticides (lindan and heptachlor) on the metabolism of *Saccharomyces cerevisiae*. Nutritive media for the growing of *Saccharomyces cerevisiae* were formulated with the same concentrations of organic acids and insecticides as would have the molasses samples obtained in yeast factories and used for the growth of *Saccharomyces cereviasiae*.

MATERIALS AND METHODS

Microorganism

Baker's yeast *Saccharomyces cerevisiae* DTN from the collection of the Faculty of Technology in Novi Sad was used. The pure culture was maintained on a slant of Parulekar et al. (1986) medium solidified with 5.5% of agar, and was stored at 4°C after incubation at 30°C for 48 h. For inoculation of the fermentor one loopful of stock culture was transferred to 50 ml of Parulekar et al. (1986) medium in a 250 mL Erlenmeyer flask. The culture was incubated with orbital shaking at 3.3 Hz at 30°C for 24 h before inoculation of the fermentor.

Equipment and fermentation

Fermentation was carried out in a 10 L fermentor (Chemap, Volketswil, Switzerland), consisting of a top-driver stirrer, a water-cooled condenser on

the air outlet, and built-in foam, temperature, pH and dissolved oxygen (DO) control system. Dissolved oxygen and pH were measured with a model 900 DO probe (New Brunswick, NI, USA) and pH electrode (Ingold, UK), respectively. The working volume for all the experiments was 5.0 L. Good mixing was assured by the rotation of two flat-brade agitators set at 10 Hz. The air flow rate was 240 lh. Temperature was maintained at 30°C. The fermentor was equipped with an additional unit (chemostat). In order to achieve the optimal state in the fermentor, every fermentation was first performed with five exchanges of fermentor content (using nutritive media without and with organic acids and insecticides).

Substrate preparation

Organic acids (acetic, butyric) and insecticides (lindan, heptachlor) were added under aseptic conditions to the sterilized liquid media P a r u l e k a r (1986), and the obtained nutritive media contained 0.00; 0.50; 1.00; 1.50; 2.00 g/L of acetic acid; or 0.00; 0.10; 0.20; 0.30; 0.40 g/L of butyric acid; 0.00; 0.10; 0.20; 0.30; 0.40 μg/L of lindan, and 0.00; 0.02; 0.04; 0.06 μg/L of heptachlor. Acetic and butyric acids, lindan and heptachlor were od analytical grade, Spelco, Pennsylvania, U. S. A.

Analytical methods

During continuous cultivation, samples of yeast were taken for the determination of dry matter (P o r r o et al., 1988), of trehaloze and glycogen (T r e v e l y a n and H a r r i s o n, 1956), proteins (B r o n n, 1986), and content of total ribonucleic acids by the method of S e i n et al. (1969). On the basis of determined dry matter content of yeast during growth and multiplication and used sugar, the yields on sugar were calculated (D a w i s, 1972).

RESULTS OF INVESTIGATIONS

The influence of acetic acid content in the nutritive media on specific growth rate, yield and composition of *Saccharomyces cerevisiae* cells is presented in Table 1.

Table 1. Fermentation parameters and composition of *Saccharomyces cerevisiae* cell with different initial content of acetic acid in nutritive media

Acetic acid g/L	Maximum biomass g/L	μ* h^{-1}	Y** g/g	Proteins % DM***	Trechalose % DM	Glycogen % DM	Total ribonucleic acids % DM
0.00	4.32	0.29	0.495	50.25	4.70	19.95	4.82
0.50	4.18	0.25	0.470	50.00	4.79	19.99	4.76
1.00	4.01	0.21	0.420	49.80	5.20	20.52	4.52
1.50	3.85	0.18	0.390	49.00	5.40	21.60	4.40
2.00	3.03	0.13	0.370	48.70	5.70	22.05	4.35

* Specific growth rate, h^{-1}
** $\frac{g\ Dry\ weight\ of\ yeast}{g\ Utilized\ sugar}$
*** Yeast dry matter

Acetic acid added to the nutritive media for *Saccharomyces cerevisiae* growth, under continuous conditions, has an inhibitory effect on growth parameters (specific growth rate and yield). At the same time, the content of proteins and ribonucleic acid is decreasing and that of trechalose and glycogen increasing.

The influence of butyric acid in nutritive media on fermentation growth parameters and composition of *Saccharomyces cerevisiae* cells is presented in Table 2.

Table 2. Fermentation parameters and composition of *Saccharomyces cerevisiae* cells with different initial content of butyric acid in nutritive media

Butyric acid g/L	Maximum biomass g/L	μ* h^{-1}	Y** g/g	Proteins % DM***	Trechalose % DM	Glycogen % DM	Total ribonucleic acids % DM
0.00	4.35	0.29	0.495	50.25	4.70	20.01	4.95
0.10	4.25	0.25	0.461	50.01	4.90	21.06	4.80
0.20	4.17	0.22	0.390	49.70	5.05	22.00	4.65
0.30	4.00	0.19	0.375	49.60	5.30	22.90	4.50
0.40	3.90	0.16	0.320	49.00	5.50	23.10	4.25

The quantity of butyric acid added to the nutritive media was ten times lower than that of acetic acid. Namely, molasses contains butyric acid in significantly lower concentrations than acetic acid (F a t t o h i, 1994). Butyric acid (even in such low concentrations) acts like an inhibitor of fermentation parameters (specific growh rate and yield) and affects the decrease of protein and ribonucleic acids content and increase of reserve carbohydrates content in *Saccharomyces cerevisiae* cells.

The aim of this work was to investigate the effect of lindan on *Saccharomyces cerevisiae* cells. The influence of lindan on fermentation parameters

and composition of *Saccharomyces cerevisiae* cells during continuous growth is presented in Table 3.

Table 3. Fermentation parameters and composition of *Saccharomyces cerevisiae* cells with different initial content of lindan in nutritive media

Lindan μg/L	Maximum biomass g/L	μ* h^{-1}	Y** g/g	Proteins % DM***	Trechalose % DM	Glycogen % DM	Total ribonucleic acids % DM
0.00	4.40	0.295	0.495	50.25	4.70	20.02	4.95
0.10	4.35	0.267	0.472	50.25	5.01	20.37	4.72
0.20	4.10	0.250	0.460	49.72	5.30	21.70	4.42
0.30	4.05	0.236	0.390	49.50	5.40	21.80	4.37
0.40	3.98	0.192	0.360	49.30	5.65	22.00	4.25

The fermentation parameters and composition of *Saccharomyces cerevisiae* cells cultivated under continuous condition in nutritive media with different heptachlor concentration are presented in Table 4. The chosen concentrations of heptachlor in the nutritive media were in accordance with concentrations in molasses, having in mind that in yeast factories molasses is diluted 8 times.

Table 4. Fermentation parameters and composition of *Saccharomyces cerevisaie* cells with different initial content of heptachlor in nutritive media

Heptachlor μg/L	Maximum biomass g/L	μ* h^{-1}	Y** g/g	Proteins % DM***	Trechalose % DM	Glycogen % DM	Total ribonucleic acids % DM
0.00	4.35	0.296	0.495	50.26	4.70	19.99	4.95
0.02	4.29	0.270	0.469	50.25	4.80	20.50	4.72
0.04	4.10	0.252	0.432	49.90	4.99	21.60	4.37
0.06	3.90	0.242	0.390	49.50	5.30	22.30	4.25

DISCUSSION

The addition of acetic acid to the nutritive media (0.00—2.00 g/L) decreases the maximal content of *Saccharomyces cerevisiae* biomass by 70%. As a consequence, the specific growth rate (μ) is decreased 2.23 times, and the biomass yield calculated on the used sugar decreases also. The obtained results are in accordance with the results presented by P h o w c i n a et al. (1995). The acetic acid affected the *Saccharomyces cerevisiae* cell composition, and the decrease of proteins and total ribonucleic acids content (the latter being important for the biosynthesis of proteins). The content of trechalose and glycogen in the cells increased with the increase of acetic acid concentration in the nutritive media. The increase of trechalose content in yeast cells can be

explained by the fact that cells synthesize the trechalose when in inadequate conditions (J o s e et al., 2000). Trechalose in yeast cells increases stress resistance (J o s e et al., 2000).

Butyric acid affects negatively the maximal concentration of biomass in the fermentor, even in concentrations 10 times lower compared to acetic acid, e. g. the specific growth rate and yield of work (production) microorganism *Saccharomyces cerevisiae*. Trechalose and glycogen content is higher in cells cultivated on nutritive media with butyric acid compared to cells cultivated on nutritive media with acetic acid.

The increase of lindan content in the nutritive media (0.1; 0.2, 0.3 and 0.4 μg/L) decreased specific growth rate (μ), content of protein and total ribonucleic acids and yield of biomass. The decrease of proteins can be explained by the fact that their synthesis depends on ribonucleic acids. The lower the nucleic acids content, the lower the synthesis rate of proteins (O u r a, 1972). The increase of lindan in the nutritive media is followed by the increase of reserve carbohydrates content (F r a n s o i s and P a r r o u, 2001). The content of trechalose and glycogen increased with the decrease of growth rate "μ". This finding is in accordance with the results of K u e n z and F i c h t e r (1972). The increase of trechalose in yeast cells is most probably the reaction of cells to stress caused by presence of lindan in nutritive media (V a n D i j c k et al., 1995; P a a l m a n et al., 2003).

The effect (influence) of heptachlor on growth parameters and composition of *Saccharomyces cerevisiae* cells was also investigated. The chosen concentrations of heptachlor in the nutritive media were in accordance with the concentrations in molasses, having in mind that in yeast factories molasses is dilluted 8 times. The increase of heptachlor concentrations in nutritive media decreases the specific growth rate "μ", however, this decrease is significantly lower compared to the effect of lindan in the nutritive media. The content of proteins and total ribonucleic acids in *Saccharomyces cerevisiae* cells decreased, while the content of trechalose and glycogen increased with the increase of heptachlor concentration in the nutritive media.

On the basis of the obtained results, it can be concluded that acetic and butyric acids, and organochlorinated insecticides (lindan and heptachlor), in concentrations found in molasses, may have an inhibitory effect on fermentation parameters and the composition of *Saccharomyces cerevisiae* cells. In that way, they affect negatively the capacity of yeast factories and also the quality of the yeast which is offered to the consumers.

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УТИЦАЈ ОРГАНСКИХ КИСЕЛИНА И ОРГАНОХЛОРНИХ ИНСЕКТИЦИДА НА МЕТАБОЛИЗАМ *SACHAROMYCES CEREVISIAE*

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Резиме

У производњи *Saccharomyces cerevisiae* (пекарског квасца) главна сировина је меласа. У меласи се могу наћи органске киселине као продукти метаболизма микроорганизама који се налазе у њој у вегетативном или спорогеном облику. Међу овим продуктима органске киселине сирћетна и буерна се налазе у највишим концентрацијама. Због тога је у раду испитан утицај сирћетне и бутерне киселине на метаболизам *Saccharomyces cerevisia* у концентрацијама које се реално могу наћи у подлогама за умножавање у фабрикама за производњу *Saccharomyces cerevisiae*. Сирћетна киселина је додавана под асептичним условима у стерилне подлоге у концентрацијама: 0.00; 0.50; 1.00; 1.5 и 2.0 g/L. Додатак сирћетне киселине смањио је максимални садржај биомасе у подлози током умножавања за 70%, тако да се специфична брзина раста смањила 2, 3 пута, што је имало за последицу смањење приноса биомасе на утрошени шећер. Сирћетна киселина утицала је на састав ћелија *Saccharomyces cerevisiae*, смањивао се садржај укупних рибонуклеинских киселина и садржај протеина, а повећавао се садржај резервних угљених хидрата трехалозе и гликогена. Бутерна киселина се додавала у концентрацијама: 0.00; 0.10; 0.20; 0.30; 0.40 g/L. Највиша концентрација бутерне киселине у подлози 0.40 g/L била је пет пута нижа од највише концентрације сирћетне киселине. И тако знатно ниже концентрације бутерне киселине инхибиторно су деловале на параметре ферментације и састав ћелија *Sasccharomyces*

cerevisiae. Линдан је додаван у хранљиву подлогу у концентрацијама: 0.10; 0.20; 0.30 и 0.40 μg/L, а хептахлор у концентрацијама 0.02; 0.04 и 0.06 μg/L. Са повећањем садржаја линдана и хептахлора у подлогама смањивале су се максимална концентрација биомасе, специфична брзина раста и садржај укупних рибонуклеинских киселина, а повећавао се садржај резервних угљених хидрата трехалозе и гликогена.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 217—227, 2005

UDC 664.2.03:577.152.33

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INFLUENCE OF ADDITION OF AMYLASE PREPARATION TO DOUGH ON FERMENTATIVE ACTIVITY OF BAKER'S YEAST

ABSTRACT: Dough samples with different content of amylases were investigated, immediately after mixing and after 7, 14 and 30 days of frozen storage. The obtained results show that the fermentation time is shorter, both in fresh and frozen samples, when amylase sample 1 was added, compared to dough without enzymes. The addition of amylase 2 to dough resulted in minimal decrease of "rising" time, both iz frozen and fresh dough samples. The rising time of fresh samples was shorter when amylase 3 was added to dough. The specific fermentative activity of fresh dough samples is increasing by about 10% compared to the control sample, for all amounts of amylase 1 and 2 added to the dough. The fermentative activity of yeast in frozen samples increased by 5—10%, after keeping of dough with the addition of amylase 1 for 14 days. The specific fermentative activity of fresh dough samples increased compared to the control, for all amounts of added amylase 3 to the dough. In frozen dough samples the fermentative activity of yeast decreased by 10% for all added amounts of amylase 3. Baked goods made of fresh and frozen dough, prepared with the addition of amylase 1, are better than the ones made of control dough sample, considering all evaluated parameters.

KEY WORDS: amylase, baker's yeast, fermentative activity

INTRODUCTION

The properties of yeast cells with improved freeze tolerance were investigated and the connection between freeze tolerance and cell composition (content of trechalose, amino-acids, lipids in cell membrane, level of thermoresistent proteins, respiratory cell capacity) was found (Tanghe, 2002). Sharadananant and Khan (2003a) have found that the addition of hydrophilic gumms (gum arabica and carboxy methyl cellulose) decreases the content of water in the dough that can be frozen, while the addition of κ-carrageenan affects negatively the frozen dough. The developed CO_2 content in dough depends on the content of fermentable sugars and amylose activity. Starch is

hydrolyzed in the presence of amylases and sugars are formed as the result (maltose and other). The amount of sugars depends on the content of amylolytic enzymes and state of starch in the dough (A u e r m a n L. J., 1988). The ability of flour water suspension to produce a certain amount of maltose at certain temperature and certain time is known as the amylolytic activity of flour. The ripe wheat grain contains practically only β-amylase, while germinated wheat contains both β- and active α-amylase. Temperature and pH affect the α- and β-amylase in different way. The optimal activity of α-amylase is at higher temperatures (but is also inactivated). At the same time, β-amylase is more stable at lower pH values. The lower the pH value, the lower the inactivation temperature of amylases, especially of α-amylase. The higher the water content and lower the starch concentration, the lower the temperature of optimal effect and inactivation of amylase. The inactivation temperature of amylases is affected by the rate and duration of heating of dough where amylolisys takes place. The optimal temperature of β-amylase is 62—64°C, in dough made of Type 850 flour with baker's yeast (pH 5,9), and of α-amylase 70—74°C. The complete inactivation of β-amylase and α-amylase was 82—84°C and 97—98°C, respectively . A u e r m a n (1988) reported that certain activity of α-amylase was found in the center of the crumb of bread made of such dough. The action of enzymes depends on dimensions of flour particles, dimensions of starch grains and degree of mechanical damage during grinding of grain, e.g. specific free surface of starch globules, where β-amylase can act. When the flour particles and starch grains are smaller and more damaged, the flour is more favourable for action of β-amylase (A u e r m a n L. J., 1988). The addition of β-amylase to wheat flour increases insignificantly the ability of maltose production, pointing to slight excess in flour. The addition of α-amylase in the same amounts, increase several times the ability of maltose production, proportionally to the added amount of α-amylase. Most probably, α-amylase splits starch to low-molecular dextrins which can be easily transformed to maltose, under the influence of excess amounts of β-amylase in the flour. The oxidation of sulphydril group (-SH), present both in α- and β-amylase, decreases signifficantly the activity of these enzymes. The activity of α-amylase depends, to a certain degree, on primary amino groups.

MATERIAL AND METHODS

Wheat flour, of average quality, usually found at the market, was used for dough production. No additives were used for control dough samples, while commercial amylase was mixed into the dough for some trials. The analyses were performed according to the Regulations on methods of physical and chemical analysis for quality control of cereals, milling and baked products, pasta and frozen products (Yugoslav Official Register 74/88): content of moisture, ash, proteins, acid value, wet gluten content (K a l u đ e r s k i G., F i l i p o v i ć N., 1998), farinographic, extensographic and amylographic characteristics. The fermentative activity of yeast was determined by modified method for Determination of baker's yeast activity (JUS E.M8.024, Official Register

SFRJ 56/87). The fermentation time of dough was determined according to standard method JUS E.M8.020. The differential method was used for experimental baking; chosen to obtain small bakery goods (100 g) of best quality under the given conditions. The following parameters of baked goods were investigated: mass and volume of baked products, height/diameter ratio, circumference. The penetrometric value was determined using the following method: Use of SUR penetrometer PNR 6 for the evaluation of physical characteristics of crumb of bread and baked goods. The Dallman number was also determined (K a l u đ e r s k i , F i l i p o v i ć, 1988). The amount of yeast for mixing was 2%, calculated on flour, using fresh baker's yeast with 30% of dry matter. The amount of salt was 1,8%, compared to flour. The amount of added water depends on the water content of used yeast, and is calculated in such a way that the water content of dough is constant. Amylases were added to dough, amylase 1 (recommended for the improvement of volume and freshness of baked goods, especially in case of frozen doughs) in concentrations 0,008%, 0,014% and 0,02%; amylase 2 (recommended for the improvement of volume, color and flavor of baked goods), in concentrations 0,002%, 0,007% and 0,012% and amylase 3 (recommended for improvement of flour quality, e.g. quality of finished baked goods), in concentrations 0,002%, 0,0035% and 0,005%). All dough constitutents were tempered at 35°C, as regulated in the method used for the analysis of fermentative activity of yeast. The dough was prepared in the mixer, and after the mixing dough samples were frozen. The time and conditions of freezing and defrosting were chosen on the basis of the results obtained during investigations of freezing dynamics (T o p o l i ć, 2004).

RESULTS AND DISCUSSION

The biggest decrease of volume of small bakery goods was found after 7 days of keeping the dough in frozen state (Table 1).

Table 1. Evaluation of small bakery goods without additives

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	101,77	101,75	102,43	99,18
Volume of small bakery goods [ml]	336,67	248,00	228,00	252,00
Height/diameter ratio [1]	0,56	0,55	0,56	0,49
Circumference, O_1/O_2 [cm]	26,5/27,2	24,2/24,6	24/23,5	23,97/24,37
Penetrometric number	94,7	63,3	48,3	44,5
Dallman number	7—8	8	8	7—8

After 14 days of keeping, the volume of small bakery goods decreased further by 20 ml, e.g. 32,28% compared to the fresh sample. According to height/diameter ratio, the form of the small bakery goods was not changing significantly after 14 days of keeping in frozen state. However, after 30 days of keeping in frozen state and defrosting, the height/diameter ratio is decreased. The decrease of penetration value shows that the keeping in frozen state

affects the rheological characteristics of dough. The Dallman number, rather uniform in all samples, points to the fact that freezing, keeping of dough in frozen state and defrosting have no significant influence on shape and distribution of pores, e.g. appearance of crumb. The addition of amylase sample 1 to dough, results in an increase of specific fermentative yeast activity in fresh — nonfrozen samples. The biggest increase was in dough where 0,0014% of enzyme was added, while different amounts of enzymes did not result in different specific fermentative yeast activity (Figure 1).

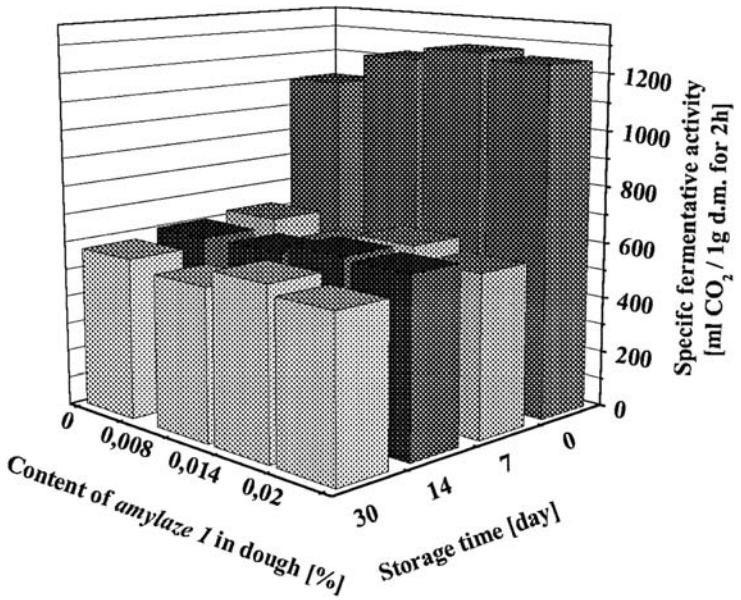

Figure 1. Influence of amylase 1 content in dough and storage time at –18±1°C on specific fermentative yeast activity

In samples prepared with 0,0014% of enzyme, kept at —18±1°C for different period of time, the increase of specific fermentative activity of yeast was minimal compared to dough samples without enzyme.

Table 2. Evaluation of small bakery goods obtained from dough with the addition of 0,008% of amylase 1

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	102,00	103,10	102,78	101,43
Volume of small bakery goods [ml]	410,00	268,00	228,00	260,00
Height/diameter ratio [1]	0,57	0,56	0,60	0,55
Circumference, O_1/O_2 [cm]	28,8/28,6	24,9/25,0	24,5/23,9	24,4/24,7
Penetrometric number	152,2	75,5	51,7	68,5
Dallman number	7	7—8	8	7—8

The volume of small bakery goods decreases during freezing, keeping at low temperatures and defrosting. The biggest decrease of volume was found after 7 days of frozen storage, while the smallest volume was determined in small bakery goods made of dough kept in frozen state for 14 days. According to height/diameter ratio, the shape of small bakery goods is not changing significantly with the exception of small bakery goods obtained from dough kept in frozen state for 14 days. The same can be concluded on the basis of the measured circumference. The freezing process affects significantly the penetrometric value, and it decreased almost by 50% in small bakery goods made of dough kept for 7 days in frozen state compared to small bakery goods made of fresh dough. Freezing, keeping of dough in frozen state and defrosting have no effect on shape and distribution of pores, e.g. appearance of crumb, as concluded on the basis of almost constant value of Dallman number.

Table 3. Evaluation of small bakery goods obtained from dough with the addition of 0,014% of amylase 1

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	101,00	102,48	99,93	100,83
Volume of small bakery goods [ml]	430,00	280,00	270,00	270,00
Height/diameter ratio [1]	0,58	0,54	0,56	0,56
Circumference, O_1/O_2 [cm]	28,4/29,0	25,3/25,4	24,7/25,3	24,7/25,5
Penetrometric number	168,5	85,5	57,8	87,3
Dallman number	7	7	7—8	7—8

The biggest decrease of volume, 34,88%, was registered in small bakery goods made of dough kept at –18°C for 7 days (Table 3). The time of keeping at low temperatures has no significant effect on the change of volume of baked small bakery goods. The biggest change of shape, e.g. smallest "rising" was registered in small bakery goods made of dough kept for 7 days in frozen state. Freezing, keeping of dough in frozen state and defrosting have no effect on shape and distribution of pores, e.g. appearance of crumb, as concluded on the basis of almost constant value of Dallman number.

Table 4. Evaluation of small bakery goods obtained from dough with the addition of 0,02% of amylase 1

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	100,85	103,25	99,48	101,93
Volume of small bakery goods [ml]	450,00	312,00	276,00	260,00
Height/diameter ratio [1]	0,54	0,52	0,52	0,54
Circumference, O_1/O_2 [cm]	28,5/29,0	26,1/26,3	25,1/25,4	24,7/25,2
Penetrometric number	155,8	85,3	67,7	72,7
Dallman number	7	7	7—8	7—8

Keeping of dough in frozen state for 14 and 30 days affects the volume of final bakery products and the registered decrease was 38,66% and 42,22%

respectively, compared to small bakery goods made of fresh dough (Table 4). The freezing process affects the decrease of circumference of small bakery goods, however, this parameter is not changing significantly with the time of keeping in frozen state. The addition of amylase 2 to dough (Fig. 2) affects the fermentative activity of yeast in the dough. The fermentative activity increased in fresh samples, however, the amount of amylase added to the activity of yeast in the dough. The specific fermentative activity decreased almost to half of the initial value, for all applied amounts of amylase 2, as the consequence of freezing process. It was also found that the time of keeping at low temperatures has no or very weak effect on fermentative activity of yeast.

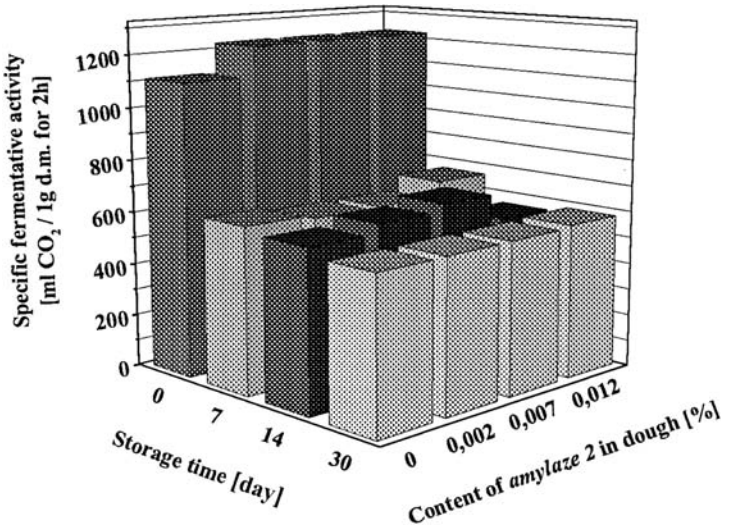

Figure 2. Influence of amylase 2 content in dough and keeping time at –18±1°C on specific fermentative yeast activity

Table 5. Evaluation of small bakery goods obtained from dough with the addition of 0,002% of amylase 2

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	101,75	103,45	104,90	103,93
Volume of small bakery goods [ml]	320,00	252,00	272,00	240,00
Height/diameter ratio [1]	0,57	0,58	0,57	0,53
Circumference, O_1/O_2 [cm]	27,5/27,2	24,5/24,5	24,2/24,6	24,4/23,5
Penetrometric number	126,8	69,0	72,2	58,7
Dallman number	7	7	7—8	7—8

The volume of small bakery goods made of dough kept for 7 days at low temperatures was by 21,25% smaller compared with the volume of small bakery goods obtained from fresh dough (Table 5). The volume decrease was smaller in small bakery goods made of dough kept for 14 days in frozen state compared to the control sample. The height/diameter ratio of small bakery go-

ods made of fresh and frozen dough (kept 7 and 14 days in frozen state) was rather uniform, confirming that the freezing process is not the cause of deformation of small bakery goods. The freezing process affects significantly the penetrometric value, and this value decreased by 50% in small bakery goods made of dough kept for 7 days in frozen state compared to small bakery goods made of fresh dough. Dallman number is uniform in all samples of baked small bakery goods.

Table 6. Evaluation of small bakery goods obtained from dough with the addition of 0,007% of amylase 2

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	102,02	102,98	103,93	103,18
Volume of small bakery goods [ml]	325,00	286,00	284,00	280,00
Height/diameter ratio [1]	0,61	0,48	0,55	0,46
Circumference, O_1/O_2 [cm]	26,2/26,6	25,3/25,8	24,7/25,0	25,0/25,7
Penetrometric number	114,7	88,2	79,5	73,2
Dallman number	7	7	7	7

The biggest decrease of volume was found in small bakery goods made of dough kept for 7 days after freezing, and it was 12% (Table 6). Longer keeping of dough in frozen state does not affect the volume of final baked products. According to the change of height/diameter ratio, the biggest deformation of shape — lowest "rising", was registered in small bakery goods made of dough kept for 7, e.g. 30 days in frozen state. The freezing of dough, keeping in frozen state and defrosting have no significant influence on shape and distribution of pores, e.g. form of crumb.

Table 7. Evaluation of small bakery goods obtained from dough with the addition of 0,012% of amylase 2

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	102,00	100,63	103,73	101,45
Volume of small bakery goods [ml]	350,00	272,00	252,00	250,00
Height/diameter ratio [1]	0,58	0,51	0,56	0,53
Circumference, O_1/O_2 [cm]	27,7/27,1	25,3/25,4	24,1/23,8	24,3/24,1
Penetrometric number	101,7	58,8	57,3	47,3
Dallman number	7	7—8	8	7—8

During longer keeping of dough in frozen state the volume of baked small bakery goods is decreasing by 0,07% e.g. 0,01% compared to previously analyzed samples (Table 7). The shape of small bakery goods is changing rather slightly, as showed by height/diameter ratio. Penetrometric value also significantly decreased in samples with the addition of 0,012% of amylase 2, as the result of freezing. Keeping of dough in frozen state has no significant effect on elasticity and compressibility of crumb.

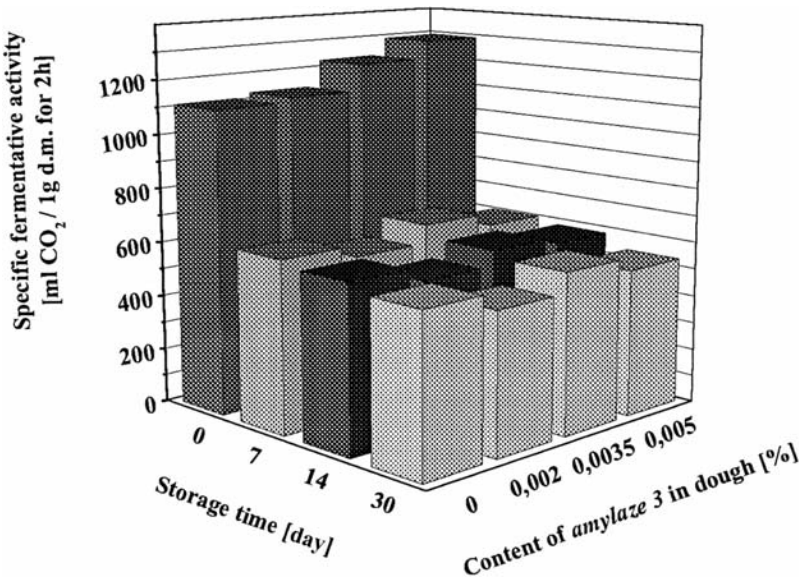

Figure 3. Influence of amylase 2 content in dough and keeping time at –18±1°C on specific fermentative yeast activity

The influence of amylase 3 content in dough and time of keeping at —18+1°C on specific fermentative activity of yeast is presented in Figure 3. The addition of amylase 3 to dough results in an increase of specific fermentative yeast activity in fresh samples and this increase is proportional to the increase of amylase amount. In samples with the addition of lowest and highest recommended concentration of amylase 3, the specific fermentative yeast activity in all frozen dough samples was lower than of the control sample (without enzymes), independently on keeping time.

Table 8. Evaluation of small bakery goods obtained from dough with the addition of 0,002% of amylase 3

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	102,00	100,63	103,73	101,45
Volume of small bakery goods [ml]	350,00	272,00	252,00	250,00
Height/diameter ratio [1]	0,58	0,51	0,54	0,53
Circumference, O_1/O_2 [cm]	21,1/21,1	25,3/25,4	24,1/23,8	24,3/24,1
Penetrometric number	101,7	58,8	57,3	51,3
Dallman number	7	7—8	8	7—8

The volume of small bakery goods made of dough kept for 7, e.g. 14 days after freezing at 18±1°C was by 22,29, e.g. 28,57% lower compared to the control sample (Table 8). The uniform values of height/diameter ratio of small bakery goods made of dough kept for 7, 14 and 30 days at low temperatures show that keeping at low temperatures does not result in deformation of

small bakery goods. However, the difference of height/diameter ratio of frozen and fresh samples is significant pointing to negative effect of freezing process. Longer time of keeping of dough in frozen state affects insignificantly the compressibility and elasticity of the crumb of finished bakery products, as presented by the minimal change of penetrometric value.

Table 9. Evaluation of small bakery goods obtained from dough with the addition of 0,0035% of amylase 3

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	101,65	104,30	104,58	101,08
Volume of small bakery goods [ml]	365,00	258,00	258,00	260,00
Height/diameter ratio [1]	0,60	0,53	0,55	0,52
Circumference, O_1/O_2 [cm]	27,2/27,9	24,4/24,6	24,17/24,6	24,9/25,0
Penetrometric number	114,3	60,2	59,3	57,4
Dallman number	7	7	8	7—8

After 7 days of keeping in frozen state, the volume of small bakery goods was by 29,31% lower compared to the control samples (Table 9). Longer keeping of dough has no negative influence on the change of volume. The biggest deformation, e.g. lowest "rising" determined on the basis of height/diameter ratio, was found in small bakery goods made of dough kept in frozen state for 7 e.g. 30 days. The penetrometric value is decreasing as the result of freezing, as presented by value measured in small bakery goods made of dough kept for 7 days in frozen state and compared to the control sample. The Dallman number has the same value in all samples.

Table 10. Evaluation of small bakery goods obtained from dough with the addition of 0,005% of amylase 3

Evaluated parameter	0. day	7. day	14. day	30. day
Mass of small bakery goods [g]	101,57	105,15	104,83	102,00
Volume of small bakery goods [ml]	380,00	264,00	256,00	256,00
Height/diameter ratio [1]	0,56	0,52	0,56	0,52
Circumference, O_1/O_2 [cm]	28,0/28,4	24,7/24,9	24,7/24,6	24,9/24,7
Penetrometric number	126,7	65,0	61,0	63,5
Dallman number	7	8	8	8

The biggest decrease of volume was in small bakery goods made of dough kept for 7 days in frozen state (Table 10). Longer keeping time had no significant effect on the change of this quality characteristic. The determined values of height/diameter ratio and circumference of baked small bakery goods show that freezing affects the change of form in a lesser degree. It was found that longer storage of dough has no negative influence on elasticity and compressibility of crumb. The Dallman number is uniform in all investigated samples.

CONCLUSION

The fermentation time of fresh and frozen samples was shorter when amylase 1, 2 and 3 were added to the dough. The specific fermentative yeast activity of fresh samples increased by about 10% compared to the control sample, for all added amounts of amylase samples 1, 2 and 3. In frozen samples, the specific fermentative yeast activity decreased by 10% for all added amounts of amylase 3. All evaluated parameters of baked goods made of fresh dough containing amylase 1, were better compared to samples made of the control dough. The characteristics of small bakery goods made of dough prepared with the addition of amylase 1 are better than of the control sample. Regarding all evaluated parameters of the baked goods made of dough with amylase 2, the ones containing 0,007% of enzyme are outstanding. Keeping time of frozen dough containing amylase 2 has no effect on quality of baked goods. The addition of amylase 3 to the dough does not result in significant improvement of quality of baked good made of both fresh and frozen dough.

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УТИЦАЈ ДОДАТКА ПРЕПАРАТА АМИЛАЗА ХЛЕБНОМ ТЕСТУ НА ФЕРМЕНТАТИВНУ АКТИВНОСТ ПЕКАРСКОГ КВАСЦА

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Резиме

Испитивани су узорци са различитим садржајем амилаза у тесту и то непосредно по замесу и након 7, 14 и 30 дана складиштења у замрзнутом стању. Резултати ових истраживања показују да се у незамрзаваним и замрзаваним узорцима време дизања теста скраћује додатком амилазе 1, у односу на тесто без додатка ензима. Код замрзаваних и незамрзаваних узорака теста, додатком амилазе 2 у тесто минимално се скраћује време дизања теста. Додатком амилазе 3, време дизања теста се, код незамрзаваних узорака, скраћује. Специфична ферментативна активност незамрзаваних узорака теста повећава се за око 10% у односу на контролни узорак, и то за све количине амилазе 1 и 2 додате у тесто. Додатком амилазе 2 у тесто, специфична ферментативна активност квасца у замрзаваним узорцима повећава се за 5—10%, након 14 дана чувања теста у замрзнутом стању. Специфична ферментативна активност незамрзаваних узорака теста повећава се у односу на контролни узорак, за све количине амилазе 3 додате у тесто. Специфична ферментативна активност квасца опада до 10% за све додате количине амилазе 3. Од незамрзаваног и замрзаваног теста, у које је додата амилаза 1, добија се пециво које је према свим оцењиваним показатељима боље од пецива добијеног од контролног узорка теста.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 229—238, 2005

UDC 663.45

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FERMENTATION TEMPERATURE AND WORT COMPOSITION INFLUENCE ON DIACETYL AND 2,3-PENTANEDIONE CONTENTS IN BEER

ABSTRACT: Diacetyl and 2,3-pentanedione are important constituents of beer sensory properties. A new GC/MS method for diacetyl and 2,3-pentanedione content determination was developed. This method was applied for the determination of diacetyl and 2,3-pentanedione contents during beer fermentation (primary fermentation and maturation). Primary fermentations were carried out at different temperatures (8°C and 14°C). Primary fermentation temperature had a great influence on diacetyl and 2,3-pentanedione formation and reduction. Formation and reduction rates increased with the primary fermentation temperature increasment. Diacetyl and 2,3-pentanedione contents also increased with the corn grits increasment. Fermentations were carried out with *Saccharomyces cerevisiae* pure culture, specially prepared for each fermentation. This GC/MS method for diacetyl and 2,3-pentanedione determination was valuable for analysing the influence of wort composition or fermentation conditions such as primary fermentation temperature on their formation and reduction.

KEY WORDS: corn grits, Diacetyl, fermentation, temperature, 2,3-pentanedione, wort composition

INTRODUCTION

Diacetyl (2,3-butanedione) is the main aroma constituent of beer (Stewart, 1977). The beer has a sweet, nonspecific taste, when diacetyl content in beer is higher than the flavour threshold and the increased contents result in buttery aroma. Diacetyl flavour threshold is 0.05—0.10 mg/L (Wainwright, 1973; Fix, 1993, Sigsgaard, 1994; Linko et al., 1998). 2,3-pentanedione is similar to diacetyl, however, the flavour threshold is higher (near 1.0 mg/L) resulting in honey-like aroma (Fix, 1993). Reduction of diacetyl and 2,3-pentanedione is simultaneous with other processes during maturation of

beer, so it can be considered as an important criterion for maturity level estimation of a certain beer. The formation and reduction of vicinal diketones take place in three stages: formation of α-acetohydroxy acids, oxidative decarboxylation of α-acetohydroxy acids to vicinal diketones and reduction of vicinal diketones (Hansen and Kielland-Brandt, 1996; Boulton and Quain, 2001). The formation and reduction of diacetyl and 2,3-pentanedione are influenced by: yeast strain and growth rate (Haukeli and Lie, 1972; White and Wainwright, 1975; Engan, 1981; Inoue, 1981; Bamforth and Kanauchi, 2004), fermentation temperature (Engan, 1981; Kunze, 1998), wort composition and treatment (Portno, 1966; Fix, 1993, Lodahl, 1993; Pugh et al., 1997; Boulton and Quain, 2001), aeration and concentration of dissolved oxygen (Engan, 1981; McCarthy, 1995; Tenge and Geiger, 2004), pH value (Haukeli and Lie, 1971; Pajunen and Makinen, 1975, White and Wainwright, 1975), infection (Engan, 1981, Fix, 1993) and pressure (Liebs et al., 1970; Sepelová et al., 2003).

Diacetyl and 2,3-pentanedione quantitative analysis during beer fermentation is difficult because of their high volatility, low contents and the interference of the other matrix compounds such as ethanol and acetoin (Martineau et al., 1994; Landaud et al., 1998). Methods for diacetyl and 2,3-pentanedione determination and quanitification include colorimetric (MEBAK, 1993; European Brewery Convention Analytica — EBC, 1998), fluorometric (McCarthy, 1995) and gas chromatographic procedures (Martineau et al., 1994; MEBAK, 1996; European Brewery Convention Analytica — EBC, 1998; Dupire, 1998, Horák et al., 2001). Using a GC/MS method for the diacetyl quanification (Martineau et al., 1998), the analysis for 2,3-pentanedione was developed (Landaud et al., 1998). Derivatizaton procedure was also modified.

Because of the complexity of beer composition and in view of the fact that retention time depends on sample composition and chromatographic conditions, chromatograph with mass selective detector was used. In addition to retention times this instrument gives also mass spectra of the investigated compounds. Thus, their identification is much more reliable than with other types of detectors. To determine the compounds that are present at low contents, which is the case with diacetyl and 2,3-pentanedione, it is necessary to apply a preconcentration method (for example, solid phase extraction — SPE).

Since the fermentation yields formation of the diacetyl and 2,3-pentanedione precursors, i.e. α-acetolactate and α-acetohydroxybutyrate, to determine the total potential of vicinal diketones in beer samples it was necessary to oxidize these compounds to diacetyl and 2,3-pentanedione. In the course of the experiments it was also necessary to optimize the amount of derivatization agent (1,2-diaminobenzene) and pH of the samples before transferring them onto the SPE columns. The effect of different organic solvents used as eluents of the investigated compounds was also investigated.

In view of the above the aim of the work was to study of the effect of fermentation temperature and wort composition on diacetyl and 2,3-pentanedione contents.

MATERIALS AND METHODS

Chemicals and supplies

Diacetyl (2,3-butanedione), 2,3-pentanedione, 2,3-hexanedione (for synthesys), 1,2-diaminobenzene and dichloromethane were purchased from Merck and SPE Octadecyl (C_{18}) columns N° 7020-02, volume 3 mL and packed with 200 mg of octadecylsilane per column were purchased from J. T. Baker.

Gas chromatograph with mass selective detector (GC/MS) and analytical conditions

The GC/MS analyses were carried out using a Hewlett Packard model G 1800 A GC SYSTEM combined with Hewlett Packard mass selective detector (range 45—425 Da) with autosampling and autoinjection (Hewlett Packard 6890). The GC separation was performed on a Hewlett Packard 5 MS (methyl syloxane) column (30 x 0.25 μm and 0.25 μm film thickness). The carrier gas was helium at constant flow rate 0.8 mL/min. The following GC temperature program was used: injector and detector temperatures 250°C and 280°C, respectively, initial temperature 50°C, without holding, temperature increase rate: 40°C/min to 200°C, without holding at 200°C followed by 20°C/min to 280°C, and held at this temperature for 7.25 min. The whole cycle lasted for 15 min. The retention times of diacetyl, 2,3-pentanedione and 2,3-hexanedione (internal standard) derivatives e.g. 2,3-dimethylquinoxaline, 2-ethyl-3-methylquinoxaline and 2-methyl-3-^{n}-propyl-quionoxaline under separation conditions were: 4.60; 4.90 and 5.20 minutes, respectively. The injected sample volume was 1.00 μL (in splitless mode). Quantification was performed by referring to standard curves obtained from the analysis of known diacetyl and 2,3-pentanedione contents added to worts. 2,3-Dimethylquinoxaline and 2-ethyl-3-methylquinoxaline peak area ratios to the internal standard were used for standard curves and quantification.

Determination of diacetyl and 2,3-pentanedione contents during fermentation

Diacetyl and 2,3-pentanedione contents were determined based on standard curves performed in aqueous solutions and worts containing 0.0050—1.0000 mg/L of diacetyl and 0.0050—1.0000 mg/L of 2,3-pentanedione. 2,3--Hexanedione was used as internal standard. To achieve precise determination of diacetyl and 2,3-pentanedione contents in the course of fermentation of different worts, for each wort composition (produced from malt and with 10, 20, 30, and 40% of corn grits) standard curves were prepared under identical conditions as with the aqueous solutions. Diacetyl, 2,3-pentanedione and 2,3-hexanedione were derivatized with the aid of 1,2-diaminobenzene, yielding 2,3--dimethylquinoxaline, 2-ethyl-3-methylquinoxaline and 2-methyl-3-*n*-propyl-quinoxaline. Internal standard content in all determinations was 0.5000 mg/L.

Diacetyl, 2,3-pentanedione and internal standard derivatives were extracted by SPE columns and then measured by GC/MS. The procedure for diacetyl and 2,3-pentanedione quantitative determination was previously described by Pejin et al. (2004).

Fermentation

Ten fermentations were performed: five at 8°C and five at 14°C using the worts produced from malt and with 10, 20, 30 and 40% of corn grits. Each fermentation was carried out using pure industrial yeast culture, deposited at the Chair of Microbiology of the Faculty of Technology Novi Sad. Medium for the inoculum growth was the wort prepared for each fermentation separately, i.e. for the first fermentation the inoculum was propagated on the wort produced from malt, and for other fermentations the inoculum was multiplied in the wort containing 10, 20, 30 and 40% corn grits as a substitute for malt. The wort was prepared according to the method for the determination of malt extract-Congress mash (European Brewery Convention, Analytica — EBC, 1998). The worts containing corn grits were prepared by replacing part of the malt with 10, 20, 30 and 40% of corn grits. After adding hops the wort was cooled, aerated and inoculated under sterile conditions in the EBC fermenter. After inoculation the count of cells in the EBC fermenter was 10^7 cells/mL.

Before preparation of wort, malt, hops and corn grits were analyzed for usual quality technological parameters. During fermentation the following parameters were determined: real attenuation, colour, pH value and number of yeast cells. Final beers were analyzed for usual quality technological parameters (European Brewery Convention, Analytica — EBC, 1998).

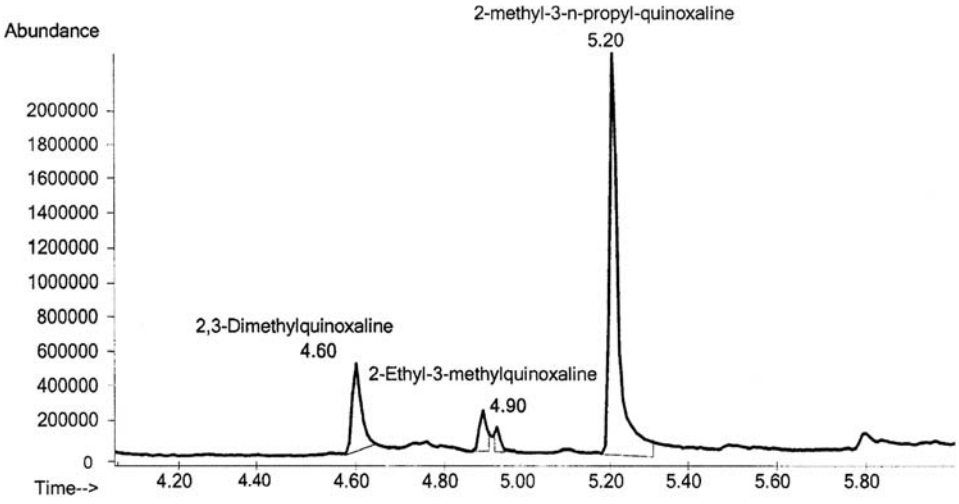

Fig. 1. Total ion chromatogram (SIM mode) of diacetyl, 2,3-pentanedione and internal standard derivatives extracted from fermenting wort

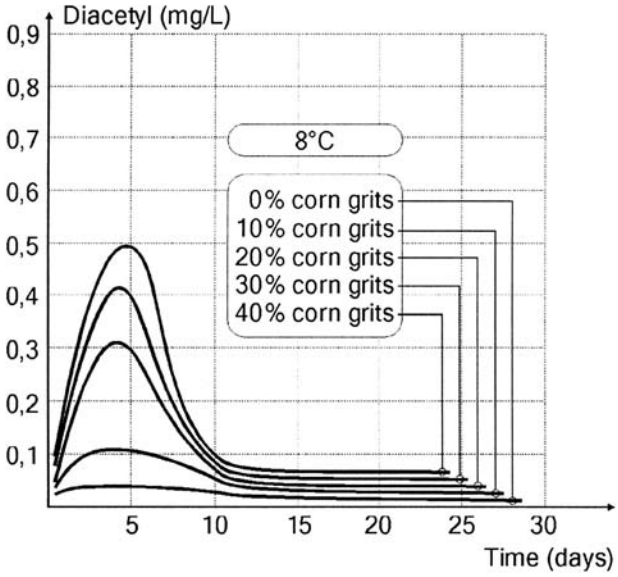

Fig. 2. Formation and reduction of total diacetyl during fermentation and maturation (fermentation at 8°C)

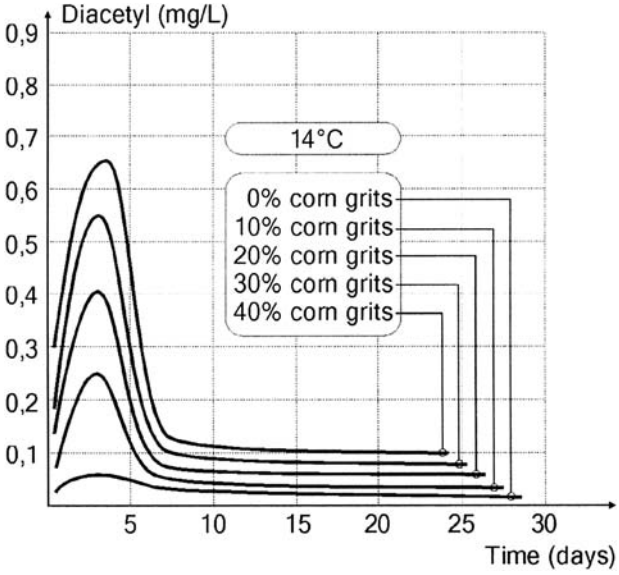

Fig. 3. Formation and reduction of total diacetyl during fermentation and maturation (fermentation at 14°C)

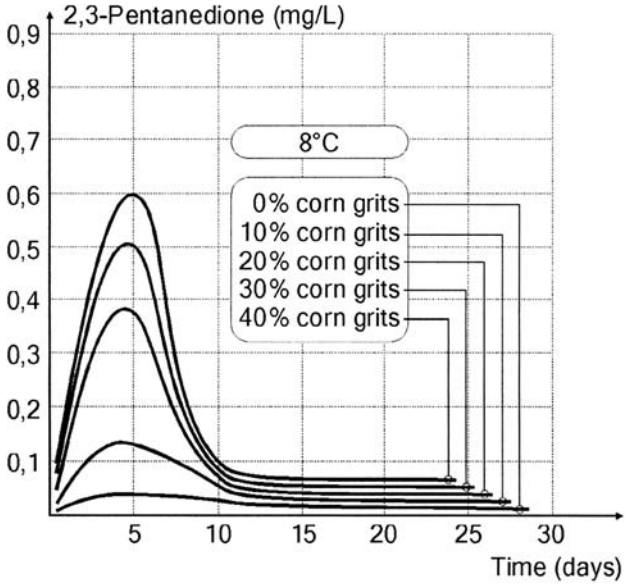

Fig. 4. Formation and reduction of total 2,3-pentanedione during fermentation and maturation (fermentation at 8°C)

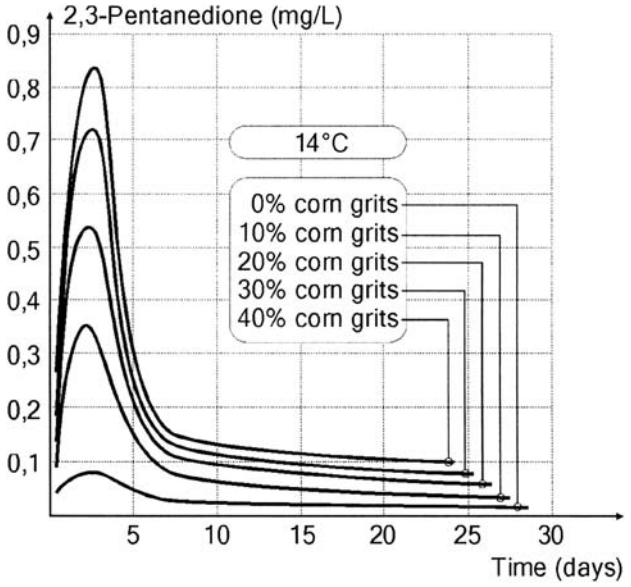

Fig. 5. Formation and reduction of total 2,3-pentanedione during fermentation and maturation (fermentation at 14°C)

RESULTS

In Figure 1 there is given the total ion chromatogram of diacetyl, 2,3-pentanedione and internal standard derivatives extracted from fermenting wort.

Diacetyl and 2,3-pentanedione contents during fermentation

In Figures 2—5 there are given the cumulative results for diacetyl and 2,3-pentanedione contents in the course of fermentation at 8°C and 14°C and maturation at 0°C of the worts produced from malt and with the addition of 10—40% of corn grits.

DISCUSSION

As can be seen from the presented results for diacetyl and 2,3-pentanedione measured during the fermentation at 8°C, the contents of both compounds increased to the fifth day of fermentation and then decreased to the end of the fermentation process. With the increase in content of corn grits diacetyl and 2,3-pentanedione contents showed an increase, too. For example, the maximal diacetyl and 2,3-pentanedione contents in the course of fermentation of the wort produced from malt were 0.0285 mg/L for diacetyl and 0,0380 mg/L for 2,3-pentanedione, and in the fermentation of the wort with 10% of corn grits maximal diacetyl content increased to 0.1081 mg/L and that of 2,3-pentanedione to 0.1425 mg/L. Thus, it can be concluded that the contents increased by about 3—3.5 times. Further increase in the content of corn grits in the wort caused an increase in the maximal diacetyl and 2,3-pentanedione contents by about 10 times compared to those found in the fermentation of the wort produced from malt. In the fermentation of the wort produced with 30% of corn grits diacetyl and 2,3-pentanedione contents increased by 15—20 times. The highest values of diacetyl (0.4933 mg/L) and 2,3-pentanedione (0.6069 mg/L) contents were obtained in the fermentation of the wort produced with 40% of corn grits.

The results obtained for diacetyl and 2,3-pentanedione in the course of fermentation at 14°C show that the contents of both compounds increased to the third day of fermentation and then decreased to the end of the fermentation. With the increase in the content of corn grits in the wort, there increased also the contents of the investigated compounds. Maximal diacetyl and 2,3--pentanedione contents in the fermentation of the wort produced from malt were 0.0558 mg/L for diacetyl and 0.0787 mg/L for 2,3-pentanedione, while in the fermentation of the wort produced with 10% of corn grits they increased to 0.2475 mg/L and 0.3460 mg/L, respectively, that is the contents increased by about 6 times. In the fermentation of the wort containing 20% of corn grits the maximal diacetyl and 2,3-pentanedione contents increased by about 8 times compared with the fermentation of the wort produced from malt. With the further increase in the content of corn grits (30%) in the wort diacetyl and

2,3-pentanedione contents increased by about 12 times compared with the fermentation of the wort produced from malt. The highest values of diacetyl and 2,3-pentanedione contents were obtained in the fermentation of the wort containing 40% of corn grits (0.6365 mg/L for diacetyl and 0.8192 mg/L for 2,3-pentanedione).

In all fermentation 2,3-pentanedione contents were significantly higher compared with those of diacetyl. At the end of maturation, diacetyl and 2,3-pentanedione contents were below the flavour threshold (about 0.1 mg/L for diacetyl and 1.0 mg/L for 2,3-pentanedione).

The increase in the fermentation temperature from 8°C to 14°C resulted in the increased diacetyl and 2,3-pentanedione contents and these results are in agreement with the findings reported in the literature (Garcia et al., 1994; Landaud et al., 1998; Šmogrovičova and Dömény, 1999).

However, in the available literature no data could be found concerning diacetyl and 2,3-pentanedione contents in the wort with partial substitution of malt with corn grits.

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УТИЦАЈ ТЕМПЕРАТУРЕ ФЕРМЕНТАЦИЈЕ И САСТАВА СЛАДОВИНЕ НА DIACETYL И PENTADION У ПИВУ

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Резиме

Диацетил и 2,3-пентандион су важне компоненте сензорних особина пива. У раду је развијена GC/MS метода за одређивање садржаја диацетила и 2,3-пентандиона. Ова метода је примењена за одређивање садржаја диацетила и 2,3-пентандиона током ферментације пива (главне и накнадне). Главне ферментације су извођене на различитим температурама (8 и 14°C). Ферментације су извођене чистом културом *Saccharomyces cerevisiae* припреманом за сваку ферментацију. Температура главне ферментације имала је значајан утицај на настајање и редукцију диацетила и 2,3-пентандиона. Брзине настајања и редукције су се повећавале са повишењем температуре главне ферментације. Садржаји диацетила и 2,3-пентандиона су били виши са повишењем садржаја кукурузне крупице. Највише вредности садржаја диацетила и 2,3-пентандиона током ферментација на 8°C добијене су у ферментацији сладовине са 40% кукурузне крупице (0,4933 mg/l за диацетил и 0,6069 mg/l за 2,3-пентандион). Највише вредности садржаја диацетила и 2,3-пентандиона током ферментација на 14°C добијене су у ферментацији сладовине са 40% кукурузне крупице (0,6365 mg/l за диацетил и 0,8192 mg/l за 2,3-пентандион). Добијени садржаји 2,3-пентандиона били су знатно виши од садржаја диацетила у свим ферментацијама. На крају свих ферментација садржаји диацетила и 2,3-пентандиона су били нижи од прага осетљивости укуса (за диацетил око 0,1 mg/l, а за 2,3-пентандион око 1,0 mg/l). GC/MS метода за одређивање диацетила и 2,3-пентандиона је била значајна за анализирање утицаја састава сладовине или услова ферментације као што је температура главне ферментације.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 239—245, 2005

UDC 663.14.038.3

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INVESTIGATION OF ZINC BIOSORPTION BY BREWER'S YEAST CELLS

ABSTRACT: The highest amount of zinc (= 90%) is bound after 3 hrs of contact at low initial (total) concentrations of zinc in suspension of yeast, 10—100 mg/l at 10—30°C. The equilibrium between bound and free zinc ions is established after 6 hrs of contact time, independently on the total zinc concentration in yeast milk. No bigger changes of content of zinc bound to brewer's yeast cells was determined at temperatures 10°C and 30°C. 40% of bound zinc in the equilibrium state is bound during the first 15 min of contact of zinc ions and brewer's yeast cells at all initial (total) zinc concentrations in suspension of yeast both at 10°C and 30°C. The "KEKAM" equation can be used for the description of kinetics of zinc biosorption by waste brewer's yeast cells, for the ranges of zinc concentration 10—100 mg/l at 30°C (mean correlation coefficient 0,96) and 60,0—100 mg/l at 10°C (mean correlation coefficient 0,95).

KEY WORDS: Biosorption, "KEKAM", waste brewer's yeast, zinc

INTRODUCTION

Biosorption is the capability of dead biomass, mostly of microbiological origin, to bind and accumulate metals from relatively dilute solutions. Of special interest are "industrial" heavy metals for their toxicity in the environment or metals of technological interest. Regarding the microbiologic biomass, the ones available in bigger amounts are more important. The activities of industrial fermentation are often connected with the problem of waste removal, i.d. use of biomass. The biosorption involves the phenomena connected with the biomass and metal ions, when the concentration of those ions in the media is higher than physiologically necessary for certain microorganism. Biosorption is the accumulation of metals without the active binding by cells (Volesky, 1994). The conditions of culture growth may affect the metal uptake capacity which is for non-living and live yeast cells Zn > (Cd) > U > Cu and Zn > Cu > (Cd) > U, respectively (Volesky, May-Philips, 1995). Due to the complex structure of microorganisms there is a number of ways of metal binding to the cells (Veglio, Beolchini, 1997). Two widely accepted mo-

dels of absorption isothermes can be linearized, as recommended by Langmuir and Freundlich (V o l e s k y, 1994). Both models while capable of describing many biosorption isotherms can hardly have a meaningful physical interpretation in biosorption. However, the parameters of these models are not acceptable for the accurate physical interpretation. In addition to equilibrium studies, the kinetics of biosorption has to be determined in order to establish the time course of the metal uptake. Rapid uptake of the metal by the biosorbent is desirable providing for a short solution-biosorbent contact time in the actual process. The type and dimensions of the contact equipment depend on the contact time, further directly affecting the total and processing price of the process. The equilibrium and kinetic characterization of biosorbent material are also important for the quantitative investigation of its characteristics and for the process design (V o l e s k y, 1991). Topochemical reactions are localized at the surface of active centres of solid career. A group of Russian scientists has developed a global kinetic equiation known as "KEKAM" (Kolmogorov-Erofeev-Kozeeva-Avrami-Mampel), which can describe the topochemical reactions (A v r a m i, 1939, 1940). The paper presents the investigation of kinetics model of zinc ion biosorption by waste yeast from brewery (waste brewer's yeast), in the range of zinc concentration 1,0—15,0 g/l at 10°C and 30°C. The global kinetic equation which can describe the topochemical reactions "KEKAM" was used.

MATERIALS AND METHODS

Waste brewer's yeast, purchased directly in a national brewery, kept at +4°C, was used for the preparation suspension of yeast. The content of dry biomass of the yeast was determined by drying till constant mass and was 30,8%. The final content of dry biomass in the prepared suspension of yeast was 20 g/l. Zinc solution, was added to suspension of yeast till the final zinc concentration amounting 10,0; 20,0; 30,0; 40,0; 60,0; 80,0 and 100,0 mg/l. $ZnSO_4$ x 7 H_2O (Merck) and bidistilled water were used for the preparation of the solution, which was sterilized by filtration (0,22 sssmm). The percentage of non-living brewer's yeast cells was determined by microscoping of cells in 0,01% solution of methylene blue. The non-living cells were dark blue coloured, while the live ones were non-dyed or very light blue. The percentage of dead cells was 1,5%, meaning that live cells of brewer's yeast were used. The pH value of the prepared yeast milk was 4,4—4,6, and was not corrected. This value enables the presence of zinc in the form of Zn^{2+} ions in the suspension of yeast. Aliquots of 50 ml of prepared suspension of yeast with known zinc concentrations were poored into 100 ml plastic Erlenmayer flasks. Incubation was performed on the rotary shaker at 30°C and 150 rpm. Lower temperature of incubation, 10°C, was used for suspension of yeast samples containing 60,0; 80,0 and 100,0 mg/l of total zinc. 5 ml of suspension of yeast samples were taken for every zinc concentration and incubated 0,25; 0,5; 1; 3 and 24 hrs. Each sample was analyzed in three replications. The samples were filtered (0,45 sssmm) and the obtained filtrates analyzed for residual zinc content. Wet

digestion method was used for the preparation of samples in mixture of cc H_2SO_4 and cc HNO_3 (G o r s u c h, 1970). The zinc content was determined by atomic absorption spectrophotometry (Varian AA10, Australia). All chemicals used for atomic absorption spectrophotometry were ultra-pure grade ("Suprapur", Merck). The content of zinc bound by brewer's yeast cells (V e g l i o et al., 1997) is expressed by the equation: $q = (C_0—C_e) / X$, where:

q — content of bound zinc (mg/g d.m.), Co — content of total zinc (mg/l), Ce — content of free zinc (mg/l), X — content of dry biomass (g/l). The degree of biosorption of zinc by yeast cells is expressed as (K n e ž e v i ć et al., 1998): $\alpha = q/_{qmax}$, where: α — degree of biosorption, q — content of bound zinc (mg/g d.m.), q_{max} — maximal content of bound zinc to yeast cells as the value after 24 h incubation (mg/g dm). The linear form of "KEKAM" equation was used for the processing of experimental data:

$$\ln (-\ln (1-\alpha)) = \ln k + n \ln t$$

In the ln (-ln (l-α)) — Int system "KEKAM" equation represents a straight line. The parameters lnk and n can be determined from the cut and slope and of the given function, respectively.

RESULTS AND DISCUSSION

The kinetics of zinc ions binding by yeast cells is presented in Figure 1, at different total (initial) content of zinc in suspension of yeast, at 30°C and 10°C. The kinetic curves can be divided into three parts: the first part is linear,

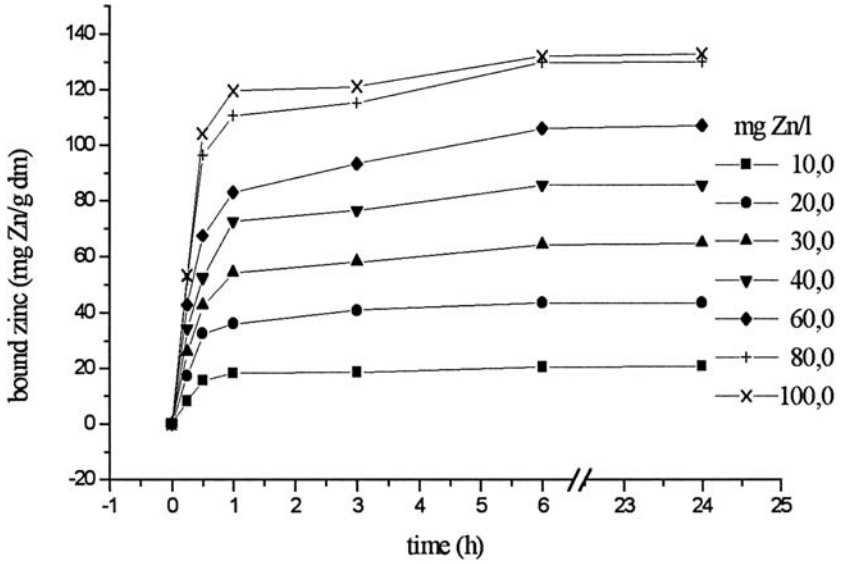

Figure 1. Kinetics of zinc ion binding to yeast cells at 30°C

the amount of bound zinc is increasing proportionally with time, the second part, where the increase of bound zinc amount is very slight and the third — equilibrium part, when the biosorption is not time-dependent.

The largest amount of zinc ions (= 90%) is bound within the first 3 hrs of contact, at all initial (total) zinc concentrations in the suspension of yeast. The equilibrium between the bound and free zinc ions was established after 6 hrs of contact, independently on the total zinc concentration in suspension of yeast. The change of total zinc content for yeast biomass in the last 24 hrs of contact was insignificant. The biosorption was also investigated at 10°C for total zinc concentrations 60, 80 and 100 mg/l in suspension of yeast. The aim was to determine the influence of temperature on the biosorption of zinc ions by yeast cells. The results show that the difference between trials at 10°C and 30°C was rather insignificant. This finding is in accordance with literature data (F a i l l a et al., 1976), which state that temperature range 4—40°C has no significant influence on biosorption, i.d. binding of metal ions to yeast cells surface. At all initial (total) zinc concentrations in suspension of yeast, both at 10°C and 30°C, 40% of final uptake of zinc (equilibrium) was bound within the first 15 min of contact. Figure 2 presents the kinetic curves $\alpha = f(t)$, obtained by using the experimental results, degree of biosorption and time of contact at 30°C and 10°C, respectively. The kinetic curves are of sigmoidal character, and belong to the family of curves with zero initial rate. The presented kinetic curves are the initial bases for the investigation of topochemical reactions.

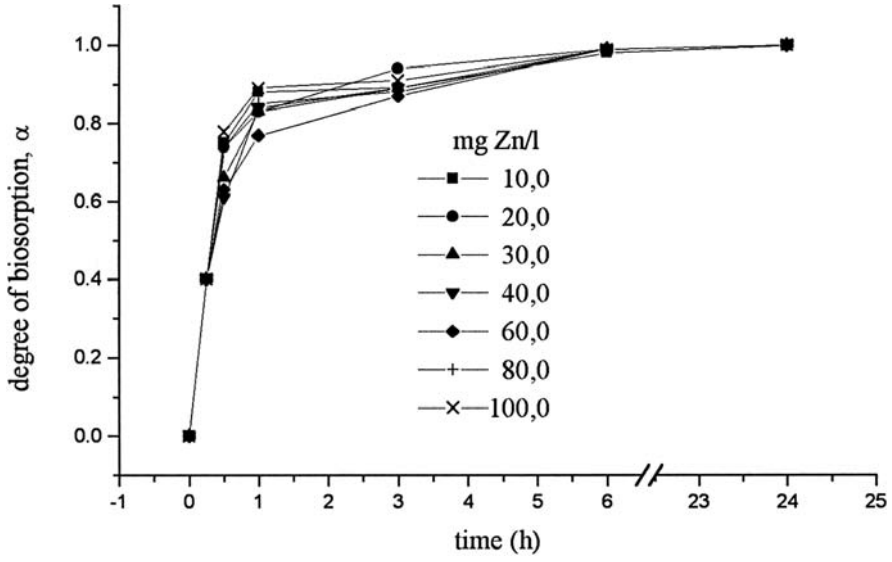

Figure 2. Dependence of biosorption level on incubation time of suspension of yeast at 30°C

The results presented in Figure 3, and the kinetic parameters and correlation coefficients given in Table 1, lead to the conclusion that the linearization of "KEKAM" equation is acceptable for the experimental results. This means

that the theory of topochemical reactions can be used for the description of flow of zinc ions biosorption by brewer's yeast cells, in experimental conditions used during this work. Reproducibility of the "0" samples analyses, expressed as relative standard deviation (average value) was 3,9%. During the biosorption the analyses reproducibility was not changed significantly.

Table 1. Kinetic parameters, correlation coefficient and standard deviation for "KEKAM" equation

Range content of zinc [mg/l]	Temperature of incubation [°C]	k	n	Correlation coefficient	Standard deviation [%]
10,0—100,0	30	1,38	0,588	0,96	3,9
60,0—100,0	10	1,38	0,586	0,95	3,8

Evaluation of the equilibrium sorption performance needs to be supplemented by process-oriented studies of its kinetics and eventually by dynamic continuous-flow tests. The rate of the sorption process, together with the hydrodynamic parameters, determines the size of the contact equipment. Reaction engineering concepts apply for the experimental approach leading to expressing the values of key process parameters used for comparative, process design and scale-up purposes. The most widely used contacting device for sorption processes is the fixed-bed reactor configuration and its modifications. The principles and methodology of deriving and evaluating the key process parameters have been dealt with extensively in the chemical engineering literature (V o l e s k y, 1994). The values of kinetic parameters for "KEKAM" equation, for both incubation temperatures, confirm that temperature does not affect the kinetic of zinc ions biosorption by waste brewer's yeast cells, in the

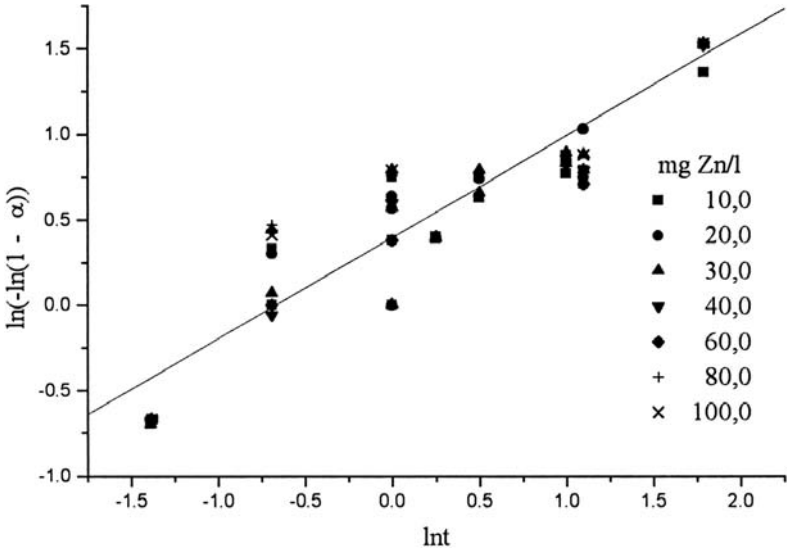

Figure 3. Linearized "KEKAM" equation for the range of zinc concentrations 10—100 mg/l at 30°C

mentioned experimental conditions. It is important to mention that the conditions (medium) of yeast culture growth (obtaining of biomass), significantly affect the "physiological state" of yeast cells influencing further the possibility of heavy metals binding.[2] Therefore, it is difficult to compare the "yield of biosorption" with the literature data, since it is hardly possible to perform the trial under the same conditions, as well as to find biomass in the appropriate "physiological state".

CONCLUSION

The dynamic equilibrium between the free zinc ions in suspension of yeast and ions bound by brewer's yeast cells is established after 6 hrs of incubation in all zinc concentrations investigated. After 3 h of incubation, for all total zinc concetrations in suspension of yeast, the (≈ 90%) of maximal amount of bound zinc is bound by brewer's yeast cells. No significant change of bound zinc ions content was estimated in the temperature range 10—30°C, e.g. in these trial conditions the temperature does not affect the biosorption. The linearization of "KEKAM" equation is satisfactory for the obtained experimental results (mean correlation coefficient 0,96). This means that the theory of topochemical reactions can be applied (used) for the description of zinc ions adsorption to brewer's yeast cells in zinc concentration range 10—100 mg/l, at incubation temperature 30°C. The "KEKAM" equation can be used as well for the description of kinetics of biosorption for the zinc concetration interval 60—100 mg/l in brewer's yeast cell at 10°C.

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ИСПИТИВАЊЕ БИОСОРПЦИЈЕ ЈОНА ЦИНКА ЋЕЛИЈАМА ПИВСКОГ КВАСЦА

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Резиме

У раду је испитивана биосорпција јона цинка ћелијама пивског квасца (отпадни пивски квасац из једне домаће пиваре) у интервалу концентрације јона цинка од 10 до 100 mg/l у суспензији пивског квасца са концентрацијом суве биомасе пивског квасца 20 g/l (вредност pH 4,5), на температурама 10°C и 30°C. Количина слободног цинка у суспензији пивског квасца одређивана је атомским апсорпционим спектрофотометром (Varian A10, Australia). Након 3 h контакта ћелија пивског квасца и јонова цинка, око 90% укупне количине цинка се веже за ћелије пивског квасца и то на обе испитиване температуре. Равнотежно стање, између везаног цинка за ћелије пивског квасца и слободних јонова цинка у суспензији, успоставља се након 6 h контакта. У погледу количине везаног цинка за ћелије пивског квасца нема значајне разлике на температури 10°C и 30°C. Након 15 минута контакта за ћелије пивског квасца веже се око 40% укупне количине цинка присутне у суспензији, и то на обе испитиване температуре. Кинетика биосорпције јона цинка ћелијама пивског квасца, под условима примењеним у овом раду, може се описати „КЕКАМ" једначином: $\ln(-\ln(1-\alpha)) = \ln k + n \ln t$; где је α — степен биосорпције јона цинка (однос количине везаног цинка изражене по граму суве материје пивског квасца у неком времену t и максималне количине везаног цинка изражене по граму суве материје пивског квасца која се остварује након 24 h контакта), t — време контакта, k и n — кинетички параметри биосорпције. Применом наведене једначине, у испитиваном интервалу концентрација јона цинка у суспензији пивског квасца, на температури од 30°C остварује се коефицијент корелације 0,96, а на температури од 10°C остварује се коефицијент корелације 0,95.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 247—254, 2005

UDC 663.14.038.7:664.6

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ACTIVATION OF WASTE BREWER'S YEAST *SACCHAROMYCES CEREVISIAE* FOR BREAD PRODUCTION

ABSTRACT: The waste brewer's yeast *S. cerevisiae* (activated and non-activated) was compared with the commercial baker's yeast regarding the volume of developed gas in dough, volume and freshness stability of produced bread. The activation of waste brewer's yeast resulted in the increased volume of developed gas in dough by 100% compared to non-activated brewer's yeast, and the obtained bread is of more stable freshness compared to bread produced with baker's yeast. The activation of BY affects positively the quality of produced bread regarding bread volume. The volume of developed gas in dough prepared with the use of non-activated BY was not sufficient, therefore, it should not be used as fermentation agent, but only as an additive in bread production process for bread freshness preservation. Intense mixing of dough results in more compressible crumb 48 hrs after baking compared to high-speed mixing.

KEY WORDS: activation, baker's yeast, bread, waste brewer's yeast

INTRODUCTION

Brewer's yeast (BY) as the by-product in brewery ends mostly in waste waters, both in the world and our country (B a r a s, 1991). The chemical oxygen consumption of waste brewer's yeast (WBY) is about 5,30 g/l, therefore this by-product is considered to be one of the greatest pollutants of the environment (K u n z e, 1998). WBY is used as feed, in pharmaceutical industry (production of ergosterol, enzymes, nucleic acids, amino acids and B group vitamins), in fermentation industry (yeast autolysate as additive to media) and in food industry (food additive) (P e p l e r, 1970). Due to high content of proteins, B-complex vitamins and minerals, BY is very important in food processing industry. The flavor of WBY is very bitter and the direct use in food industry would affect negatively the sensory characteristics of food products. The bitterness originates from the presence of resin and tannins adsorbed to

the cell surface, in the fermentation step during beer production (N a n d, 1987). Therefore, the debittering of WBY is necessary before it can be used in food industry (K u n z e, 1998). No available literature data were found on the use of waste BY as the leavening agent for dough in bakery industry. Our brewing industry produces about 6.000 t of waste yeast per year, with cca 15% dry matter. At the same time, the use of baker's yeast is sbout 50.000 t. It can be assumed that bakery products of similar quality could be produced with double portion of BY in the composition of dough. WBY could be used in bakery industry after appropriate processing. Only 3% of used baker's yeast would be replaced by waste brewer's yeast, however, the decrease of dumping problem of WBY would be a significant contribution to environmental protection. The direct use of debittered BY as dough component does not result in appropriate effects, so it is necessary to activate the brewer's yeast in suitable media. The aerobic procedure activates the enzyme complex of BY cells, enabling the fermentation and dough rising (D o d i ć, 2002). The paper presents the investigation of possible use of WBY (*Saccharomyces. cerrevisiae*) as raw material for bread production. BY was compared to commercial baker's yeast regarding the volume of developed gas in dough and some characteristics of bread, as final product.

MATERIAL AND METHODS

BY *Saccharomyces. cerrevisiae* (waste yeast from a domestic brewery) and commercial pressed baker's yeast *Saccharomyces. cerrevisiae* with 27% of dry matter ("Fermin", Senta, Serbia and Montenegro) were used for the investigations. BY was debittered using 2N solution of NaOH (N a n d, 1987) and separated by centrifugation, 20 min at 7000 rpm (Westfalia separator). After the separation, the dry matter content of BY biomass was 27%. In our previous investigations of waste brewer's yeast revitalization for use in baking industry, the optimization process, which included composition of medium, process parameters and fermentation technique, was developed. The activation of WBY for use in bread production, applied in this work, was also defined. The debittered BY was activated for 45 min in media containing 5% (m/v) of malt extract (obtained from a domestic brewery, 80% dry matter, out of which 65% of sugars) at 30°C, mixing rate 300 rpm and specific aeration rate 4,5 l/l min (D o d i ć, 2002). The activation was performed in the laboratory fermentor Chemap-Pec (Mannedorf, Swiss), working volume 10 l. The biomass of BY was centrifuged for 20 min at 7000 rpm (Westfalia separator) to the final dry matter content of 27%. The investigated yeasts were: non-activated BY, activated BY and control baker's yeast. Dough was mixed in farinograph mixer (Brabender, OHG, Duisburg, Germany), using 100% of white flour, 2% of salt, 2% of baker's yeast or 8% of non-activated BY or 8% of activated BY and water in an amount necessary for the achieving of constant dough consistency of 500 FU (farinologic units) after 5 min of mixing. The dynamics and volume of gas developed in the dough were determined using the fermentograph (Brabender, OHG, Duisburg, Germany) according to the recommenda-

tions given by the producer. Dough samples (flour 100%, yeast 2%, table salt 2%, vegetable fat 0.7%, and water according to farinographic water absorption) were prepared using the intense mixing process (mixer Stephan, 100 s, 1400 rpm, dough fermentation in mass 10 min) and high-speed mixing process (kneading machine DIOSNA, 1 min at 8 rpm and 7 min at 120 rpm, dough fermentation in mass 60 min). Bread was baked at 230+5°C for 30 min. The weight of bread was 500 g. Important elements for quality evaluation are: volume of bread and compressibility of crumb, e.g. freshness stability of bread for a certain period after baking. Crumb compressibility of bread samples prepared with the investigated yeasts was evaluated on the basis of PN, as the measure of compressibility, 8, 24 and 48 hrs after baking. The PN of crumb was determined with SUR Penetrometar (PNR 6) as the mean value of PN determined at 3 places on the cross section of bread (Jančić, Beleslin, 1979). Bread volume was determined by volume meter with millet (Instrumentaria, Zagreb, Hrvatska), 24 hours after the baking. All determinations were performed in triplicate. The standard error and T-test were analyzed. MS Origin program was used for data analysis.

RESULTS AND DISCUSSION

BY was evaluated on the basis of influence on dough properties and stability of bread freshness. Dough properties defined by gas developing dynamics (ml CO_2) during fermentation were registered on fermentograph, while

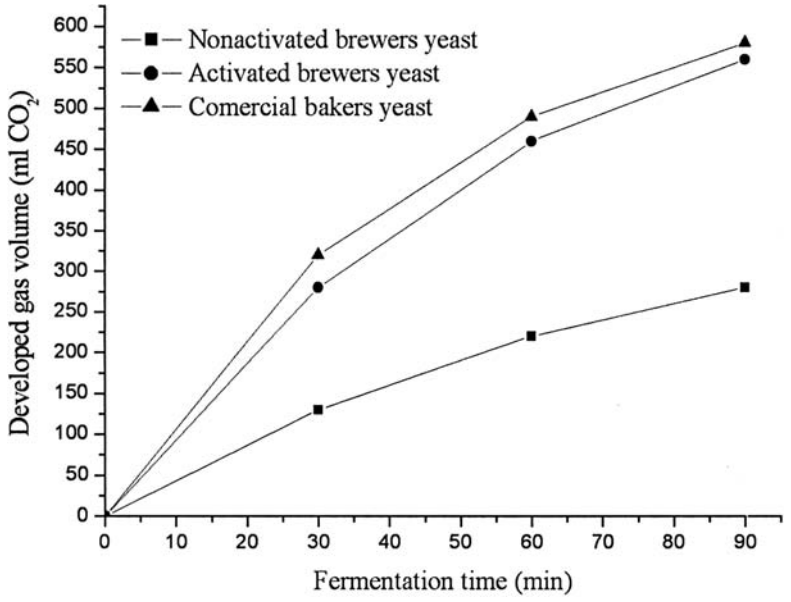

Figure 1. Change of developed gas volume (ml CO_2) during fermentation of dough prepared with different kinds of yeasts

bread freshness stability was monitored on the basis of crumb compressibility and volume of bread obtained during experimental baking. The fermentative activity of the investigated yeasts during dough fermentation, registered on fermentograph, are presented in Figure 1.

The increase of developed gas volume (ml CO_2) during dough fermentation was registered for all kinds of investigated yeasts. However, the developed gas volume in dough prepared with the use of non-activated BY is rather small, after 30 min and 90 min of fermentation the developed gas volume of this sample was by 60% e.g. 50% smaller compared to dough prepared with control baker's yeast. The use of activated BY resulted in significantly increased gas voume, practically by 100%, in dough prepared with this sample, compared to dough prepared with non-activated BY. Most probably the activation of BY resulted in shorter or lack of lag phase during fermentation of dough. The volume of developed gas after 30 min of fermentation of dough prepared with activated BY is by 10% smaller compared to dough made with baker's yeast (control), and after 90 min of fermentation the difference was less than 5%. Regarding the technological aspect, the minimal difference in volume of developed gas during fermentation of dough prepared with activated BY and control baker's yeast, is no barrier for the use of activated BY in production of appropriate quality bread. The activation of BY results in the activation of enzyme cell complex, necessary for the fermentation of fermentable sugars in flour and development of sufficient amount of CO_2 that affects directly and significantly the quality of dough and of bread, as final product. The quality of bread samples made with baker's, activated and non-activated BY by intense and high-speed mixing was evaluated through bread volume (Fig. 2).

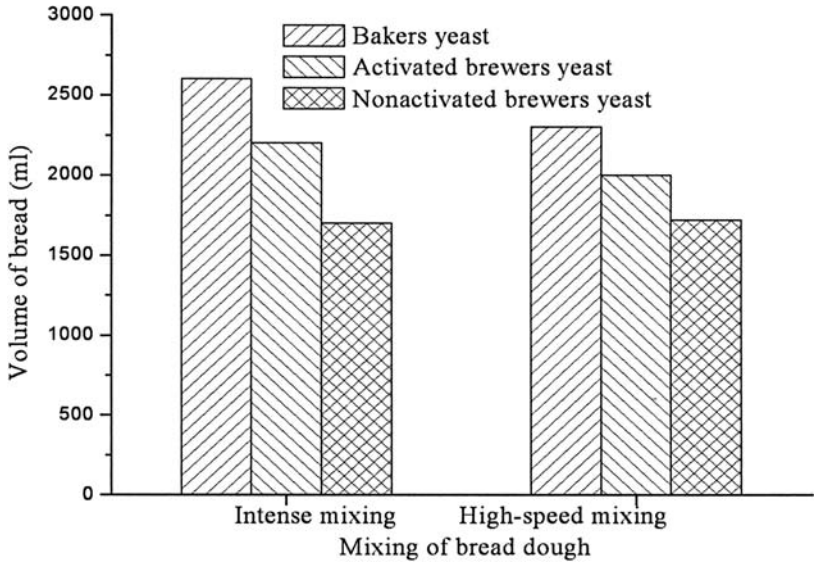

Figure 2. Volume of bread samples made with baker's, activated and non-activated brewer's yeast by intense and high-speed mixing

Volume of bread samples made applying the intense mixing process, with activated and non-activated BY was by 15% e.g. 40% smaller compared to bread made with baker's yeast. The activation of BY results in improved bread quality, regarding bread volume. Applying the high-speed mixing process and using the three kinds of investigated yeasts, somewhat lower values were found for bread volume, in average by about 10%, compared to the bread made applying the intense mixing process. The activation of BY affects positively the bread quality in this case as well. The influence of BY use on crumb compressibility, evaluated by PN of samples determined 8, 24 and 48 hours after baking is presented in Figures 3 and 4.

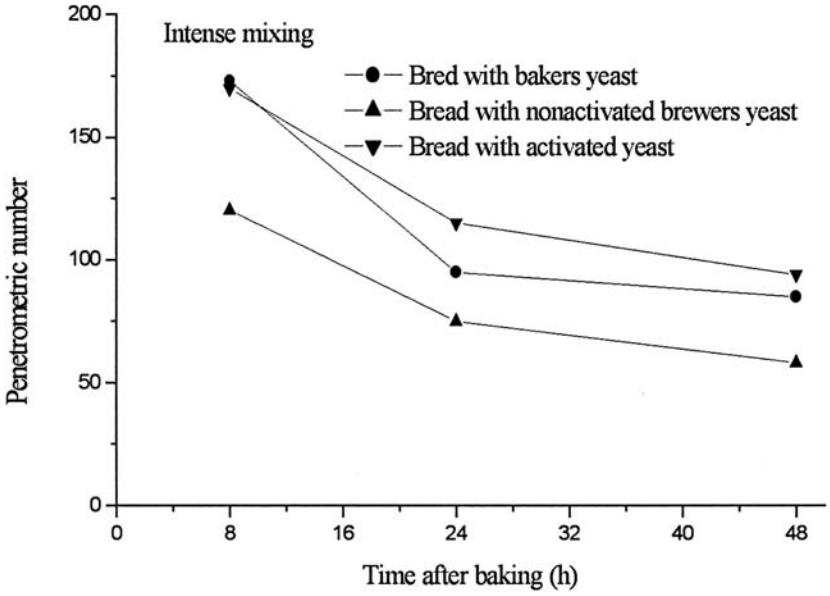

Figure 3. Change of PN of bread with time after baking of dough prepared by intense mixing

The results presented in Figure 3 show that the PN values of bread samples, 8 hours after baking of dough obtained by intense mixing, and with the use of activated BY and baker's yeast are similar. On the basis of this finding, it can be concluded that crumb compressibility of bread made with activated BY, determined 8 hours after baking, e.g. bread freshness is almost the same as of control bread prepared with baker's yeast. The use of activated BY in the high-speed mixing of dough, results in slower decrease of PN, in the period 8 to 24 hours after baking. The PV of control bread decreased by about 45% and of bread with activated BY by about 30% compared to values measured 8 hours after baking. The slower aging is a very important characteristic of bread made with the use of activated BY. The analysis of RV change in the subsequent period (24—48 hours after baking) shows that freshness stability of bread made with activated BY is significantly pronounced, e.g. the aging of this bread sample is clearly slower compared to bread samples made with ba-

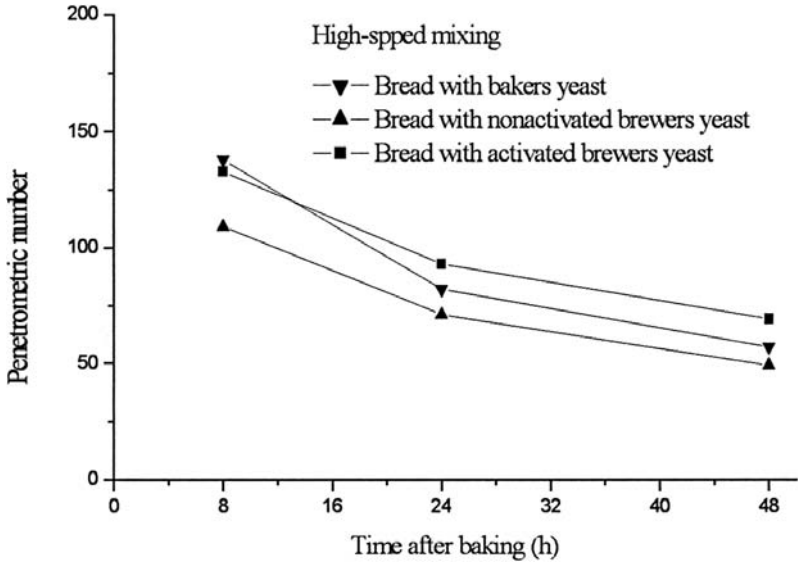

Figure 4. Change of PV after baking of bread samples prepared by high-speed mixing

ker's yeast. Bread made with the use of non-activated brewer's yeast is characterized by slower aging compared to bread made with baker's yeast. However, the shortage of brewer's yeast use is the insufficient volume of developed gas in dough.

The PV of dough prepared by high-speed mixing process, are in average by 25% lower compared to intense mixing. The PV of bread (8 hours after baking) made by high-speed mixing, with activated BY, is similar to control bread with baker's yeast. So, the crumb compressibility of bread made with activated BY and of control bread are almost the same. Bread made with non-activated BY is characterized by a tougher crumb e.g. aging is more pronounced compared to bread with activated BY and control bread (measured 8 hours after baking). 24 hours after baking, the PV of control bread and of bread with non-activated BY decreases by about 40%, and of bread with activated BY by about 30% of the value determined 8 hours after baking. This confirms the conclusion from the experiment with the use of intense mixing, i.d. that crumb compressibility is more expressed in control bread compared to bread with activated BY. In the subsequent period after baking (24—48 hours), the dynamics of PV decrease is almost the same for all three bread samples. The biggest crumb compressibility was again found in bread with activated BY, followed by control bread, and bread with non-activated BY, at the end. The mean values of developed gas volume in dough were analyzed (T-test), as well as the standard error of developed gas volume during fermentation (90 min) (Table 1) and of PV, e.g. crumb ST of bread samples prepared with the investigated yeasts. No significant difference (significance factor 0,05) was found in developed gas volume during 90 min of fermentation when baker's yeast and activated BY were used. However, the volume of developed

gas during fermentation of dough prepared with non-activated BY is significantly different (significance factor 0,01) compared to other two bread samples. Values of standard measuring error of developed gas volume of dough, bread volume and PV of crumb are in acceptable range.

Table 1. T-test and standard error (+%) during determination of developed gas volume, bread volume and PV.

Uzorak	T — test					Standard error (±%)							
	Fermentographic investigation					Intense mixing				High-speed mixing			
							Penetrometric number						
	Baker's yeast	Activated brewer's yeast	Fermentation of bread dough (min)			ml bread	Time after baking (h)						ml bread
			30	60	90		8	24	48	8	24	48	
Baker's yeast	—	—	3,1	3,0	3,4	0,9	2,8	3,0	3,1	2,5	2,3	2,6	0,8
Nonactivated brewer's yeast	**	**	3,4	3,1	3,3	1,1	2,4	2,9	2,4	2,4	3,0	2,8	0,9
Activated brewer's yeast	NS	—	2,9	3,2	3,1	1,2	2,6	2,8	2,2	2,4	2,9	3,0	0,9

All determinations were performed in triplicate.

CONCLUSIONS

On the basis of the analysis of results obtained in this work, the following can be concluded: The fermentative activity of debittered BY (from brewery) without activation is not satisfactory in dough compared to commercial baker's yeast, so this yeast can be used as the additive for bread stability (freshness) improvement. The use of activated BY (45 min in media with 5% (m/v) at 30°C, 300 rpm, specific aeration rate 4,5 l/l min) results in an increased developed gas volume in dough by about 100% compared to non-activated brewer's yeast, but the developed gas volume is by 5—10% smaller compared to dough made with commercial baker's yeast. The activation of BY affects positively the quality of bread regarding the volume of the final product. The use of activated BY in bread production affects positively the crumb ST, e.g. freshness stability preservation.

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АКТИВАЦИЈА ОТПАДНОГ ПИВСКОГ КВАСЦА *SACCHAROMYCES CEREVISIAE* ЗА ПРИМЕНУ У ПРОИЗВОДЊИ ХЛЕБА

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Резиме

Извршена је оцена отпадног пивског квасца *Saccharomyces cerevisae* (активираног и неактивираног) у односу на комерцијални пекарски квасац са аспекта запремине развијеног гаса у хлебном тесту, запремине и одрживости свежине произведеног хлеба. Поступком активације отпадног пивског квасца запремина развијеног гаса у хлебном тесту се повећава за око 100% у односу на неактивиран пивски квасац и добија се хлеб са постојанијом свежином у односу на хлеб са пекарским квасцем. Поступак активације пивског квасца позитивно утиче на квалитет произведеног хлеба и то са аспекта запремине хлеба. Неактивиран пивски квасац не даје потребну запремину развијеног гаса у хлебном тесту и не може се користити као средство за дизање теста него само као адитив у производњи хлеба у циљу одржања свежине хлеба. Интензивни замес хлебног теста даје стишљивију средину хлеба током 48 h након печења у односу на брзоходни замес.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 255—267, 2005

UDC 582.28:631.416(497.13 Fruška gora)

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MACROELEMENTS AND HEAVY METALS IN SOME LIGNICOLOUS AND TERICOLOUS FUNGI

ABSTRACT: Considering the importance of *Basidiomycotina* fungi as bioindicators and the increasing tendency of air and soil contamination nowadays, content of macroelements: *N, P, K, Ca, Mg* and *Na*, and some of heavy metals (microelements): *Fe, Pb, Cu, Cr, Zn* in sporocarps of 22 species of macrofungi from National park Frushka Gora was analyzed in this work. The majority of them are lignicolous species from the class: *Homobasidiomycetes* order: *Aphyllophorales s. lato* and two of them belong to subdivision *Ascomycotina*. Special attention was given to the medically important fungal species widely distributed in this area: *Ganoderma lucidum*, *Ganoderma applanatum*, *Coriolus. versicolor*, *Flammulina velutipes*, *Meripilus giganteus* and *Omphalotus olearius*. Since fungi uptake macro- and microelements from the substrate, the soil and tree samples were also analyzed. The aim of this study was to determine the common concentration data of these elements in unpolluted area. According to the obtained results, fungi tended to accumulate K, P and N with lower variability recorded than that for Ca, Mg and Na due to essential importance of these elements in fungal metabolic processes, independently from ecological group affiliation and habitats. The analysing substrate (wood) contain higher concentration of Ca and Pb in relevance to fungi. As a result of transfer factors (TFs) that were estimated from the ratio of "concentration in fungi on dry weight basis" to "concentration in the tree or soil on dry weight basis" analyzed fungi tended to accumulate Cu and Zn, and partially Cr. Among the species that accumulate microelements the following species could be emphasized: *Meripilus giganteus* (exept for Zn), *Schizophyllum commune* (except for Pb), *Ganoderma applanatum* (except for Fe and Zn). Superaccumulators of Fe were the lignicolous, medically imortant species *M. giganteus*, *G. lucidum*, *Sch. commune*, and tericolous ones: *C. atramentarius*, *F. velutipes*, and *P. vernalis*. Good Cu accumalators were tericolous species: *Psathyrella vernalis*, *Morchella vulgaris*, and *Coprinus atramentarius*, then the species possesing the rhizomorphs: *Armillaria polymyces* and *Omphalotus olearius*, and finally lignicolous species: *Ganoderma applanatum* and *Pseudotrametes gibbosa*. The highest accumulation of Zn was recorded for the species *Schizophyllum commune*, while the fungal species expressed the smallest tendency of accumulation of Pb, except one tericolous, saprophytic species *C. atramentarius*. The greatest accumulation of Cr was found in tericolous species especially *C. atramentarius*, too.

Statistical cluster analysis classified fungi mostly by location, by which the urban site is distinguished from the others, where the analyzed tericolous species were found. These data indicate that the accumulation ability is not only genetically coded, but also influenced by environmental factors. This data could be used in toxicology, pharmacology and environmental protection.

KEY WORDS: *Basidiomycotina*, bioindication, Frushka Gora, fungi, heavy metals, macroelements, sporocarps

INTRODUCTION

Fungi are ubiquitous in natural environments and important in industrial processes, which, together with other microbial groups can accumulate metals and radionuclides from their external environment by means of the physicochemical and biological mechanisms. The principal factors influencing the accumulation of heavy metals in macrofungi are *environmental factors* (metal concentrations in the soil and substrate, pH, organic matter, and contamination by atmospheric deposition) and *fungal factors* (fungal structure, biochemical composition, decomposition activity, development of mycelium and sporocarps, morphological properties), although physiological mechanisms of its uptake are still not determined. Fungi are very important in natural cycle of metal ions due to many reasons: they are very good in accumulation of metals and could be used as bioindicator species of heavy metals in soils (Dietl et al., 1987). Due to mycelium, which has great surface of hyphae that could adsorb and accumulate metals, the majority of them (Cu, Zn, Cd) are captured in fungal biomass in the humus zone. Fungi *Basidiomycotina* degrade the upper layer of humus e.g. polyphenolic compounds (lignin, humin acid, fulvic acid, humin) by enzymes (phenoloxidases) which are responsible for effective binding of ions by means of ion exchange and forming the chelates. Hence, the high tolerance against heavy metals can be expected among the fungi that have the ability of polyphenol degradation (Hoiland, 1995).

High concentrations and bioaccumulations of different major and trace elements were reported in European forests (Byrne, 1976; Gadd, 1993; Vetter, 1994; Kalac et al., 1991) and also in Japanese forests (Yoshida & Maramatsu, 1996, 1998). Many of them demonstrated that mushrooms tended to accumulate Cu, Zn, Rb, Cd and Cs. Kalac et al. observed that Hg, Pb, and Cu were accumulated by tericolous *Lepista nuda* and *Lepiota rhacodes*. Higher concentrations of Pb, Cd, Zn and Hg are found in macrofungi (prevalent ectomycorhizal that are in tense contact with roots of wood) from urban or industrial areas (Rűhling & Söderström, 1989; Melgar et al., 1998; Fellner & Pešková, 1995), but also in wood ecosystems, which are influenced by contaminants. From this point of view lignicolous fungi have not been examined, probably due to their main role in decaying of wood substrata. In the present study the mineral concentrations of certain lignicolous and tericolous species were investigated. The aims of this study were: 1) the comparison of fungi on the basis of their mineral content; and 2) to determine whether these species have the availability for bioaccumulation of micro- and macroelements.

MATERIAL AND METHODS

Fungi and soil sampling: The sporocarps of the investigated fungal species were collected from four locations (*L1 — Elektrovoyvodina* (Irishki Venac), *L2 — Mali spomenik* (Irishki Venac), *L3 — Zmayevac*, *L4 — Paragovo*) in the National park Frushka Gora and at one urban location *L5 — Ribarsko*

ostrvo. The locations on Frushka Gora are situated within the area under the first degree of protection, distant from the potential pollution sources (industrial facilities or heavy car traffic). The sampling sites were visited in autumn 1999 and spring 2000. Predominantely sporocarps of species growing on stumps, fallen logs, tree-stump roots etc. were collected and identified. Species used in the present study are listed in Table 1. with notes about date, location and substrate on which they were found. The samples were cleaned by using a brush and the attached soil and humus were gently removed. They were not rinsed with water in order to avoid possible loss of part of the elements by leaching. After cleaning, samples were air dried, pulverized in a mill and then dried in a dry-kiln (105°C) to constant mass. Since fungi uptake macro- and microelements from the substrate, the tree and soil samples were also taken and prepared following the same procedure like fungal samples. Soil samples were taken to a depth of 25 cm randomly, covering the surface of approximately 300 m in diameter and in the centers within locations. At every location twenty soil samples were taken. These samples were mixed in one composite sample for each location and air-dried. After removal of stones and plant materials, the air-dried samples were ground in a mill to pass a 1 mm sieve.

Table 1. Species, location, date, ecology, utility, and habitat of mycelium of investigated lignicolous fungi

Species	Locality, date	Eco. group	Habitat of mycelium	Utility
Meripilus giganteus (Pers. ex Fr.) Karst	L1 $_1$07.10.99	Ter/Lig/sap	soil by stump *Acer*	*/L
Pseudotrametes gibbosa (Pers. ex Pers.) Bond. & Sing.	L1 $_2$07.09.99	Lig/sap (p)	stump *Fagus*	( )
Coriolus versicolor (L. ex Fr.) Quel.	L1 $_3$07.09.99	Lig/sap	dead trunk *Fagus*	( )/L
◊*Ganoderma applanatum* (Pers. ex Wall r.) Pat	L1 $_3$07.09.99	Lig/sap	dead trunk *Acer*	( )/L
+*Stereum hirsutum* (Wild. ex Fr.) S. F. Gray	L1 $_3$07.09.99	Lig/sap (p)	dead branch *Fagus*	( )
α*Pholiota squarrosa* (Muller. ex Fr) Kummer	L1 $_4$10.10.99	Lig/p (sap)	lived trunk *Fagus*	*
◊*Ganoderma lucidum* (Curt. ex Fr.) Karst	L1 $_2$07.09 99	Lig/sap	dead trunk *Acer*	( )/L
Daedaleopsis confragosavar. tricolor (Bull.) Bond.	L1 $_3$07.09.99	Lig/sap	dead branch *Tilia*	( )
▽*Armillaria polymyces* (Pers. ex S. F. Gray) Sing.	L2 $_1$10.10.99	Ter/Lig/sap	base of trunk *Querqus*	*/L
Daedalea quercina L. ex Fr.	L2 $_2$10.10.99	Lig/sap (p)	stump	( )
Pseudotrametes gibbosa (Pers. ex Pers.) Bond. & Sing.	L2 $_3$10.10.99	Lig/sap (p)	fallen trunk *Querqus*	( )
Coriolus versicolor (L. ex Fr.) Quel.	L2 $_3$10.10.99	Lig/sap	dead trunk *Prunus*	( )/L
+*Stereum hirsutum* (Wild. ex Fr.) S. F. Gray	L2 $_3$10.10.99	Lig/sap (p)	dead brunch *Fagus*	( )

$^{\delta}$*Omphalotus olearius* (D. C. ex Fr.) Sing.	L2 $_4$10.10.99	Lig/Ter/sap (p)	the base of trunk *Fagus*	++/L
$^{\chi}$*Morchela conicavar. costata* Pers. (Focht)	L5 $_1$8/4/00	Ter /sap	soil, sand	**
$^{\Theta}$*Coprinus atramentarius* (Bull.) Fr.	L5 $_1$9/4/00	Sap	soil, sand	*
$^{\Theta}$*Psathyrella vernalis* (Lge.) Mos	L5 $_1$9/4/00	Ter /sap	soil	( )
$^{\chi}$*Morchella vulgaris* Pers.	L5 $_1$16/4/00	Ter/sap	soil, sand	**
$^{\nabla}$*Flammulina velutipes* (Curtis) Karst	L5 $_3$9/1/00	Lig /sap	dead trunk *Salix*	**/L
**Laetiporus sulphureus* (Bull. ex Fr.) Murr.	L3 $_4$27/4/00	Lig/ p	lived trunk *Prunus*	**
$^{\beta}$*Schizophyllum commune* Fr.	L1 $_3$27/4/00	Lig/sap	dead branch	( )/L
$^{\infty}$*Pleurotus ostreatus* (Jacq. ex Fr.) Kummer	L1 $_3$27/4/00	Lig/sap	dead trunk *Fagus*	**
$^{\infty}$*Panus tigrinus* (Bull. ex Fr.) Singer	L1 $_3$27/4/00	Lig/sap	dead trunk *Fagus*	( )
$^{\nabla}$*Panellus stypticus* (Bull. ex Fr.) Karst	L1 $_3$27/4/00	Lig/sap	dead branch	( )
**Laetiporus sulphureus* (Bull. ex Fr.) Murr.	L4 $_4$2/5/00	Lig/P	lived trunk *Prunus*	**
$^{\bullet}$*Polyporus tuberaster* (Pers.) Fr.	L4 $_3$2/5/00	Lig/sap	dead brunch *Fagus*	( )

Legend: sap = saprophytic species, p = parasitic species, Ter = tericolous species, Lig = lignicolous species, * = edible species, M = medically important, ( ) = not edible, ++ = poisonous, L1 = species collected on locality 1, L2 = species collected on locality 2, L3 = species collected on locality 3, L4 = species collected on locality 4, L5 = species collected on locality 5, microhabitat of mycelium L_1 = soil, L_2 = stump, L_3 = dead trunk, L_4 = lived trunk or soil near the lived trunk. Fungi that are marked Lig/Ter forming rihzomorphs. $^{\bullet}$*Fam. Polyporaceae s. stricto* **Fam. Polyporaceae s. lato,* $^{\delta}$*Fam. Ganodermataceae,* $^{\nabla}$*Fam. Tricholomataceae,* $^{+}$*Fam. Stereaceae,* $^{\infty}$*Fam. Pleurotaceae,* $^{\Theta}$*Fam. Coprinaceae,* $^{\alpha}$*Fam. Strophariaceae,* $^{\beta}$*Fam. Schizophyllaceae,* $^{\chi}$*Fam. Morchellaceae,* $^{\delta}$*Fam. Omphalotaceae*

Mineralization of mushroom tissue and substrates. Approximately 3 g aliquots of homogenized dry mushrooms or substrates were weighed to 0.1 mg accuracy, placed in a porcelain crucible and ashed, first on a stove to total carbonization and then in an oven at 425—440°C for 2 h. After cooling, 1 ml H_2O_2 was added to each crucible and ashed in oven for 30 min. In each vessel 10 ml 25% HCl was added and slowly heated on a stove to evaporate 1/2 to 1/3 of acid volume. The residues were transferred into a 50 ml volumetric flasks making up the level with boiling deionized water (S a r i ć et al., 1990). All samples were performed in triplicate.

Soil. A content of heavy metals was analyzed by atomic absorption spectrometry (AAS, Varian spectra 600 type). Soil mineralization was done by the method of A l l o w a y (1995) with HNO_3.

Analyses. Concentrations of K were analyzed by flame photometry and concentrations of P were determined by spectrophotometer. Trace elements (Pb, Cu, Cr, and Zn) and macroelements (Fe, Ca, and Mg) were measured by atomic emission spectrometry in the Lab of Physical Chemistry, Institute of

Nuclear Sciences, Vincha, Belgrade. Direct-current U-shaped arc plasma was used as the excitation source. A PGS-2 plane grating spectrograph (Carl Zeiss, Jena) with an attachment for photoelectric detection having a single (laboratory-made) exit slit was used as the monochromator. The solution was aspirated by an argon stream through a concentric glass Meinhard type nebulizer and the aerosol obtained was introduced into the plasma column (Kuzmanović et al., 1996). The original sample solutions were diluted 2.5 times for mushroom samples and 1.5 times for substrate samples. These solutions also included a fixed concentration of 0.5% KCl as buffer. The reference and blank solutions also included the same concentration of buffer.

RESULTS AND DISCUSSION

Contents of ash. Mineral content of fungi could be determined via analyses of ash mineral content. The soluble fraction was 6.46% (0.70—22.77%), while the unsolubile fraction was 4.72% (0.32—42.14%). We assume that the expressed variability in the ash content is determined by the specific species features, because the same species from different sites have very similar values (e.g. *S. hirsutum*, *C. versicolor*, *L. sulphureus*). In general, lignicolous species contain less amount of ash contents then tericolous species which means that they have less concentrations of mineral elements. Species with rizomorphs (*A. polymyces*) had higher ash content then strictly lignicolous ones, due to mycelial cords along which the accumulation influx of mineral elements is significantly getting stronger than the environmental substrates (aproximately 10 times).

Concentrations of macroelements and microelements. The results obtained are shown in the following figures: Fig. 1 (The content of N, P, K); Fig. 2 (The content of Ca, Mg, Fe); Fig 3 (The content of Cu, Cr); Fig. 4 (The content of Pb, Zn).

a) Concentrations of macroelements: The fungi expressed the affinity for the accumulation of **K** (0.05% in *Daedalea quercina* to — 6.38% in *Psathyrella vernalis* in average **1.83% d.m.**), and **P** (0.02% in *D. quercina* — 0.53% in *M. giganteus* in average **0.3% d.m.**) followed by **N** (1.43% in *C. versicolor* to 5.19% in *Morchella vulgaris* in average **3.08% d.m.**), with lower variability recorded than for **Ca** (156.44 mg/kg d.m. in *Pholiota squarrosa* — 11946.07 mg/kg d.m. in *Coprinus atramentarius* — in average **2226.85 mg/kg d.m.**), **Mg** (377.73 mg/kg d.m. in *D. quercina* to 4531.94 mg/kg d.m. in *C. atramentarius* — in average **1384.24 mg/kg d.m.**), and **Na** (1.42 mg% in *Panellus stypticus* to 120.42 mg% in *Omphalotus olearius* — **in average 15.09 mg%**). This indicates the essential importance of these elements in fungal metabolic processes, independently from ecological fungal group and habitats, so the fungal sporocarps contained them in higher concentrations then the substrate they grow on.

b) Protein content: As a result of the examined N content in analysed fungal species, the protein content varied from 6.26—22.73%, according to Breene, 1990. The highest protein content was recorded for edible species:

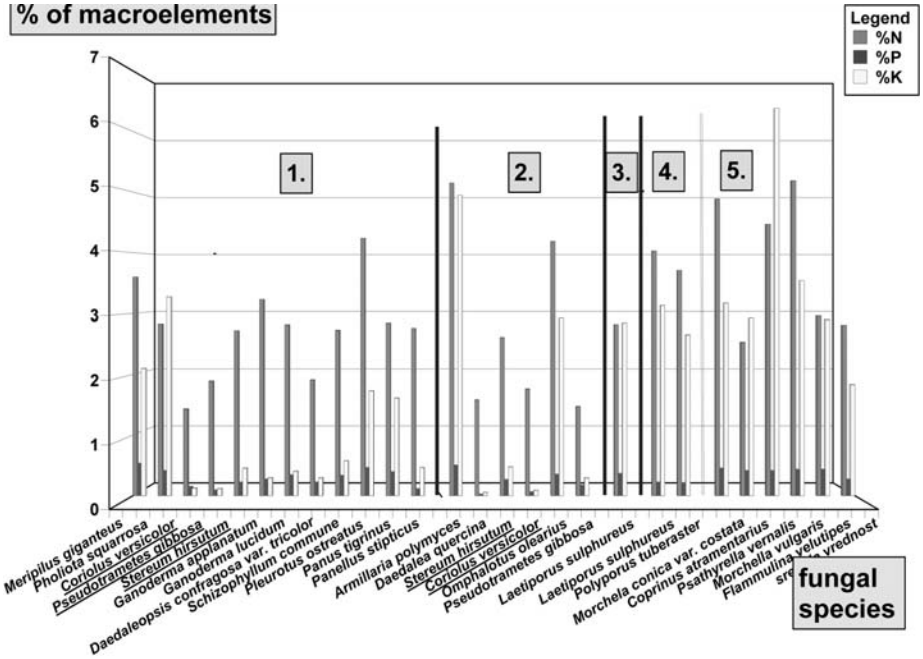

Fig. 1. Content of N, P and K on dry mass of analyzed fungal sporocarps (%)

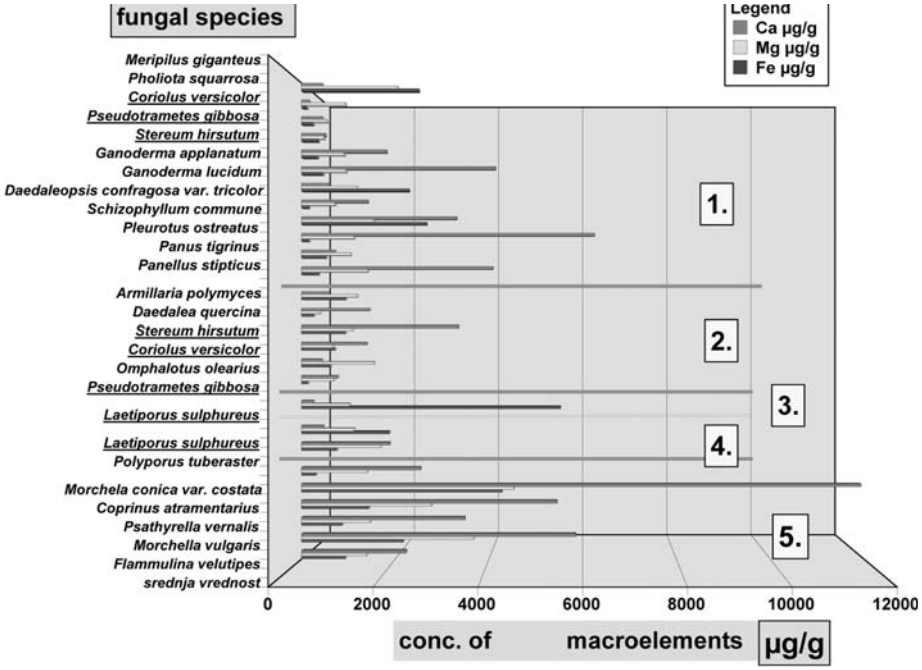

Fig. 2. Content of Ca, Mg and Fe on dry mass in analyzed fungal sporocarps (µg/g)

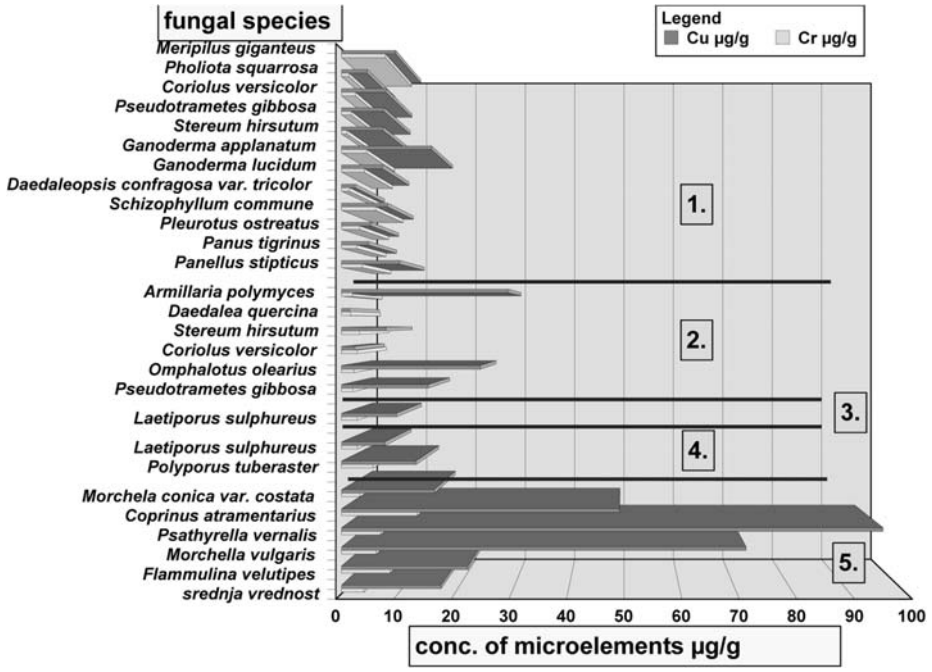

Fig. 3. Content of Cu and Cr on dry mass in analyzed fungal sporocarps (µg/g)

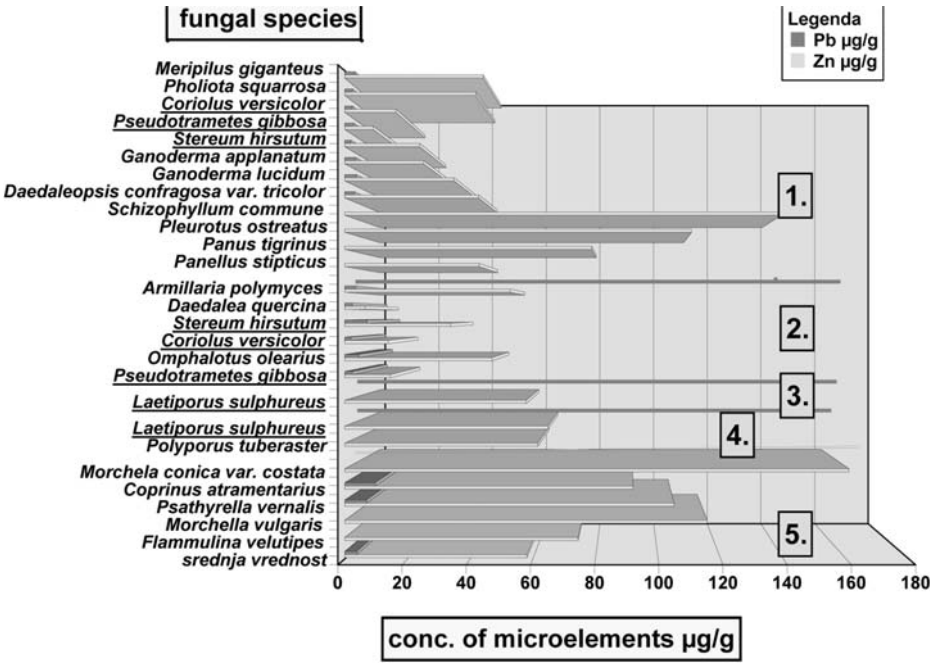

Fig. 4. Content of Pb and Zn on dry mass of analyzed fungal sporocarps in (µg/g)

M. vulgaris (22.73%), *A. polymyces* (22.56%), *P. ostreatus* (18.57%) and the poisonous ones *O. olearius* (18.09%). High content of N in examined sporocarps could be the consequence of N translocation from mycelium to metabolically active sporocarp's hyphae, while the young part of vegetative body has intensive cell divisions and contain higher N concentrations due to intensive sinthesis of proteins. Generally, the analyzed tericolous fungi had higher N concentrations than lignicolous ones.

According to literature data (B o ž a c, 1996; V e t t e r, 1993; B r e e n e, 1990), the analyzed sporocarps contained smaller average concentrations of N and P, similar values for K and Na and higher concentrations of Ca (10 times) and Mg. Such a high content of Ca was probably the consequence of the fact that lignicolous species were dominant in total number of the analyzed species and because they use wood as a substrate characterized by a higher Ca importance in relation to fungi, and which concentration is increasing with the age. Tericolous species contain high concentration of Ca (10.51% $CaCO_3$) probably due to high concentration of this element in the soil as their substrate.

Species *M. giganteus* could be considered as a good accumulator of all of macroelements, (except Ca), while *P. ostreatus* was found to be a good accumulator only for N and P. *P. squarrosa* and *P. vernalis* distinguish themselves by the accumulation of K, while the extreme accumulators of Ca are the species: *G. applanatum* and *P. stypticus*, and especially *S. hirsutum* (superaccumulator). The species *C. atramentarius* has shown the highest accumulation of Ca and Mg, and species *P. vernalis* the highest accumulation of K and Na. Rich Na content was found in species: *A. polymyces* and *O. olearius*, too. Lignicolous species, which accumulated a small content of the majority of macroelements, had good uptake of Ca, such as *D. quercina*, *C. versicolor*, *P. gibbosa* (indicator species). Only the species that have direct contact with soil via the rizomorphs (Lig/Ter) or partly by the mycelium, could be considered as good macroelement accumulators, such as: *A. polymyces*, *O. olearius*.

c) Concentrations of microelements: Considering content of microelements, in investigated fungal species the domination of Fe was recorded: *Fe* (102.67 in *Ph. squarrosa* — 4276.61 *C. atramentarius* μg/g; A.v. = 928.03 μg/g), followed by content of *Zn* (6.57 in *D. quercina* — 160.96 in *M. conica var. cristata* μg/g; A.v. = 58.14 μg/g), *Cu* (1.40 in *D. quercina* — 95.86 in *P. vernalis* μg/g; A.v. = 17.60 μg/g), *Cr* (0.96 in *S. hirsutum* — 13.36 in *C. atramentarius* μg/g; S.v. = 3.94 μg/g) and *Pb* (1.93 in *S. hirsutum* — 9.72 in *C. atramentarius* μg/g; A.v. = 3.88 μg/g). The tericolous species from the site 5 showed the highest concentrations both for the majority of microelements and macroelements concentration as well as for the total ash content. According to these results, the chemical composition in different fungal species (ecotypes), as well as within the same species originating from different localities, was found to be specific and depended dominantly on availability of these elements from their substrates. These are specific accumulators (ecotypes) of specific element (superaccumulator, accumulator, bioindicator), which created the defense mechanisms in the course of the evolution, or as a consequence of stress adaptation by which they could exclude or amortise unfavorable effects of heavy metals presence in the environment.

In comparison with the previous studies (Božac, 1996; Breene, 1990, Yoshida and Maramatsu, 1998) recorded results were high for Fe and Pb, but also for Cu, Cr i Zn in some species. Species wich contained 1000 μg/g **Fe** (what would be toxic for plants) are considered as *superaccumulators*, such as some lignicolous, medically important fungal species: *M. giganteus*, *G. lucidum*, and *Sch. commune*, and some tericolous species: *C. atramentarius*, *F. velutipes*, and *P. vernalis*. The highest accumulation that was noticed for the species *C. atramentarius* from the location 5, was not just the consequence of species feature as superaccumulators, but also the result of specific chemical copmposition of soil. However, the fact that saprophytic species could change physico-chemical condition of their environment, revealed their influence on availability of other elements in substrate and their accumulation in metabolically active hyphae.

The content of Pb in investigated tericolous species was found to be twice higher in relation to the mean values characteristic for fungi, as well as for plants, but none of the found concentration was critical nor toxic, exept for *C. atramentarius* (Tf — 7.22). The Pb uptake was found to be exclusively *species specific*, which was predictable due to the fact that Pb belongs to the group of "elements of secondary importance in metabolic processes", which in low concentrations are not dangerous, but could be toxic if they are present in a higher concentrations. As a rule, lignicolous fungi was not found to accumulate microelements, except *G. applanatum*, for Cu, *Sch. commune* for Zn and *A. polymyces*, *O. olearius* for both of them. The content of Cr in the species *C. atramentarius* was found in concentration that could be considered as a toxic one.

There are data about the synergistic effect of accumulation of different metals (more efficient accumulation from the mixture than from the pure solution of elements) on the fungal cell surface, which was confirmed with our results. The highest significant correlation was noticed for the synergistic uptake of Fe and Cr (0.83) and for Fe with Cu and Zn. As accumulators of microelements, we could separate the following species: *M. giganteus* (except for Zn), *Sch. commune* (except for Pb), and *G. applanatum* (except for Fe and Zn). As species that accumulate all of microelements, there were recorded species with increased absorptive surface due to the increased micelium surface via developed ryzomorph structures, such as: *A. polymyces* and *O. olerius*, and one strictly lignicolous fungus — *S. hirsutum*, that could be considered as a superaccumulator species of microelements, especially for Pb and Cr. The best tericolous accumulator species were: *C. atramentarius* (especially for Fe, Pb and Cr) and *P. vernalis* (especially for Pb and Cu). While the concentration of Pb on the urban location (Sampling site No 5) was higher in relation to other localities due to the heavy traffic, these two species could be used as bioindicators of the soil Pb load.

The content of all of microelements varied significantly among the species, but differs within the same species depending on their environment (supstrate), especially in regard with the Fe and Pb content (i.e. *C. versicolor*, *S. hirsutum*). According to these data, it could be assumed that different ecotypes

within the same species differ considering their ability of metal ions uptake from the substrata (intraspecific antagonism?).

d) Fungal mineral content versus the contents of minerals of wood as their substrata: According to our results, the concentrations of macro- and microelements were found to be lower in wood substrata than in fungal tissue, especially for P, K i N. Only the content of Ca and Pb was significantly higher in wood substrata. Lignicolous fungi have not been accumulating Fe from wood, except the species *G. lucidum* (Tf = 19, which was 3—5 fold higher than in other fungal species).

e) Fungal mineral content versus the contents of minerals of soil as their substrata: As a result of Tf values in relation to soil, the analyzed fungi tended to accumulate **K, P, N, Cu and Zn, and partially Cr.** As very good accumulators of **Cu** in relation to the investigated tericolous fungi, the species: *P. vernalis*, *M. vulgaris*, and *C. atramentarius*, were recorded, followed by species forming rysomorphs: *A. polymyces* and *O. olearius*, and finally strictly lignicolous fungi: *G. applanatum* and *P. gibbosa*. While the content of Cu in soil of all of the investigated locations was found to be in normal, usual average concentrations, it could be assumed that fungal Cu accumulation was determined by morpho-ecological properties of fungal species, first of all by the surface of mycelium. These species could be considered for the use in elements remove from polluted areas. The highest accumulation of Zn was recorded for saprophytic lignicolous fungi *Sch. commune* (Tf = 10.48). In relation to Pb, fungi showed lower tendency of accumulation, so higher concentration of this element could be expected only in heavily polluted areas near the frequent traffic

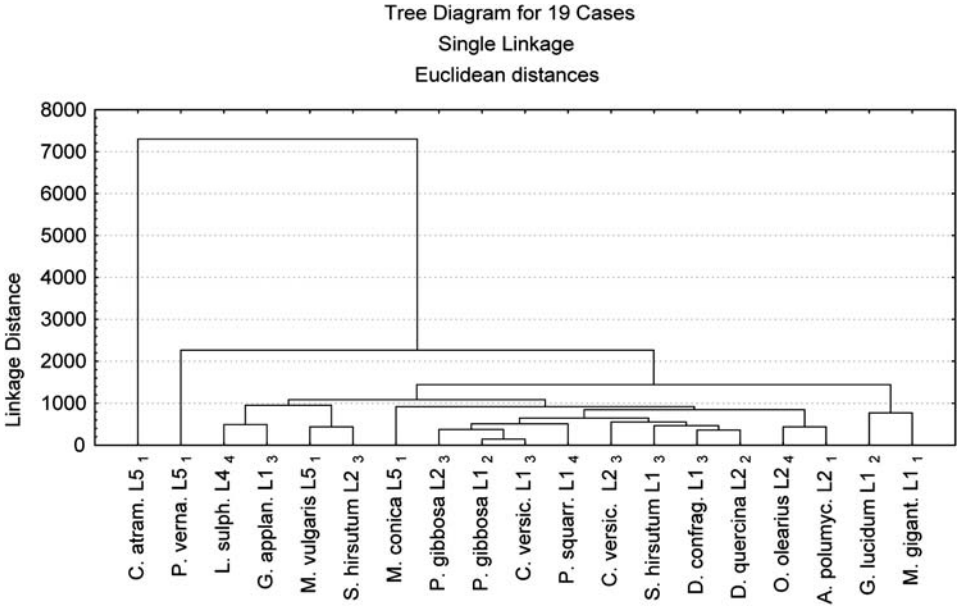

Fig. 5. Cluster analysis of anlyzed fungi according to content of macroelements and heavy metals

zones. The highest recorded Tf values were found in *S. hirsutum* (0.46) and *C. atramentarius* (0.40).

f) Cluster analysis (Fig. 5) classified fungi mostly by location. Some species from different locations (*Stereum hirsutum*, *Coriolus versicolor*) showed different mineral contents and were classified into separated branches of the dendrogram. These data indicate that the accumulation ability is not only genetically coded, but also influenced by environmental factors.

g) The monitoring of the content of macroelements and heavy metals in biological (fungal) and their substrate (wood, soil) samples are inevitable, not only in the sense of broadening our knowledge of elementary composition in wood ecosystems, but also in order to speculate the effect of migrations of chemical elements, especially the anthropogenic radionuclides in natural materials and products common in human use, e.g. wood, edible and medicinal fungi.

ACKNOWLEDGEMENTS

We are very grateful to Pavlović M. from the Institute for Nuclear Sciense in Vinča, for measurements of the content of macro- and microelements in analyzed sporocarps, and to prof dr. Sekulić P. from Institute for crop and vegetables from Novi Sad, for the soil analyses, as well as to Kitanović Ana, dipl. biologist for the help in data analyses.

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МАКРОЕЛЕМЕНТИ И ТЕШКИ МЕТАЛИ У НЕКИМ ЛИГНИКОЛНИМ И ТЕРИКОЛНИМ ГЉИВАМА

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Резиме

С обзиром на значај *Basidiomycotina* у биоиндикацији, али и све већу контаминацију ваздуха и земљишта, у овом раду је анализиран садржај *макроелемената*: *N, P, K, Ca, Mg* и *Na*, и *неких тешких метала* (*микроелемената*): *Fe, Pb, Cu, Cr, Zn* у спорокарпима 22 врсте макрогљива из Националног парка Фрушка гора. Већина их припада лигниколним врстама из класе: *Homobasidiomycetes* реда *Aphyllophorales s. lato*, док су две врсте из подразреда *Ascomycotina*. Посебна пажња била је усмерена на медицински значајне гљиве које су широко распрострањене на овом подручју: *Ganoderma lucidum*, *Ganoderma applanatum*, *Coriolus versicolor*, *Flammulina velutipes*, *Meripilus giganteus* и *Omphalotus olearius*. Пошто гљиве усвајају макро и микроелементе из супстрата, такође су анализирани узорци земљишта и дрвета (супстрата) на којима су гљиве расле. Циљ овог рада био је добијање основних података о уобичајеним концентрацијама ових елемената у незагађеном окружењу. Према нашим резултатима, гљиве су тежиле да акумулирају K, P и N, са много мањом варијабилношћу него Ca, Mg и Na, што указује на њихов есенцијални значај у фунгалним метаболичким процесима, независно од еколошке групе и станишта. Анализирани супстрат (дрво) садржао је веће концентрације Ca и Pb у односу на гљиве. Према трансфер факторима који су добијени из односа у концентрацији ових елемената у гљивама и концентрације у супстрату изражено на суву масу (с. м.) узорка, анализиране гљиве су тежиле за акумулацијом Cu и Zn, а делимично и Cr. Међу врстама акумулаторима микроелемената могу се издвојити: *Meripilus giganteus* (осим Zn), *Schizophyllum commune*

(осим Pb), *Ganoderma applanatum* (осим Fe и Zn). Суперакумулатори Fe биле су лигниколне, медицински значајне врсте *M. giganteus*, *G. lucidum*, *Sch. commune* и териколне врсте: *C. atramentarius*, *F. velutipes* и *P. vernalis*. Добри акумулатори Cu су териколне врсте: *Psathyrella vernalis*, *Morchella vulgaris* и *Coprinus atramentarius*, затим врсте са ризоморфима: *Armillaria polymyces* и *Omphalotus olearius*, па тек онда лигниколне врсте: *Ganoderma applanatum*, *Pseudotrametes gibbosa*. Највећа акумулација цинка (Zn) уочена је код врсте *Schizophyllum commune*, док су према олову (Pb) гљиве показале малу тенденцију за усвајањем, осим териколне, сапрофитске врсте *C. atramentarius*. Значајну акумулацију хрома (Cr) утврдили смо такође код териколних врста (нарочито *C. atramentarius*).

Статистичка кластер анализа класификовала је гљиве углавном по локалитетима, при чему се посебно издваја урбани локалитет са анализираним териколним врстама. Ови подаци указују да акумулација није само одређена генетским карактерима, него је такође под утицајем фактора окружења. Ови подаци би се могли употребити у токсикологији, фармакологији и заштити животне средине.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 269—276, 2005

UDC 582.28:631.465

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LIGNINOLYTIC ENZYME PRODUCTION IN *PLEUROTUS ERYNGII* DEPENDING ON THE MEDIUM COMPOSITION AND CULTIVATION CONDITIONS

ABSTRACT: *Pleurotus eryngii* produced laccase (Lac) both under conditions of submerged fermentation (SF) and solid-state fermentation (SSF) using all of the investigated carbon and nitrogen sources, while significant peroxidases production occurred only under SSF conditions. The highest levels of Lac activity were found under SF conditions of dry ground mandarine peels (999.5 U/l). After purification of extracellular crude enzyme mixture of *P. eryngii* which was grown under SF conditions with dry ground mandarine peels, it was revealed two peaks of Lac activity and one peak of activity against phenol red in absence of external Mn^{2+} which was very low (1.4 U/l). Results obtained by purification also showed that the levels of phenol red oxidation in absence of external Mn^{2+} were higher than phenol red oxidation levels in presence of external Mn^{2+}. In the medium with the best carbon source for Lac production (dry ground mandarine peels), $(NH_4)_2SO_4$, with a nitrogen concentration of 20 mM, was the most optimum nitrogen source among 8 investigated sources.

KEY WORDS: carbon and nitrogen sources, solid-state fermentation, laccase, *Pleurotus eryngii*, peroxidases, submerged fermentation

INTRODUCTION

Pleurotus eryngii is edible and medicinal species that belong to group of white rot fungi due to its ability to produce extracellular ligninolytic enzymes: laccase (Lac), two peroxidases: Mn dependent peroxidase (MnP) and versatile peroxidase (VP), as well as aryl-alcohol oxidase (AAO) (Munoz et al., 1997a), and modify and degrade lignin. Due to this ability, *P. eryngii* can be cultivated on different lignocellulosic materials such as sawdust, paper products, and most agricultural wastes that are produced in enormous amounts worldwide (Croan, 2000).

The carbon sources in the medium play an important role in ligninolytic enzyme production. Mansur et al. (1997) showed that the use of fructose

instead of glucose resulted in a 100-fold increase in the specific Lac activity of *Basidiomycetes*. According to Master and Field (1998) and Hammel (1997), the ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen. However, in *P. ostreatus* a high concentration of nitrogen in the medium (34 mM N as glutamate) did not repress but rather slightly stimulated mineralization of lignin compared to the N-limited medium (Kaal et al., 1995).

Grapevine sawdust and mandarine peels represent very common agricultural wastes in some regions, and are prospective substrates for the bioconversion into fungal biomass and lignocellulytic enzymes. According to Tsiklauri et al. (1999) grapevine sawdust contains: 0.7—0.8% total nitrogen, 1.6—1.8% reducing substances, 12—13% hemicellulose, 23—26% cellulose, and 23—26% lignin, while mandarine peels contain: 1.1—1.3% total nitrogen, 11—14% reducing substances, 32—34% soluble carbohydrates, 12—14% cellulose, and 9—11% lignin.

Cultivation conditions which are characterized by different physico-chemical characteristics influence the levels of ligninolytic enzymes activities. SSF conditions are very similar to those existing in nature on wood substrates, while those in SF are very different and this could account for the low production of some enzymes in liquid cultures.

The purpose of this investigation was to study the effect of different carbon and nitrogen sources, as well as raw plant materials (grapevine sawdust and mandarine peels) under SF conditions of dry ground mandarine peels and SSF conditions of grapevine sawdust, on Lac and peroxidases production in *Pleurotus eryngii*.

MATERIALS AND METHODS

2.1. *Organisms and growth conditions*

Culture of *P. eryngii* var. *eryngii*, strain No. 616, is preserved on wort agar medium in the culture collection of the Institute of Evolution, University of Haifa (HAI), and documented in the Catalogue of Cultures (Wasser et al., 2002).

The whole procedure of inoculum preparing and further procedure is described previously (Stajić et al., 2004).

2.2. *The effect of different carbon sources on the Lac and peroxidases production under SF and SSF conditions*

SF was carried out at room temperature (22±2°C), on a rotary shaker at 180 rpm, in 250 ml flasks containing 50 ml of synthetic medium (Stajić et al., 2004) which was enriched with 1% of one of the investigated carbon sources (carboxymethyl-cellulose sodium salt, cellulose, glucose, maltose, D-mannitol, D-gluconic acid sodium salt, and xylan), or with 4% of dry ground man-

darine peels, as well as with microelements. The initial pH of the medium with mandarine peels as a carbon source, was adjusted to 9.4 prior to sterilization by adding 10% NaOH, so that the pH would be 6.0 after sterilization. In other cases, it was adjusted to 6.0 by adding 4% HCl. Homogenized suspensions (of 5 ml) were used for inoculation of one flask. Biomasses were separated by centrifugation (4°C, 5000 rpm, 20 min) after 5 and 7 days of cultivation and supernatants were used to estimate enzyme activity.

SSF was carried out at 25°C in 100 ml flasks containing 4 g of grapevine sawdust as a carbon source and 12 ml of synthetic medium (Stajić et al., 2004). Three ml of suspension, after inocula homogenization, were used per flask. Samples from flasks were harvested after 7 and 10 days of cultivation, and extracellular enzymes were extracted by griding in a mortar with 20 ml of distilled water during 5 minutes at the ice. This procedure was repeated 3 times and the obtained extracts were mixed (total volume 60 ml). Solids were separated by centrifugation (4°C, 5000 rpm, 10 min), and supernatants were used for measurements of the Lac and peroxidases activities.

Three replications for each investigated carbon sources were used.

2.3. *The effect of different nitrogen sources and concentrations on the Lac and peroxidases production under SF conditions*

In the synthetic medium with the best carbon source for Lac production (dry ground mandarine peels), without NH_4NO_3 and with microelements, either one of the inorganic nitrogen sources [ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium phosphate monobasic ($NH_4H_2RO_4$), ammonium sulfate $(NH_4)_2SO_4$), and potassium nitrate (KNO_3), in a nitrogen concentration of 30 mM], or one of the organic nitrogen sources [bacteriological peptone, casein acid hydrolysate vitamin free, in a concentration of 0.5%, and corn step liquor in the concentration of 0.8%] was added.

The effect of different concentrations of the best nitrogen source on the Lac production was studied in a medium with 20 mM, 40 mM, and 60 mM of nitrogen.

Three replications for each nitrogen sources and concentration were used.

2.4. *Enzyme activity assays*

Lac activity was assayed using syringaldazine as a substrate, and by measuring the increase in absorbance at 525 nm (ε_{525} = 65000 $M^{-1}cm^{-1}$) for 60 seconds. The mixture contained: 0.1 M acetic buffer (pH 5.0), 1 mM syringaldazine (dissolved in 96% ethanol), and enzyme preparation (V_{tot} = 1 ml).

Peroxidases activities were determined with 3 mM phenol red as the substrate, in a buffer with the following contents: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt, pH 4.5 (ε_{610} = 22000 $M^{-1}cm^{-1}$). The mixture contained: buffer, enzyme preparation, 2 mM

H_2O_2, and phenol red, with or without 2 mM $MnSO_4$ (V_{tot} — 500 μl). Reaction was ceased by 2 M NaOH.

2.5. *Enzymes purification*

Purifications of Lac and peroxidases were carried out on 7-day-old submerged culture of *P. eryngii*, which was grown in the medium with the best carbon sources, using FPLC AKTA explorer (Pharmacia Biotech. Sweden). The supernatant from the submerged culture after centrifugation was filtrated using a 0.45 μm filter. The fluid was then concentrated by ultrafiltration with a PM-10 membrane (10 kDa, Amicon). The concentrated fluid was loaded onto a HITrap Q anion-exchange column (5 ml column, Pharmacia Biotech.) previously equilibrated with 20 mM sodium acetate, pH 6.0. After washing with 6 column volumes (cv) of 20 mM sodium acetate buffer, the enzyme was eluted by two linear NaCl gradients: 0—0.3 M in 30 cv and 0.3—1.0 M in 10 cv. 1.5 ml of each fraction was collected. Lac activity of the separated fractions was performed with 50 mM ABTS [2,2'-azino-bis (3-ethylbenzothiazoline--6-sulphonic acid)] in phosphate buffer, pH 6.0 (ε_{436} = 29300 $M^{-1}cm^{-1}$), and peroxidases activity with 3 mM phenol red (ε^{610} = 22000 $M^{-1}cm^{-1}$).

RESULTS AND DISCUSSION

3.1. *Effect of different carbon sources on the Lac and peroxidases production*

In crude mixture, Lac activity was detected under both SF and SSF (Table 1), while peroxidases production occurred only under SSF conditions.

The highest level of Lac activity was found in submerged culture with dry ground mandarine peels as the carbon source after 7 days of cultivation (999.5±20.7 U/l). Significant levels of Lac activity were also evident under conditions of SF with xylan and D-gluconic acid sodium salt on the 5th day of cultivation (134.4±12.1 U/l and 121.5±10.3 U/l, respectively), after which they started to decrease, while low levels of Lac activity were obtained with other carbon sources in both submerged and solid-state cultures (Table 1).

Martinez et al. (1994) showed that *P. eryngii* and related species have a limited attack on cellulose, less than 15% of the initial content, while xylan degradation was parallel to lignin degradation. However, contrary to the results of Martinez et al. (1994) and Munoz et al. (1997a), where Lac production in *P. eryngii* was significantly stimulated by alkali lignin that is present in straw, in this study, it was shown that Lac activity under SSF conditions of grapevine sawdust was low (42±1 U/l and 9.3±0.2 U/l, after 5 and 7 days, respectively).

After purification of extracellular crude enzyme mixture from 7-day-old culture of *P. eryngii*, which was grown in the medium with the most optimum carbon source, it was found: two peaks of Lac activity (7411 U/l and 847 U/l).

Table 1. Laccase activity depending on the carbon source in the medium

Species	Strain (No.)	Carbon source	Period of cultivation (days)	Lac activity (U/l)
P. eryngii var. *eryngii*	616	Glucose	5	24±13
			7	4.4±0.2
		Maltose	5	17.4±6.1
			7	11.4±4.3
		Mannitol	5	49±2
			7	15.8±0.5
		D-gluconic acid sodium salt	5	121.5±10.3
			7	42.6±15.1
		Xylan	5	134.4±12.1
			7	73±8
		Cellulose	5	19±6
			7	11.8±3.4
		Carboxymethyl cellulose	5	21.7±3.4
			7	35.7±6.4
		Dry ground mandarine peels	5	162±14
			7	999.5±20.7
		Grapevine sawdust	5	42±1
			7	9.3±0.2

These results are in accordance with the results of Munoz et al. (1997a, b) who also investigated Lac of *P. eryngii* in the glucose/ammonium-tartrate medium and obtained two bands with Lac activity (Lac I and Lac II) which respond to isoenzymes.

Regarding to significant Mn^{2+} concentration in grapevine sawdust (Kilby, 1999), and the fact that only trace Mn^{2+} concentrations could be enough for MnP action (Martinez et al., 1996), in this case, it can not be spoken about MnP and VP activities, but only about activity against phenol red in presence and absence of external additional Mn^{2+}, respectively. One peak of activity against phenol red in absence of external Mn^{2+} was found after purification of crude enzyme mixture, but its activity was very low (1.4 U/l). However, Martinez et al. (1996) purified two isoenzymes of *P. eryngii* from liquid peptone medium and described them as VP, which are capable of oxidizing both Mn^{2+} and phenolic and non-phenolic aromatic substrates.

3.2. *Effect of different nitrogen sources on the Lac and peroxidases production*

Pleurotus eryngii produced Lac under SF conditions with all the investigated nitrogen sources and concentrations, but peroxidases levels were low (Table 2).

Table 2. Lac activity and activity against phenol red in presence and absence of external Mn^{2+} depending on the nitrogen source in the medium

Species	Strain (No.)	Nitrogen source	Period of cultivation (days)	Lac activity (U/l)	Activity against phenol red (U/l)	
					$+Mn^{2+}$	$-Mn^{2+}$
P. eryngii var. *eryngii*	616	Peptone	5	1.47±0.09	1.32±0.08	2.5±0.2
			7	9.4±1.2	0.7±0.2	2.7±0.3
		Casein acid hydrolysate vitamin free	5	1.0±0.2	0.7±0.2	0.9±0.3
			7	6.1±0.6	1.0±0.1	1.5±0.2
		Corn step liquor	5	57.0±8.5	2.28±0.04	2.3±0.2
			7	33.5±4.2	1.2±0	0.9±0.2
		KNO_3	5	3.9±0.1	1.3±0.1	0.7±0.1
			7	8.30±0.08	0.78±0.04	1.8±0.1
		NH_4C1	5	2.0±0.4	1.0±0.3	1.2±0.4
			7	46.0±1.4	1.03±0.07	2.4±0.4
		$(NH_4)_2SO_4$	5	1.3±0.1	0	2.7±0.1
			7	100.9±5.3	0.7±0.4	1.2±0.1
		$NH_4H_2PO_4$	5	14.3±3.4	0.7±0.1	2.3±0.2
			7	22.3±0.9	0.68±0.06	0.38±0
		NH_4NO_3	5	1.3±0.2	0.6±0.1	1.8+0.5
			7	9.4±1.2	0.7±0.2	2.7±0.3

The highest level of Lac activity was in the medium with $(NH_4)_2SO_4$ as the nitrogen source (246.4±19.8 U/l, after 7 days of cultivation) and with a nitrogen concentration of 20 mM. With an increase of nitrogen concentration, the levels of Lac activity decreased, and with a nitrogen concentration of 40 mM the Lac activity was only 68.3±16.8 U/l (approximately 72% lower). Lac production increased during cultivation in media with all the investigated nitrogen sources, except for corn step liquor.

Activity against phenol red in presence of external Mn^{2+} was low under SF conditions with all nitrogen sources, while activity in absence of Mn^{2+} was higher, especially in the medium with $(NH_4)_2SO_4$ and peptone, as nitrogen sources (Table 2). These results are in accordance with the results of Elisashvili et al. (2001) who also showed that manganese dependent peroxidase activity was 0 or very low in SF, but in SSF it was significant. Martinez et al. (1996) found MnP production in *P. eryngii, P. ostreatus, P. pulmonarius*, and *P. sajor-caju* in peptone medium, but it was not detected in medium with other investigated nitrogen organic sources.

In conclusion, this study showed that Lac and peroxidases production depends on the cultivation conditions (SF or SSF), carbon sources, as well as nitrogen sources and concentration. The highest levels of investigated ligninolytic enzymes activities were in the medium with dry ground mandarine peels as the carbon source, and because of this reason the investigated *P. eryngii*

strain can be used in the process of biotransformation of this material to feeds and fuels, as well as in paper manufacturing.

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ПРОДУКЦИЈА ЛИГНИНОЛИТИЧКИХ ЕНЗИМА КОД *PLEUROTUS ERYNGII* У ЗАВИСНОСТИ ОД САСТАВА МЕДИЈУМА И УСЛОВА КУЛТИВАЦИЈЕ

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Резиме

Pleurotus eryngii је продуковао лаказе (Lac) под условима култивације и на течном и на чврстом медијуму (SF, SSF) са свим проучаваним изворима угљеника и азота. Највиши нивои активности Lac нађени су у течним културама са сувом млевеном кором мандарина (999,5 U/l). Ова врста је продуковала пероксидазе само при култивацији на чврстом медијуму.

После пречишћавања смесе екстрацелуларних ензима које је *P. eryngii* продуковао у течним културама са сувом млевеном кором мандарина као најбољим извором угљеника, нађена су два пика активности Lac и један пик активности против фенол црвеног у одсуству спољашњег Mn^{2+} са врло ниском активношћу (1,4 U/l). Резултати добијени пречишћавањем такође су показали да су нивои оксидације фенол црвеног били виши у одсуству него у присуству спољашњег Mn^{2+}.

У медијуму са најбољим извором угљеника највиша Lac активност је била у присуству $(NH_4)_2SO_4$ као извора азота, при концентрацији азота од 20 mM.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 277—282, 2005

UDC 616-002.828

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HUMAN MYCOSES

ABSTRACT: Fungi are an independent group of plant kingdom which members do not contain chlorophyll and have no capability of photo synthesizing, meaning that they can not synthesize nutritive compounds, so they exist as the saprophytes or parasites of plants, animals and humans. Between 50.000 and 100.000 species are known, but only about 100 species cause diseases (mycoses) of humans or animals, while many other cause diseases of plants. Only the dermatophytes and species of genus *Candida* are usually transferable from human to human. The importance of fungi was certainly less than that of the bacteria and viruses for the time being, but their role as the opportunist pathogens is unavoiding, especially for immunocompromised patients. Incidence of fungal infections grows steadily, even in the countries with high level of general and health culture. Diagnosis and therapy of fungal infections are many times unadequate. Prophylaxis is still an object of discussion.

KEY WORDS: diagnosis, mycoses, therapy

INTRODUCTION

Fungi are an independent group of plant kingdom which members do not contain chlorophyll and have no capability of photosynthesizing, meaning that they can not synthesize nutritive compounds, so they exist as the saprophytes or parasites of plants, animals and humans (1). According to other authors, fungi are special group of microorganisms classified between protists and procaryotes, or between plant and animal kingdom. They have much more morphological complexity than that of the bacteria (procaryotes). They possess a true nucleus and thus are described as being eucaryotic (2). Fungi may be unicellular, (for example the yeasts) or cellular units may be connencted together to form long filaments or hyphae. Probably, the most accurate definition is that fungi are non-photosynthesizing protists which grow as the mass of branching, knitting filaments known as mycelium. Although being perforated, they allow the free passage of nuclei and cytoplasm. That means that the whole organism is a syncicium (multinuclear intracytoplasmatic mass) bordered inside many branching tubes. These tubes are made of polisaccharids such as a chi-

tin, and are chomologous to bacterial walls. Fungi are probably the evolutive branch of protozoa; they are not familiar with actinomycetes, mycelial bacteria although have some similarities at first sight. Some fungi have the ability to change their form (dimorphism) under special conditions, which is the characteristic of pathogenic fungi (2, 3, 4).

CLASSIFICATION

Fungi are clasified in the following way:

Ascomycotina (ascomycetes): A bag (ascus) is formed by the sexual fusion and contains the meiotic products — four or eight spores (*ascospores*). Asexual spores (*conidia*) are usually formed outside on the hyphal endings. Examples: *Trichophyton (Arthoderma), Microsporum (Nannizzia), Blastomyces (Ajellomyces).*

Basidiomycotina (basidiomycetes): Sexual fusion creates the organs in the form of club, named basidium with the four meiotic structures on its surface (*basidiospores*). Asexual spores (*conidia*) are usually formed outside on the hyphal endings. Example: *Cryptococcus neoformans (Filobasidiellla neoformans).*

Deuteromycotina (fungi imperfecti): This is not real phylogenetic group, but more fictitious class where all those fungi in which sexual reproduction is unknown are temporary allocated. Examples: *Epidermophyton, Sporothrix, Candida genus.*

Zygomycotina (phycomicetes): Mycelium is usually non-septated. Asexual spores are made unlimitedly inside the structures called sporangia. Sexual fusion creates the spores with the thick walls called zygospores. Example: *Rhizopus nigricans* (opportunist pathogen). The majority of pathogenic fungi are located in the group of imperfect fungi where sexual reproduction is unrecognizible (1, 4).

IMPORTANCE

It has been estimated that there are between 50.000 and 100.000 species of fungi. They are dispersed everywhere: in the air, water, but mostly in the soil. They have an active role in the processes of changing of material. Their activity for man could be either usefull or harmfull. Their capacity for producing of antibiotics such as penicillin and griseofulvin, vitamins or utilization in the beer industry are all examples of usefulness. In agriculture, fungal pathogens are responsible for losses amounting to perhaps 10% of the world crops. Saprophytic fungi cause significant damage to stored food and other foodstuffs with calamitous consequences for man's agroeconomy. Of thousands known fungi about 100 are pathogenic for humans or animals. Fungi can cause diseases in man directly by producing mycotoxins, by acting as allergens, and by direct tissue invasion (infections-mycoses) (1, 2, 5).

For practical reasons, human mycoses could be classified as superficial, cutaneous, subcutaneous and systemic mycoses. For sometime, superfitial and

cutaneous mycoses are included in the same group. Most important fungal infections are presented in Table 1. (4, 6). Superficial, cutaneous and subcutaneous mycoses of skin, hair and nails could be of chronic course and very resistant to treatment, but rarely have impact to man's general health. But, due to extraordinary extension, they have great epidemiological and socioeconomical importance. *Pityriasis (tinea) versicolor* is the most frequent of supeficial mycoses, expressed mostly during the summer months. In tropical climates the condition is more common than in temperate zones and as many as 40% of population may be affected. The disease is perhaps of cosmetical significance (2, 5). Occasionally, it has been implicated as a cause of fungal sepsis (*fungemia*) during the intravenous lipid supplementation. Skin mycoses are caused by fungi which attach to only superficial keratinized tissue (skin, hair and nails), but not the deeper tissues. Most important are dermatophytes, a group of very similar fungi with world-wide geographical distribution. They are classified into three genera: *Epidermophyton, Microsporum* and *Trichophyton* (4, 6, 7).

Table 1. The most important fungal infections and the causes

THE TYPE OF FUNGAL INFECTIONS	THE CAUSES	THE DISEASE
Superficial	Malassezia furfur	Pityriasis (tinea) versicolor
Cutaneous	*Trichophyton* sp., *Epidermophyton,* *Microsporum* sp., *Candida albicans*	Dermatophytosis (tinea of the skin, hair, nails) Candidiasis (skin and visible mucoses)
Subcutanous	*Sporothrix schenkii*	Sporotrichosis
Systemic	*Blastomyces dermatitidis* *Coccoidioides immitis* *Histoplasma capsulatum* *Paracoccidioides brasiliensis*	Blastomycosis Coccidioidomycosis Histoplasmosis Paracoccidioidomycosis
Systemic oportunistic	*Aspergillus fumigatus* and others *Aspergillus* sp. *Candida albicans* and others *Candida* sp. *Cryptococcus neoformans* *Mucor* and *Rhizopus* sp.	Aspergillosis Candidiasis Cryptococcosis Zygomycosis

Besides, they are ubiquitous, some special have higher incidence in particular world's regions. According to etiological classification dermatophytes have been divided into anthropophilic, geophilic and zoophilic species. The main reservoir of dermatophytic infection is infected man or animal. It could not be eliminated the fact that the soil was natural saprophytic habitation for all dermatophytes in the past, but modern epidemiological investigations don't give support for such assertions especially for those dermatophytes completely adapted to living as human or animal keratinophylic parasites. Micropopulation of soil destroys (lysis) very quickly the pathogenic dermatophytes in pathologic material of man or animal under laboratorial or natural conditions. Animals that most often serve like a host of zoophylic dermatophytes are: dog, cat, cattle, horse, sheep, pig, poultry, rodents (hedgehog), monkies etc. (6, 7, 8). *Ti-*

nea pedis (athlete's foot) is the most frequent dermatophytosis. Approximately 10 percent of total population can be expected to have this infection at any given time. Significant presence of foot dermatophytoses starts since 20. century with the wearing of closed foot-wear. Together with foot dermatophytoses,. next most frequent is a fungal nail infection — onychomycosis. Socioeconomical, cultural and factors from working environment have great impact on developing of these diseases. It is estimated that onychomycoses are responsible for 18—40% of all nail diseases and 30% of all fungal skin diseases. A great U.K. study in 1990. revealed that 2,73% of adult population have onychomychosis. Haneke says that frequency of onychomycoses are compatible to 50% tinea pedis and / or tinea manus approved by mycological culture. Both infections are more frequent in persons from the group with risk of fungal diseases: coalminers, workers in industry, sportsmen, soldiers, persons in boarding schools or closed collectives (1, 9). In some European countries (Poland, Croatia) the most common dermatophytoses established were tinea corporis tinea capitis, and the most common etiological agent was *Microsporum canis* (10, 11). Otherwise, in majority of other cases, tinea pedis and onychomycosis prevailed, and *Trichophyton mentagrophytes* and *T. rubrum* as the causative agents, too. During the investigation performed in the period from 1995. to 1996. in policlinic department of Clinic for skin and veneral disease Novi Sad, in 10,5% of patients a presence of some dermatomycoses was registered.

Onychomycoses were diagnosed in 1,7% of the total number of patiens examined in that period, or in 10,22% of all cases of diagnosed dermatomycoses (9). Beside negative impact on patient's quality of life and working capability, onychomycoses are probably the most recalcitrant dermatomycoses and most difficult to treat, with frequent recidives and with regular low level of clinical and mycological cure. Costs of treatment of this disease are extraordinary high. A study performed by Medicare in USA during the one-year period (1989—1990) showed that costs of treatment of onychomycoses were 43 million dollars (12). *Candida albicans*, a dimorphous yeast, is a regular inhabitant of the human gastrointestinal and genital tract, and could be isolated in high percent of healthy persons (50—80% according to study). Although a member of normal mucosal flora of respiratory, gastrointestinal and in women's genital tract, on such places *Candida albicans* and spp. could become dominant, and cause diseases. Candidiasis most commonly affects skin, nails, mucous membranes, gastrointestinal tract, but also can transverse into systemic form and harms various internal organs, causing septicemia, meningitis, endocarditis etc. Most important predisposing factors for *Candida* infection are: diabetes mellitus, complete prostration, immunodeficiency, intravenous drug abusing, venous and urethral catheterisation, using of antibiotic which change normal intestinal bacterial flora and corticosteroids. The incidence of these infections is constantly increasing, especially in Intensive Care Units (ICV) (1, 5, 13).

This tendency stems of the mid eightieth years, when the funguses (mostly *Candida* and very rarerly *Aspergillus* which has mortality nearly 100%) as the causes were significantly influencing the increase of frequency of nosocomial infections. The data for the year 1990:

— 16,1 fungal infections occurred at 1000 traumas or burns
— 10,1 fungal infections occurred at 1000 cardiosurgical patients
— 7,3 fungal infections occurred at 1000 patients in general surgery

Candidaemia (the identification some of pathogen species *Candidae*'s in at least one specimen of the blood cultures) wasn't more times established (even 50% patients with candidaemia have the negative results of hemocultures) because of the difficult diagnostic and a non-specific clinical manifestations. Besides, there are no standard techniques to detecting of *Candida* and its metabolic products (13, 14). Fungal infections present a great risk for the patients with transplanted organs as well. Today, the transplantation is used as a therapeutical choice for many acute and chronic diseases. The skin lesions are many times atypical and may be the sign as both common fungal diseases and disseminated lethal fungal diseases (15). Black funguses (dark pigmented, melanised) — pheohyphomycoses are specific abstracted by their lethality, and are widely prevalent in the nature (16). On the basis of very rare studies in the world of an autopsy materials it is evident that the incidence of systemic mycoses (mostly candiasis and aspergillosis) was about 3%, while in patients with hematological diseases it was up to 26% (17).

THE DIAGNOSIS

The diagnosis of mycoses requires, besides clinical examination, the series of procedures in the aim of the effective identifications of the pathogen agents as well. The most important procedures in the mycological diagnostic are: taking of materials (cutting of skins and nails, uprooting of hairs) and direct microscopy, the cultivation on nourishing backgrounds of causes (the most used is Saboraoud — agar), the Wood — lamp examination, the pathohistological examination, the different serological and/or immunologicals assays, the molecular-biologycal techniques (PCR), the test of inoculations at the experimentaly animals, the computer systems for the identification of yeasts, etc. (1, 4). In the perspective it should be expected the special development of the molecular-biologycal techniques which give the possibility for fast identification in the clinical samples of the nicety microorganisms that are difficult to be cultivated in microbiological laboratories, and what may be of essential significance for the adequate therapy, especially for the therapy of the severe form of mycoses (4, 9). The computer systems for the identification of yeasts (AMS — YBC, ATB ID 32 C) represent a powerful diagnostic instrument in the work of clinical and microbiological laboratories. It sould be mentioned as the techiques of future in medical mycology and confocal microscopy, citometry, and corneofungometry, etc. (9, 18).

THERAPY

Today, it is preceded in the using the finite number of clinical antifungal agents. Many biochemical ways of antibiotics for the target structures of bacte-

ria couldn't be used by funguses, since the funguses have similar structures with the human cells because the many in vitro effective medicaments couldn't be applied in vivo due to its toxicity. The prior target structure of antifungal chemotherapeutic agents is the membrane of fungal cells, apropos the ergosterole as a predominant sterol of the membranes (4).

The most important medicaments which are used for therapy of the fungal infections are: amphoterycine B, flucitozine, griseofulvine, nystatine, 5-fluorocitozine, antifungal azoles (ketokonazole, itrakonazole, flukonazole) terbinafine, etc. In the local therapy the most used are antifungal azoles (clotrimazole, myconazole), nystatine, cyclopiroxolamine, amorolphine, terbinafine etc. In last years it is significant the discovering of vorikonazoles and their inauguration in clinical praxis, that proved successful in the therapy of the severe systemic mycoses (candidiasis, aspergillosis) by which the other antifungal medicaments were reduced fast to perfoming of resistency, especially by the rare species of *Candidae's (C. Tropicalis, C. glabrata, C. parapsilois)*. Accordingly, it is possible the retreiving of lives also in immunocompromited patients infected with species which develop fast resistibility, and some of them may even be of natural-originating resistibility (C. Krusei). It is also significant the introducing of the liposomal amphoterycine B in the empiric therapy of the systemic (deep) mycoses beyond where it couldn't be expected the results of laboratories and where it is precedent the rapidity of therapies (1, 19, 20).

МИКОЗЕ ЉУДИ

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Резиме

Гљиве су самостална група биљног царства чији припадници немају хлорофил, па тиме ни способност фотосинтезе, т. ј. не могу да синтетишу хранљиве састојке, због чега егзистирају као сапрофити или паразити биљака, животиња и људи. Има их између 50 и 100 хиљада врста, али само око сто њих узрокује обољења (микозе) људи или животиња, док многе друге врсте изазивају обољења биљака. Само дерматофити и врсте рода *Candida* обично се преносе са човека на човека. Значај гљива је досада био нешто мањи у односу на бактерије или вирусе, али њихова улога као опортунистичких патогена незаобилазна је код имуносупримираних особа. Инциденција гљивичних инфекција је у сталном порасту, чак и у земљама са високо развијеном општом и здравственом културом. Дијагностика и терапија гљивичних инфекција су често неадекватне. Профилакса је још увек предмет дискусије.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 283—289, 2005

UDC 615.28

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FLUCONAZOLE IN THE THERAPY OF PITYRIASIS VERSICOLOR

ABSTRACT: The authors present the results of the systemic application of flukonazole in therapy of Pityriasis versicolor. It was arranged for the total number of 38 patients, 18 females and 20 males. The diagnosis of diseases was established on the base of the clinical examination, the native mycological examination and by the using of Wood lamps. The therapy was passed by the using of 300 mg flukonazole in a single dose, once weekly, during two weeks. The following period amounted to one week after the passed therapy. The therapeutic efficacy was assessed with regard to the clinical and mycological healing. The clinical efficacy was assessed semiquantitative on the base of increasing of the percentage rates of the total score of disease that was computed by collecting of the numeric values for every clinical argument typical for the disease, and the mycological efficacy on the base of the mycological findings and the fluorescence finding after lightening with the Wood's lamp. The controling examinations were performed on day 0, 14 and 22. The results of investigations have shown that the complete clinical healing was achieved after two weeks of therapy in 94,74%, and the mycological healing in 92,11% patients. The rate of the mycological healing was evaluated after 1 week of following period equal to the rate of the clinical healing and it was also 94,74%. The undesirable effects of the drug applications weren't by any patient.

KEY WORDS: flukonazole, Pityriasis versicolor, therapy

INTRODUCTION

Pityriasis versicolor (PV) is one of the most frequent fungal skin infections with chronic nature. As the disease cause it is recognized lipophylic yeast *Mallassezia* that is well known as *Pityrosporum ovale* or *Pityrosporum orbiculare*, the resident of the normal skin flora are recognized as the disease cause (1). The transition from blastospore form to a mycelium makes this yeast pathogenic for the humans. The conversion in the form of mycelium occurs under the influence of variety of different predisposition factories. The most important factor are high temperatures and high humidities because of which the disease occurs with the low prevalence in the Scandinavian countries (1%) and

with the highest prevalence of 40—50% in tropical areas (2). For the apparation of disease genetical factors are significant, which is corroborated by the lack of diseases in couples and its apparation in the cousins of the first, second and third degrees of the kins.

The predisposition to frequent recurrences, the extension of skin changes, often perturb the life qualities of the patients. This fact destined the dermatologists during recent years, towards the systemic application of antimycotics in the attempt at more successful treatment (3, 4).

THE AIM

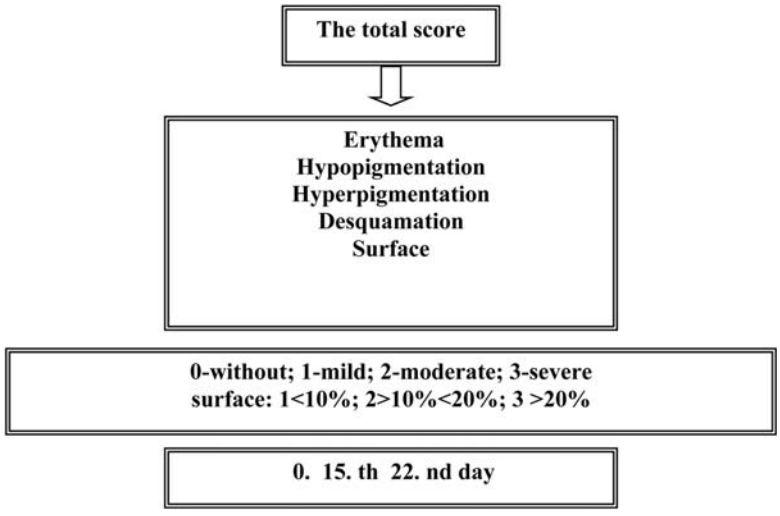
The study had the aim to check the efficacy and safety of oral application of fluconazole in the therapeutic regime of 300 mg once weekly during two weeks in the patients with PV. The following period of patients after treatment has been a week after the passed therapy.

It is arranged the total number of 38 patients, 18 females and 20 males, 24 patients with the first manifestation of the disaese and 14 with the recurrence of the disease.

It is established the diagnosis of the disease by the clinical and mycological examination and by using of Wood's lamp. The mycological examination was the obligate diagnostic procedure for all cases regarding the cognition that only *Malassezia furfur* produces indole which gives the fluorescence by lightening with Wood's lamp.

It was semiqunatitative assessment. The clinical efficacy of the drug was assessed with regard to decrease of the total score values of the diseases (in percentages) that it was computed by collecting the numeric values of the clinical parameters typical for the disease, nevertheless it is taken in consideration the size of the skin surfaces involved with the disease (Table 1).

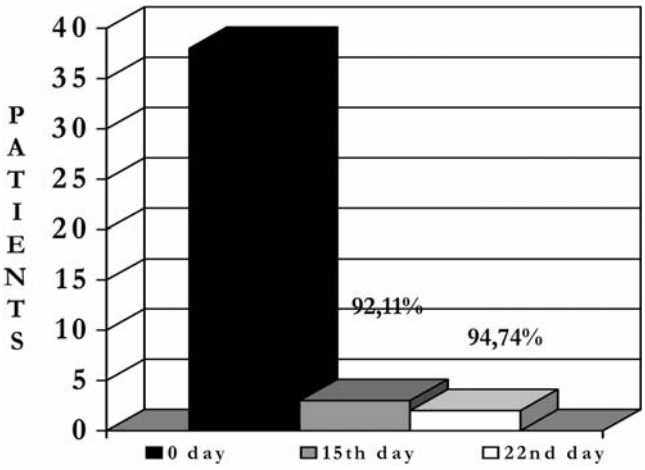
Table 1. Assessment of the clinical efficacy



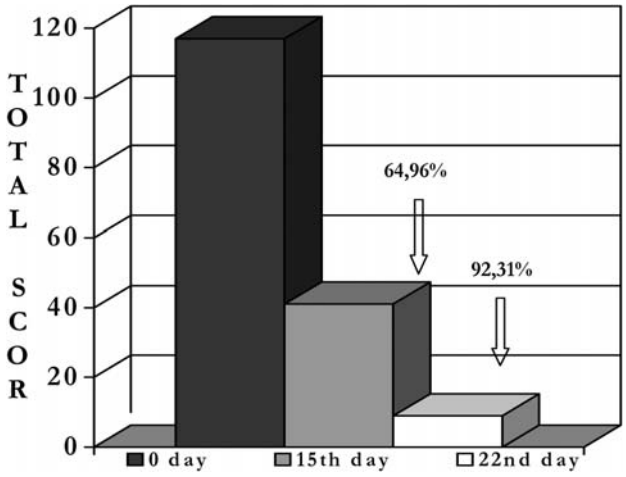
The total score was computed an 0-th, 15-th and 22-nd day, and on the 7-th day after the passed therapy. The mycological efficacy has been followed on 0-th, 15-th and 22-nd day.

RESULTS

The results of the investigations showed that after two weeks of the fluconazole applications negative mycological findings were achieved in 92,11% patients. After 7 days this percentage increased to 94,74%. As for the clinical healings, after two weeks, the total score of diseases decreased for 64,96% and after three weeks for 94,74% (Graphics 1 and 2).



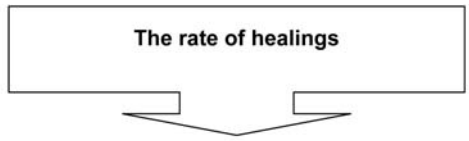
Graphic 1. The mycological efficacy



Graphic 2. The clinical efficacy

The complete healing was achieved in 86,84% patients, the clinical without micological healing in 5,26%, and the mycological without clinical healing in 7,89% patients (Table 2).

Table 2. The rate of healings



COMPLETE-33 (86,84%) patients

CLINICAL WITHOUT MYCOLOGICAL-2 (5,26%) patients

MYCOLOGICAL WITHOUT CLINICAL-3 (7,89%) patients

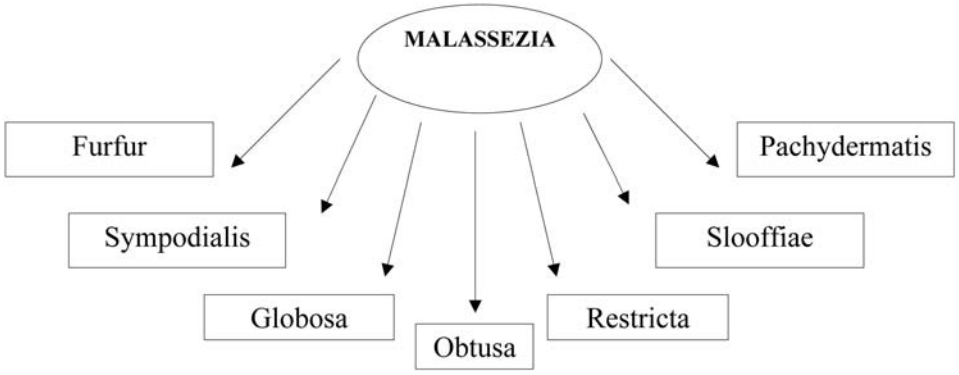
The undesirable effects of the drug applications were'nt noted in any patient.

DISCUSSION

As the causes of PV, the yeasts were recognized in 1874. (3). Malasezz described its presence in the corneal layer of skin as the oval and spheric cells into bud. Bailon named it *Malassezia furfur*, and Castellani, who first succeeded its cultivation, registered the term *Pityrosporon ovale*. In 1996. 7 distinct species were recognized, and 6 primarily were isolated from human skin (4) (Picture 1). It is only *Malassezia pachydermatis* obligatory to be lipophilic, and it was isolated primarily in animals, rarely in humans.

In the light of the recent cognitions about variety of *Malassezia* species and the passed investigation in the last five years, the most frequent causes of PV are *Malassezia furfur, Malassezia globosa* and *Malassezia sympodialis*.

The therapy of diseases could be passed as local and systemic, specific and nonspecific. Whereby the disease often recurres after the passed therapy, that it was based until last mainly on the local application of antimycotic drugs, there were the attempts of its systemic using. It was shown that the systemic using of these drugs is more effective and that they should be given during short period. Today it is recommended itrakonazole and flukonazole of the systemic antimycotics in the therapy of PV. Griseofulvine isn't effective given perorally as well as terbinafine, that is effective in the form of the local preparations (the form of creams, gels and solutions). Ketokonazole isn't recommended because of the hepatotoxic acting of drugs. The systemic antimycotics are recommended for the prevailing and recurred infections, nevert-



Picture 1. *Malessezia* species

heless the clinicians makes the choice of oral antimycotics that are able to eradicate funguses in optimal dose, well passable and safe for using.

We decide to assess the efficacy and the safety of flukonazole in this study by the outset of the flukonazole pharmacodynamic profiles and of facts that it progresses the multiple higher concentration in the skin than in the plasme, and the concentration higher of the minimal inhibitory concentration for the majority of dermatophytes and non-dermatophytes causing the fungal diseases in human kind.

The administration of flukonazole in the therapy of PV hitherto has shown the excellent therapeutic results with regime of 300 mg weekly during two weeks. The results of the previous investigations confirmed the high rate of the mycological and clinical healing, in the range 80—98% for the mycological healing and 91—98% for the clinical healing, which is in accordance with the results of our investigations (5, 6). The fact of the increased rates of the clinical and mycological healing 7 days after the passed therapy could be explained by the pharmacodynamic profile of drugs and its presence in the skin 10 days after withdrawal of therapies.

In this study settings weren't the undesirable actions, though it is well known that flukonazole causes in 6% cases the undesirable actions.

CONCLUSION

It could be said with full right in the conclusion that the therapeutic regime of flukonazole's of 300 mg weekly during two weeks is effective, elegant and safe and that it is the pharmacodynamic rational regime of the therapies *Pityriasis versicolor.*

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ФЛУКОНАЗОЛ У ЛЕЧЕЊУ PITYRIASIS VERSICOLOR

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Резиме

Аутори износе резултате системске примене флуконазола у лечењу Pityriasis versicolor. Укупно је обрађено 38 болесника, 18 жена и 20 мушкараца. Дијагноза болести постављена је на основу клиничког прегледа, нативног миколошког прегледа и применом Wood-ове лампе. Терапија је спровођена применом 300 mg флуконазола у једној дози, једампут недељно, 2 недеље. Период праћења износио је недељу дана након спроведене терапије. Терапијска ефикасност процењивана је са аспекта клиничког и миколошког излечења. Клиничка ефикасност процењивана је семиквантитативно на основу процентуалног смањења тоталног скора болести који је израчунаван сабирањем нумеричких вредности за поједине клиничке параметре карактеристичне за болест, а миколошка ефикасност на основу миколошког налаза и налаза флуоресценције након осветљавања Wood--овом лампом. Контролни прегледи вршени су 0, 14. и 22. дана. Резултати истраживања показали су да је након две недеље терапије комплетно клиничко излечење постигнуто у 94,74%, а миколошко у 92,11% болесника. Након недељу дана праћења стопа миколошког излечења се посве изједначила са стопом клиничког излечења и износила је такође 94,74%. Нежељених дејстава примене лека није било ни код једног болесника.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 291—299, 2005

UDC 616.5-002.828

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MODIFIED SABOURAUD DEXTROSE AGAR FOR ISOLATION AND IDENTIFICATION OF DERMATOPHYTES

ABSTRACT: The most common causative agents of dermatomycoses are fungi belonging to genders *Trichophyton, Microsporum* and *Epidermophyton*. Media mainly used for isolation of dermatophytes are mycobiotic agar, dermatophyte test medium, Sabouraud agar (original formula or modification by Emmons) with or without antibiotics and cycloheximide.

Peptones are the most important components of the media, which enable adequate reproductivity in identification of dermatophytes. Standard medium for isolation of dermatophytes is not produced in our country. The aim of the study was to create an optimal, easily accessible and economic medium which enables isolation and identification of dermatophytes according to criteria for morphological diagnosis provided by identification guides.

We examined 57 strains of *Trichophyton*, 24 of *Microsporum* and 5 of *Epidermophyton floccosum* (*E. floccosum*). Each strain was seeded on Sabouraud dextrose agar (Torlak, Serbia and Montenegro), Sabouraud maltose agar (Torlak), two experimental modified Sabouraud dextrose agar media marked as SA-2 and SA-3 (Torlak), Sabouraud-Chloramphenicole agar (Biomerieux, France), Sabouraud-Chloramphenicole agar (Himedia, India), Glucose-peptone agar (Himedia, India) and Sabouraud Emmons dextrose Agar with Chloramphenicole and Cycloheximide (Biolife, Italy).

Colony morphology of *Trichophyton mentagrophytes* (*T. mentagrophytes*) was uniform on all the media, while morphology of *Trichophyton rubrum* (*T. rubrum*) and *Microsporum canis* (*M. canis*) depended more on the media type. Colonies of *E. floccosum* were typical and uniform on all the media, as were the control species of *Trichophyton schoenleinii* (*T. schoenleinii*) and *Trichophyton soudanense* (*T. soudanense*).

Experimental modified Sabouraud dextrose agar (Torlak) marked as SA-3 demonstrated the best results in identification of dermatophytes in this study.

KEY WORDS: dermatophytes, identification, isolation, media

INTRODUCTION

Dermatomycoses are diseases widespread throughout the world. Diagnosis of dermatomycoses is based on a mycological examination of skin and/or skin adnexa scrapings and finding of mycelial filaments in the material, as well as on isolation of a causative agent from the material. The most common cause of dermatomycoses are fungi belonging to the genera *Trychophyton, Microsporum* and *Epidermophyton*. Media mainly used for isolation of dermatophytes are mycobiotic agar and dermatophyte test agar (7, 9), as well as Sabouraud agar with original formula (glucose 4%, peptone 1%) or Emmons' modification of Sabouraud medium (glucose 2%, peptone 1%). These media can be made in house or purchased as ready-made.

Bacteria and saprophytic moulds which colonize the skin and/or it's adnexa (hair and nails) could interfere with isolation of dermatophytes since dermatophytes grow more slowly. Therefore, media for isolation of dermatophytes are supplemented with antibiotics (chloramphenicole, gentamycine) and antimycotic cycloheximide (actidione), which prevents growing of saprophytic moulds. The number of onychomycoses caused by saprophytic moulds like *Aspergillus, Scopulariopsis* etc. is increasing (5, 12), therefore nail scrapings should be seeded both on media with actidione and media without it.

Standard medium for the isolation of dermatophytes is not manufactured in our country. The aim of this study was to create an optimal, easily accessible and economic medium which would enable isolation and identification of dermatophytes according to criteria for morfological diagnosis provided by identification guides (3, 6, 8).

MATERIALS AND METHODS

We examined a total of 86 strains of dermatophytes: 57 strains belonging to genus *Trichophyton* (*T. mentagrophytes* 36, *T. rubrum* 16, *T. megninii* 1, *T. equinum* 1, *T. soudanense* 1, *T. schoenleinii* 1 and *T. tonsurans* 1), 24 strains belonging to genus *Microsporum* (*M. canis* 20, *M. audouinii* 1, *M. gypseum* 1, *M. persicolor* 1, *M. species* 1) and 5 strains of *Epidermophyton floccosum* (*E. floccosum*). Control strains of dermatophytes that had been tested on mycobiotic agar (Mycosel, Oxoid, UK) (*T. schoenleinii, T. soudanense, T. equinum, T. megninii, T. tonsurans, M. audouinii* and *M. persocolor*) were obtained thanks to Dr. Mary Moore (Department for Mycology, St. John's Institute for Dermatology, St. Thomas Hospital, London, UK).

Each strain was seeded on Sabouraud dextrose agar (SDA) (Torlak); Sabouraud maltose agar (SMA) (Torlak); two experimental media made by modification of composition of standard Sabouraud dextrose agar, termed SA-2 and SA-3 (Torlak); Sabouraud-Chloramphenicole agar (Biomerieux, France); Sabouraud-Chloramphenicole agar (Himedia, India); glucose-peptone agar (GPA) (medium made in our laboratory with peptone manufactured by Himedia, India) and Sabouraud Emmons Dextrose Medium with Chloramphenicole and Cycloheximide (BL) (Biolife, Italy). The experimental media SA-2 and SA-3

were modified Sabouraud dextrose media: SA-2 with changed peptone composition and SA-3 with changed peptone composition and concetration of glucose (2%). Media were poured in Petri dishes of 19 cm in diameter, about 5 mm thick. Fungal innocula of pinhead size were seeded using a needle, on three spots. Cultures were incubated in the dark at 27°C. Growth has been observed for 3 weeks, and examination of colonies was done after 7,14 and 21 days. Examination of colonies included: measuring of colony diameter depending on the duration of incubation, morphology of colony surface (with detailed record of the following parameters: uniformity, typical morfology regarding identification keys, and pigment), pigment on the reverse of the colony and microscopic findings.

The criteria for evaluation of the tested media were based on common morphology of dermatophytes on mycobiotic agar (Mycosel, Oxoid, UK), and on the identification keys (3, 6, 8).

RESULTS

Results of examination of *Trichophyton mentagrophytes* strains are shown in Table 1.

Table 1. *Trichophyton mentagrophytes* (n = 36)

Parameters	Details	Colony morphology
colony diameter (7 days)	without AB*	21—48 mm
	with AB	14—33 mm
colony morphology	uniformity typical appearance colour	stable within species not depending on the medium after 7 days center folded or bulged
pigment on the reverse of the colony	colour most intense	white cream (2 species: yellow and greyish) brownish red (varies from yellow to brown) BL, SA-3, SA-2
microscopic examination	optimal identification	SA-3 best (macroconidia, microconidia and spiral hyphae), on SA-2 no macroconidia, on BL chlamydospores dominate

* AB — antibiotic (chloramphenicole and/or actidione)

Notes:

1. After the second week colony diameter equalizes, independent on the presence of antibiotics in the media.
2. No connection was found between the shape of the center and the type of medium.
3. Diagnosis of *T. mentagrophytes* can be made in one week.

Results of examination of *Trichophyton rubrum* strains are shown in Table 2.

Table 2. *Trichophyton rubrum* n = 16

Parameters	Details	Colony morphology
colony diameter (7 days)	without AB with AB	10—28 mm 5—20 mm
colony morphology	uniformity	partial: all species are the same on the same medium; morphology depends on the medium not on the species
	typical appearance colour	SA-2, SA-3, SDA
pigment on the reverse of the colony	colour most intense	white (9 species: yellow, greyish or reddish after 2 weeks) brownish red (varies from yellow to brown after 2 weeks) SA-2, SA-3; best after 7 days
microscopic examination	optimal identification	SA-3 the best (macro i microconida, spirals), on SA-2 no macroconidia, SDA, on BL chlamydospores dominate

Note: Diagnosis of *T. rubrum* can be made after two weeks.

Results of examination of *Microsporum species* strains are shown in Table 3.

Tabela 3. *Microsporum species* (*canis, audouinii and species*) n = 23

Parameters	details	colony morphology
colony diameter (7 days)	with and without AB note	10—46 mm great variation, does not depend on AB in the medium
	uniformity	no*
colony morphology**	typical appearance	partial, species within gender difficult to differentiate
	colour	white-orange
pigment on the reverse of the colony	colour most intense	orange variable SA-3, SA-2
	note	intensity of pigment depends on a species
microscopic examination	after 2 to 3 weeks	SDA for 7 days macroconidia, a few days later also on SA-2, SA-3

* Four types of colonies could be differentiated: aberant, with fine mycelium grown in the medium, orange white colonies and white with aboundant mycelium (sterile).
** Morphology depended on the type of medium, not on the species. Typical morphology was present on SA-2 and SA-3, on SMA not satisfactory. On BL medium 5 of 11 species had satisfactory morphology.

Note: *M. audouinii* had most typical colonies on SMA and BL.

On all the media the appearance of *Epidermophyton floccosum* was uniform and typical. Colony diameter was 6 to 11 mm after 7 days on media with antibiotics, and 11 to 15 mm on media without antibiotics. Microscopic identification was possible after 7 to 14 days.

Comparative analyses of the control strains on the mycobiotic agar (Mycosel, Oxoid, UK) and our tested media

M. persicolor

The diameter of a colony after 7 days on mycobiotic agar was 25 mm, and on our tested media 22—25 mm without antibiotics, and 16—22 mm with antibiotics. On all the media colonies granulated surface and cream white colour with bulging center, except on SMA where the colour was rose. Pigment on the reverse of the colony was brown on mycobiotic agar, rose on SMA, and various intensity of yellow-brown on the other media, the strongest on SA-3. Microscopic examination of colonies grown on the mycobiotic agar revealed characteristic microconidia, macroconidia and spiral hyphae. Macroconidia and microconidia were found in colonies from SA-3, SDA and GPA, and only microconidia in colonies grown on the rest of the tested media.

T. equinum

The diameter of a colony after 7 days on mycobiotic agar was 30 mm, and on our tested media 25 mm without antibiotics and 18—20 mm with antibiotics. On all the media colonies were almost identical: flat, disheveled, with edges immersed in the media, coloured white to yellow. Pigment on the back of the colony was brown in the center and yellow on the circumference. The findings on SA-3 matched the most with the findings on the mycobiotic agar. Characteristic microconidia were found by microscopic examination of a colony from mycobiotic agar, while macroconidia in addition to microconidia of a colony from SA-3.

T. megninii

The diameter of a colony after 7 days on mycobiotic agar was 12 mm, and on our tested media 10—16 mm not depending on the presence of antibiotics. On mycobiotic agar the colony was flat, white, velvety, developing a rose pigment only after the second week. On our tested media colonies were uniform, white coloured with bulging center and the formation of radial grooves after the third week of incubation. Pigment on the reverse of the colony was characteristic light red only on the mycobiotic agar, and on our tested media no pigment was produced. Beside findings on the mycobiotic agar, microscopic examination was characteristic on SMA (macroconidia and microconidia), and only microconidia on SDA, SA-3 and BL.

T. tonsurans

The diameters of colonies after 7 days were about the same on all the media, including mycobiotic agar. Colonies were powdery, cream to brown

coloured, also identical on all the media. The pigment on the reverse of colonies was characteristic — yellow-brown — on the mycobiotic agar. On our tested media the production of pigment was satisfactory on SA-3, SDA and BL. Besides typical microscopic findings on mycobiotic agar (microconidia, baloon forms and chlamydospores), the same morphology was found on SA-2 and SA-3.

T. schoenleinii and *T. soudanense* were of the same morfology on all the tested media, including the mycobiotic agar.

Comparing the parameters of examination we found that the presence of antibiotics in a medium, no matter whether they were antibacterial (Chloramphenicol) or antimycotic (Actidione), had influence on growth of the majority of dermatophytes in the first week of incubation, reflecting on diameters of colonies, with exception of *T. tonsurans, T. megninii* and genus *Microsporum.*

Morphology of all the strains of *T. mentagrophytes* was uniform on all the media, in contrast to *T. rubrum* where morphology was more depending on the type of the medium. Therefore, usage of a non-standard medium in practice could lead to mistakes in diagnosis of *T. rubrum*.

Examination of 20 strains of *M. canis* established that colonies were distinguished as members of the genus *Microsporum*, but variations in appearance were present. We noted that the difference in morphology was due to the type of medium, and not to strain type.

Colonies of *Epidermophyton floccosum* were typical and uniform on all the media, as were control strains of *T. schoenleinii* and *T. soudanense*. Other control strains of *M. persicolor, T. tonsurans* and *T. equinum* were of almost identical morphology on all the media, including mycobiotic agar (Mycosel, Oxoid, UK), in contrast to *T. megninii* which showed somewhat different morphology on mycobiotic agar than on the other media.

The colour of colonies of all the tested strains corresponded to the description in identification guides. The control strain of *M. persicolor* was rose coloured on SMA, but cream on all the other media. For that reason SMA could be used for differential diagnosis of *T. mentagrophytes* and *M. persicolor.*

All tested strains of dermatophytes, except *T. megninii* and *T. schoenleinii*, produced pigment of various intensity depending on the type of medium or on the strain, which diffused into the media. SA-2 and SA-3 were most stimulative for the pigment production in *T. mentagrophytes, T. rubrum* and *Microsporum species*. Besides, SA-2 and SA-3 media performed best results in the production of characteristic structures for asexual reproduction of dermatophytes, which contributed to clarity of microscopic morphology of all the strains of *Trychophyton*, except *T. megninii*. The control strain of *T. megninii* developed more complete motphology on SMA. Microscopic finding of *Microsporum* strains was better on SDA, where typical macroconidia developed in 7 days, though they did develop on SA-2 and SA-3 in the second week of incubation. The control strain of *M. audoinii* demonstrated the best morphology on SMA.

DISCUSSION

The number of dermatophyte species is large, but only most commonly isolated in our country were included in this study (2).

Specialized laboratories in the world mostly use mycobiotic agar for isolation of dermatophytes (7). DTM (Dermatophyte Test Medium) and a new DIM (Dermatophyte Identification Medium) are used for quick diagnosis of dermatophytes (11). Diagnostic principle in these media is based on production of alcaline metabolic products during growth — a feature that distinguishes dermatophytes from saprophytic moulds. That leads to change in colour of a medium (indicator is phenol-red in DTM, and brom-cresol-purple in DIM). These media are mostly used in field research or in non-specialized laboratories.

Dermatophytes are identified on the basis of colony morphology, growth rate, pigment production, microscopic findings and physiological features. Most strains of dermatophytes can be identified basing on their macroscopic and microscopic features in primary culture. For further identification Lactrimel--agar and Trichophytone agars 1—7 (Difco) could be used, as well as a number of physiological tests (production of urease, hair perforation test etc.).

In addition to mentioned media, which are special for dermatophytes, standard Sabouraud agar can also be used. Standard Sabouraud agar is a medium consisting of 1% peptone, 1.5—2% agar, and 4% glucose, with final pH 5.6 (1). In practice, composition of Sabouraud media made by different manufacturers quite varies (1). Differences in media composition lead to differences in morphology and lack of reproducibility in diagnosis of colonies (1, 10). The most important component of the medium, one that morphology and especially pigment production depends on, is peptone (4). Peptones present in commercial Sabouraud media can be restrictively divided according to: enzyme used for peptone hydrolyses (pancreatic, peptic, papaic), protein source (casein, soya, meat), and according to purpose (mycological). These media are also different in final pH (5.6—6.8). Mycobiotic agar has similar composition as Sabouraud agar, but it contains plant source peptone (phytone peptone) (1%), dextrose (1%), actidione (0,04%) and chloramphenicole (0,005%) (7).

In our country only a few laboratories were able to perform isolation of dermatophytes on mycobiotic agar. One of the authors (N. B.) spent most of her long-standing experience working with Sabouraud dextrose agar supplemented with penicillin, streptomycin and cycloheximide for isolation of dermatophytes. Differential diagnosis of dermatophytes on that medium was not simple. Therefore, in this study, modifications of present Sabouraud dextrose agar (Torlak) were undertaken, which were supposed to enable faster and more accurate diagnosis of dermatophytes on an easily accessible and economic medium.

By changing the peptone type and dextrose concentration in Sabouraud dextrose agar (Torlak) we obtained a new medium SA-3, which enabled isolation and identification of majority of species of dermatophytes in our region according to identification guides.

Conclusion: Experimental medium SA-3 (Torlak) demonstrated the best results in identification of dermatophytes in this study. Macroscopic and microscopic morphology of dermatophytes on this medium corresponds the most with morphology of these fungi on standard media that contain mycological peptone as a main component. Sabouraud dextrose agar (Torlak), which was used in our country for primary isolation of dermatophytes, can be used for identification of *M. canis* because it supports production of macroconidia already in the first week of incubation. On all the media *T. mentagrophytes* can be diagnosed after 7 days, while for the identification of other species of dermatophytes more than 14 days is necessary.

AKNOWLEDGEMENTS

Authors are grateful to dr Diana Tomić and Nada Boljević (Institute for Microbiology, Military Medical Academy, Belgrade) for their help in collecting the strains of dermatophytes, as well as Kosović Lidija, Milekić Slavica and Božić Milojka on technical support.

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МОДИФИКОВАНИ САБУРО ДЕКСТРОЗНИ АГАР ЗА ИЗОЛАЦИЈУ И ИДЕНТИФИКАЦИЈУ ДЕРМАТОФИТА

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Резиме

Дерматомикозе су широко распрострањена обољења свугде у свету. Најчешћи узрочници дерматомикоза су гљиве из родова *Trichophyton, Microsporum* и *Epidermophyton*. За изолацију дерматофита углавном се користе микобиотски агар, дерматофит-тест агар, Сабуро агар са оригиналном формулом или Емонсова модификација Сабуро агара са и без антибиотика и циклохексимида. Пептони су најзначајнија компонента подлога која омогућава адекватну репродуктивност у идентификацији дерматофита. У нашој земљи се не производи стандардна подлога за изолацију дерматофита. Циљ овог рада је био да се креира оптимална, лако доступна и економична подлога, која омогућава изолацију и идентификацију дерматофита према критеријумима за морфолошку дијагностику предвиђеним водичима за идентификацију.

Прегледано је 57 сојева рода *Trichophyton*, 24 сојева рода *Microsporum* и 5 сојева *Epidermophyton floccosum* (*E. floccosum*). Сваки сој је засејан на Сабуро декстрозни агар (Торлак), Сабуро малтозни агар (Торлак), две експерименталне подлоге настале модификовањем састава стандардног Сабуро декстрозног агара под радним називом SA-2 и SA-3 (Торлак), Сабуро-хлорамфеникол агар (Biomerieux, Француска), Сабуро-хлорамфеникол агар (Himedia, Индија), глукозо пептонски агар (са пептоном Himedia, Индија) и Сабуро Емонс декстрозни агар са хлорамфениколом и циклохексимидом (Biolife, Италија). Критеријуми за оцену испитиваних подлога установљени су на основу познате морфологије коју сојеви дерматофита показују на микобиотском агару (Mycosel, Oxoid, Велика Британија) и на основу кључа за идентификацију гљива.

Морфологија колонија *Trichophyton mentagrophytes* (*T. mentagrophytes*) била је униформна на свим подлогама, док је морфологија *Trichophyton rubrum* (*T. rubrum*) и *Microsporum canis* (*M. canis*) више зависила од врсте подлоге него од соја. Колоније *E. floccosum* су биле типичне и униформне на свим испитиваним подлогама, као и контролни сојеви *Trichophyton schoenleinii* (*T. schoenleinii*) и *Trichophyton soudanense* (*T. soudanense*). Експериментална подлога SA-3 показала је најбоље резултате у идентификацији дерматофита у овој студији. Макроскопска и микроскопска морфологија дерматофита на овој подлози највише одговара морфологији ових гљива на стандардним подлогама у којима се као главна компонента налази миколошки пептон. Сабуро декстрозни агар (Торлак), који се досада у нашој земљи користио за примарну изолацију дерматофита, може се користити у идентификацији *M. canis* јер потпомаже продукцију макроконидија већ у првој недељи инкубације. На свим подлогама *T. mentagrophytes* се може дијагностиковати након 7 дана, док је за идентификацију осталих врста дерматофита потребно више од 14 дана.

Зборник Матице српске за природне науке / Proc. Nat. Sci, Matica Srpska Novi Sad, № 108, 301—307, 2005

UDC 633.13:632.26

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EFFICIENCY OF *PC* GENES OF RESISTANCE TO PREVALENT PATHOTYPES OF *PUCCINIA CORONATA AVENAE*

ABSTRACT: This paper deals with efficiency of some *Pc* genes of resistance to prevalent pathotypes of crown rust causer in oat. It was found that in seedlings stage the most efficient were genes *Pc 68* and *Pc 39* (100%). Satisfying degree of efficiency demonstrated genes *Pc 38* and *Pc 55* (87,5%). Majority of genes expressed partial resistance. Efficiency of *Pc* genes of resistance to prevalent pathotypes of pathogen in both years of investigation was satisfying and it was 61,45%.

KEY WORDS: efficiency, oats, *Pc* genes, *Puccinia coronata avenae*, resistance

INTRODUCTION

Leaf rust of oat, with its causer *Puccinia coronata avenae*, is frequent and economically very important oats disease (Harder and Haber, 1992; Šebesta et al., 1996; Chong and Aung, 1996; Aung et al., 1996). There is small number of data for appearance and damages caused by this parasite in SCG. This disease is appearing frequently, while strong attack was noticed in 1959 (Kostić, 1965). Stojanović et al., (1995) pointed out that crown rust is broadly distributed in Serbia, but the intensity of its development is different in some years and locations. In conditions of epidemic, grain yield is lower from 30 to 50% (Hohrjakova and Fedorova, 1975).

Different strategies were applied in breeding of oats for resistance to this pathogen. Basic goal was in generally to control intensity of disease development on low level, and in that way to make possible a stabile production. One of significant strategies is incorporation of more major genes for resistance in new cultivar (Mundt, 1990; Bošković et al., 2000).

The goal of these investigations was to establish efficiency of some *Pc* genes of resistance to prevalent pathotypes of *P. coronata avenae*.

MATERIAL AND METHODS

Investigations were carried out during 1999 and 2000 year in Center for Small Grains in Kragujevac. Establishment of virulence formulas of prevalent pathotypes was performed by set of isogenic lines of oats with famous genes for resistance (*Pc 38, Pc 39, Pc 48, Pc 50, Pc 50-2, Pc 54-1, Pc 54-2, Pc 55, Pc 56, Pc 58, Pc 59, Pc 60, Pc 61, Pc 62, Pc 63, Pc 64, Pc 67* and *Pc 68*), according to methods of Green, 1965; Šebesta and Harder, 1983).

Resistance of seedlings of genotypes with known *Pc* genes for resistance was examined in controlled conditions (growth chamber). Inoculation of seedlings by pure cultures of prevalent pathotypes with pathogen formulae (A/V): *38, 39, 48, 50-2, 54-1, 54-2, 55, 58, 60, 61, 62, 67, 68/50, 56, 59, 63, 64* and *38, 39, 48, 54-1, 55, 56, 58, 59, 60, 63, 68/50, 50-2, 54-2, 61, 62, 64, 67*, was performed in stage of full development of the first leaf, and after 12—14 days it was estimated the type of their reaction by determination of types of infection from 0 to 4 (Stakman et al., 1962).

Efficient were those genes, which in interaction with genes for virulence of pathogen made possible expression of resistant (0—2), and inefficient those that caused expression of susceptible types of infection (3—4).

RESULTS OF INVESTIGATIONS

The obtained results show that efficiency of *Pc* genes to causer of *P. coronata avenae* was different, which is noticeable from the data presented in Table 1.

In 1999 season, the most efficient were genes *Pc 39, Pc 39, Pc 54-1* and *Pc 68* with 100% efficiency to prevalent pathotypes of the mentioned pathogen. On the other side, the lowest efficiency demonstrated genes *Pc 39, Pc 63, Pc 64* and *Pc 67* (25%). Efficiency of genes *Pc 48, Pc 50, Pc 56* and *Pc 58* was 50%, and genes *Pc 50-2, Pc 54-2, Pc 55, Pc 60, Pc 61* and *Pc 62*, 75%, which points out their medium efficiency.

In the 2000 season, genes *Pc 39, Pc 48, Pc 55, Pc 56, Pc 59* and *Pc 68* demonstrated the highest efficiency (100%), which means that for these genes of resistance in population of pathogen were not present adequate alleles of virulence. High efficiency expressed gene *Pc 58*, which was 87,5%. The lowest efficiency (0%) had genes *Pc 50-2* and *Pc 54-2*, for which, all pathotypes were virulent. Very low efficiency (12,5%) demonstrated also genes *Pc 61, Pc 64* and *Pc 67*. The rest genes *Pc 38, Pc 50, Pc 54-1, Pc 60, Pc 62* and *Pc 63* were middle efficient to population of pathogen and their efficiency ranked from 25% to 75%.

The most efficient in both years of investigations were genes *Pc 39* and *Pc 68* (100%). High efficiency expressed genes *Pc 38* and *Pc 55* which was

Table 1. Efficiency of *Pc* genes of resistance to prevalent pathotypes of *Puccinia coronata avenae* in 1999 and 2000 seasons

Pc genes	1999.				2000.				Average
	R*		S*		R		S		
	№	%	№	%	№	%	№	%	
38	4	100.00	0	0.00	6	75.00	2	25.00	87.50
39	4	100.00	0	0.00	8	100.00	0	0.00	100.00
48	2	50.00	2	50.00	8	100.00	0	0.00	75.00
50	2	50.00	2	50.00	2	25.00	6	75.00	37.50
50-2	3	75.00	1	25.00	0	0.00	8	100.00	37.50
54-1	4	100.00	0	0.00	5	62.50	3	37.50	81.25
54-2	3	75.00	1	25.00	0	0.00	8	100.00	37.50
55	3	75.00	1	25.00	8	100.00	0	0.00	87.50
56	2	50.00	2	50.00	8	100.00	0	0.00	75.00
58	2	50.00	2	50.00	7	87.50	1	12.5	68.75
59	1	25.00	3	75.25	8	100.00	0	0.00	62.50
60	3	75.00	1	25.00	5	62.50	3	37.50	68.75
61	3	75.00	1	25.00	1	12.50	7	87.50	43.75
62	3	75.00	1	25.00	3	37.50	5	62.50	56.25
63	1	25.00	3	75.00	6	75.00	2	25.00	50.00
64	1	25.00	3	75.00	1	12.50	7	87.50	18.75
67	1	25.00	3	75.00	1	12.50	7	87.50	18.75
68	4	100.00	0	0.00	8	100.00	0	0.00	100.00
Mean		63.88				59.02			61.45

* R — resistant; S — susceptible

87,5%. Resistance of majority of the rest *Pc* genes ranked from 18,75% to 81,25%. Genes *Pc 64* and *Pc 67* were very low efficient (18,75%).

Average efficiency of genes of resistance in the first year of investigations was 63,88% and it was something higher than the average efficiency of genes in the season 2000, which was 59,02%. Efficiency of *Pc* genes of resistance to prevalent pathotypes of pathogen in both years of investigations was sufficient and it was 61,45%.

DISCUSSION

Resistance of oats to *P. coronata avenae* is controlled by different genes, which were marked by starting fonts of pathogen (*Pc*). Existence of good sources of resistance, as well as knowledge of efficiency of *Pc* genes, present the basis for oats breeding in the goal of creation of resistant cultivars against this pathogen.

The number of genes, which control resistance of oat to causer of crown rust in some cultivar is great and can vary from one to several tens. Si-

mons et al. (1978) in their catalogue presented the list of 61 *Pc* genes of resistance. According to recent knowledge, today over 90 *Pc* genes of resistance (Chong and Brown, 1996) are famous.

Efficiency of *Pc* genes was the object of many investigations in the world (Šebesta et al., 1987, 1995, 1997, 2003; Manisterski and Wahl, 1995; Chong, 1996; Chong and Aung, 1996; Leonard and Anikster, 1996; Dilkova and Forsberg, 1996), as well as in our country (Kostić, 1964; Stojanović et al., 1995; Staletić et al., 2002). In dependence on virulence of population of pathogen, efficiency of identified genes of resistance was different.

Our results demonstrate that the most efficient in both years of the investigation were genes *Pc 68* and *Pc 39* (100%). Manisterski and Wahl (1995) emphasized that line with *Pc 68* gene was completely resistant in stage of seedlings, which is in agreement with the obtained results. Šebesta et al., (1996) pointed out that genes *Pc 39, Pc 55, Pc 58* and *Pc 68* are very efficient in seedlings stage. Also, significant efficiency of gene *Pc 68* (99%) found Leonard and Anikster (1996) in USA, as well as Bonnett (1996) in Australia. During the period from 1995 to 2001, those genes in Europe demonstrated very high efficiency, *Pc 68* (99%) and *Pc 39* (94%), (Šebesta et al., 2003).

In our study, except of genes *Pc 68* and *Pc 39*, as efficient appeared genes *Pc 38* and *Pc 55* (87,5%) and *Pc 54-1* (81,25%), too. Line with gene *Pc 38*, in former studies (Kostić, 1964; Stojanović et al., 1995) expressed high resistance, which is in agreement with the obtained results, however, in recent years in our population of pathogen appeared pathotypes with alleles of virulence for *Pc 38* gene (Staletić et al., 2002).

The greatest number of genes expressed partial efficiency which was from 37,5% to 75%. Low efficiency expressed genes *Pc 64* and *Pc 67* (18,75%).

The results related to efficiency of genes for resistance are not universal, since they can be different depending on areas and of specific structure of population of pathogen, as well as ecologic factors (Lampert et al., 1990). Observing efficiency of genes per year it can be noticed that some genes (*Pc 38, Pc 39, Pc 54-1* and *Pc 68*) in 1999 and genes (*Pc 39, Pc 48, Pc 59* and *Pc 68*) in 2000 expressed high efficiency, that is, they did not have alleles of virulence in population of pathogen. Yukhnina et al., (1996) emphasized significance of line with gene *Pc 59*, which possesses high resistance to causer of crown rust of oat, but also, excellent agronomic characteristics. In the 2000 year, gene *Pc 54-2* was completely inefficient (0%). In breeding programs for resistance there are being used those genes to which the 90% of pathotypes of pathogen are avirulent (Krivčenko and Sukhanberdina, 1978).

On the basis of the obtained results it could be concluded that the efficiency of *Pc* genes for resistance to prevalent pathotypes of *P. coronata avenae* was middle (61,45%), which points at a need for invention of new donors of *Pc* genes of resistance because of their usage in oats breeding for resistance to this pathogen.

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ЕФИКАСНОСТ *PC* ГЕНА ОТПОРНОСТИ ПРЕМА ПРЕВАЛЕНТНИМ ПАТОТИПОВИМА *PUCCINIA CORONATA AVENAE*

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Резиме

У раду је дат преглед ефикасности неких *Pc* гена отпорности према превалентним патотиповима проузроковача лисне рђе овса. Утврђено је да су у фази сејанаца најефикаснији били гени *Pc 68* и *Pc 39* (100%). Задовољавајући степен ефикасности испољили су и гени *Pc 38* и *Pc 55* и она је износила 87,50%. Већина гена је испољила парцијалну отпорност. Ефикасност *Pc* гена отпорности према превалентним патотиповима патогена у обе године испитивања била је задовољавајућа и износила је 61,45%.

Зборник Матице српске за природне науке издаје Матица српска
Излази двапут годишње
Уредништво и администрација: Нови Сад, Улица Матице српске 1
Телефон: 021/420-199
e-mail: zmspn@maticasrpska.org.yu
www.maticasrpska.org.yu
Proceedings for Natural Sciences published by Matica Srpska
Published twice a year
Editorial and publishing office: Novi Sad, Ul. Matice Srpske 1
21000 Novi Sad, SCG
Phone: +381-21/420-199

The editors of the Matica srpska *Proceedings for Natural Sciences*
Completed the selection for Issue 108/2005 on February 7, 2005
Editorial Staff Secretary: Julkica Boarov and Mirjana Zrnić
Managing editor: Dr. Slavka Gajin
English text proof-reader: Srđan Vraneš and Vera Vasilić
Technical design: Vukica Tucakov
Published in March 2005
Publish by: Mladen Mozetić, GRAFIČAR, Novi Sad
Printed by: „Ideal", Novi Sad

Публиковање овог броја помогли су
Министарство науке и заштите животне средине Републике Србије,
Покрајински секретаријат за науку и технолошки развој,
Научни институт за ратарство и повртарство и
Научни институт за ветеринарство, Нови Сад.
The edition and printing of the Proceedings has been financially supported by the
Ministry of Science and Environmental Protection of Republic of Serbia,
Institute of Field and Vegetable Crops and
Scientific Institute for Veterinary Medicine, Novi Sad.