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MYCOTOXINS IN POULTRY PRODUCTION

ABSTRACT: All poultry is sensitive to mycotoxins. This partly depends on the type, age and production categories of poultry, their living conditions and nutritive status and partly on the type, quantity and duration of mycotoxin ingestion. The presence of mycotoxins results in significant health disorders and a decrease in production performances. This leads to considerable economic loss for the poultry industry — either direct losses, i.e. death of the poultry or the indirect ones, i.e. the decrease in body mass, number and quality of eggs, greater food conversion, and immunosuppression. Immunosuppression results in increased sensitivity to infective agents and a bad vaccinal response. Morevover, mycotoxin residues in poultry meat, eggs and products derived from them pose a threat to human health.

In order to prevent and reduce the negative implications of mycotoxins in the poultry production, it is necessary to create both global and national strategies for combatting mycotoxins, advance diagnostic techniques and procedures, intensify the control of food quality, introduce new limits on the maximum amount of mycotoxins allowed in food and poultry feed used for certain species and categories of animals, and synchronise it with the European standards.

KEY WORDS: immune response, mycotoxins, mycotoxicoses, poultry, production results

INTRODUCTION

Safe food is an imperative in food production worldwide. Poultry meat, eggs, and products derived from them are crucial in the safe food chain. As far as safety is concerned, special attention is directed towards possible contamination of food and poultry feed with fungi and to the risk of mycotoxin contamination.

There are numerous mycotoxins in the food chain that cause unwanted biological effects inside human and animal organisms upon ingestion (B r y d e n, 2007). High level of mycotoxins in food and feed results in the appearance of acute mycotoxicoses and high mortality rate. Lower levels cause the occurrence of chronic mycotoxicoses with or without manifested clinical symp-

toms, but followed by a considerable decrease in production performance, immunosuppressive effects, and the occurrence of residues in poultry meat and eggs (S i n o v e c, 2006).

Due to the fact that mycotoxins have largely distinct structures, metabolisms, and consequently the level of toxicity, it is impossible to formulate a general attitude towards both the changes they cause in the organism and their influence on the poultry production parameters. This is why mycotoxins (ochratoxin, trichothecenes, aflatoxin, etc.) which are most frequently detected as food and poultry feed contaminants, will be discussed separately. Toxicity of mycotoxins primarily depends on the species of mycotoxins, quantity and duration of ingestion, type, sex and age of the animal, general health and immune status, as well as environmental factors (zoohygenic and zootechnological normatives) and nutritive status (B i n d e r, 2007). Since fungi frequently produce more than one mycotoxin, the animal simultaneously takes in more mycotoxins through ingestion. So far, any discussions about the interaction between two or more mycotoxins inside an organism, have most frequently been related to either their negative effects, or the way they can cause some other effects that have neither been fully explained, nor confirmed yet.

OCHRATOXIN

Ochratoxin is a relatively frequent cause of decrease in production results in poultry industry, whereas the degree of decrease depends on dosage and duration.

After resorption, the largest quantity of ochratoxin can be found inside kidneys and liver, and to a considerably smaller extent in the musculature. Inside the liver, ochratoxin A is hydrolysed to OTα and L-phenylalanine, and only an insignificant part of it to dp 4-hydroxy-OTA, whereby the newly formed metabolites are less toxic.

It is characteristic of poultry to possess a capacity for more efficient and faster excretion of this toxin than other animals, thus whithin 48 hours aproximately 90% of the ingested OTA is secreted and the semi-life of ochratoxin A in the serum is approximately 4 hours (G a l t i e r et al., 1981). It is considered that the toxic effect of ochratoxin A is based on numerous direct and several indirect effects. Primary effects are most probably connected with the influence of OTA on enzymes participating in the phenylalanine metabolism (phenylalanine-transferase, phenylalanine-hydroxylase, phenylalanine-lipoperoxide) and the functions of mitochondries.

Secondary mechanism of the toxic effect is based on increased lipid peroxidation in liver and kydney microsomes (F u c h s, 1988). Ochratoxin A stimulates NADPH-dependent microsomal and ascorbate-dependent lipid peroxidation with iron as an essential cofactor, i.e. it stimulates lipid peroxidation by complexing with iron and facilitates its reduction in this manner. The formed complex of OTA and iron produces highly toxic hydroxyl radicals in the presence of NADPH cytochrome of the P-450 reductase system.

The third mechanism of OTA's toxic effect is based on the inhibition of respiration in mitochondries (U r a g u c h i and Y a m a z a k i, 1978), where it acts as a competitive inhibitor of the carrier's proteins, localised on the inner membrane of mitochondries. Furthermore, it is considered that OTA represents a potent teratogenic agent for chickens, but not for other domestic animals (S i n g h and H o o d, 1985).

Ochratoxin A also manifests immunomodulator effects (M u l l e r at al., 1999; D w i v e d i and B u r n s, 1984). Reduction in the number of lymphoid cells was observed after OTA ingestion, especially in the thymus, bursa Fabricii and spleen of poultry. This indicates a potential suppression of cell-mediated immunity. The inhibitive effects are especially prominent in the number of T and B lymphocites, which confirms that OTA possesses immunosuppressive characteristics. Reduction in serum immunoglobulin and fagocyte capacity of leucocytes and neutrophyl (D w i v e d i and B u r n s, 1984) also occur, resulting, naturally, in reduced resistance to viral, bacterial, and parasitic infections.

The unspecific clinical image of chronic ochratoxicosis in poultry is followed by a decrease in egg production of laying hens and parent flocks, whereas, as far as broilers are concerned, their growth is hindered and conversion of food is weakened. The egg shell often becomes thin and fragile, with different discoloration appearing on the surface.

Growth inhibition is connected with malabsorption syndrome, as confirmed by the presence of hypocarotenoidemia. The minimum amount of ochratoxin leading to reduced growth also causes reduced bone firmness and poor pigmentation, whilst for the reduced bone diametar larger quantities of ochratoxin are necessary (D u f f at al., 1987).

Nephropathies need not be clinically manifested, although polydepsia accompanied by a substantial amount of moist excrement most frequently appears. Changes inside the kidneys are followed by the occurrence of glomerulonephritis with increased glomerules and dilated capillaries, with a decrease in the relative mass of kidneys. A decrease in the concentration of proteins, triglycerides, cholesterol, calcium, neorphanic phosphorus and potassium is followed by an increase in the level of uric acid and creatinine and a decrease in glomerular filtration (N e d e l j k o v i ć at al., 1999).

Turkey is somewhat more resistant to ochratoxin effects, but dosage and time-dependent effects can also be detected in them.

TRICHOTHECENES DEOXYNIVALENOL (DON), T-2 TOXINS, DIACETOXYSCIPRENOL (DAS) AND HT-2 TOXIN

Trichothecene poisoning in poultry manifests acutely or chronically. The acute form, unlike other mycotoxins, has a characteristic clinical picture and is easily diagnosed. However, the chronic form manifests unspecific clinical symptoms.

Upon peroral ingestion, T-2 toxin is very rapidly resorbed in the lower parts of the digestive tract (before jejunum), and only one hour after the inge-

stion it reaches maximum concentration in the blood (U r a g u c h i and Y a m a z a k i, 1978).

After this phase, a slower ensues one during which T-2 toxin and the formed metabolites are distributed to certain tissues. In comparison with trichothecenes, T-2 toxin is resorbed more rapidly. Resorption is performed by means of active transport, during which T-2 toxin is rapidly transported to ribosomes through the cell membrane of enterocytes.

After 3-4 hours, the biggest portion of T-2 toxin and its metabolites can be found in the majority of organs, whereas it takes then approximately 12 hours to reach the muscles, skin, and gall. After 24 hours, the largest portion of T-2 toxin is inside the excretion organs — the gall-bladder (gall), liver, kidneys, and intestines. In the liver, toxin T-2 is rapidly transformed into different metabolites (B a u e r, 1995), less toxic than the mother compound, and it is eliminated from the organism without accumulation.

The mechanism of T-2 toxin's agency has not yet been sufficiently explained. To become biolologically active, T-2 toxin does not demand biotransformation upon entering the organism. Primarily, T-2 toxin inhibits DNA replication (U e n o, 1983), whilst the degree of inhibition depends on the amount of mycotoxin and sensitivity of the species.

DNA synthesis is secondarily inhibited by disorders of protein synthesis, but certain disorders in cellular organisation can, under the influence of T-2 toxin, affect the synthesis of nucleic acids. Changes caused by T-2 toxin in DNA are of reversible nature. Nevertheless, it is considered that T-2 toxin, when its action is prolonged, can induce mutagenic, teratogenic, and carcinogenic effects (K r i v o b o k et al., 1987).

The inhibition of protein synthesis is observed in various tissues and it is characterised by either polysomic alterations and desegregation, or the induction of structural changes in chromosomes. Mycotoxins can cause both of these effects either simultaneously or successively. Numerous trichothecenes inhibit synthesis of proteins by blocking the elongation of the polypeptide chain, at the position of peptidyl-transferase on the 60S ribosomal subunit.

Accute intoxication of poultry with trichothecenes occurs as digestive and nervous system disorders, with hyperpnea accompanied by lethargy and loss of balance. Basic changes can be manifested as haemorrhages in the digestive tract and muscles. Local epithelial-necrotic effects in the form of stomatite, necrosis, and ulceration inside the mouth are very prominent (S i n o v e c et al., 2006).

One of the primary effects of T-2 toxin in poultry are weakened immunity and resistence of the organism. T-2 toxicosis is characterised by the decay of lymphoid cells inside the thymus, spleen, and bone marrow, which inhibits the cellular immune response. T-2 toxin has a distinctly negative effect on the immune system, which manifests itself as reduced resistence of poultry to infective deseases, especially to salmonelosis and E. coli infections (B o o n c h u v i t et al., 1975). The toxic effect also manifests itself as reduced proliferation of limphocites stimulated by phytohaemagglutin and lipopolysaccharide (R a f a i et al., 2000). Furthermore, T-2 toxin hinders the protective func-

tion of intestinal mucosis, which enables the penetration of bacteria and occurrence of secondary infections.

AFLATOXIN

Aflatoxin is the most studied mycotoxin, due to both its toxicity to animals and people and its rather high carcinogenic potential. Poultry is considerably resistant to aflatoxin, due to which the acute intoxication is relatively rare. Chronic intoxication with aflatoxin demands ingestion of aflatoxin for several weeks (one week minimum). The clinical picture is the consequence of the mechanism of aflatoxin effects in the organisms of poultry.

The toxic effect of aflatoxin manifests itself on the level of interaction with genetic material. The aflatoxin molecule penetrates the cell and the nucleus, subsequently placing itself between the base pairs of DNA. The inserted aflatoxin molecules decelerate to a great extent the process of DNA information transfer. Mistakes in DNA transcription are very frequent (R e s a n o v i ć, 2002), which result in the inhibition of protein synthesis, i.e. "wrong" proteins are synthesised. The immunosuppressive effect of aflatoxin has been proved, although the mechanism has not yet been fully explained. Negative effects of aflatoxin on complement, interferon, and serum proteins are probably the result of liver damages and the inhibition of protein synthesis. Aflatoxin performs suppression of nonspecific substances (complement and interferon) in charge of humoral immunity, as well as the suppression of fagocites through macrofagus.

Apart from this, aflatoxin also causes aplasia of the thymus, spleen, and bursa Fabricii in chicken, whereas larger quantities (0,6—10,0 ppm) cause the suppression of class G and A immunoglobulins during immunisation (K a r a m a n et al., 2005). It is interesting that doses of aflatoxin which do not affect the level of antibodies after vaccination have a strong effect on the cell-mediated immune response, which is manifested as a decrease in the total number of lymphocites and T effector cells, as well as a decrease in fagocite activity of monocytes (G h o s h et al., 1991).

Acute aflatoxin poisoning leads to impaired coordination of movement, vertigo, and paresis, followed by diarrhea with admixtures of blood, haemorrhages, tumescences, jaundice, coma, and death.

The clinical picture of chronic aflatoxicosis in poultry is dominated by a considerable decrease in body mass, reduced food consumption, bad conversion, decrease in egg-laying ability, reduced percentage of hatching, as well as disbalanced immunogenosis and exitus. Disruptions in blood coagulation occur and the prothrombic period is considerably elongated. Due to this occurrence, heamorrhages appear on the musculature, which in turn decreases the usability of such torsos. Poultry exposed to aflatoxins is pallid (its cockscombs, wattles, and legs), as a consequence of poor pigmentation, which is the result of reduced ingestion, resorption, and transport of carotenoid due to the presence of aflatoxin.

The disorder in metabolism of group B vitamins and amino acids manifests itself as a decrease of their concentration in the plasma, liver, and gall. In addition to this, aflatoxin affects bone mineralization by impairing the resorption of calcium and phosphorus inside kidneys, which leads to the metabolic vitamin D3 deficiency.

Steatorrhea is one of the cruicial symptoms of aflatoxicosis, caused by decreased concetration of gall, which leads to an increase in unabsorbed lipid content in the cecum. Thus in the case of chronic aflatoxicosis of poultry, food conversion is significantly increased.

ZEARALENON

Poultry is very resistent to zearalenon. Of all species of poultry, turkey is the most sensitive to the effects of zearalenon, which can cause a decrease in their egg-laying ability even up to 20% (Allen et al., 1983). The immunosuppressive effects of zearalenon in poultry have not been proved so far.

CYCLOPIAZONIC ACID

Cyclopiazonic acid is not a common contaminator of food and poultry feed. However, when detected, cyclopiazonic acid can, depending on the quantity and duration of ingestion, cause a very dramatic clinical picture of the central nervous system disorder — manifesting itself as ataxia, paresis, paralysis, and opisthotonus. Prominent cumulative toxicity of cyclopiazonic acid can be observed.

A decrease in the weight of bursa Fabricii followed by an incerease in the weight of liver, kidneys, and forestomach can also be detected. The decrease in the weight of bursa Fabricii leads to a weakened immune response after vaccination.

FINAL OBSERVATIONS

Mycotoxins have a very strong influence both on animal and human health. Total losses for the poultry industry caused by the presence of mycotoxins in food and the changes they cause on living material are very difficult to integrate and estimate. Numerous strategies for the prevention of harmful effects of mycotoxins in poultry farming have been applied, but none of these gave sufficiently good results.

In order to prevent and reduce the negative implications of mycotoxins in the poultry production, it is necessary to create both global and national strategies for combatting mycotoxins, advance diagnostic techniques and procedures, intensify the control of food quality, and introduce new limits on the maximum amount of mycotoxins allowed in food and poultry feed.

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МИКОТОКСИНИ У ЖИВИНАРСКОЈ ПРОИЗВОДЊИ

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Резиме

Сва живина је осетљива на микотоксине у зависности од врсте, старосне и производне категорије, услова амбијента и нутритивног статуса, са једне стране, и врсте, количине и дужине уношења микотоксина, са друге стране. Присуство микотоксина резултира значајним поремећајем здравља и падом производних перформанси, а самим тим и значајним економским губицима у живинарској индустрији, како директним, који се очитују угинућем живине, тако и индиректним, у виду пада телесне масе, броја и квалитета јаја, веће конверзије хране и имуносупресијом. Имуносупресија резултира повећаном осетљивошћу на инфективне агенсе и лошим вакциналним одговором. Опасност по људско здравље представљају резидуе микотоксина у живинском месу, јајима и производима добијеним од њих.

Да би се предупредиле и смањиле негативне импликације микотоксина у живинарској производњи потребно је формирати како глобалне тако и националне стратегије за борбу против микотоксина, унапредити дијагностичке технике и процедуре, пооштрити контролу квалитета хране, увести нове лимите за максималне количине микотоксина у храни и хранивима за поједине животињске врсте и категорије и ускладити их са европским стандардима.

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DYNAMICS OF DEOXYNIVALENOL AND ZEARALENONE PRODUCTION BY *FUSARIUM GRAMINEARUM* UNDER LABORATORY CONDITIONS

ABSTRACT: Toxicological investigations encompassed two cultures of *Fusarium graminearum*: (i) D2 isolate, originating from air was obtained on Sabouraud medium during a routine control of laboratory sterility conditions at the Department of Microbiology of the Center for Bio-Ecology in 2006, and (ii) GZ-LES control isolate, a well known producer of zearalenone (ZON) and deoxynivalenol (DON), was isolated from maize kernel collected at Leskovac in 1975. Preliminary analysis of fungal potential for the production of DON and ZON were performed by the modified rapid screening method of Filtenborg et al. (1983). Dynamics of DON and ZON biosynthesis was tested under different conditions of isolate cultivation: (i) in a basic liquid semi-synthetic medium with 2% yeast extract and 15% sucrose, pH 6.5 (YES), (ii) in broth with same concentrations of yeast extract and sucrose supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O, pH 6.5 (YES^{Zn}) and (iii) on natural solid substrates such as wet sterilized maize and rice kernels. The quantitative determination of DON and ZON was performed in both liquid and natural solid substrates with thin-layer chromatographic methods (TLCs).

The maximum yield of DON was recorded after three weeks of cultivation on maize kernels at 27±1°C. Contrary to the D2 isolate, which did not show the potential for the DON biosynthesis, the control isolate GZ-LES produced 645.6 ppb of the same type B trichothecene under previously mentioned conditions. The ZON biosynthesis by the isolate D2 (1.2 ppb) was observed after 2 weeks of the stationary cultivation in YES and YES^{Zn} at room temperature (17—19°C). The same isolate produced 0.74 ppb and 17.35 ppb ZON on maize and rice kernels after only 7 and 28 days of cultivation at the room temperature ranging from 17 to 19°C and from 15 to 23°C, respectively.

KEY WORDS: *Fusarium graminearum*, DON, ZON

INTRODUCTION

Fusarium graminearum, beside *F. culmorum*, is the main causal organism of fusarium head blight (FHB) or scrib, a disease that leads to severe losses of

yield and quality of cereal grain. During the development of FHB these pathogens commonly contaminate grain with mycotoxins deoxynivalenol (DON) and zearalenone (ZON). These mycotoxins that can be the cause of health--threatening toxicoses (M a r a s a s et al., 1984), represent at least two biochemical origins. ZON with the estrogenic activity in mammals is biosynthesized through a polycetidic pathway, while 8-ketotrichothecenes DON and nivalenol (NIV) are derived from the condensation of three mevalonate units (B l a c k - w e l l et al., 1985).

F. graminearum isolates can be broadly divided into two chemotypes based on the production of host specific virulence factors DON and NIV (I l - g e n et al., 2008). According to K i m et al. (2003) small cereal grains grown in Korea produced either DON or NIV, whereas isolates from corn grown in the United States produced DON only. In England and Wales (J e n n i n g s et al., 2004) DON is predominant chemotype (75% of tested *F. graminearum* cultures), as well as, in Argentina where only chemotype IA (DON and 3-acetyl-DON) was observed (M o l t o et al., 1997).

There are only a few reports about simultaneous presence of DON and ZON in Serbian crops (J a j i ć et al., 2007; B a g i et al., 2008). In order to determine the occurrence of DON and ZON, this study was carried out with *F. graminearum* isolates cultivated on different substrates under laboratory conditions.

MATERIAL AND METHODS

Microorganisms. Two isolates of *F. graminearum* were under present investigation. Isolate D2, originating from air, was obtained on Sabouraud medium during a routine control of laboratory sterility conditions at the Department of Microbiology of the Center for Bio-Ecology in 2006, and control isolate GZ-LES, a well known producer of DON and ZON (J a j i ć et al., 2007), was isolated from maize kernel collected at Leskovac in 1975. Stock cultures of the fungi were maintained on the potato-sucrose agar at 4—6°C.

Preliminary analysis of fungal potential to produce deoxynivalenol (DON) and ZONralenone (ZON) were performed according to the rapid screening method of F i l t e n b o r g et al. (1983) modified by B o č a r o v - S t a n č i ć et al. (in press) on the following media: YESA (2% yeast extract, 15% sucrose and 2% agar, pH 6.5), $YESA^{Zn}$ (2% yeast extract, 15% sucrose, 0.23 mg/l $ZnSO_4$ x 5 H_2O, and 2% agar, pH 6.5), PPSA (2% peptone-1, 5% sucrose and 2% agar, pH 6.5), $PPSA^{Zn}$ (2% peptone-1, 5% sucrose, 0.23 mg/l $ZnSO_4$ x 5 H_2O and 2% agar, pH 6.5) and PDA (potato-dextrose agar, pH 6.9).

Liquid media and conditions for the toxin production. The *F. graminearum* isolate D2 and the control isolate GZ-LES were grown in the yeast extract-sucrose broth (YES, pH 6.5) (S a m s o n and v a n R e e n e n - H o - e k s t r a, 1988) as well as in yeast extract-sucrose medium supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O (YES^{Zn}, pH 6.5) (M ü h l e n c o e r t, 2004). Both media (YES and YES^{Zn}) contained 2% yeast extract and 15% sucrose. Media (250 mL each) were poured into 500 mL Erlenmeyer flasks and were inoculated with five fragments (5 x 5 mm) of the fungus, that were grown on po-

tato-sucrose agar (PSA) in Petri dishes at 27±1°C for seven days. After inoculation of the media, the Erlenmeyer flasks were kept stationary at the room temperature (17—19°C) for 14 days. The pH value was measured after cultivation of the isolate. The samples for the analysis were taken every week during the cultivation period. All cultivations were performed in two replications.

Solid media and conditions for the toxin production. Both isolates of *F. graminearum* were grown on wet (>45%) sterilized maize and rice kernels (50 g per Roux bottle). Each substrate was inoculated with five fragments (5 x 5 mm) of the fungus that was grown on the potato-sucrose agar (PSA) in Petri dishes at 27±1°C for seven days. Inoculated maize samples were cultivated during a 4-week period under room (17—19°C) and chamber (27±1°C) conditions. On other hand, during 14, 21 and 28 days the inoculated rice samples were cultivated under room (15—23°C) and chamber conditions (27±1°C). All cultivations were performed in two replications.

Determination of fusariotoxins. Qualitative and quantitative DON determinations in filtrates of fungal cultures were carried out by applying the thin-layer chromatographic (TLC) method developed by Cvetnic et al. (2005), and ZON after Bočarov-Stančić et al. (2003). In accordance with these methods, liquid cultures of fungus were filtered after a stationary cultivation. Furthermore, after the cultivation on maize and rice kernels, the samples were dried for 24 h or more at 60°C, until constant weight was achieved. After a pulverization of dried samples, the DON determination was done according to the TLC method of Pepeljnjak and Babić (1991), and ZON after Balzer et al. (1978). Thin-layer chromatography was performed with toluene/ethyl acetate/formic acid developing saturated solvent (5:4:1, v/v/v). Only in case of the ZON, determination in benzene/glacial acetic acid developing solvent (90+10 v/v) was used parallely. After developing plates and air drying in the dark fume extractor the plates were examined under long wave UV light (366 nm). DON and ZON were visualized by the use of 20% $AlCl_3$ in 60% ethanol. All analyses were done in three replications.

RESULTS AND DISCUSSION

Results of the present investigation are shown in Tables 1 — 4.

Tab. 1. Fusariotoxins production by *F. graminearum* isolates on different agar media and temperatures

Medium	Aver. temp. (°C)	Days	Intensity of fusariotoxins production			
			D2 isolate		GZ-LES control islate	
			DON	ZEN	DON	ZEN
YESA	19.0	7	–	+	–	++
		14	–	+	–	++
		21/28	–/–	+++/+++	–/–	+++/+++
	24.9	7	–	+	–	+
		14	–	+	–	++/+++
		21/28	–/–	++/+++	+/+	+++/+++

YESA^{Zn}	19.0	7	–	–	–	+
		14	–	+	–	+++
		21/28	–/–	+++/+++	–/–	+++/++++
	24.9	7	–	+	–	–
		14	–	+++	–	+
		21/28	n.a.	n.a.	+/+	+/+
PPSA	19.0	7	–	–	–	–
		14	–	+	–	–
		21/28	–/–	++/+++	–/–	+++/+++
	24.9	7	–	–	–	–
		14	–	+	–	–
		21/28	–	+++/++++	+/+	++/++
PPSA^{Zn}	19.0	7	–	–	–	–
		14	–	++	–	–
		21/28	–/–	++/++++	–/+	+++/++++
	24.9	7	–	–	+	–
		14	–	++	+	–
		21/28	n.a.	n.a.	+/+	–/–
PDA	19.0	7	–	–	–	–
		14	–	–	–	–
		21/28	–/–	–/–	–/–	–/–
	24.9	7	–	–	–	–
		14	–	–	–	–
		21/28	–/–	–/–	–/+	–/–

Legend: n.a. — not analyzed; – not detected; + low intensity; ++ moderate intensity; +++ high intensity; ++++ very high intensity

Screening of the fusariotoxin production under different conditions of fungal cultivation *in vitro* (Table 1) revealed that only *F. graminearum* GZ-LES had the capability to produce DON. The biosynthesis of this type B trichothecene was observed in almost all cases at higher temperature (average 24.9°C). The exception was cultivation of this isolate on PPSA^{Zn} when DON was recorded after prolonged cultivation (28 days) at lower temperature (average 19.0°C). Dynamics of the DON production was as follows: early detection (after 7 days) on PPSA^{Zn}, after 21 days on PPSA, YESA, and YESA^{Zn}, and after 4 weeks on PDA.

ZON was biosynthesized on agar media by both tested isolates of *F. graminearum* isolates (Table 1) but under different cultivation conditions. PDA was not applicable for testing the ZON production. ZON was observed on PPSA^{Zn} and PPSA after 2 weeks of D2 isolate cultivation, regardless of the applied temperature, while in the case of GZ-LES isolate after 3 weeks cultivation at a lower temperature (average 19.0°C). After 7 days, ZON was biosynthesized on YESA^{Zn} by the D2 isolate at higher temperatures (average 24.9°C), and by GZ-LES at lower temperatures (average 19.0°C), respectively. The best results were achieved on YESA where both isolates of *F. graminearum* produced ZON after the 7-day cultivation regardless of the temperature conditions. A prolonged cultivation of fungi resulted in higher ZON yields.

Tab. 2 — Quantity of fusariotoxins produced by D2 isolate of *F. graminearum* stationary cultivated in liquid media

Temperature (°C)	Days	Medium	pH value	Toxin yield (mg/l)	
				DON	ZON
17—19	7	YES	—	n.d	n.d.
		YES^{Zn}	—	n.d	n.d.
	14	YES	4.97	n.d	1.20
		YES^{Zn}	4.73	n.d	1.50
	21	YES	4.90	n.d	1.50
		YES^{Zn}	4.50	n.d	1.50
	28	YES	5.20	n.d	0.90
		YES^{Zn}	4.60	n.d	1.20

Legend: n.d. — not detected (< 0.037 and < 0.097 mg/l of ZON and DON, respectively)

After the incubation period, a decrease of pH value was determined in both tested liquid media (Table 2).

Investigations of the DON and ZON production in liquid media (YES and YES^{Zn}) revealed that the control isolate GZ-LES *F. graminearum* did not biosynthesize any of the tested fusariotoxins after 28 days of cultivation at room temperature (15—23°C). The explanation for this finding is that the applied temperature was maybe too high for the ZON production, but, on the other hand, too low for the DON biosynthesis under stationary cultivation conditions.

In the case of D2 isolate, it was not surprising that it did not produce DON (Table 2), because it also was not a producer of the same fusariotoxin on agar media of the same composition (Table 1). The ZON biosynthesis by the same culture was recorded after 14 days of cultivation in both tested liquid media (YES and YES^{Zn}) at lower room temperatures (17—19°C). The maximal yield of ZON (1.5 mg/l) was observed after 21 day cultivation in both applied liquid media (Table 2). The supplementation of the trace element Zn to YES (0.23 mg/l $ZnSO_4$ x 5 H_2O) resulted in a more outstanding decrease of the initial pH 6.5 to the final pH 4.6, as well as, in higher ZON yields after 14 (1.50 mg/l in comparison to 1.20 mg/l) and 28 days of cultivation (1.20 mg/l in comparison to 0.90 mg/l).

The use of different liquid media for testing the toxin production by isolates of *F. graminearum* is reported also by other authors (Miller and Greenhalgh, 1985; Pestka et al., 1985). Pestka et al. (1985) obtained 5.50 mg/l of DON after 20 days by the use of the glucose-yeast extract-peptone nutrient medium for cultivation of the strain R6576.

The results of fusariotoxin yields and dynamics of biosynthesis by isolates of *F. graminearum* cultivated on natural solid substrates (Table 3. and 4) show significant differences regarding the temperature conditions and the type of substrate.

Tab. 3. Quantity of fusariotoxins production by two isolates of *F. graminearum* cultivated on maize kernels

Temperat. (°C)	Days	Moisture (%)	Toxin yield (mg/kg)			
			D2 isolate		GZ-LES	
			DON	ZON	DON	ZON
17—19	7	46.2	n.d.	0.74	0.58	n.d.
	14	48.0	n.d.	0.94	1.44	0.37
	21	50.0	n.d.	1.29	n.d.	0.37
	28	46.8	n.d.	1.84	n.d.	0.37
27±1	7	43.0	n.d.	n.a.	1.44	n.d.
	14	50.6	n.a.	n.a.	1.44	n.d.
	21	48.4	n.a.	n.a.	1.73	n.d.
	28	49.8	n.a.	n.a.	2.02	n.d.

Legend: n.d. — not detected (d55of ZON and DON, respectively); n.a. — not analyzed

Tab. 4. Quantity of fusariotoxins production by two isolates of *F. graminearum* cultivated on rice kernels

Temperat. (°C)	Days	Moisture (%)	Toxin yield (mg/kg)			
			D2 isolate		GZ-LES	
			DON	ZON	DON	ZON
15—23	28	42.3	n.d.	17.35	n.a.	n.a.
27±1	14	37.9	n.a.	n.a.	302.5	n.d.
	21	—	n.a.	n.a.	645.6	1.26
	28	—	n.a.	n.a.	400.0	3.13

Legend: n.d. — not detected (dof ZON and DON, respectively); n.a. — not analyzed

DON production was observed only in the GZ-LES isolate, regardless of the applied temperature and on both types of cereal substrate (Tables 3. and 4). Much higher quantity of this type B trichothecene was detected after 28 days of cultivation on rice grain at 27±1°C (400.0 mg/kg) than on maize kernels (2.02 mg/kg). Biosynthesis of the maximum DON quantities on maize kernels was detected after cultivation for two weeks at room temperature (17—19°C), and four weeks at 27±1°C, 1.44 mg/kg and 2.02 mg/kg, respectively (Table 3). On rice kernels maximal yield of DON (645.6 mg/kg) was achieved after three weeks of cultivation at 27±1°C (Table 4). Other authors also established that a higher incubation temperature (28°C) favored the DON production (Greenhalgh et al., 1983; Liorens et al., 2004).

After 28 days of cultivation at room temperature the D2 isolate of *F. graminearum* produced more ZON on rice grain, then on corn kernels (Tables 3. and 4). During the cultivation on maize kernels, the yield of this estrogenic substance succesively increased from initial 0.74 mg/kg (after 7 days) to final 1.84 mg/kg (after 28 days) (Table 3).

In contrast to the D2 isolate the GZ-LES isolate of *F. graminearum* biosynthesis of ZON was detected after longer cultivation (14 days) on maize kernels at room temperature (17—19°C) and its yield did not change during

further cultivation; it was constantly 0.37 mg/kg. During cultivation at higher temperature (27±1°C), isolate GZ-LES did not produce ZON at all on the same substrate (Table 3), while on rice grains the biosynthesis of the same mycotoxin (1.26 mg/kg) was detected after three weeks (Table 4). The ZON yield increased during cultivation period at 27±1°C (Table 4). The highest quantities of ZON that can be obtained at lower temperatures, such as 19.5°C or room temperature ranging from 17 to 21°C, are indicated by other authors too (Greenhalgh et al., 1983; Lori et. al., 1990).

CONCLUSIONS

The temperature and nutrient media affected significantly the mycotoxin production, although the tested isolates (D2 and GZ-LES) responded differently to the same cultivation conditions.

The best medium for screening ZON, regardless of the temperature conditions, was YESA, and for DON screening it was $PPSA^{Zn}$ and higher temperatures (about 25°C).

In liquid media (YES and YES^{Zn}) at room temperature (17—19°C) only the ZON biosynthesis occurred by *F. graminearum* D2 isolate.

Higher incubation temperatures (27±1°C) favored the DON production on wet cereal kernels, while lower temperatures (17 to 21°C) favored the ZON biosynthesis.

The isolate *F. graminearum* GZ-LES evidently belongs to the DON chemotype contrary to D2, because the last one did not possess the capability to biosynthesize DON.

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ДИНАМИКА ПРОИЗВОДЊЕ ДЕОКСИНИВАЛЕНОЛА И ЗЕАРАЛЕНОНА КОД ИЗОЛАТА *FUSARIUM GRAMINEARUM* У ЛАБОРАТОРИЈСКИМ УСЛОВИМА

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Резиме

Испитивањем су били обухваћени новоизолована култура *F. graminearum* (D2) неиспитаног токсиколошког профила и контролни изолат исте врсте гљиве (GZ-LES) добро познат произвођач деоксиниваленола (DON) и зеараленона (ZON). Прелиминарне анализе DON-а и ZON-а су извршене према модификованој методи Filtenborg-а и сар. (1983). Динамика биосинтезе DON-а и ZON-а је праћена гајењем изолата гљива у/на четири различита типа подлоге: течној полусинтетичкој подлози са 2% екстракта квасца и 15% сахарозе (YES pH 6,5), подлози истог састава са додатком 0,23 mg/l $ZnSO_4$ x 5 H_2O (YES^{Zn} pH 6,5) и стерилисаним влажним зрнима кукуруза и пиринча.

Квантитативно одређивање DON-а у култури изолата гљива гајених у течној подлози је извршено танкослојном хроматографијом према Цветнићу и сар. (2005), а у чврстој подлози применом поступка аутора Пепељњака и Бабића (1991). Потенцијал за биосинтезу ZON-а код изолата гајених у течној подлози је одређиван поступком танкослојне хроматографије према Бочаров--Станчић и сар. (2003), а изолата гајених на чврстој подлози према Балзеру и сар. (1978).

Максимална концентрација DON-а (645,6 ppb) је детерминисана после три недеље култивације контролног изолата *F. graminearum* GZ-LES на зрну кукуруза и при 27±1°C. За изолат исте врсте гљиве D2 је утврђено да не поседује способност биосинтезе DON-а, с обзиром да није производио овај трихотецен типа Б ни у једном од тестираних услова култивације. Производња ZON-а је констато-

вана код изолата D2 (1,2 ppb, односно 1,5 ppb) после две недеље стационарне култивације у течним подлогама (YES и YES^{Zn}) на собној температури од 17 до 19°C. На природним чврстим супстратима (зрно кукуруза и пиринча) исти изолат је биосинтетисао ZON већ после седам дана култивације на зрну кукуруза и собној температури од 17 до 19°C (0,74 ppb) или после 28 дана култивације на зрну пиринча и собној температури од 15 до 23°C (17,4 ppb).

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INVESTIGATION OF TOXIGENIC POTENTIAL OF FUNGAL SPECIES BY THE USE OF SIMPLE SCREENING METHOD

ABSTRACT: Potential for the biosynthesis of aflatoxin B1 (AFLB1), ochratoxin A (OTA), diacetoxyscirpenol (DAS), T-2 toxin (T2), and zearalenone (ZON) was investigated in different fungal species belonging to the genera: *Aspergillus*, *Fusarium* and *Penicillium*. The majority of investigated isolates originated from cereal grains, crushed oil soybean seed and fodder mixtures. The simple screening method developed by Filtenborg et al. (1983) was applied with few modifications concerning the type of the medium and cultivation temperature. In order to optimise the biosynthetic conditions for different mycotoxins, the following control cultures, known as mycotin producers were used: OTA — *A. ochraceus* CBS 108.08, DAS — *F. semitectum* (SL-B i SL-C), T2 — *F. sporotrichioides* (ITM-391, M-1-1, R-2301) and ZON — *F. graminearum* (GZ-LES). The fungi were cultivated on the standard medium (YESA — 2% yeast extract, 15% sucrose and 2% agar, pH 6.5), three modifications of the basic medium ($YESA^{Zn}$ — the standard medium supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O; PPSA — the medium in which yeast extract was replaced with peptone-1; $PPSA^{Zn}$ — the medium in which yeast extract was replaced with peptone-1 and supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O), and the potato-dextrose agar (PDA).

The earlier biosynthesis of tested mycotoxins was recorded under the following cultivation conditions of fungal species: AFLB1 — after 14 days on PDA at 27±1°C, OTA — after 10 days on YESA and $YESA^{Zn}$ at 27±1°C, DAS — after 10 days on PPSA and $PPSA^{Zn}$ at 27±1°C, T2 — after 7 days on $PPSA^{Zn}$ and PPSA at room temperature (20—24°C), and ZON — after 1 week on YESA and $YESA^{Zn}$ at room temperature (21—24°C).

KEY WORDS: *Aspergillus*, *Fusarium, Penicillium*, screening, mycotoxins

INTRODUCTION

It is strongly recommended by many authors (Samson and van Reenen-Hoekstra, 1988) that dominant mycobiota of important food and feed in every country should be identified. Correct identification of dominant species can indicate which mycotoxin can be expected in a given food or feed item under given environmental conditions. In order to get the accurate data about mycotoxin profile of determined species, the simple screening method for toxigenic fungi was developed by few authors including Filtenborg et al. (1983).

In order to determine toxigenic profiles of fungal isolates from feed components and fodder mixtures in Serbia the present investigation was carried out. Considering the climatic conditions in our country, the first modification of the screening method of Filtenborg et al. (1983) was done in accordance with lower temperature for testing fusariotoxin production. Another modification of the same method was the addition of the trace element Zn in order to investigate its impact on the mycotoxin biosynthesis under tested laboratory conditions, and the third one was the replacement of yeast extract with peptone-1 in nutrient medium for screening fungal toxicity.

MATERIAL AND METHODS

Test microorganisms. Potential for the biosynthesis of aflatoxin B1 (AFLB1), ochratoxin A (OTA), diacetoxyscirpenol (DAS), T-2 toxin (T2), deoxynivalenol (DON) and zearalenone (ZON) was investigated in different fungal species belonging to the genera: *Aspergillus* (11), *Fusarium* (7) and *Penicillium* (3). Most investigated isolates, determined according to Samson and van Reenen-Hoekstra (1988), originated from cereal grains and fodder mixtures and were obtained during routine mycrobiological analyses in the Center for Bio-Ecology in 2008. Stock cultures of the fungi were maintained on the potato-dextrose agar at 4—6°C.

Control microorganisms:

— *Aspergillus ochraceus* **CBS 108.108**, the strain that produces OTA;

— *F. graminearum*, the isolate **GZ-LES** obtained in 1975 from corn kernels in Leskovac, that synthesises ZON and DON (Jajić et al., 2007);

— *Fusarium sporotrichioides* isolates, known as T2 toxin producers (Bočarov-Stančić et al., 2007): **ITM-391**, leg. dr A. Bottalico, Consiglio Nazionale delle Richerce, Istituto Tosssine e Micotossine da Parassiti Vegetali, Bari, Italy; **M-1-1** from soybeans, leg. dr Y. Ueno, Faculty of Pharmaceutical Sciences, Tokyo, Japan and **R-2301**, leg. dr D. Latus, Germany;

— *Fusarium semitectum* cultures **SL-B** and **SL-C** that biosynthesise DAS, isolated from unfaded alfalfa in 2004 (Bočarov-Stančić et al., 2005).

Stock cultures of the fungi were maintained on the potato-dextrose agar at 4—6°C.

Media and conditions for screening toxin production. The fungi were cultivated on the standard (Samson and van Reenen-Hoekstra,

1988) yeast extract-sucrose agar (YESA — 2% yeast extract, 15% sucrose and 2% agar, pH 6.5), with three modifications of that basic medium: (i) the yeast extract-sucrose agar supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O ($YESA^{Zn}$, pH 6.5) (Müchlencoert, 2004), (ii) the medium in which the yeast extract was replaced with peptone-1 (PPSA — 2% peptone-1, 5% sucrose and 2% agar, pH 6.5) and (iii) the medium in which yeast extract was replaced with peptone-1 and supplemented with 0.23 mg/l $ZnSO_4$ x 5 H_2O ($PPSA^{Zn}$, pH 6.5), as well as on the potato-dextrose agar (PDA, pH 6.9).

In most cases, the temperature of 27±1°C was used for the fungal cultivation, although in the case of testing *Fusarium* isolates for fusariotoxins production, the cultivations were done parallely at room temperature. The cultivation period ranged from 7 to 14 days, depending on a type of mycotoxin and temperature conditions.

Determination of mycotoxins. Agar plugs were cut out of the colony center with sterile metal borer (diameter 6 mm), removed from the agar plate and placed with a sterile needle or tweezers in sterilized Petri dish with a mycelial side up. Circular plugs were wetted with 10—20 μL of chloroform/methanol (2:1 v/v) and after few seconds rapidly extracted mycelial side was gently applied against the TLC plate (MN Kieselgel G, Machery-Nagel) with sterile tweezers (Filtenborg et al., 1983). After drying the application spot, another one of the same colony was applied nearby together with 5 μl of the working standard of the tested mycotoxin (internal standard). The thin-layer chromatography was performed in saturated tanks with toluene/ethyl acetate/formic acid developing solvent (5:4:1, v/v/v) for all tested mycotoxins. Besides this solvent, only in the case of the ZON determination, benzene/glacial acetic acid developing solvent (90+10 v/v) was used. After developing plates and air drying in dark fume extractor the plates were examined under long wave UV light (366 nm). T2 and DAS were visualised by the use of 20% sulphuric acid in methanol, and ZON with 20% $AlCl_3$ in 60% ethanol. All analyses were done in three replicates.

RESULTS AND DISCUSSION

Results of the present investigation are shown in Tables 1—4.

Aflatoxin B1 (AFLB1). The ability to produce this mycotoxin was tested in 6 *Aspergillus* spp. isolates from crushed oil soybean seed and different types of fodder. Besides 3 *A. flavus* isolates (Table 1), one *A. clavatus* (T0001C/08), one *A. fumigatus* (RST-1/46), and one *A. versicolor* (RST-4/46) were also tested.

Tab. 1 — Production of aflatoxin B1 by *Aspergillus flavus* isolates

Sample designation	Temper. (°C)	Days	Medium				
			$PPSA^{Zn}$	PPSA	PDA	$YESA^{Zn}$	YESA
GD-16/06	27±1	14	—	—	+	—	—
L09661/08	27±1	14	—	—	—	—	—
T0001F/08	27±1	14	—	—	—	—	—

Legend: — no biosyinthesis; + low intensity of biosynthesis

The obtained results show that only one *A. flavus* culture (GD-16/06) biosynthesised AFLB1 and only on PDA medium (Table 1). Other tested *Aspergillus* spp. isolates did not express potential for AFLB1 biosynthesis.

The cultivation time was exceeded to 14 days, because no traces of AFLB1 were observed after 10-day cultivation under the above conditions. In order to confirm the obtained results, another cultivation experiment was carried out with sterilised wet rice grain as a substrate for the toxin production. AFLB1 yield of 14 mg/kg of wet mass actually confirmed our results obtained by the use of modified method of Filtenborg et al. (1983), and showed once again that the optimal conditions for testing the AFLB1 production were: 27±1°C and 14-day cultivation on substrate containing starch.

Ochratoxin A (OTA). Screening of the OTA biosynthesis was done with 5 *Aspergillus* spp. and 3 *Penicillium* spp. isolates from crushed oil soybean seed, a complete mixture for calf growth and other feed types. Besides 3 *A. ochraceus* isolates (Table 2), one *A. alutaceus* (ZM/R), one *A. niger* (MA--K1/08), two *P. aurantiogriseum* (GD-8/06, MS/4a), and one *P. viridicatum* (OZK/R) were also tested. They all had the same toxigenic profile as *A. ochraceus* isolates — none of them produced OTA.

Tab. 2 — Production of ochratoxin A by *Aspergillus ochraceus* isolates

Sample designation	Temper. (°C)	Days	Medium				
			$PPSA^{Zn}$	PPSA	PDA	$YESA^{Zn}$	YESA
GD-16/06	27±1	10	—	—	—	—	—
RST-2/46	27±1	10	—	—	—	—	—
T00010/08	27±1	10	—	—	—	—	—
CBS 108.08*	27±1	10	+	—	+++	++++	++++

Legend: * control strain, — no biosynthesis; + low intensity of biosynthesis, +++ high intensity, ++++ very high intensity

The *A. niger* isolate was included in our investigation because it was known from literature (Tjamos et al., 2004) that this species could produce various amounts of OTA in Corinth raisins and wine-producing vineyards in Greece. The only producer of this mycotoxin was a control strain *A. ochraceus* CBS 108.108 which optimal conditions for OTA biosynthesis were media with yeast extract and high sucrose content (YESA and $YESA^{Zn}$) (Table 2).

Fusariotoxins. Ability to produce ZON, DAS and T2 was investigated in 7 *Fusarium* isolates (*F. solani* — 1, *F. oxysporum* — 3, and *F. verticillioides* — 3) obtained from crushed oil soybean seed, different types of feed and soil for the indoor plant cultivation.

Tab. 3 — Production of zearalenone by *Fusarium* spp. isolates

Sample designation	Species	Temper. (°C)	Days	Medium				
				PPSA^{Zn}	PPSA	PDA	YESA^{Zn}	YESA
RST-3/46	*F. oxysporum*	21—24	7	—	—	—	—	+
			10	—	—	—	—	+
			14	—	—	—	—	+
MA-7A/08	*F. oxysporum*	21—24	7	—	—	—	—	—
			10	—	—	—	+	+
			14	—	—	—	++	++
MA-9/08	*F. oxysporum*	21—24	7	—	—	—	—	—
			10	—	—	—	—	+
			14	—	—	—	++	++
GZ-LES	*F. graminearum**	21—24	7	—	+	—	+	++
			14	—	+	—	+++	++

Legend: * control strain, — no biosynthesis; + low intensity of biosynthesis, ++ moderate intensity, +++ high intensity

Zearalenone (ZON) biosynthesis was detected only by *F. oxysporum* isolates and the control *F. graminearum* culture GZ-LES. At room temperature varying from 21 to 24°C, earlier formation of ZON was detected in some isolates after 7 days of cultivation, or after 10 days in others on YESA (Table 3), although higher quantities of the same fusariotoxin were detected after a prolonged cultivation (14 days). Other authors also reported the formation of the maximum amount of ZON after 12 days at 20°C by *F. oxysporum* and other *Fusarium* species isolated from cereal grains (El-Kady and El-Maragh y, 1982). In the control strain GZ-LES some smaller quantities of ZON were observed in the PPSA medium in which the yeast extract was replaced with the same concentration of peptone-1.

Diacetoxyscirpenole (DAS). None of the tested isolates of *Fusarium* spp. biosynthesised this mycotoxin of type A trichothecene. Results obtained with 2 control strains of *F. semitectum* (Table 4) point out that the best medium for screening the ability to produce DAS under laboratory conditions was PPSA in which the yeast extract was replaced with the same concentration of peptone-1 (2%). These results are, to a certain degree, surprising because our previous investigations (Bočarov-Stančić et al., 2003) showed that the isolate SL-B could produce DAS in the liquid media with yeast extract and peptone-1. We assume that the cultivation temperature (27±1°C), higher than in previous investigations, is the reason for such a result.

Tab. 4 — Production of trichothecenes of type A by *Fusarium* spp. control isolates

Species	Sample design.	Temp. (°C)	Days	Medium				
				$PPSA^{Zn}$	PPSA	PDA	$YESA^{Zn}$	YESA
Diacetoxyscirpenol								
F. semitectum	SL-B	27±1	10	++	+++	—	—	—
F. semitectum	SL-C	27±1	10	+	++	—	—	—
T-2 toxin								
F. sporotrichioides	ITM-391	20—24	7	+++	++	n.a.	++	++
			10	+++	++++	n.a.	+++	++++
		27±1	10	++	+++	+	n.a.	n.a.
F. sporotrichioides	M-1-1	20—24	7	++	+	n.a.	+	+
			10	++	+++	n.a.	++	++
		27±1	10	++	++	+	n.a.	n.a.
F. sporotrichioides	R-2301	20—24	7	++++	+++	n.a.	+++	+++
			10	++++	++++	n.a.	++++	++++
		27±1	10	+++	++++	n.a.	n.a.	n.a

Legend: n.a. — not analysed; + low intensity of biosynthesis, ++ moderate intensity, +++ high intensity, ++++ very high intensity

T2 toxin. As in case of DAS, none of the tested isolates of *Fusarium* spp. biosynthesised this another mycotoxin of type A trichothecene (Table 4), although according to Marasas et al. (1984), our previous investigations (Mašić et al., 1997), and Sokolović et al. (2008) some isolates belonging to the species *F. solani*, *F. oxysporum* and *F. verticillioides* can produce T2.

In the case of control *F. sporotrichioides* isolates, the earliest T2 biosynthesis was observed after 7 days of cultivation on all media with 15% of sucrose, although the highest yields of this fusariotoxin were observed after a prolonged cultivation (10 days) at room temperature (20—24°C). The medium PDA was unsuitable for screening the ability to produce T2 (Table 4). On the other hand, the best results were obtained after cultivation on PPSA or YESA, i.e. media with high sucrose concentration, regardless of the use of peptone-1, or the yeast extract as an N atom source. The addition of a small Zn concentration (0.23 mg/l $ZnSO_4$ x 5 H_2O) did not affect the toxin production although some authors point out that trace microelements can significantly influence the mycotoxin production (Mühlencoert, 2004).

CONCLUSIONS

For screening ability to produce different mycotoxins, the optimal cultivation conditions are as follows:

— AFLB1 — 14 days on PDA and 27±1°C;

— OTA — 10 days on YESA and $YESA^{Zn}$ at 27±1°C;

— ZON — 2 weeks on YESA and $YESA^{Zn}$ at room temperature (21—24°C);

— DAS — 10 days on PPSA and $PPSA^{Zn}$ at 27±1°C;

— T2 — 7 days on $PPSA^{Zn}$ and PPSA at room temperature (20—24°C).

Trace element Zn (0.23 mg/l $ZnSO_4$ x 5 H_2O) did not affect the mytoxin production in tested conditions.

Analysed fungal isolates from cereals and fodder in Serbia were mainly non toxic — out of 21 only one culture of *A. flavus* produced AFB1, and three *F. oxysporum* cultures produced ZON.

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ИСПИТИВАЊЕ ТОКСИГЕНОГ ПОТЕНЦИЈАЛА ГЉИВА ПРИМЕНОМ ЈЕДНОСТАВНОГ ТРИЈАЖНОГ МЕТОДА

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Резиме

Потенцијал за биосинтезу афлатоксина Б1 (АФЛБ1), охратоксина А (ОТА), диацетоксисцирпенола (ДАС), Т-2 токсина (Т2) и зеараленона је испитан код изолата гљива које припадају родовима *Aspergillus*, *Fusarium* и *Penicillium*. Изолати су углавном били пореклом са зрна житарица и сточне хране. Примењена је једноставна тријажна метода Filtenborg-a и сар. (1983) код које су извршене извесне модификације у смислу типа подлоге и температуре култивације гљива.

У циљу оптимизације услова за тестирање токсигеног профила одабраних гљивичних изолата употребљене су контролне културе за које је претходно доказано да су произвођачи следећих микотоксина: ОТА-а *A. ochraceus* CBS 108.08, ДАС-а *F. semitectum* (СЛ-Б и СЛ-Ц), Т2 — *F. sporotrichioides* (ITM-391, M-1-1, R-2301) и ZON-а *F. graminearum* (ГЗ-ЛЕС). Гљиве су гајене на стандардној подлози (ЕКСА — 2% екстракта квасца и 15% сахарозе, pH 6,5), три модификације основне подлоге (EKSA^{Zn-} — стандардна подлога са додатком 0,23 mg/l $ZnSO_4$ x 5 H_2O; ППСА — подлога у којој је екстракт квасца замењен пептоном-1; ППСА^{Zn} — подлога у којој је екстракт квасца замењен пептоном-1 и којој је додато 0,23 mg/l $ZnSO_4$ x 5 H_2O; pH 6,5) и кромпир декстрозној подлози (pH 6,5).

Биосинтеза испитаних микотоксина је најраније констатована при следећим условима гајења гљива: АФЛБ1 — после 14 дана култивације на КДА и 27±1°C, ОТА — после 10 дана култивације на ЕКСА и EKSA^{Zn} и 27±1°C, ДАС-а после 10 дана култивације на ППСА и ППСА^{Zn} на 27±1°C, Т2 — после 7 дана на ППСА и ППСА^{Zn} и собној температури (20—24°C), и ZON-а после недељу дана на ЕКСА и ЕКСА^{Zn} и собној температури (21—24°C).

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FUSARIUM SPECIES: THE OCCURRENCE AND THE IMPORTANCE IN AGRICULTURE OF SERBIA

ABSTRACT: *Fusarium* species have been isolated from over 100 plant species in Serbia. From the economic aspect, they have been and still are the most important for the production and storage of small grains and maize, and are exceptionally important for some other species. Total of 63 species, 35 varieties (var.) and 19 specialised forms (f. sp.) of basic species, particularly of *F. oxysporum* (4 var. and 12 f. sp.) and *F. solani* (7 var. and 3 f. sp.) were identified. Species *F. langsethiae* and *F. thapsinum*, recently identified, have been isolated from wheat and sorghum seeds, respectively. *F. graminearum* is the most important pathogen for wheat, barely and maize, while *F. poae* is also important for wheat and barely. Furthermore, species of the section *Liseola* (*F. verticillioides*, *F. subglutinans* and *F. proliferatum*) are important for maize and sorghum. In recent years, species of the section *Liseola* have been increasingly occurring in wheat and barley. The June—October period in Serbia is the most critical period for quality maintenance of stored maize, as the abundance and frequency of fungi, particularly of toxigenic species of the genus *Fusarium*, are the greatest during that period.

In general, there is a lack of data about fusarioses of industrial crops in Serbia. There are mere descriptions of specific cases in which the development of *Fusarium* species was mostly emphasised by agroecological conditions. The presence of recently determined *Fusarium* species in kernels of these plant species indicates their importance from the aspect of the yield reduction and grain quality debasement and the mycotoxin contamination.

Root rot and plant wilt are characteristic symptoms of fusarioses for forage and vegetable crops, while pathological changes in fruits provoked by *Fusarium* species are less frequent. *F. oxysporum* and its specialised forms prevail in these plant species.

KEY WORDS: *Fusarium*, diseases, cereals, industrial crops, forage crops, vegetables

INTRODUCTION

The importance of *Fusarium* species is mostly estimated through damages that they cause either by destroying crops, grain, nursery plants, stored fruits, finished products, processed products, or by causing the decrease in the livestock production or death of animals, human diseases, etc. The majority spe-

cies of the genus *Fusarium* are capable of causing diseases (mycoses) in plants, animals and humans or mycotoxicoses in animals and humans. These fungal species parasitise on living cells, tissues or organs that are often weakened by other factors and cause infections of a mycosis type. These fungi form mycotoxins in infected plants and if these plants are involved in a food chain they can cause intoxication of humans and animals known as mycotoxicoses.

There is almost not a single place in Serbia where fungi of the genus *Fusarium* were not observed (Lević, 2008). They are identified as pathogens of wheat, industrial, forage, vegetable, fruit, forest, ornamental, aromatic, medicinal and weed plants, then as parasites of fungi, parasitic flowering plants and insects. Furthermore, they are isolated from the human eye, feed and food, soil, water and air. More important secondary metabolites (fusariotoxins) formed by certain species of the genus *Fusarium* were determined, as well as the effects of food contaminated by these metabolites on human and animal health.

Although *Fusarium* species have been isolated from over 100 plant species in Serbia, they have been and still are, from the economic aspect, the most important for the production and storage of small grains and maize, and are exceptionally important for some other crops (Lević, 2008; Lević et al., 2008b). Total of 63 species, 35 varieties (var.) and 19 specialised forms (f.sp.) of basic species, particularly of *F. oxysporum* (4 var. and 12 f. sp.) and *F. solani* (7 var. and 3 f. sp.) were identified. There are over 225 publications that discuss *Fusarium* spp., but it is not known whether in the described species are only the identified ones or maybe there are unidentified new species. Such a great number of unidentified species is a result of insufficient experience of the majority of researchers, who easily determine the observed fungi up to the genus, but not to a species. In spite of such a great number of identified species, not more than ten are the most distributed, while this number is much smaller with regard to the economic and mycotoxicological importance.

The first occurrence of species of the genus *Fusarium* was determined in Serbia as teleomorph forms (*Gibberella pulicaris* (Fries) Sacc., *Nectria episphaeria* (Tode), *Gibberella saubinetii* (Mont.) Sacc.) at the end of the 19th and the beginning of the 20th century, while the anmorph form (*Fusarium roseum* Link) was distinguished as late as 1911 (Ranojević, 1902, 1910; 1914).

Numerous species of the genus *Fusarium* were isolated from different fodder components and mixtures. These species contribute to the yield reduction and quality debasement of fodder, especially in regard to mycotoxins (Bočarov-Stančić et al., 2008, 2001; Jajić et al., 2007; Stanković et al., 2008d). Zearalenone and its derivatives, as well as type A trichothecenes (T-2 toxin, DAS) were mainly studied and determined in Serbia, while type B trichothecenes (deoxynivalenol) or fumonisines were significantly less observed and determined (Lević et al., 2004; Bočarov-Stančić et al., 2008). In recent times, greater attention has been given to these and other fusariotoxins, such as beauvericin, fusaproliferin and moniliformin (Abramović et al., 2005; Stanković et al., 2007, 2008a, 2008b, 2008c, 2008d).

The importance of species of the genus *Fusarium* in Serbia can be considered on the basis of their distribution, abundance, pathogenic properties rela-

ted to crops, consumption of contaminated products, damages caused by natural infections, mycotoxin contamination and the number of published papers. The most numerous studies state results obtained on the occurrence of species on certain parts of different plants species and various substrates depending on biotic and abiotic factors. There are the least data on human diseases (endophthalmitis) caused by species of the genus *Fusarium* (S r d i ć et al., 1993).

This study encompasses the occurrence and importance of certain *Fusarium* species in agriculture with regard to cereals, industrial, forage and vegetable plants.

CEREALS

Due to great economic damages, species of the genus *Fusarium* were the most important agents causing root and stalk rots of maize (*Zea mays* L.) and wheat (*Triticum vulgare* L.) in Serbia up to the mid-1980s (D r a g a n i ć, 1978; B a l a ž et al., 1985). In the majority of cases of root and stalk rots of both maize and wheat, the species *F. graminearum* was the most frequent pathogen. Due to the intensive activities of phytopathologists and breeders on the development of resistant genotypes, the symptoms of maize stalk lodging could hardly be recorded in fields in Serbia since the 1990s (L e v i ć et al., 1995). As far as wheat is concerned, root and stem rot is of a weaker intensity than earlier, but it can occur more intensively under certain conditions, for instance under conditions of wheat-maize rotation and without the fertiliser application (B a l a ž et al., 1998).

In Serbia, besides root and stalk diseases, *Fusarium* species used to be and still are the most important agents causing ear roots and fusarioses of spikes of both wheat and barley (*Hordeum vulgare* L.). Barley is one of the first crops for which a compulsory phytopathological seed test was established in Serbia in 1930 in order to prevent the introduction and distribution of the species *F. roseum* Link (Report on the activities of the Agricultural Experimental and Testing Station from 1919 to 1932, published in 1936).

The presence of the *Fusarium* species on maize ear or spikes of small grains is noticed during each year, but with different infection intensity or frequency of certain species. The literature data on their distribution and frequency are numerous, sometimes even drastically different although they were obtained during the same year by various researches. These great differences are the result of effects of particular or combined factors, and are mostly related to: (i) impacts of agroclimatic conditions during the growing season; (ii) the susceptibility of observed genotypes; (iii) the representability of samples; (iv) the number of analysed samples and deficient information on each sample; (v) the number of locations and years of investigation; (vi) applied methods for the isolation and the identification of fungi; (vii) the routine in the identification of certain species; and (viii) the impossibility of the conservation of isolated cultures in an appropriate way so that the confirmation of the identification or re-identification could be done by reanalyses.

In the last decades, the amount of local precipitation during the wheat flowering time had a significant effect on the variation of the intensity of

Fusarium head blight (FHB) and on the wheat grain yield in Serbia. In such a way, it was determined in 2005, when the intensity of spike fusariosis was, on average, high, that the grain yield reduction varied from 3.5 to 38.3%, depending on the agroecological conditions and resistance of wheat varieties (Lević et al., 2008c).

The type of isolation media for fungi is of a special importance for the identification of a particular *Fusarium* species. For instance, potato dextrose agar (PDA) is a medium most suitable for distinguishing *F. langsethiae* and *F. thapsinum* from *F. poae* and *F. verticilioides*, isolated from wheat and sorghum kernels, respectively. On PDA, *F. langsethiae* forms a powdered colony whose colour varies from white to dark red, while *F. thapsinum* forms a colony with a yellow pigment in the medium. These types of colonies are never formed by *F. poae* and *F. thapsinum*.

The medium containing 2% agar is a very efficient medium for the determination of the *Fusarium* species in the maize kernel, as fungi abundantly sporulate, but scarcely form aerial mycelium. This provides *in situ* identification of numerous *Fusarium* species both on and around kernels.

The distribution and frequency of *F. oxysporum* can be used as an example of differences in results obtained in the studies of mycobiota in wheat and maize kernels. Dopuđa and Lević (2004) and Stanković et al. (2007) established that this species was present up to 3%, i.e. that it was absent in the tested samples of wheat kernels. On the contrary, the results obtained by Stojanović et al. (2005) show that this fungus was present in wheat kernels even in 45.7% of samples. Studies carried out by Bočarov-Stančić (2001) point out that different results were obtained when the kernel infection with *Fusarium* species was determined in dependence on the fact whether kernels were surface sterilised or not. In cases when kernels were not surface sterilised, the obtained results are many-fold higher.

The intensity of the occurrence of *F. oxysporum* has been changed in maize. During the 1980s, *F. oxysporum* spread pretty much in stored maize. In some cases, even 56% of surface sterilised maize kernel samples was infected (Muntañola-Cvetković, 1982; Noory (1983). However, recent results gained by Lević et al. (2003) indicate that this species is very seldom isolated from surface sterilised maize kernels.

In spite of the stated differences in the determination of the intensity of frequency of *Fusarium* species, conclusions on their frequency and importance for cereals can be drawn according to the results of long-term analysis. Data presented in Tables 1 (unpublished data) and 2 point out that the most important pathogenic species for wheat, barley and maize is *F. graminearum*. Furthermore, *F. poae* is also important pathogenic species for wheat and barley, while species of the section *Liseola* (*F. verticillioides*, *F. subglutinans* and *F. proliferatum*) are also important for maize and sorghum (Lević et al., 1997, 2008). Under agroecological conditions of Serbia, *F. graminearum* is mainly developed along the rachis (Lević et al., 2008c), which points out that cultivated varieties of wheat do not possess the type II resistance or resistance to the spread of pathogens on spikes. On the other hand, *F. poae* is the

most often developed on the rachis tip, and although it is very frequent it does not express pathogenic effects on wheat seedlings (L e v i ć et al., 2008d).

Tab. 1 — The number of positive samples[a] with determined occurrence of *Fusarium* spp. in kernels of wheat, barley, maize and sorghum in the 2004—2007 period

Fusarium species	Wheat	Barley	Maize	Sorghum
F. avenaceum		1/29		
F. culmorum		1/29		
F. equiseti		5/29	4/67	1/60
F. graminearum	71/75	26/29	21/67	
F. langsethiae	3/75			
F. oxysporum		1/29		
F. poae	37/75	18/29	2/67	
F. polyphialidicum		2/29	1/67	
F. proliferatum	10/75	4/29	25/67	32/60
F. sambucinum		1/29		
F. semitectum		5/29		2/60
F. solani			1/67	6/10
F. sporotrichioides	9/75	21/29	8/67	2/60
F. subglutinans	75/5	1/29	24/67	8/60
F. thapsinum				34/60
F. tricinctum	4/75	2/29		
F. verticillioides	27/75	1/29	38/67	60/33

[a] Number of samples infected with *Fusarium* spp./total number of tested samples. A total of 100 kernels per each sample was tested — kernels were rinsed under tap water for two hours, then surface sterilised with 1% NaOCl for 10 seconds, then they were washed with sterile water three times and dried out on soft sterile paper.

The frequent occurrence of *F. verticilioides* and *F. proliferatum* in wheat kernels is an interesting data from the toxicological aspects, as it was thought that fumonisins, synthetised by these fungi, were less important for wheat than for maize (V i s c o n t i and D o k o, 1994). In accordance with these results, it was determined that 59.3% of samples of wheat harvested in 2005 and 2006 were contaminated with fumonisins, provided that the FB_1 concentration was > 20,000, 2,300—16,700 and < 2,000 ppb in 50.0, 20.8 and 29.2% samples, respectively (L e v i ć et al., 2008a; S t a n k o v i ć et al., 2008d). These results point out that it is necessary to determine natural contaminations of wheat and barley kernels with fumonisins under agroecological conditions of Serbia.

Tab. 2 — Occurrence of *Fusarium* species (given in descendent values) in kernels of cereals in Serbia[a]

Plant species			
Wheat	Barley	Maize	Sorghum
Occurrence > 16%			
F. graminearum	*F. graminearum*	*F. graminearum*	
F. poae	*F. poae*		
	F. sporotrichioides		
		F. subglutinans	
		F. verticillioides	
			F. proliferatum
			F. thapsinum
Occurrence 6—15%			
F. proliferatum	*F. proliferatum*	*F. proliferatum*	
F. verticillioides	*F. verticillioides*		*F. verticillioides*
F. subglutinans	*F. subglutinans*		
F. avenaceum		*F. avenaceum*	
F. oxysporum		*F. oxysporum*	
F. sporotrichioides			
F. culmorum			
		F. poae	
Occurrence < 5%			
F. semitectum	*F. semitectum*	*F. semitectum*	*F. semitectum*
F. equiseti	*F. equiseti*	*F. equiseti*	*F. equiseti*
F. tricinctum	*F. tricinctum*	*F. tricinctum*	
F. chlamydosporum		*F. chlamydosporum*	
	F. crookwellense	*F. crookwellense*	
	F. avenaceum	*F. avenaceum*	
	F. culmorum		
	F. oxysporum		
	F. polyphialidicum		
	F. sambucinum		
F. acuminatum			
F. arthrosporioides			
F. compactum			
F. langsethiae			
F. anthophilum			
		F. solani	*F. solani*
		F. sporotrichioides	*F. sporotrichioides*
			F. subglutinans

[a] Data source: L e v i ć (2008).

The June—October period in Serbia is the most critical period for quality maintenance of stored maize, as the abundance and frequency of fungi, particularly of toxigenic species of the genus *Fusarium*, are the greatest during that period (K r n j a j a et al., 2007b). The positive correlation (r = 0.598**) between the dynamics of the occurrence and the frequency of isolated fungi indicates that fungi with greater frequency of occurrence maintain longer in maize kernels during storage. According to these authors, *F. verticillioides* and *F. subglutinans*, out of total six identified species of the genus *Fusarium*, are

present in kernels during the whole year and with the highest frequency (24.7% and 5.9%, respectively). During the year, *F. verticillioides* (22.0/39.5%) and *F. subglutinans* (8.012.5%) are mainly present from February to October and April to October, respectively, while both species are the least isolated during the winter period (December—January — 4.0—8.0% and 0.5—1.0%, respectively). *F. graminearum* is the third toxigenic species of the genus *Fusarium* that could be significant from the mycotoxicological aspect for the June—September period when it occurs in the highest percent (5.0—11.0%). Lately, the occurrence of the remaining species of the genus *Fusarium* has been sporadic (1.3% *F. proliferatum*, 1.0% *F. sambucinum* and 0.5% *F. poae*).

INDUSTRIAL CROPS

In general, fusarioses of industrial plants, have been very little studied in Serbia. Exceptionally, there are sporadic literature data on excess cases that are most often caused by impacts of agroclimatic conditions. *Fusarium* species mainly cause plant wilt in industrial plants as a result of root rot (Table 3). *F. oxysporum* is the species most often isolated from roots of these plants.

In Serbia, for a long time, *Fusarium* species have not been considered the important causative agents of diseases of sugar beet root (*Beta vulgaris* L. var. *saccharifera* Lange). However, the occurrence of leaf chlorosis, caused by necrosis and rotting of roots, has been very frequent during the last decade. Leaf chlorisis has caused great economic damages (J a s n i ć et al., 2005a). *F. oxysporum* was mainly isolated from infected plants, while *F. solani* was not often isolated (Table 4). This root rot occurrence is attributed to the effects of droughts, which have been very frequent in Serbia during the growing season of sugar beet.

There is a lack of results obtained from the studies of soybean (*Glycine max* (L.) Merrill) fusarioses in Serbia, although several *Fusarium* species that cause root and stem rots, wilting and infections of pods and seeds, have been isolated and identified during the last several years (Table 3). *Fusarium* species, especially *F. oxysporum* and *F. semitecum*, are the most frequently isolated from soybean seeds (Table 4). The pathogenicity test shows that *F. oxysporum* caused the decrease in seed germination and plant emergence and also led to the increase of rotten soybean kernels (J a s n i ć et al., 2005b).

Sunflower (*Helianthus annuus* L.), as well as soybean is not a sufficiently studied industrial plant from the aspects of diseases caused by *Fusarium* spp., although the most recent studies show that these diseases can be important due to a frequent and sometimes intensive, occurrence, in kernels (Tables 3 and 4), (B o č a r o v, 1983). The first more important data about sunflower fusariosis was described more than four decades ago, when *F. avenaceum* caused important diseases of sunflower plants during rainy summer months.

Complex of species of the genus *Fusarium* (*F. oxysporum, F. verticillioides, F. proliferatum, F. solani*, etc.) often cause root rot and wilting of hop (J a s n i ć et al., 1996). In addition, it was revealed that *Fusarium* species isolated from hop are potentially toxigenic species (S t a n k o v i ć et al., 2008b). The mycotoxin production of strains shows that all isolates of *F. proliferatum* and

F. verticillioides produced fumonisin B_1 (250,000—300,000 ppb), five out of six isolates of *F. proliferatum* produced beauvericin (400,000—500,000 ppb), three strains of *F. proliferatum* produced fusaproliferin (400.000—450,000 ppb) and all isolates of *F. verticillioides* produced fusaproliferin (up to 400,000 ppb).

Tab. 3 — Fusarioses of industrial plants[a]

Plants	Symptoms	Causative agents
Sugar beet	Seedling wilt, root necrosis	*F. oxysporum var. orthoceras, F. oxysporum*
	Chlorosis, wilt and death of plant, bearded root and root rot	*F. oxysporum, F. graminearum F. equiseti*
Soybean	Necrosis of root and above ground stem part and plant wilt	*F. oxysporum F. graminearum,*
	Infection of pod and kernel	*F. semitectum*
Sunflower	Plant wilt and root rot	*F. avenaceum*
	Infection of kernel	*F. verticillioides*
Hop	Rot of root and above ground stem part, leaf chlorosis, wilt	*Complex Fusarium species*
Tobacco	Wilt of young plant, white loose fungal mycelium in leaf and seed	*F. argillaceum*

[a] Data sources: Lević (2008); Jasnić et al. (2005a, 2005b); Balaž and Stojšin (1997); Lević (2008, unpublished data).

Tab. 4 — *Fusarium* species in industrial plants in Serbia[a]

Plants			
Sugar beet	Soybean	Sunflower	Hop
Prevailing *Fusarium* species			
F. oxysporum	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
	F. semitectum		
			F. culmorum
Additionally occurring *Fusarium* species			
F. graminearum	*F. graminearum*	*F. graminearum*	
F. acuminatum		*F. acuminatum*	*F. acuminatum*
F. solani		*F. solani*	*F. solani*
F. proliferatum			*F. proliferatum*
	F. sporotrichioides	*F. sporotrichioides*	
		F. verticillioides	*F. verticillioides*
F. oxysporum f. sp. betae			
F. semitectum			
F. culmorum			
F. equiseti			
	F. poae		
	F. equiseti		
		F. avenaceum	
		F. camptoceras	
		F. dimerum	
		F. equiseti	
		F. roseum	
		F. semitectum var. majus	

[a] Data source: Lević (2008).

F. oxysporum and *F. solani* are the most frequent causative agents of root rots and plant wilt of lucerne (*Medicago sativa* L.) and clover (*Trifolium* spp. L.), while *F. verticillioides* causes the greatest damages to seeds (Tables 5 and 6). Strong wilt of red clover was recorded only in one case, in an area intended for the production of fodder and seed in the region of Kruševac in 1997 (Urošević et al., 1999).

F. culmorum, *F. semitectum* and *F. sporotrichioides* were isolated from 16.7 to 33.0% of samples of healthy lucerne plants and plants with wilting symptoms (Bočarov-Stančić et al., 2005). Lucerne wilt is caused by several species of the genus *Fusarium*, and according to some authors a specialised form of *F. oxysporum* f. sp. *medicaginis* is the most important (Todorov et al., 1995). A smaller number of isolates of *F. oxysporum*, originating from lucerne roots, were observed by the vegetative compatibility group method and these observations did not confirm the presence of *F. oxysporum* f. sp. *medicaginis* in Serbia (Krnjaja et al., 2007a). This indicates that further comprehensive studies on lucerne fusarioses and the identification of specialised forms of widely spread species *F. oxysporum* are necessary.

Tab. 5 — Fusarioses of forage plants[a]

Plants	Symptoms	Causative agents
Clover	Tracheomycosis plant wilting	*F. oxysporum*
	Seed infection	Complex of *Fusarium* species
Lucerne	Root rot and plant wilting	*F. oxysporum, F. solani*
	Seed infection	Complex of *Fusarium* species

[a] Data sources: Lević (2008); Krnjaja and Lević (2005); Krnjaja et al. (2005a, 2005b).

Tab. 6 — *Fusarium* species in forage plants in Serbia[a]

Occurrence of *Fusarium* spp.	Plant species	
	Clover	Lucerne
Prevailing species:		
	F. oxysporum	*F. oxysporum*
		F. oxysporum f. sp. medicaginis
	F. solani	*F. solani*
Additionally occurring species:		
	F. proliferatum	*F. proliferatum*
	F. subglutinans	*F. subglutinans*
	F. verticillioides	*F. verticillioides*
		F. acuminatum
		F. arthrosporioides
		F. avenaceum
		F. equiseti
		F. graminearum
		F. semitectum
		F. sporotrichioides
		F. tricinctum

[a] Data sources: Lević (2008); Bočarov-Stančić et al. (2005); Krnjaja et al. (2005a, 2005b, 2007a).

VEGETABLES

In Serbia, *Fusarium* species periodically cause significant diseases of onion (*Allium cepae* L.), garlic (*A. sativum* L.), tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.) and watermelon (*Citrullus vulgaris* Schrad.), especially wilting type of disease (table 7). Usually, *Fusarium* species rarely occur in the majority of vegetables, and if they occur they are often of a weaker intensity. *F. oxysporum* is the most often causative agent for these diseases (Table 8).

The significance of the *Fusarium* basal rot of onion has been increasing in Serbia. The disease is important not only for the green onion production, but also for the production of onion sets and bulbs. Especially expressed damages in the onion production occur in onion continuous cropping or in a short-term crop rotation. Species of the genus *Fusarium* can cause rots up to 53.2% of onion seedlings (K l o k o č a r - Š m i t et al., 1988). The following species were most often isolated from infected bulbs, onion sets and seedlings, as well as from soil: *F. oxysporum* Schlecht. f. sp. *cepae* (Hanz.) Snyd. & Hans. and *F. solani*, upon that *F verticillioides* (syn. *F. moniliforme*) and *F. oxysporum* (K l o k o č a r - Š m i t et al., 1990).

F. proliferatum, *F. oxysporum* and *F. solani* were isolated from cloves of garlic (Table 8). *F. proliferatum* caused golden-yellow to tan spots on the inoculated plants, while w a t e r - s o a k e d, soft and tan necrotic spots with mildly w r i n k l e d tissue occurred on cloves (S t a n k o v i ć et al., 2005). Isolates of *F. proliferatum*, originating from garlic, are good mycotoxin producers (S t a n k o v i ć et al., 2007).

Wilting, vessel necrosis and death of plants of tomato, aubergine and pepper caused by *Fusarium* species were determined during the 1980s (*cit.* after L e v i ć, 2008). *F. oxysporum* f. sp. *lycopersici* is stated as the most often causative agent of tomato fusariosis, although it was not identified in all cases with certainty. It is considered that the application of systemic fungicides in the production of this vegetable is a reason for rare occurrence of fusarioses on tomato in Serbia.

Two out of several cases of the intensive occurrence of fusarioses in potato tubers are the most important in Serbia. The first case was during the 1960s with the infection level up to 50% (M a r t i n o v i ć, 1961), when *F. coeruleum* was identified as a principal pathogen that caused dry rot of tubers (mummified tubers). In the second case during 1992, there was an outbreak of tuber rot in all regions of Serbia at the end of the growing season of potato. The infection intesity in some plots was 30.0%, but in some smaller plots it ranged from 1.0 to 80.0% (S t o j š i n and M a r i ć, 1995). Infected tubers may completely rot, shrivel, and become mummified. *F. oxysporum* Schel. var. *tuberosi* Snyd. & Hans. and *F. solani* were mainly isolated from such tubers, while *F. solani* var. *coeruleum* and *F. avenaceum* were rarely identified very rare.

Tab. 7 — Fusarioses of vegetable plants[a]

Plants	Symptoms	Causative agents
Onion	Root and bulb rot, death of seedlings	*F. oxysporum* var. *cepae, F. solani*
Garlic	Water-soaked, soft and tan necrotic spot with mildly wrinkled tissue of clove	*F. proliferatum*
Cabbage	Wilting, rot of root and root collar	*F. oxysporum* f. sp. *conglutinans*
Pepper	Wilting, root necrosis	*F. annuum, F. oxysporum*
Watermelon	Wilting and dying of plant, seed and fruit infection	*F. oxysporum* f. sp. *niveum*
Cucumber	Rot of lower part of stems, wilting and dying of plant	*Fusarium* spp.
Tomato	Necrosis of vascular vessels, wilting	*F. oxysporum* f. sp. *lycopersici*
Bean	Root rot and chlorosis from the base to the top of the plant	*F. oxysporum* f. sp. *phaseoli*
Pea	Necrosis and rot of root	*F. solani* var. *redolens*
Potato	Dry rot, dark tissue of the central part of tuber, mummified tuber	*F. coeruleum, F. oxysporum* var. *tuberosi, F. solani*
Broad bean	Rolling and wrinkling of leaf, parchment appearance, root rot	*F. oxysporum, F. oxysporum* f. sp. *fabae, F. oxysporum* f. sp. *pisi*

[a] Data sources: Lević (2008); Ivanović et al. (1987, 1997); Stojšin and Marić (1995); Stanković et al. (2007).

The first outbreak of Fusarium wilting of watermelon was recorded in the 1970s, when *F. oxysporum* f. sp. *niveum* caused the disease in 60.0—70.0% of watermelon plants, especially those cultivated in continuous cropping (*cit.* after Lević, 2008). The disease symptoms in grown watermelon plants also occurred in fruits. Due to the application of watermelon grafting onto rootstock of gourd that is resistant to *Fusarium* species, cases of fusarium diseases of watermelon in Serbia have been occurring less and less since the 1990s.

Tab. 8 — *Fusarium* species (given in descendent importance) in vegetable plants in Serbia[a]

Plant species			
Onion	Garlic	Tomato	Potato
F. oxysporum	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
F. solani	*F. solani*		*F. solani*
F. proliferatum	*F. proliferatum*		
F. acuminatum			
F. cepae			
F. equiseti			
F. oxysporum f. sp. *cepae*			
F. verticillioides			
		F. incarnatum	
		F. oxysporum f. sp. *lycopersici*	
		F. semitectum	
			F. coeruleum
			F. avenaceum
			F. oxysporum var. *tuberosi*
			F. solani var. *coeruleum*
			F. oxysporum f. sp. *solani*
			F. sambucinum

[a] Data source: Lević (2008).

Fusarium rot of pepper roots and root collars and fusarium wilt occurred in the intensity of up to 10% in the late 1950s and 1970s (*cit.* after Lević, 2008). At the end of 1990s, Todorović and Horvat (1997) also pointed out to *Fusarium* diseases of pepper grown under controlled conditions. *F. annuum* and *F. oxysporum* f. sp. *lycopersici* were identified.

Data on fusarioses of bean (*Phaseolus vulgaris* L.) and string bean are scarce (*cit.* after Lević, 2008). The occurrence of root rots, necrosis of vascular vessels of the above ground parts of stems and plant wilting were recorded during the 1980s. Infected plants obviously lagged in growth and were of a chlorotic appearance. Chlorosis spread from the base to the top of a plant until the plant died. *F. oxysporum* f. sp. *phaseoli* was mainly isolated from infected plants.

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FUSARIUM ВРСТЕ: ПОЈАВА И ЗНАЧАЈ У СРБИЈИ

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Резиме

Врсте рода *Fusarium* су у Србији изоловане са преко 100 биљних врста, а са економског аспекта, биле и остале, најзначајније за производњу и чување стрних жита и кукуруза. Само изузетно су значајне и за неке друге биљне врсте. Укупно је до сада идентификовано: 63 врсте, 35 варијетета (var.) и 19 специјализованих форми (f. sp.) основних врста, посебно врсте *F. oxysporum* (4 var. и 12 f. sp.) и *F. solani* (7 var. и 3 f. sp.). *F. langsethiae* и *F. thapsinum* су новоидентификоване врсте изоловане са зрна пшенице, односно сирка. *F. graminearum* је најзначајнији патоген пшенице, јечма и кукуруза, а *F. poae* и патоген пшенице и јечма. Врсте из секције *Liseola* (*F. verticillioides*, *F. subglutinans* и *F. proliferatum*) значајни су и патогени кукуруза и сирака, мада је последњих година утврђена све чешћа појава ових врста на зрну пшенице и јечма. У Србији је период јун—октобар најкритичнији за очување квалитета ускладиштеног кукуруза јер су бројност и учесталост гљива у том периоду највеће, посебно токсигених врста рода *Fusarium* (43,5—62,5%).

Генерално, фузариозе индустријских биљака су мало проучаване у Србији. Описи су само појединачни случајеви у којима је развој *Fusarium* врста најчешће потенциран повољним агроклиматских условима. Присуство *Fusarium* врста на семену индустријских биљака, које је последњих година све чешће утврђено, указује да ове патогене гљиве у Србији могу бити значајне са становишта смањења приноса и квалитета зрна, посебно у погледу контаминираности микотоксинима.

Трулеж корена и увенулост биљака су карактеристични симптоми фузариоза крмних и повртарских биљака, док је појава патолошких промена на плодовима ређа. На овим биљним врстама доминирају *F. oxysporum* и њене специјализоване форме.

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EXAMINATION OF THE EFFICACY OF VARIOUS FEED ADDITIVES ON THE PATHOMORPHOLOGICAL CHANGES IN BROILERS TREATED WITH T-2 TOXIN

ABSTRACT: A 21-day-long experiment was performed on 160 one-day-old "Ross" broiler chicks. This research was done with the aim of investigating pathomorphological changes in broilers exposed to a relativly small amount of T-2 toxin (2 ppm) and the possibility of prevention and/or alleviating adverse effects of T-2 toxin using various feed additives. Pathohistological examination showed negative consequences of T-2 toxin in all examined organs as degenerative changes developed in small intestine mucosa, enterocites and hepatocites necroses, as well as lymphocites depletion in bursa of Fabricius. Disparately from inorganic (Minazel-plus, Mz) and organic (Mycosorb, Ms) adsorbents, which did not provoke protective effects, in liver, small intestine and bursa of Fabricius of broilers who were given feed with T-2 toxin and mixed adsorbent (Mycofix-plus, Mf), mostly preserved structure of these organs could be noted.

KEY WORDS: T-2 toxin, broiler, pathomorphological changes, adsorbents

INTRODUCTION

Trichothecenes inhibit protein synthesis and from the pathological and immune point of view they are of extreme importance for poultry. When animals ingest relatively small amounts of T-2 toxin, clinical symptoms can miss and performances can remain unalterable during longer period. In this way toxin might be undiscovered although it causes subclinical changes that could be found at the pathomorphological examination.

A new approach to mycotoxin control is to alleviate and/or prevent harmful effects of mycotoxins in feed. Different feed additives are in use today, which either adsorb mycotoxins on their surface or they provide enzyme degradation of mycotoxins. The efficacy of alleviating harmful effects depends mostly on chemical structure of adsorbent, as well as on the type of mycotoxin.

Adsorbents are substances nonresorbable from the gut, which can physically bind some chemicals and thus block their resorption. Mineral adsorbents are commonly in use (active charcoal, hydrated sodium calcium aluminosilicate, sodium bentonit, dietary clay and zeolites) (T o m a š e v i ć - Č a n o v i ć et al., 2003). The feasibility of utilizing organic adsorbents is also examined, particularly esterified glucomanane which is isolated from the inner layer of yeast cell wall and which possesses significant capability of mycotoxin adsorption (D e v e g o w d a et al., 1996). Recently a new type of additive, containing microorganisms with the ability to inactivate mycotoxins by enzyme modification of their structure (F u c h s et al., 2002), has been developed.

The primary objective of this study was to overhaul the feasibility of alleviating and/or preventing pathohistological changes in broilers exposed to T-2 toxin by utilizing different feed additives.

MATERIAL AND METHODS

Pure T-2 toxin isolate, obtained under laboratory conditions by cultivation of *Fusarium sporotrichoides*, from 4 isolates: ITM-496, KF-38/1, M-1-1 i R-2301 (B o č a r o v - S t a n č i ć and R a d o š e v i ć, 1991) was used for feed contamination. To achieve proper homogenisation of T-2 toxin in the feed, ethil-acetate extract containing 2 ppm of T-2 toxin was 3 times sprayed at certain amount of feed that was afterwards mixed into the rest of the feed necessary for the experimental period (1—21. days).

After proper preparation of the feed samples, the determination of the present amount of T-2 toxin was done using ELISA method with monoclonal T-2 toxin antibodies (B a r n a - V e t r o i sar., 1994).

Minazel-plus (Modified clinoptilolite, ITNMS Beograd), *Mycosorb* (Esterified glucomanane, Altech, USA) and *Mycofix-plus* (Biomin, Austria) were added to the feed in the amount of 0,2%.

In vivo trial 21 days long was performed on 160 one-day old "Ross" broiler chicks of both sexes. Animals were divided into 8 experimental groups, each containing 20 broilers: group 1: negative control, free from T-2 toxin and additives; group 2: positive control, 2 ppm T-2 toxin without binders; group 3: 2 ppm T-2 toxin + 0,2% Minazel-plus; group 4: 2 ppm T-2 toxin + 0,2% Mycosorb; group 5: 2 ppm T-2 toxin + 0,2% Mycofix-plus; group 6: 0,2% Minazel-plus without T-2 toxin; group 7: 0,2% Mycosorb without T-2 toxin; group 8: 0,2% Mycofix-plus without T-2 toxin.

Animal sacrifice was done in all experimental groups on the 21st day of the trial by method of cervical dislocation. Pathohistological examination was performed on liver, small intestine and bursa of Fabricius samples, embedded in paraffin, and sections of 3—5 m thickness were cut, stained using the standard hematoxylin eosin method (S c h e u e r et al., 1986) and observed under the optical microscope (Olympus BX-41).

RESULTS AND DISCUSSION

T-2 toxin affects almost all tisues and organs. Labile cells are mostly attacked by structural changes due to T-2 toxin cytotoxic effects. This mycotoxin causes pathomorphological alterations in liver, digestive system, bone marrow, skin, lungs, heart, reproductive organs, spleen, bursa of Fabricius and nervous tissue (K u b e n a et al., 1998, B a i l e y et al., 1998).

Pathohistological examination of liver from broilers fed with T-2 toxin contaminated feed without the adsorbents, revealed massive focal disseminated necrosis and numerous focal lymphocyte aggregates, which is in agreement with the findings of numerous other authors (H o e r r et al., 1981, H o e r r et al., 1982, G r i z z l e et al., 2004). Similar finding of periportal focal necrosis and hepatocyte degeneration was found in broilers that, besides T-2 toxin, also received inorganic and organic adsorbents Minazel-plus and Mycosorb with feed. Absence of protective effects in cases of T-2 toxicosis in broilers fed with diets containing zeolite or organic adsorbent, based on the pathohistological examinations, was also noted by G a r s i a et al. (2003) and D v o r s k a and S u r a i (2001).

In the liver of broilers which were given feed with T-2 toxin and mixed adsorbent Mycofix-plus, the liver moderate hepatocytes degeneration was observed, mostly in the form of the intracellular oedema and in fewer cases, lymphocyte infiltration in portal area was present. Preventive effect of Mycofix-plus was also noted in the research of G a r s i a et al. (2003) who observed toxic effects in liver at the bile ducts epithelium in the portal area. This beneficial effect is based on the enzymatic inactivation of the 12,13-epoxide ring of the trichothecenes and flavonoligands which are some of the components of Mycofix-plus and have the role to protect liver by blocking receptors in cell membrane of hepatocytes.

In the small intestine of broilers who were fed with T-2 toxin contaminated feed, on the 21st day, degeneration and descquamation of enterocytes, as well as limphocyte infiltration between intestine glandules was observed, which is in agreement with other investigations (H o e r r et al., 1981, H o e r r et al., 1982, G r i z z l e et al., 2004). Similar finding of degeneration and descquamation of enterocytes was noted in broilers that were, besides T-2 toxin, given adsorbents, inorganic and organic.

Contrary to these results, in the small intestine of broilers fed with feed contaminated with mixed adsorbent Mycofix-plus, protective effects were noticable as in the case of pathohistological examination, only moderate enterocytes descquamation could be seen, but the lenght and the structure of small intestine villi remained normal.

Pathohistological inspection of bursa of Fabricius of broilers who were given T-2 toxin without adsorbents in feed showed atrophy of lymphoid tissue in the cortex and medulla, hereby observing vacuolisation in cortex lymphoepithelium, while lymphocyte depletion was expressed in medulla. This finding is compatibile with observations of H o e r r et al. (1981), H o e r r et al. (1982), G r i z z l e et al. (2004), F a z e k a s et al. (2000). Similarly, protective failure was noted in tissue of bursa of Fabricius in broilers who were fed

with feed contaminated with added inorganic binder Minazel-plus, as well as organic adsorbent Mycosorb. This was in accordance with conclusions of Kubena et al. (1998) and Bailey et al. (1998).

Differing from organic (Mz) and inorganic (Ms) adsorbents, which application did not show protective effects in the tissue of bursa of Fabricius from broilers who were given feed with added toxin and mixed adsorbent Mycofix-plus preserved medullar and cortex structure, with dense lymphocyte population, especially expressed through diffuse proliferation of T-lymphocytes in cortex of follicles, was observed.

The protective effect of mixed adsorbent can be explained by the fact that enzymes, de-epoxidases, partially degraded the ingested T-2 toxin by selective destruction of its toxic 12,13-epoxy group (Fuchs et al., 2002). Besides the biological constituent, Mycofix-plus contains inorganic binder with the adsorption based on the production of hidrating connection between the mycotoxin and the adsorbent, as well as flavonoligands, terpenoid complexes and fycofite components which reduce inflammation, stimulate immune response and accelerate metabolic processes.

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ИСПИТИВАЊЕ УТИЦАЈА РАЗЛИЧИТИХ ДОДАТАКА ХРАНИ НА ИНТЕНЗИТЕТ ПАТОМОРФОЛОШКИХ ПРОМЕНА КОД БРОЈЛЕРА ТРЕТИРАНИХ Т-2 ТОКСИНОМ

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Резиме

Експеримент је изведен на 160 једнодневних бројлерских пилића провенијенције "Ross", у трајању од 21 дан. Истраживања су била усмерена на омогућавање детаљнијег увида у патоморфолошке промене код бројлера изложених дејству релативно ниских доза Т-2 токсина (2 ppm), као и могућности превенције или ублажавања штетних ефеката коришћењем различитих адсорбената. Патохистолошком анализом исечака танког црева, јетре и Фабрицијеве бурзе, уочени су ефекти Т-2 токсина у свим испитиваним органима, у виду дегенеративних промена на слузници танког црева, некрозе ентероцита и хепатоцита, као и деплеције лимфоцита у Фабрицијевој бурзи. За разлику од неорганског (Minazel-plus,

Mz) и органског (Mycosorb, Ms) адсорбента чијом применом није дошло до заштитног ефекта, у ткивним исечцима јетре, црева и Фабрицијеве бурзе код бројлера који су путем хране добијали Т-2 токсин и мешовити адсорбент (Mycofix, Mf), запажа се углавном очувана структура испитиваних органа.

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EVALUATION OF THE EFFICACY OF DIFFERENT FEED ADDITIVES TO ADSORBE T-2 TOXIN *IN VITRO*

ABSTRACT: In the trial, *in vitro* HPTLC — High Performance Thin Layer Chomatography was used to determine the amount of "free", i.e. unbound or non-decomposed T-2 toxin. Mean adsorption or degradation levels of T-2 toxin in examined feed aditives, in *in vitro* conditions, ranged from 26.06 to 34.84% and did not significantly differ among used adsorbents: inorganic (Minazel plus — Mz), organic (Mycosorb — Ms) and mixed (Micofyx — Mf). All these additives showed better adsorption ability in the acidic environment (pH 3).

KEY WORDS: T-2 toxin, glucomanane, modified clinoptilolite, mycofix, HPTLC

INTRODUCTION

Trichotecenes are toxic products of secondary metabolism of several *Fusarium* fungi and over 170 are isolated and identified so far. For veterinary medicine the most important among them is T-2 toxin which is the most toxic (Humphreys, 1988). Its presence has been recorded all over the world implying that T-2 toxicoses are a very serious problem everywhere.

A new approach to mycotoxin control is to alleviate and/or prevent harmful effects of mycotoxins in feed. Different feed additives are in use today which either adsorb mycotoxins on their surface or provide enzyme degradation of mycotoxins. Efficacy of alleviating harmful effects depends mostly on the chemical structure of adsorbent, as well as on the type of mycotoxin.

Adsorbents are substances nonresorbable from the gut which can physically bind some chemicals and thus block their resorption. Mineral adsorbents are commonly in use (active charcoal, hydrated sodium calcium aluminosilicate, sodium bentonit, dietary clay and zeolites) (Tomašević-Čanović et al., 2003). The feasibility of organic adsorbent utilization was also examined, particularly that of esterified glucomanane which is isolated from

the inner layer of yeast cell wall and possesses significant capability of mycotoxin adsorption (D e v e g o w d a et al., 1996). Recently, a new type of additive has been developed which contains microorganisms with the ability to inactivate mycotoxins by enzymatic modification of their structure (F u c h s et al., 2002).

MATERIAL AND METHODS

In an *in vitro* trial, pure crystal T-2 toxin was used, *Fusarium* sp., concentration 99%, product of Biopur, Austria.

As mycotoxin binders were used:

a) modified clinoptilolite (*Min-a-zel Plus*, ITNMS Beograd) obtained by processing of clinoptilolite from Zlatokop zeolite.

b) esterified glucomanane (*Mycosorb*, Alltech USA) which is isolated from the inner layer of yeast cell wall.

c) mixed adsorbent, which contains inorganic binder, bacteria, enzymes, as well as phytogenic material extracted from plants (*Micofix Plus*, Biomin, Austria).

The *in vitro* investigation of the efficacy of different feed additives to adsorb or deactivate T-2 toxin was conducted according to standard procedure: addition of basic standard T-2 toxin solution (1 mg/ml in ethil-acetate) and 100 mg of tested additives. The contact between toxin and additives was reached in a magnetic mixer, in the buffer solution at pH3 and pH7, at 37°C during 1 h. Separation of unbound toxin remainder from the complex adsorbent-mycotoxin was done by filtration through quantitative filter paper Filtrak 391. Testing of wach feed additive was performed in 5 replications.

Determination of "free" i.e. unbound and non-degradated T-2 toxin was accomplished by TLC technique (HPTLC — High Performance Thin Layer Chromatography) according to the procedure of B a t a et al. (1983). The prepared samples were inflicted on the chromatographic panes Silikagel 60 F254 HPTLC Merck. Chromatogram development was done in the toluen-ethyl acetate-formic acid (50:40:10 v/v/v) system. Fluorescens was rised by panes splashing with 25% solution of sulfuric acid in methanol. Densitometric determining of T-2 toxin amount at 254 and 366 nm was performed with Camag TLC Scanner denzitometer with the software system Cats III.

Determination of toxin-adsorbent binding percentage was calculated from the difference between the total toxin amount that was added to the buffer solution and the amount of the unbound toxin remainder obtained by HPTLC.

RESULTS

Results received in the *in vitro* conditions are shown in Table 1.

Tab. 1 — The amount and the percentage of T-2 toxin adsorbed by different feed additives

Sample* (T-2 exp)	Amount of the unbound T-2, mg (T-2 unb), %	Amount of the bound T-2, mg, %	Average value of the bound T-2 toxin, %**
T-2 Mz, pH 7	35.24	14,56	29.24
T-2 Mz, pH 3	34.21	15.59	31.30
T-2 Ms, pH 7	34.98	14.82	29.76
T-2 Ms, pH 3	32.45	17.35	34.84
T-2 Mf, pH 7	36.82	12.98	26.06
T-2 Mf, pH 3	34.26	15,54	31.20

* Cone. T2 empiric 50 mg/sample, experimentaly determined amount 49,8 μg (T-2 exp)

** $\frac{\text{(T-2 exp — T-2 unb)}}{\text{T-2 exp}} * 100\%$

As it could be seen from the table, amounts of the adsorbed T-2 toxin did not differ significantly between the samples tested in the solutions of the same acidity. Somewhat larger adsorbance percentage was noted in the solutions with lower acidity — pH 3.

The average value of adsorbed T-2 toxin by the innorganic binder Minazel Plus (Mz) was 29,24% at pH 7 and 31,3% at pH 3. The highest adsorbtion was achieved by organic adsorbent Mycosorb (Ms) — 29,76% at pH 7 and 34,84% at pH 3. On the other hand, the lowest adsorbtion was noted in the trials with the mixed binder Mycofix (Mf) — 26,06% at pH 7 and 31,20% at pH 3.

DISCUSSION

Although an attempt was made to simulate the conditions in the gastrointestinal tract during the trial, it was impossible to reproduce the conditions existing in real system. During the experiment specific problem was the calculation of the adsorbed mycotoxin amount. Ledoux and Rottinghaus (1999) stated that in the *in vitro* trials only adsorbents with the ability to bind over 80% of mycotoxins should be taken in account.

In the performed experiments, in the solutions of the same acidity no significant differences in the adsorbed amounts of T-2 toxin were found. Higher level of adsorption was noted at the lower acidity (pH 3), when better contact between the adsorbent and toxin was reached. After the extraction of T-2 toxin by ethylacetate, layers which are easier and faster to split off were obtained and the segregated organic phase with T-2 toxin was plain giving clear spots without filth.

The obtained results indicated that T-2 toxin was highly adsorbed by the inorganic binder Minazel Plus (Mz) in *in vitro* conditions (29,24% at pH 7 and 31,3% at pH 3), as trichotecens do not possess polar functional group and natural zeolites are active only toward polar mycotoxins, especially aflatoxins. Results of Garsia et al. (2003) and Avantaggiato et al. (2005) showed that most of the tested adsorbents are not able to bind *Fusarium* mycotoxins. The reason for the relatively high level of T-2 adsorption was the

fact that adsorbent of the III generation (Minazel Plus), obtained from the processed natural zeolite with more than 80% of clinoptilolite and with balanced ratio of cations Ca/K/Na was used in the trials. By organic modification of its surface with surfactants (long-chain organic cations), i.e. changing of the surface polarity and hydrofobicity, new active centres have been created, as double layer of organic ligand which adsorbes not only polar, but also non-polar organic molecules, like T-2 toxin, while all basic characteristics of the material were preserved (Tomašević-Čanović et al., 2003).

Analysing the results regarding the adsorption of T-2 toxin by esterified glucomanane in *in vitro* conditions (29,76% at pH 7 and 34,84% at pH 3) similar conclusion was made to that reported by Raju and Devegowda (2002), Dawson (2001) and Devegowda et al. (2004) who claimed that, depending on the T-2 toxin dosage and duration of contact between the adsorbent and contaminated feed, percentage of adsorbed T-2 toxin by esterified glucomanane was 15—32%. Mycosorb exerts its protective effect through high adsorptive capacity of esterified glucomanane toward T-2 toxin and other mycotoxins, due to its large surface — aproximately 2,2 ha/kg EGM (Devegowda et al., 1996).

The obtained results showed somewhat higher level of adsorption or deactivation of T-2 toxin by Mycofix (26,06% at pH 7 and 31,20% at pH 3) as compared to *in vitro* inestigations done by Garcia et al. (2003) who noted 3,28% T-2 adsorption by Mycofix at pH 7. Biological detoxification of mycotoxins comes as a result of their degradation by enzymes or their biotransformation by interfering with whole microorganism cell or single enzyme system (Karlovsky, 1999). Besides enzyme or microbiological degradation of mycotoxins ("biotransformation") Mycofix contains adsorption component, innorganic binder, while the adsorption is based on the generation of hydratic junction between the mycotoxin and the binder.

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ИСПИТИВАЊЕ ЕФИКАСНОСТИ АДСОРПЦИЈЕ Т-2 ТОКСИНА РАЗЛИЧИТИМ АДСОРБЕНТИМА У УСЛОВИМА *IN VITRO*

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Резиме

In vitro испитивањем одређивана је количина „слободног" тј. невезаног или неразграђеног Т-2 токсина техником танкослојне хроматографије (HPTLC — High Performance Thin Layer Chromatography). Просечне вредности адсорпције или деградације Т-2 токсина испитиваним адсорбентима у условима *in vitro* нису се значајније разликовале у зависности да ли је био умешан неорганski (Minazel plus, Mz), органски (Mycosorb, Ms) или мешовити адсорбент (Micofyx, Mf) и износиле су од 26,06 до 34,84%. Сви испитивани адсорбенти су показали већу способност адсорпције овог токсина у киселој средини (pH 3).

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EFFECTS OF FERTILISERS ON WINTER WHEAT INFECTION CAUSED BY *FUSARIUM* SPECIES

ABSTRACT: Effects of fertilisers on the grain yield (t ha^{-1}) and the development of mycobiota, especially of the *Fusarium* species, in winter wheat kernels were observed. Fertilisers were applied in the following variants: T1 — 30 m^3 of slurry (pre-sowing treatment) and 25 m^3 of slurry (top-dressing), T2 — 22.500 kg of manure (pre-sowing treatment) and 220 kg of urea (top-dressing), T3 — 300 kg of urea (top-dressing) and T4 — the control without application of fertilisers.

The average wheat grain yield was the highest in the treatment T2 (6.9 t ha^{-1}), then in T3 (6.3 t ha^{-1}) and T1 (6.2 t ha^{-1}), while the lowest wheat grain yield (4.3 t ha^{-1}) was registered in the control (T4). Fungi of genera *Alternaria* (88.8—96.3%) and *Fusarium* (3.7—11.1%) were mainly isolated from all wheat kernel samples collected after harvest (T1—T4). Species of the genus *Dreschlera* were isolated in treatments T1 (0.3%) and T2 (0.2%). The species *Stemphylium botryosum* was isolated only in the treatment T1 (0.2%). *F. graminearum* (3.5—10.8%) was isolated from wheat kernels in all observed treatments, while *F. sporotrichioides* was isolated in treatments T1 (0.6%), T2 (0.5%) and T3 (0.3%). *F. poae* (0.3%) and *F. subglutinans* (0.2%) were isolated in the treatment T2, while *F. graminearum* was predominantly present in treatments T3 (10.8%), T1 (8.7%) and T2 (7.8%), and in the control T4 (3.5%). The obtained results point out that the application of urea affected the decrease in the intensity of *Alternaria* species infestation, but it also affected the increase of frequency of *Fusarium* species, especially *F. graminearum*, in comparison with the control and other treatments. Negative correlation was registered between the grain yield and the intensity of infestation of *Fusarium* species (collectively) and *F. graminearum* (individually).

KEY WORDS: N-fertilisers, slurry, manure, urea, wheat, yield, *Fusarium*, incidence

INTRODUCTION

Fusarium head blight (FHB) or scab, caused by *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schw.) Petch) is one of the most devastating diseases of wheat, barley and other cereals worldwide. The disease reduces grain yield through a number of ways including floret sterility, poor

seed filling and reduced seed size (S u b e d i et al., 2007). The infection does not only reduce yield as a result of shrunken kernels, but also reduces milling and malting quality and contaminates kernels with mycotoxins. Mycotoxins are hazardous to animal and human health. Therefore, guidelines and legislation are already in place, or under consideration, in most countries to protect consumers and animal welfare. As fusarium mycotoxins are produced within the growing crop, it is important to understand how cropping practices affect mycotoxin contamination of kernels. The evidence is provided to show the importance of a choice of cultivar, crop rotation, soil cultivation, fertiliser and the chemical and biological control of insects, weeds and fungi.

Due to the development of FHB in Serbia in years favourable for such development, as it was in 2005, the wheat grain yield loss can vary from 1.8% to 38.3%, or it is, on the average, lower by 6.9%, depending on the reaction of wheat varieties to FHB and environmental conditions at the site of observations (L e v i ć et al., 2008).

Cropping practises can also influence susceptibility to the disease. The type and application rates of fertilisers, especially nitrogen (N), can affect the disease incidence and severity in some crops for some pathogens, although it is difficult to make generalisations (R e i d et al., 2001). The nutrients in both inorganic and organic fertilisers are able to cause the incidence and severity of biotic plant diseases, pest and weed populations and their impact on the crop. The majority of information on this aspect deals with the effect of nutrients via plant. Individual elements have different roles; in general, it can be stated that they change the losses caused by pests by influencing plant resistance, alter the plant growth and in this way they change the microclimate in the stand. The increased nutrition used to be prescribed as the first measure to control plant diseases. The most important aspect of this is an increase in the ability of a crop to compensate losses. The effect of individual nutrients is very complex — the severity of one plant disease can be decreased, while that of others can be increased (V e v e r k a et al., 2007).

As it is important to understand the effect of fertilisation on the presence of the casual organism of head blight of wheat, the objective of the present study was not only to observe the effects of fertilisers on the frequency of pathogenic fungi, especially *Fusarium* species in wheat kernels sampled after harvest, but also to record wheat grain yield under the same conditions of fertilising.

MATERIALS AND METHODS

The trial wheat field was set up in 2007 on chernozem soil at the Institute for Animal Husbandry, Belgrade, Serbia. The winter wheat variety Pobeda was sown in a field that had previously been sown with maize. The experiment was set up according to the randomised complete block design with three replications. The fertiliser was applied prior to sowing in autumn 2007 and in top-dressing in spring 2008. The following four treatments were observed:

T1 — 30 m^3 of pig slurry, in the pre-sowing treatment and 25 m^3 of pig slurry in top-dressing;

T2 — 22.500 kg sheep and dairy manure, in the pre-sowing treatment and 220 kg of urea in top-dressing;

T3 — 300 kg of urea in top-dressing;

T4 — control without application of fertilisers.

The wheat grain yield at 10.7% moisture and the incidence of *Fusarium* species in wheat kernels at three fertiliser rates with a different N source, beside the control (without fertiliser) plots, were investigated. A total of 2400 wheat kernels, 600 kernels per a treatment, were observed in regard to the presence of pathogenic fungi species, especially of the genus *Fusarium*. After a superficial disinfection in sodium hypochlorite, wheat kernels were placed on a 2% agar surface, 10 kernels per Petri dish, and incubated for 7—10 days at temperature of 25°C. According to the methods developed by Burgess et al. (1994) and Watanabe et al. (1994), fungi genera were determined with a special focus on the determination of species of the *Fusarium* genus. The wheat grain yield (t ha^{-1}) was determined at harvest.

RESULTS AND DISCUSSION

The highest wheat grain yield (6.9 t ha^{-1}) was recorded when manure and urea were applied in the pre-sowing treatment and top-dressing, respectively, and then when plants were treated with urea in top-dressing (6.3 t ha^{-1}) or when slurry was applied as a split application (pre-sowing treatment and top-dressing) (6.2 t ha^{-1}). The grain yield obtained in the control (without fertilisers) amounted to 4.3 t ha^{-1}, which is statistically significantly lower than the yields obtained in treatments with fertilisers (Table 1).

Tab. 1 — Wheat grain yield (t ha^{-1}) in treatments with different fertilisers (T1—T4)

Treatments[a]	Yield (t ha^{-1})
T1	6.2**
T2	6.9**
T3	6.3**
T4	4.3**
$LSD_{0.05}$	1.165
$LSD_{0.01}$	1.765

[a] T1 = 30 m^3 and 25 m^3 of pig slurry ha^{-1}, pre-sowing treatment and top-dressing, respectively; T2 = 22.500 kg cattle manure and 220 kg urea ha^{-1}, pre-sowing treatment and top-dressing, respectively; T3 = 300 kg urea ha^{-1}, top-dressing; T4 = no fertilisers applied.

The microbiological analysis of kernels collected in the wheat plots treated with different fertilisers showed a high presence of *Alternaria* (88.8—96.3%) and a relative by high presence of *Fusarium* (3.7—11.1%) in all investigated treatments (T1—T4). The presence of *Fusarium* spp. was three-fold higher in all observed treatments than in the control treatment. The low presence of *Dreschelera* spp. in the treatments T1 (0.3%) and T2 (0.2%) and *Stemphylium botryosum* in the treatment T1 (0.2%) were determined (Table 2).

Tab. 2 — Frequency (%) of fungal species in wheat kernels in all investigated treatments (T1—T4)

Fungal species	Treatments[a]			
	T1	T2	T3	T4
Alternaria spp.	90.2	91.0	88.8	96.3
Dreschlera spp.	0.3	0.2	—	—
Fusarium spp.	9.3	8.8	11.1	3.7
Stemphylium botryosum	0.2	—	—	—

[a] T1 = 30 m^3 and 25 m^3 of pig slurry ha^{-1}, pre-sowing treatment and top-dressing, respectively; T2 = 22.500 kg cattle manure and 220 kg urea ha^{-1}, pre-sowing treatment and top-dressing, respectively; T3 = 300 kg urea ha^{-1}, top-dressing; T4 = no fertilisers applied.

The following four species of the genus *Fusarium* were identified: *F. graminearum* Schwabe, *F. sporotrichioides* Sherbakoff, *F. poae* (Peck) Wollenweber and *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas. Of the four identified *Fusarium* species, *F. graminearum* was the most frequent in all the investigated treatments (3.5—10.8%), followed by *F. sporotrichioides* in the treatments T1 (0.6%), T2 (0.5%), and T3 (0.3%) and *F. poae* (0.3%) and *F. subglutinans* (0.2%) in the treatment T2. *F. graminearum* was mainly present in the treatment T3 (10.8%), followed by T1 (8.7%) and T2 (7.8%) and its lowest presence was detected in the treatment T4 (3.5%) (Table 3).

According to the results obtained by Stanković et al. (2007), mycobiota of fungi of genera *Alternaria* and *Fusarium* are highly frequent in wheat kernels and *F. graminearum* has the highest frequency. According to data of Lemmens et al. (2004) and Heier et al. (2005) nitrogen fertilisation significantly increased the FHB severity and amounts of mycotoxin deoxynivalenol (DON) in winter wheat.

Tab. 3 — Frequency (%) of *Fusarium* species in wheat kernels in all investigated treatments (T1—T4)

Fusarium spp.	Treatments[a]			
	T1	T2	T3	T4
F. graminearum	8.7	7.8	10.8	3.5
F. poae	—	0.3	—	—
F. sporotrichioides	0,6	0.5	0.3	—
F. subglutinans	—	0.2	—	0.2

[a] T1 = 30 m^3 and 25 m^3 of pig slurry ha^{-1}, pre-sowing treatment and top-dressing, respectively; T2 = 22.500 kg cattle manure and 220 kg urea ha^{-1}, pre-sowing treatment and top-dressing, respectively; T3 = 300 kg urea ha^{-1}, top-dressing; T4 = no fertilisers applied.

This study shows that the application of nitrogen fertiliser (urea) also significantly affected the increase of the intensity of incidence of the *Fusarium* species, particularly of *F. graminearum*.

CONCLUSIONS

The application of nitrogen organic (slurry and manure) and mineral fertilisers (urea), individually or in a combination, affected the following:

— the increase of the wheat grain yield in treatments with fertilisers from 2.0 (T3) to 2.6 t ha^{-1} (T2) in relation to the control (T4);

— the intensities of infestation of species of the genus *Fusarium* in treatments with urea (T3), which were higher by 7.4, 2.4 and 1.8% than in treatments T4, T2 and T1, respectively;

— the intensity of infestation of species of the genus *Alternaria* in the treatment T3 (urea), which was lower by 7.5, 2.2 and 1.4% than in treatments T4 (control), T2 (manure + urea) and T1 (slurry, split application), respectively;

— the intensity of infestation of *F. graminearum* in the treatment T3 (urea), which was higher by 7.3, 3.0 and 2.1% u than in treatments T4, T2 and T1, respectively.

Obtained results indicate a negative correlation between the grain yield and the intensity of infestation of species of the genus *Fusarium* (collectively) and *F. graminearum* (individually).

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УТИЦАЈ ЂУБРИВА НА ИНФЕКЦИЈУ ЗРНА ОЗИМЕ ПШЕНИЦЕ *FUSARIUM* ВРСТАМА

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Резиме

У раду је проучаван утицај ђубрива на принос зрна (t ha^{-1}) и развој микобиоте зрна озиме пшенице, посебно врста рода *Fusarium*. Ђубрива су примењена у следећим варијантама: Т1 — 30 m^3 (предсетвено) и 25 m^3 осоке (у прихрањивању), Т2 — 22.500 kg стајњака (предсетвено) и 220 kg уреe (у прихрањивању), Т3 — 300 kg уреe (у прихрањивању) и Т4 — контрола без примене ђубрива.

Примена азотних органских (осока и стајњак) и минералних ђубрива (уреа), појединачно или у комбинацији, утицала је на:

— повећање приноса зрна пшенице у третманима с ђубривима од 2.0 (Т3) до 2.6 t ha^{-1} (Т2) у односу на контролу (Т4);

— већи интензитет напада врсте рода *Fusarium* у третману са уреом (Т3) за 7.4, 2.4 и 1.8% у поређењу с третманима Т4, Т2 и Т1;

— мањи интензитет напада врсте рода *Alternaria* у Т3 третману (уреа) за 7.5, 2.2 и 1.4% у односу на третмане Т4 (контрола), Т2 (стајњак + уреа) и Т1 (осока, двократна примена);

— већи интензитет напада *F. graminearum* у третману са уреом (Т3) за 7.3, 3.0 и 2.1% у поређењу с третманима Т4, Т2 и Т1.

Добијени резултати указују на негативну корелацију између приноса зрна и интензитета напада врста рода *Fusarium* (збирно) и *F. graminearum* (појединачно).

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HISTOCHEMICAL EVALUATION OF T-2 TOXIN-INDUCED CARDIOTOXICITY IN RATS: SEMIQUANTITATIVE ANALYSIS

ABSTRACT: In this study female Wistar rats were treated with T-2 toxin (1 LD_{50} 0.23 mg/kg *sc*) and sacrificed on days 1, 3, 5, 7, 14, 21, 28 and 60 after the treatment. Control groups of rats were treated by saline (1 ml/kg 0.9% NaCl). At each time-schedule, control groups of animals were sacrificed, too. Pathohistological alterations of the heart were evaluated in whole visual fields stained by haematoxylin and eosin (HE), periodic acid-Schiff's (PAS), Masson-Trichrom's (MT) and Giemsa (GIM) methods. The changes observed were scored by using semiquantitative grading scale. The heart alterations detected in T-2 toxin-treated animals ranged from focal parenchymal or hyaline degeneration (HE = 2.5 — 4.0; $p < 0.05$ vs. control) to diffuse necrosis of muscle cells (HE = 5.0; $p < 0.05$ vs. control and 1st day after T-2 treatment). The myofibrils were slightly PAS-positive during the first week of the study (PAS = 2.0 — 3.2; $p < 0.05$ vs. control and 1st day after T-2 treatment), while a diffuse distribution of glycogen granules in endo- and perimisium were observed from day 21 to 60 in the whole heart' tissue (PAS = 4.0; $p < 0.05$ vs. control and 1st day after T-2 treatment). Massive hemorrhagic foci associated with diffuse accumulation and degranulation of MCs were the most intensive from day 28 to 60 of the study (MT = 5.0; $p < 0.05$ vs. control and 1st day after T-2 treatment). During the whole study period, irregular distribution of glycogen granules, intensity and total number of haemorrhages were in correlation with the degree of heart structural lesions, which showed the higest coefficient of correlation (r = 0.8750; $p < 0.001$). Our results indicate that basic histohemical methods can be a useful tool for evaluation of T-2 toxin-induced cardiac damage, which is

probably a result of complex inflammatory mechanisms, eventually leading to vascular lesions and myocardial necrosis, as well as for some potential cardioprotectors in the future.
KEY WORDS: T-2 toxin, Rats, Cardiotoxicity, Pathohistology

INTRODUCTION

Administration of T-2 toxin to various animals produced the signs of a shock-like syndrome characterised by massive haemorrhages, immunological failure, cardiomyopathy and death (Anonymous, 2003). The exact causal mechanism of T-2 toxin-induced cardiomyopathy remains unclear. Many investigators consider its cardiotoxic effects just as results of particular myocardial structural alterations, capillary damages, haemorrhages and a focal accumulation of inflammatory cells (Borison et al., 1991; Jaćević, 2005). Its toxic effects on the plasma membrane caused an increase of membrane permeability, which eventually led to irreversible cell injury (Sherman et al., 1987). T-2 toxin also has profound effects on ribosome, sarcoplasmatic reticulum functions and mitochondrial respiration (Feurstein et al., 1985; Ueno, 1984; Pestka et al., 2004; Speijers and Speijers, 2004).

Some authors showed that pro-inflammatory action of T-2 toxin is probably the most important mechanism of its acute cardiotoxicity (Jaćević, 2005; Newton et al., 1997; Bondy and Pestka, 2000). Regarding all these facts, it seems that T-2 toxin-induced blood vessel and myocyte damages as a result of activation of a large number of mast cells (Jaćević et al., 2003).

The aim of this study was to evaluate basic histohemical methods as a useful tool for analysis of T-2 toxin-induced cardiotoxicity in rats.

MATERIALS AND METHODS

The experiment was performed on adult female Wistar rats, weighing 180—220 g (Animal House, Military Medical Academy, Belgrade). The animals were housed in plastic cages, under standard laboratory conditions (21°C, 12/12h light/dark cycle, commercial food and tap water *ad libitum*). In this study rats were treated with T-2 toxin (1 LD_{50}, 0.23 mg/kg *sc*) and sacrificed on days 1, 3, 5, 7, 14, 21, 28 and 60 after the treatment. Control groups of rats were treated with saline (1 ml/kg 0.9% NaCl). At each time-schedule, control groups of animals were sacrificed, too. Each group consisted of 8 animals. The study protocol was based on the Guidelines for Animal Study no. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Republic of Serbia). T-2 toxin used in these experiments was produced under laboratory conditions from *Fusarium sporotrichoides* fungi, cultivated on synthetic GPY (glucose 5%, peptone 0.1%, yeast extract 0.1%, pH 5.4) medium. Extraction and crude purification of the toxin were performed by filtration, while definite purification and determination of T-2 toxin content were performed by gas chromatography with electron capture detection (GC-ECD) (Romer et al., 1987). T-2 toxin was preliminarily tested on animals in order to obtain its

LD_{50} value (Litchfield and Wilcoxon, 1949). The hearts were excised after sacrification, and the samples were fixed in 10% neutral formalin for 5 days. Tissue samples were dehydrated in graded alcohol, xylol and embedded in paraffin blocks. Finally, 1-mm thick paraffin sections were stained by haematoxylin and eosin (HE), periodic acid-Schiff's (PAS), Masson-Trichrom's (MT) and Giemsa (GIM) methods. Pathohistological alterations of the heart were analysed in whole visual fields. The changes observed were scored by using semiquantitative grading scale (scoring scale of various myocardial lesions by using HE, PAS and MT staining) (Table 1). The mast cells (MCs) were counted with standard microscope.

Tab. 1 — Pathohistological grades of myocardial lesions in rats treated with T-2 toxin (scoring scale for myocardial degenerations — HE, glycogen distribution — PAS and vascular alterations — MT

Grade	Definition
0	Normal histological structure of myofibrills
1	Mild damage — Small groups of cells with early myofibrillar loss and normal nuclei (HE). Glicogen fragmentation (PAS). Oedema and hyperemia (MT).
2	Moderate damage — Groups of cells (more than 50%) with marked myofibrillar loss and cytoplasmatic vacuolisation (HE). Glicogen deposit in the cytoplasm (PAS). Small focal heamorrhages in the endomisium (MT).
3	Severe focal damage — Majority of cells with homogenisation and hyalinisation of cytoplasm with karyopicnosis and focal accumulation of inflammatory cells (HE). Large glicogen deposit on the pole of myocites (PAS). Thickening of blood vessels with vacuolisation of endothelial cells (MT).
4	Severe diffuse damage — Diffuse discoidal fragmentation of citoplasm with karyorrhexis or karyolysis and diffuse accumulation of inflammatory cells (HE). Irregular distribution of glicogen granules in the sarcoplasm (PAS). Diffuse and massive haemorrhages in the myocard (MT).
5	Tissue necrosis (HE). Glicogen accumulation in the endo- and perimisium (PAS). Heamorrhagic infiltartion of the heart tissue (MT).

Statistical evaluation was performed using commercial statistical software (Stat for Windows, R.4.5, Stat Soft, Inc., USA, 1993). In table, all results are shown as the mean (χ) ± the standard deviation (SD). Comparison of data was done by one-way ANOVA + post-hock analysis (Tuckey's test) and Pearson's test. The differences with values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered significant.

RESULTS

Myocardial alterations detected in T-2 toxin-treated animals ranged from focal parenchymal degeneration (HE = 2.5; $p < 0.05$ vs. control) or hyaline degeneration (HE = 4.0; $p < 0.05$ vs. control and 1st day after T-2 toxin treatment) to diffuse necrosis of muscle cells (Table 2).

Tab. 2 — Severity of myocardial lesions in T-2 toxin treated rats

	Treatment ($\chi \pm SD$)					
	Control (1 ml/kg *sc* 0.9% NaCl)			T-2 toxin (0.23 mg/kg *sc* T-2 toxin, $1LD_{50}$)		
Days	HE	PAS	MT	HE	PAS	MT
1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.5 a	2.0 ± 0.5 a	3.0 ± 0.5 a
3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.4 a	2.5 ± 0.5 a	3.5 ± 0.5 a
5	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	3.0 ± 0.5 a	2.8 ± 0.4 a	3.5 ± 0.5 a b
7	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	3.2 ± 0.5 a	3.2 ± 0.5 a b	4.0 ± 0.5 a b
14	0.2 ± 0.4	0.2 ± 0.4	0.2 ± 0.4	4.0 ± 0.5 a b	3.5 ± 0.5 a b	4.5 ± 0.5 a b
21	0.4 ± 0.8	0.4 ± 0.8	0.4 ± 0.8	4.5 ± 0.5 a b	4.0 ± 0.5 a b	4.5 ± 0.5 a b
28	0.4 ± 0.8	0.4 ± 0.8	0.4 ± 0.8	5.0 ± 0.5 a b	4.0 ± 0.5 a b	5.0 ± 0.5 a b
60	0.4 ± 0.8	0.4 ± 0.8	0.4 ± 0.8	5.0 ± 0.5 a b	4.0 ± 0.5 a b	5.0 ± 0.5 a b

Statistical evaluation was performed using Tuckey's test.
a — $p < 0.05$ vs control; b — $p < 0.05$ vs 1st day after treatment.
* According to scoring scale (Table 1).

Necrotic areas were most prominent in the inner part of the myocardium and in all layers of endocardium on 28th and 60th day of the experiment (HE = 5.0; $p < 0.05$ vs. control and 1st day after T-2 toxin treatment). These myofibrils were slightly PAS-positive during the first week (PAS = 2.0—3.2; $p < 0.05$ vs control), while a diffuse distribution of glycogen granules in endo- and perimisium were observed from day 21 to 60 in the whole heart' tissue (PAS = 4.0; $p < 0.05$ vs. control and 1st day after T-2 toxin treatment) (Table 2). From day 3, interstitial haemorrhages appeared uniformly in each of the sections examined, and were located in the middle myocardial or subendocardial areas. Massive hemorrhagic foci associated with a diffuse accumulation of inflammatory cell infiltrates were the most intensive from day 28 to 60 of study (MT = 5.0; $p < 0.05$ vs. control and 1st day after T-2 treatment) (Table 2). During the whole study period, irregular distribution of glycogen granules, intensity and total number of haemorrhages were in correlation with the degree of heart structural lesions, which showed the higest coefficient of correlation (r = 0.8750; $p < 0.001$) (Table 3).

Tab. 3 — Time dependent frequency of myocardial lesions — HE, distribution of glycogen granules — PAS and intensity of haemorrhages — MT in T-2 toxin-treated rats

Parameters	X variable	Y variable	Pearson (r)	Probability (p <)
T-2 (HE)	Days after treatment 0—60	Myocardial degenerations — HE	0.8750	0.001
T-2 (PAS)	Days after treatment 0—60	Glycogen distribution — PAS	0.5218	0.001
T-2 (MT)	Days after treatment 0—60	Vascular alterations — MT	0.2258	0.05

Statistical evaluation was performed using Pearson's test.

The total number of MCs had only a negative coeficient of correlation (r = — 0.329; $p < 0.01$) (Table 4). The total number of all type of MCs were the highest on the 20th day of the study (results not shown).

Tab. 4 — Time dependent frequency of myocardial lesions (HE) and total number of mast cells — GIM in T-2 toxin-treated rats

Parameters	X variable	Y variable	Pearson (r)	Probability (p <)
T-2 (HE)	Days after treatment 0—60	Myocardial degenerations — HE	0.8750	0.001
T-2 (GIM)	Days after treatment 0—60	Total number of mast cells — MCs	–0.3293	0.05

Statistical evaluation was performed using Pearson's test.

DISCUSSION

The present study demonstrates that the subcutaneous administration of a single dose of 0.23 mg/kg (1 LD_{50}) of T-2 toxin to Wistar rats results in myocardial damage, especially in massive hemorrhagic lesions and intensive degranulation of MCs, and provides new insights into the pathogenesis of these alterations. Moreover, our results point to the marked complexity of the pathogenesis of these lesions. Signs of inflammation, degeneration and rapid loss of normal cell architecture, could be found in the heart samples of T-2 toxin treated rats stained by HE method. Similar pathohistological alterations, vacuolisation and hyaline degeneration of myocytes with karyopicnosis, have been reported in the heart of humans and animals during the acute T-2 toxin poisoning (Sudakin, 2003; Jaćević et al., 2006). If the examination lasted long enough, as our experiment did, for 60 days, pathohistological changes in the heart of T-2 toxin-poisoned rats ranged from hyaline degeneration to myocardial necrosis with focal accumulation of mononuclear cells. In this period, diffuse distribution of glycogen granules were observed in PAS-positive endo- and perimisium of the whole heart' tissue. These results suggested that T-2 mycotoxin has a direct toxic effect on capillaries and increases their permeability, as well as on the plasma membrane which led to irreversible cell injury (Borison et al., 1991; Jaćević, 2005; Sherman et al., 1987). In contrast, a total number of MCs was in the correlation with cardiac damages until to day 21 of the study, and could probably contribute to the vascular damages and myofibrilar necrosis. Induction of necrosis by mast cells was suggested by the findings of increased numbers of these cells and extrusion of their granules in areas of vascular injury as early as 24 hours after the treatment with T-2 toxin (Jaćević, 2005; Jaćević et al., 2006). Tissue mast cells possess granules containing substances that can have profound effects on vascular integrity. The release of inflammatory mediators (e.g., histamine) and pro-inflammatory cytokines (IL-1a, IL-1b, IL-6, IL-8, and TNF-a) by mast cells is thought to be involved in vascular permeability changes, induction of leukocyte-endothelium interactions, and acute vascular inflammation (Galli, 1993; Gavrisheva and Tkachenko, 2003). Thus, it seems likely that all of the factors cited here could contribute to the myocardial necrosis and vascular lesions induced by T-2 toxin.

CONCLUSION

Our results indicate that basic histohemical methods can be a useful tool for evaluation of T-2 toxin-induced cardiac damage, which is probably a result of complex inflammatory mechanisms, eventually leading to vascular lesions and myocardial necrosis, as well as for some potential cardioprotectors in the future. Further morphological investigations are needed in order to elucidate further these processess.

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ХИСТОХЕМИЈСКО ИСПИТИВАЊЕ КАРДИОТОКСИЧНИХ ЕФЕКТА КОД ПАЦОВА ТРЕТИРАНИХ Т—2 ТОКСИНОМ: СЕМИКВАНТИТАТИВНА АНАЛИЗА

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Резиме

У овом раду су испитани токсични ефекти на срцу Wistar пацова акутно трованих Т-2 токсином. Животиње, једнократно третиране Т-2 токсином у дози од 0,23 mg/kg *sc* (1 LD_{50}), жртноване су 1, 3, 5, 7, 14, 21, 28. и 60. дана после апликације отрова. Контролне групе животиња третиране су физиолошким раствором (1 ml/kg 0,9% NaCl) и жртноване у истим временским интервалима. Процена патохистолошких промена извршена је на узорцима ткива срца, бојених стандардним хистохемијских методама: хематоксилин и еозин (HE), Гимза (GIM), перјодна киселина Schiff-ов реагенс (PAS) и Masson trichrom (MT), применом семиквантитативне анализе. У срцу пацова третираних Т-2 токсином уочене су промене од фокалне паренхиматозне и хијалине дегенерације миофибрила (HE = 2,5—4,0; $p < 0,05$ у поређењу са контролом) до фокалне или дифузне некрозе мишићних ћелија (HE = 5,0; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина). Током прве недеље испитивања миофибриле су биле благо PAS-позитивне (PAS = 2,0—3,2; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина), док је дифузна дистрибуција гранула гликогена у ендо- и перимизијуму запажена од 21. до 60. дана (PAS = 4,0; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина). Масивна хеморагична поља, окружена многобројним инфламаторним ћелијама, нарочито су изражена у периоду од 28. до 60. дана испитивања (MT = 5,0; $p < 0,05$ у поређењу са контролом и 1. даном после апликације Т-2 токсина). Током целог периода испитивања, неправилна дистрибуција гранула гликогена, интензитет крварења и укупан број мастоцита су били у корелацији са степеном оштећења ткива срца ($r = 0,8750$; $p < 0,001$). Добијени резултати су потврдили раније изнету тезу да су кардиотоксични ефекти Т-2 токсина вероватно резултат комплексних инфламаторних механизама.

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THE FREQUENCY OF OCCURRENCE OF AFLATOXIN M_1 IN MILK ON THE TERRITORY OF VOJVODINA

ABSTRACT: Aflatoxin is one of the most common mycotoxins which can be found in milk. It represents a natural metabolite of aflatoxin B_1 that occurs as a result of animal metabolism and the body's attempt to detoxificate it. It is excreted in milk, feces and urine of animals that consumed contaminated feed with aflatoxin B_1. The carry-over from feed to milk depends on many factors, ranging from 0.3 to 6.2%. Aflatoxin M_1 is in the first group of carcinogens according to the IRAC classification from 2002, but it is considered to have only 10% of carcinogenicity from its precursor aflatoxin B_1. Legislation in member countries of European Union for this mycotoxin in milk intended for people is 0.05 μg/l, while the rest of the countries that also have legislation for this mycotoxin allow the concentration that is ten times higher, and that is 0.5 μg/l.

In this paper, we have tried to provide on insight into the quality of milk, food often consumed by children, from the standpoint of mycotoxicology, and to compare the obtained data with data available from literature, from countries in the region that have similar climatic and agricultural conditions. From a total of 65 samples of processed milk, aflatoxin M_1 was found in 18 samples and none of the samples exceeded the level of 0.05 μg/l, which is allowed by the legislation of the European Union.

KEY WORDS: aflatoxin M_1, milk

INTRODUCTION

Mycotoxins are large group of compounds, secondary metabolites of fungi, in which aflatoxins are one of the most frequent and most toxic. The first time aflatoxins were heard about was in 1961, when Blount described Turkey "X" disease (B l o u n t, 1961), which is now known to be caused by aflatoxin B_1 and most likely cyclopiazonic acids. Since that time, a golden era studies of mycotoxins as possible contaminants of food for people and animals begins. Mycotoxins rarely act acutely as toxins, and are usually found in small quantities in food for people or animals. Since their action chronic in character, they are often called the silent killers.

Aflatoxins usually generate fungi from the genus *Aspergillus*, most often *A. flavus* and *A. parasiticus*. Aflatoxin M_1 is 4-hydroxy derivative of aflatoxin

B_1, which occurs in the body of animals during metabolism in the liver. Cytochrome responsible for this biotransformation is the CYP450, which is accompanied with two dominantly present isoforms, CYP3A4 and CYP1A2. Both isoforms of enzyme catalyse biotransformation of aflatoxin B_1 in high reactive form of exo-aflatoxin B_1 8,9-epoxide (G u e n g e r i c h, 1998), and later in aflatoxin M_1. Aflatoxin M_1, according to the classification of IRAC (IRAC, 1993), was first classified in the second group of carcinogens (2B), but in the year 2002, the same organization classified it in the first group of carcinogens (IRAC, 2002). All aflatoxins, as well as aflatoxin M_1, are very stable compounds during processing and do not reduce significantly. Even more, during the production of cheese and other milk products it comes to the multiple increase of the amount of aflatoxin M_1 (depending on the type of cheese coefficient the increase ranges for soft cheeses from 2.5 to 3.3, and from 3.9 to 5.8 for hard cheeses). Average consumption of milk and milk products in the European Union per day is about 340 g per person, while the average consumption of milk is 290 g (H e n r y S. H. et al., 2004).

It is believed that the rate of samples with aflatoxin B_1, and the aflatoxin M_1 in Europe, due to climate conditions (for fungi of the genus *Aspergillus*, the most appropriate regions with subtropical and tropical climate) are relatively poorly represented, and that aflatoxin B_1, found in food for people and animals, comes mainly from food imported in the European countries from the subtropical and tropical regions. However, recent data from the EFSA (European Food Safety Authority) publications (EFSA, 2004) raised serious doubts. In the area of North Italy, due to unfavourable climate factors (rotation of drought and rainy interval) and insect damage caused contamination of corn with significant concentration of aflatoxin B_1, which resulted in increased aflatoxin M_1 in milk in the amount which exceeds allowed level of 6%, and that percentage increased to 7.3% at the end of the same year. The usual practice in the countries of the European Union is monitoring of milk and milk products for the presence of aflatoxins M_1. Legislation of this toxin in the European Union is one of the most restrained legal regulations in the world and is 0.05 mg/l (Commission Regulation (EC) N. 466/2001), while other countries that also have legislation for this mycotoxin (including Serbia) allow the value that is ten times higher and amounts 0.5 μg/l.

MATERIALS AND METHODS

All 65 samples were taken from September to December 2008. Method used to determine aflatoxin M_1 combined clean up process with immunoaffinity columns and TLC determination (G r o s s o et al., 2004; S h u n d o L. and S a b i n o M., 2006). The milk samples were centrifuged for 15 min and the upper fat layer was discarded. The skimmed milk (100ml) was passed throught an immnuoaffinity column (AFLAPREPM$_1$ R-Biopham, Rhone Ltd). Column was washed with distillated water (40 ml). Bound aflatoxin M_1 in the immunoaffinity column was released by the elution with 2.5 ml acetonitrile--methanol (3:2; v/v) and 2.5 ml methanol, the elute was evaporated to dryness

using rotary evaporator. Concentrate of AFM_1 was resuspended in 1 ml acetonitrile and evaporated again. The last concentrate was resuspended in 200 ml toluene acetonitrile (9:1; v/v).

In accordance with the TLC procedure, concentrate samples from 50 and 100 ml were used and TLC plate samples were applied (TLC aluminum sheets. 20x20 cm, Silica gel 60). The plates were developed in chlorophorm: acetone:isopropanol (87:10:3; v/v). After the plate had dried, it was read under long wave (366nm) light. The concentration of AFM_1 was determined by taking into account the spots of samples and spots of standards.

RESULTS

A total of 65 samples of commercial milk brought into the local markets from different manufacturers (a dairy), and with different content of milk fat (fat content of milk varied from 0.9% to more than 3.2%, depending on the characteristics of the producer) were analysed. From a total of 65 analysed samples, 34 samples were pasteurized milk, while the 31 samples of milk were long-term UHT milk. In eighteen milk samples, different concentrations of aflatoxin M_1, were found while in other samples content was less than the limit of detection (0.01 mg/l). None of the samples exceeded the allowed concentration of 0.5 μg/l, but also a single sample is not exceed allowed level by the legislation of European Union (0.05 μg/l).

Tab. 1 — Overview of the analyzed samples of pasteurized and UHT milk

Type of milk	< 0,01 μg/l	od 0,0125—0,05 μg/l	> 0,05 μg/l	Σ
pasteurized	27	7	0	34
UHT	20	11	0	31
Summary	47	18	0	65
Percent	72.31	29.69	0	100

As seen in Table 1, from a total of 65 samples of analyzed commercial milk, in 18 samples (269,69%) certain amount of aflatoxin M_1 was detected (ranging from 0.01 mg/l to 0.03 mg/l). The mean value for aflatoxin M_1 was 0.014 mg/l.

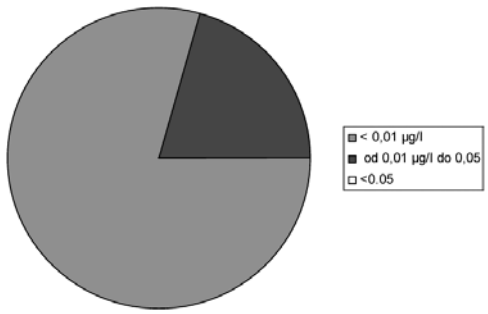

Fig. 1 — Graphically presented results

DISCUSSION

There are a lot of accessible data on the content of aflatoxin M_1 in milk and milk products from the countries of the European Union, because there are long-term monitoring on the presence of this mycotoxin, however, there is little or no data about this problem on the territory of Serbia or even beyond, in the territory of former Yugoslavia. From 11,831 processed samples from the territory of the European Union, 280 were from individual small farms, the concentration of aflatoxin M_1 in all the samples greater than 0.05 μg/l was detected in only 0.06% of samples, which indicates that the incidence of this mycotoxin are extremely low (EFSA, 2004). Countries in the region that are not members of the EU, which implemented some kind of monitoring, have higher values of concentration aflatoxin M_1 in milk and milk products then the member countries. In the study taken in Albania on samples of milk from individual farms, 42% of samples had lower value then 0.05 μg/l, while 58% of samples had higher concentrations of aflatoxin M1. Maximum concentration found was 0.85 μg/l (Panariti, 2001). The study made in Turkey in the UHT milk showed that 47% of samples exceeded the allowed limit of the EU. The average value found was 0.108 μg/l (Unusan, 2006). We must also take into account the data taken from the EU before introduction the regulation that sets 0.05 μg/l as a limitin 2003. Data from work Dragacci & Frémy, 1993 in which results of 15-year observation was summarized, regarding milk quality in France over period 1978—1992, there were two periods of high contamination with aflatoxin M_1 in milk, the first was 1978—1984 and another in the winter 1988—1991 year. Very contaminated samples, were found, some of reached 5 mg/kg aflatoxin in M1 (in the period 1978—1983). In the north of Italy in the period of 2003 surprisingly high concentration of aflatoxin B_1 in food for feeding milk cows were found resulted in higher concentration of aflatoxin M_1 in milk (EFSA, 2004). As a result of the situation in the country, strategy of surveillance and control was erected which is already near timeframe and so far has given towards the good results reduction of the percentage of contaminated samples of food and milk (Decastelli et al., 2006). Also, in the analysis of the results from this work, it should be noted that the samples analyzed were commercially available milk products, which means that this milk was produced from merging milk from a large number of producers, thus the contribution of the singular manufactures to the aflatoxin burden of milk products was masked. It is not surprising to obtain such results, since our previous work showed that 28% of milk samples collected from individual forms, exceeded the maximum concentration allowed for aflatoxin M_1 (Polovinski et al., 2008).

CONCLUSION

Although the results presented in this paper are encouraging, from the standpoint of mycotoxicological quality of milk in our market, it is certainly of general interest to introduce system of regular monitoring in order to always

have available data. The variation in the content of aflatoxin B_1 and M_1 are large and depend on the year and even within one year there may be significant variations depending on many factors. As a country which is clearly aimed at joining, establishing systems of monitoring to achieve better food and feed quality is a significant determinant.

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УЧЕСТАЛОСТ ПОЈАВЉИВАЊА АФЛАТОКСИНА M_1 У КОНЗУМНОМ МЛЕКУ НА ТЕРИТОРИЈИ ВОЈВОДИНЕ

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Резиме

Афлатоксин M_1 је један од најчешћих микотоксина који може да се јави у млеку. Представља природни метаболит афлатоксина B_1 који настаје као последица метаболизма животиње и покушаја организма да детоксикује афлатоксин B_1. Излучује се путем млека, фецеса и урина животиња које су конзумирале храну контаминирану афлатоксином B_1, проценат преласка зависи од многобројних фактора и креће се од 0.3 до 6.2%. Афлатоксин M_1 се убраја у прву групу карциногена према IARC класификацији из 2002. године, али се сматра да поседује само 10% од канцерогености свог прекусора афлатоксина B_1. Законска регулатива у земљама чланицама Европске уније за овај микотоксин у конзумном млеку намењеном за исхрану људи износи 0.05 μg/l, док у осталом делу земаља које поседују законску регулативу за овај токсин (у које спада и Србија) дозвољена концентрација је десет пута већа и износи 0.5 μg/l.

У овом раду смо покушали да дамо увид у стање квалитета млека, као животне намирнице коју чешће конзумирају деца, са становишта микотоксикологије, и упоређивањем добијених података са подацима приступачним из литературе, из земаља у окружењу са сличним климатским и пољопривреним условима. Од укупно 65 обрађених узорака млека, у 18 узорка је пронађен афлатоксин M_1, и ни у једном узорку није премашивао дозвољену количину по законској регулативи Европске Уније од 0.05 μg/l.

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MYCOTOXIC PORCINE NEPHROPATHY AND SPONTANEOUS OCCURRENCE OF OCHRATOXIN A RESIDUES IN KIDNEYS OF SLAUGHTERED SWINE

ABSTRACT: In order to find information on the occurrence of mycotoxic porcine nephropathy in Serbia, during a six month period (2006/2007) samples of kidney from individual healthy slaughtered pigs were collected (n=90) and analyzed by HPLC for ochratoxin A. In addition, histological examinations were carried out. The incidence of OTA in kidney was 33,3% and varied between 0.17—52.5 ng/g. Histopathological examination of kidneys confirmed tubulopathies with oedema and cell vacuolization. In addition, hemorrhages and necrosis of proximal kidney tubulesž cells were found. These findings indicate that it is likely that most of the kidney injury is related to ochratoxin A and other nephrotoxic compounds which enhance the toxicity of OTA.

KEY WORDS: Ochratoxin A, pig, nephropathy

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by several species in the *Aspergillus* and *Penicillium* genera. It is detected widely as a contaminant of agricultural commodities, especially cereals (20). As cereals are widely used in animal feed, and because OTA is relatively stable *in vivo*, whence it is further transmitted to animal edible tissues (8), especially in kidney and liver. Major target for the toxicity of ochratoxin A in mammalian species is kidney, where the toxin primarily affects the proximal convoluted

tubules (2, 14, 17, 18). OTA has been causally associated with nephropathy in pigs and poultry. Among farmed animals, pigs are particularly sensitive to OTA. This mycotoxin plays a special role in the genesis of swine mycotoxic nephropathy, a common disease in Scandinavia (10, 16, 23). It exhibits also immunosuppressive, teratogenic, nephrotoxic and genotoxic properties in several animal species (12).

Occurrence of OTA has been recognized as a potential human health hazard and OTA contamination is of public health significance since it is associated with Balkan nephropathy, a kidney disease in humans (4) described in several rural regions of Bulgaria, Romania, Serbia, Croatia, and Bosnia, and associated with an increased incidence of tumors of the upper urinary tract. However, causality has not yet been established. Special attention has been paid to OTA since 1993, when the International Agency for Research on Cancer classified this toxin as a possible human carcinogen (group 2B) (24). Human exposure to OTA can occur directly by consumption of food containing the toxin, or indirectly by consumption of animal tissue exposed to contaminated materials (8).

Up to now, there has been little published information on the occurrence of porcine nephropathy and content of ochratoxin A in kidneys of Serbian slaughtered pigs. Therefore, the aim of this paper was to evaluate the natural occurrence of porcine nephropathy and OTA content in kidneys from healthy slaughtered pigs originating from different regions of Serbia. Also, the purpose of this paper is to briefly review risk assessments of OTA in order to highlight the critical issues that these assessments have identified.

MATERIALS AND METHODS

Reagents

OTA were purchased from Sigma-Aldrich Chemie GmbH. A working standard OTA for HPLC containing 25 ng/mL methanol was prepared daily just before starting the injection of a series of samples. Other reagents were HPLC grade. All other chemicals were reagent grade or chemically pure.

Sample collection

During six month period (September 2006/Februar 2007), sample of kidney per animal was collected from healthy slaughtered pigs (n = 90) originating from three different regions of Serbia. Slaughtered pigs were randomly sampled in the slaughterhouse during meat inspection, and whole kidneys were sampled from each pig. The samples were homogenized and stored at –18°C before analysis. No preservatives were added.

Microscopical examination: Kidney samples were fixed in 10% neutral buffered formalin and absolute alcohol for 5 to 7 days, processed by routine

methods, sectioned at 5—8 μm, and stained with hematoxylin and eosin (HE) for light microscopy.

Extraction and clean-up for ochratoxin analyses

Kidney Analyses were performed by the method of Matrella et. al (2006) (21), which briefly includes double extraction with acidic ethyl acetate. The organic phase was removed and extracted with 0.5M $NaHCO_3$ pH 8.4. The organic phase was evaporated to dryness under N_2 steam, reconstituted in 150 μl mobile phase and a 20 μl aliquot was injected. The detection limit for OTA in organs was 0.01 ng/g with a 61% mean recovery from artificially contaminated samples at 3 ng/g.

Chromatographic conditions (HPLC)

An aliquot of 50 μl for kidneys samples were injected into Waters Symmetry Shield RP 18, high pressure liquid chromatography column (length and inner diameter 150x4,6 mm, particle size 5 μm, of the HPLC system (Waters Alliance). The column was eluted with 4% acetic acid and acetonitrile (32:68 v/v) at 25°C and a flow rate of 1 mL/min. Measurements were performed by fluorescence detection at wavelengths of 334 nm (excitation) and 460 nm (emission) gain 10.

Statistical analysis

Descriptive statistics of the data set were performed with a standard programmed and included arithmetic mean, standard deviation, coefficient of variation, minimum and maximum. Statistical differences in the mean levels of OTA contamination across the three groups of positive samples were determined by one-way ANOVA ($p < 0.05$).

RESULTS AND DISCUSSION

The incidences and mean values of ochratoxin A in swine kidney and the results of pathomorphology examination are summarized in Table 1 and Figure 1 and 2.

Ochratoxin A in Kidney

The incidence of OTA in kidneys was 33.3% in the range 0.17—52.5 with the mean level 1.26 ng/g. The majority of samples (16.6%) contained OTA at low levels (0.01—1.0 ng/g). The concentrations in ten samples (11.1%) ranged between 1—5 ng/g, while ochratoxin A in five (5.5%) samples was

greater than 5 ng/g. In regard to regional distribution of OTA, the occurrence of OTA among the three regions whence samples were collected are almost similar and varied between 26.6% (region Vladimirci) and 36.6% (region Senta and Bogatić) (Table 1), but the mean level of contamination is different. The highest OTA level (52.5 ng/g, mean 2.20 ng/g), with the highest coefficient of variation (4.33) was found in the samples originating from region Bogatić. In 2.2% samples of kidneys, OTA levels were considerably higher and greatly exceeded the permissible levels for this toxin established in Serbia, including those proposed by the SCF (1998) (27), and JECFA (2001) (10 ng/g) (13).

Tab. 1 — Incidence of Ochratoxin A in kidney of slaughtered pigs

Region	N	n (%)	ng/g		
			$\bar{X}$±Sd	C.V. %	Range
Vladimirci	30	8 (26.6)	0.42±1.2	2.96	0.18—6.5
Senta	30	11 (36.6)	1.14±3.3	2.89	0.17—17.0
Bogatić	30	11 (36.6)	2.2±9.54	4.33	0.26—52.5
Total	90	30 (33.3)	1.26±5.85	4.64	0.17—52.5

N — total number of analyzed samples, n — number of positive samples, X — arithmetic mean (conc. below LOD are regarded as zero), C.V. — coeff. of variation.

Comparison of data obtained in this trial, along with theother recently published data for the occurrence of OTA in pig edible tissues, shows that the found levels are comparable with levels in other European countries or Canada (5, 7, 9, 23). The incidence of OTA in kidneys was 33.3% and almost comparable with the incidence reported by Koller (1991) in Austria (41.9%) or by Gareis and Scheuer (1999) in Germany (41.9%), and in Serbia (41.6%) (22). The mean level (1.26 ± 5.85 ng/g) was lower than those reported in Austria (2.5 ng/g) (15) and recently published data in Serbia (3.12 ng/g) (22), but higher then the mean level found in Germany (0.43 ng/g) (8).

Patomorphology Examination

Gross pathology

All 90 pigs were slaughtered during the study period. Kidneys submitted to the laboratory were pale, swollen and enlarged and changed in colour from normal mahogany to tan, as follows: 43 (47.7%) had "mottled or pale kidneys", 27 (30%) had enlarged kidneys and 11 (12.2%) were smaller than normal. The only macroscopic lesions observed in few cases were small grey--white foci on the kidney surface. No obvious difference was observed between the right and left kidney. No significant changes were seen in other organs.

Histological examination

Histological findings of renal tissues and incidence of ochratoxin A in kidneys from slaughtered pigs are summarized in Figure 1 and 2. Histological examination showed two types of changes: degenerative — affecting epithelial cells in some proximal tubules of pigs, and proliferative changes in the interstitium. The major renal histopathological changes were in the epithelium of proximal tubules (Fig. 2). Dystrophy, (moderate to obvious degenerative changes, Fig. 1A), swelling, vacuolization and lipophilic degeneration, were the main changes in the tubular epithelial cells. The majority of glomeruli exhibited mild or moderate exudates in the Bowman's capsular spaces as well as hypercellularity of vascular loops. In addition vascular changes expressed as a hyperaemia of blood vessels, moderate to marked hemorrhages of some renal cortical regions occurred occasionally (Fig. 1D). In the interstitium of the renal cortical regions, there was limited proliferation of connective tissue (Fig. 1B) and focal infiltration of mononuclear inflammatory cells which was sometimes

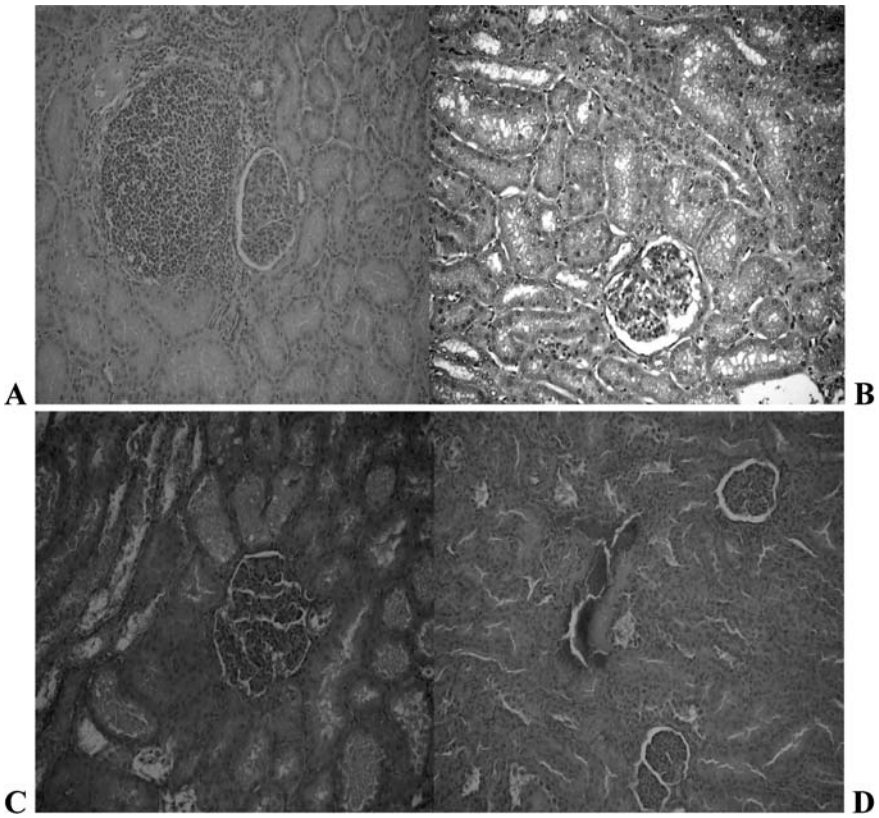

Fig. 1 — Dystrophy and vacuolar degeneration in the epithelium of proximal tubules' cells (A), Focal interstitial fibrosis (B), Necrosis of proximal tubules' cells (C) and Hemorrhages in cortex (D)

accompanied by small granulomas. OTA analysis of the kidney samples with degenerative changes of moderate to marked cloudy swelling revealed the incidence of OTA in 32.2% samples at concentration levels up to 52.5 ng/g (Fig. 2). Additionally, vascular changes, as well as fatty changes were observed in six kidneys of pigs in which ochratoxin A was detected up to 6.5 ng/g, while focal interstitial fibrosis and necrosis of proximal tubules' cells were only seen in one pig kidney in which ochratoxin A was detected up to 52.5 ng/g. The lesions produced by higher OTA levels were more severe and widespread, including degeneration, atrophy, glomerular swelling and sclerosis and interstitial nephritis (Fig. 2).

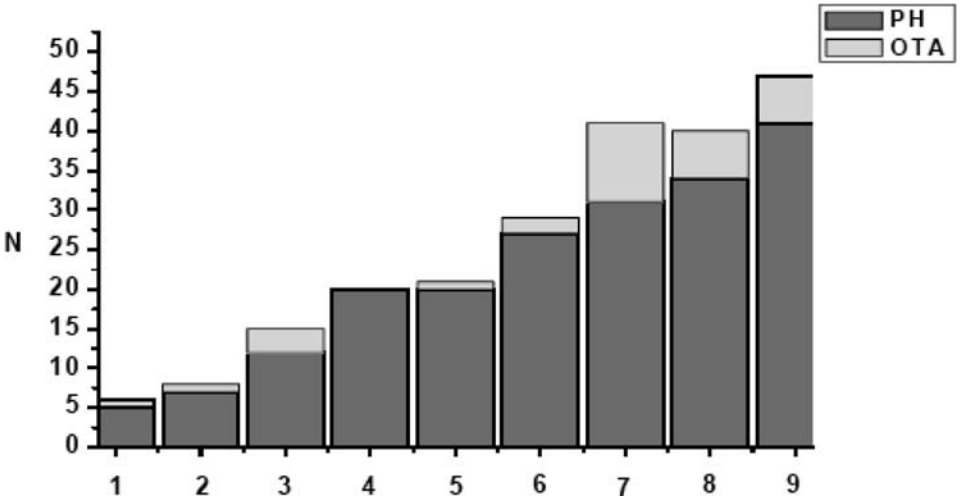

Fig. 2 — Summary of histological findings (PH) of renal tissues and incidence of OTA in kidney from slaughtered pigs (n=90). Necrosis of proximal tubules' cells (1), hypercellularity of vascular loop (2), vascular changes (3), exudates in Bowman's space (4), focal interstitial nephritis (5), dystrophy of proximal tubules' cells (6), swelling of proximal tubules' cells (7), renal hemorrhages (8), fatty changes of proximal tubules' cells

The macroscopic and microscopic changes observed in our study were more similar to those reported by other papers (28, 29, 30). These findings confirm that high affinity of OTA towards serum proteins, allows its accumulation in the organs of animals (11). Kidney is the main target of OTA. This high susceptibility of kidney is, at least partly, the result of OTA-toxicokinetics. Renal blood flow per tissue weight is extremely high, which results in the delivery of relativly large amounts of OTA in comparison to other organs. Furthermore, free OTA is secreted in the proximal tubule and subsequently reabsorbed, mainly in the proximal straight tubule, the thick ascending limb of the loop of Henle and the collecting duct (1, 6, 25, 27). The inhibition of protein synthesis and damaged energy production in the mitochondria could be considered as the most important factors for degenerative changes in the epithelial cells of proximal tubules where ochratoxin A was detected. While agreeing that the most important toxicological target of OTA in a pig is kidney, the principal descriptions of the pathology of MPN vary considerably with respect

to some other details, and according to the dosing regime and the duration of OTA exposure. Enlarged kidneys are indicative of renal inflammation and proliferative lesions following chronic exposure to OTA (28, 30). Therefore, it seems that MPN observed in Serbia may have a multitoxic aetiology because it cannot be explained by the concentration of OTA alone. The lack of a strong correlation among histopathological changes and incidence of OTA in kidney (33.3% kidney samples were positive, at levels ranging up to 52.5 ng/g) found in our trial might thus explain the result of OTA-toxicokinetics, as well as possible synergism between OTA and other nephrotoxic mycotoxins or compounds which enhance the toxicity of OTA. Such synergism between OTA and other mycotoxins under field conditions may be responsible for the MPN in Serbia, which is associated with relatively low mean contamination by OTA in feed. The production of multiple toxins by one or several fungi, which is sometimes completely normal, presents a problem that has not been sufficiently investigated.

However, it should be remembered that when comparing data factors such as climate conditions during harvest, practices for grain/feed storage etc have influence on the ochratoxin A level in edible tissues. The data obtained in this trial show that there should be more concern for the livestock industry.

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МИКОТОКСИЧНА НЕФРОПАТИЈА СВИЊА И ЗАСТУПЉЕНОСТ РЕЗИДУА ОХРАТОКСИНА А У БУБРЕЗИМА ЗАКЛАНИХ СВИЊА

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Резиме

У циљу стицања сазнања о заступљености микотоксичне нефропатије свиња и утицаја ОТА на врсту и локализацију патоморфолошких промена на бубрезима, на линији клања током ветеринарско-санитарног прегледа редовно закланих свиња пореклом са фарми из Војводине и Србије, узимани су узорци бубрега за анализу. Током шестомесечних испитивања укупно је анализирано 90 узо-

рака бубрега закланих свиња. Присуство резидуа ОТА у узорцима бубрега пореклом из испитиваних региона утврђено је код 33,3% испитаних узорака, у количини од 0,17 до 52,5 нг/г. Заступљеност резидуа ОТА била је највећа у узорцима бубрега пореклом са локалитета Сента и Богатић (36,6%), док је највећи просечан садржај резидуа ОТА (–2,2 μg/kg) утврђен у узорцима бубрега пореклом са локалитета Богатић. Патохистолошким прегледом бубрега најчешће су утврђене тубулопатије са едемом и вакуолизацијом ћелија. Такође, утврђене су хеморагије и некроза ћелија проксималних бубрежних тубула.

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MYCOTOXINS IN WINE WITH SPECIAL ATTENTION ON OCHRATOXIN A

ABSTRACT: Wine quality is a complex, multi — layered conception consisting of numerous factors such as sensory characteristics, chemical composition, legislation, market — consumer, with hygienic — toxicological factor being of special importance due to growing demands for health safe foods. This paper shows the results of studies carried out up till now concerning the mycotoxins in wine (Aflatoxins, Trichothecens, Patulin), with special attention paid to ochratoxin A, most frequently present in grapes, must and wine, and to the influence of certain technological operations and processes during wine making. Due to its high toxicity, the presence of ochratoxin A has been limited to 2 μg/l by EU EG regulation 123/2005.

KEY WORDS: wine quality, mycotoxins, Ochratoxin A

INTRODUCTION

During the last fifteen years, there has been a constant interest in the quality of food products, so wine is also often the subject of expert — scientific discussions. Wine quality is a very complex and multy — layered concept, considering the numerous factors it comprehends. Total wine quality, as an integral concept represents overall of individual qualities, and their exact determination is the only way for their comprehension and evaluation. It could be said with certainty that wine quality factors are its sensory characteristics, chemical composition, hygienic — toxicological, market — consumer factors, as well as legislation. Some of these factors, such as chemical composition and hygienic — toxicological represent so called "internal" quality, while sensory characteristics represent "external" quality perceived by senses. Apart from these, which can not be separated from wine, there are factors that do not participate directly in the structure of total wine quality, but show an indirect influence (Jovic, S., 1993; Jovic, S., Kovac, V., 1995).

With emphasised demands for health — safe food, meaning the absence of undesirable compounds and pollution agents, hygienic — toxicological quality factor is gaining importance. Some pollution agents get into wine from the outside (heavy metals, pesticides, radionucleides), while certain undesirable compounds are a consequence of activity of yeasts, bacteria or mould on grapes or in must and wine (biogenic amines, ethylcarbamate, higher alcohols, methanol and mycotoxins). Therefore, it is a constant obligation of wine producer to enrich wine with compounds that have positive influence on health and prevent formation of the harmful ones, by applying current scientific knowledge. Having this in mind, it is also a constant task of oenology to establish possible presence of certain mycotoxins in wine, stop their occurence in wine and find the way to reduce their quantity below the allowed maximum. This is, at the same time, an effort to ensure the wine keeps its epithet "The healthiest and most hygienic drink" given by the famous French chemist and biologist Luis Pasteur.

BASIC FACTS ON MYCOTOXINS

Mycotoxins are secondary products of mould metabolism that are toxic for humans, animals and plants in very small quantities, but not for micro — organisms that produced them. They are mainly low molecule, thermo — stabile compounds which, by their chemical disposition, belong to various groups of compounds. Some mycotoxins remain in mould mycelium as endotoxins, while others, such as ectotoxins, get discharged into the surrounding environment. Unlike toxins of higher plants, for a long time there has been little knowledge about mycotoxins as toxic products of hiphomycetes. Although, as far as 1980, Japanese pathologists had the knowledge about certain species of *Penicillium* that appeared on rice and produced harmful for humans. It was not until the discovery of aflatoxins in 1960. that the studies on mycotoxins started to get into full swing. Certain mycotoxins differ, among other things, in demonstrating acute or chronic toxicity. The chronic one shows carcinogenic, mutagene and teratologic actions. Diseases caused by mycotoxins are called mycotoxicosis (M ü l l e r, 1983). From about 300 known mycotoxins the most important are: aflatoxins, citrinin, patulin, ochratoxin A, fumonisin, trichohecene (dezoxynivalenol, nivalenol, T_2 — toxin, HT_2 — toxin) and zearalenone.

Mycotoxins can appear on plants and their yield, prior to harvest or after it, if the storage conditions are bad. Foodstuffs can get contaminated by mycotoxins during different phases of food chain if mould of species *Aspergillus*, *Fusarium* and *Penicillium* appear and multiply, as they are considered to be the main producers of mycotoxins. Some estimates show that as much as 25% of world products of plant and animal origin is contaminated with mould so, apart from the economic losses, there is always a danger of intoxication with mycotoxins.

First studies on mycotoxins in oenology dealt with aflatoxins at first, and then with patulin and trichotecene, but, since 1996, they have been focused mainly on ochratoxin.

FORMER STUDIES ON MYCOTOXINS IN OENOLOGY

Aflatoxins were the first mycotoxins in oenology to be studied, most probably because they are the most toxic ones, and their analytic proving methods were the first to be developed. Aflatoxins (B_1, B_2, G_1 and G_2) are produced exclusively by mould *Aspergillus flavus* and *Aspergillus parasiticus* types which require higher temperature and air humidity for their development. Aflatoxin B_1 is the main toxin which often appears together with B_2 or those from G group. The food most frequently contaminated by aflatoxins is: peanuts, hazelnuts, almonds, pistachios, wallnuts, dried fruits and spices. If stock — feed contains these toxins, aflatoxin M_1 can be found in milk and milk products. Aflatoxin B_1 is one of the strongest carcinogenes which causes liver tumor. In comparison to B_1, aflatoxin M_1 is somewhat less toxic and has far less influence on appearance of cancer.

At first, Schuller et al. (1967) made tests with 33 types of wine and aflatoxin content that was under 1 μg/l was found in two samples, but according to Franc and Eyrich (1968) some other compounds, with their fluorescence can simulate the presence of aflatoxins. Radler and Theis (1972) analised 215 samples of mouldy grapes and did not find *Aspergillus flavus*, mould that produces this toxin in any of them. Drawert and Barton (1974) did not find aflatoxins in 17 white wines from Rheinpfalz, neither did Lemperle et al. (1975) in 150 samples of Baden wines from 15 different vintages.

According to the former researches, aflatoxins have not been found on healthy or mouldy grapes, nor on grapes with noble rot (*Botrytis cinerea*). Also, their presence has not been proved in wines of all quality categories, in marc or enzyme preparations used in wine technology.

Patulin is a toxin that originates as a secondary product of metabolism of mould *Byssochlamys nivea*, *B. fulva*, *Penicillium urticae*, *P. expansum*, *P. chrysogenum*, *Aspergillus clavatus*, *A. terreus* and others. These moulds can be found on cereals, in bakery and meat products. Patulin can be found, relatively often, on fruit infected with *P. expansum* (Brown rot), and, unless rotten fruit is removed, it can be found in fruit mash and juices. It can be found mainly in apple juice, which results in poor quality and bad technology in juice production.

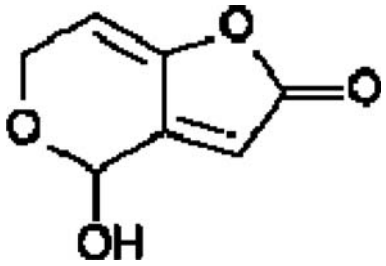
In must, obtained from grapes infected with mould, originating from Canada, Scott et al. (1977) have found patulin in quantities 30 μg/l to 4,5 mg/l, while patulin was not present in wines obtained from the sam must. Altmayer et. al. (1982) have tested 64 samples of must from Rheinpfalz, and 22% of the samles contained patulin in quantities below 50 μg/l, while 16% of the samples contained 50 μg/l and more, with the highest value being 280 μg/. Mortimer et al. (1985) have tested 13 samples of white and red grape juice and no contamination with Patulin was found at detection limit of 5 μg/l. During alcohol fermentation, decomposition of Patulin occurs faster at higher temperatures. So, Woller and Mayerus (1986) established that fermentation at 17°C completely decomposes 1000 μg/l of Patulin in three days, at 13°C in seven days, while at 8°C two weeks were necessary for its

complete decomposition. Patulin has inhibitory effect on numerous types of microorganisms, which is why it was studied as antibiotic at first, but due to its high acute toxicity for humans, animals and plants it is classified as mycotoxin. It does not show carcinogenic effect, but damages DNA and inactivates enzymes with SH group.

Chemically, Patulin is anhidro-3-hydroxymethilen-tetrahydro-1,4-piron-2--carboxyl acid. At pH between 3,0 and 6,5 it is stable, and at higher pH values lactonic ring opens and toxicity disappears. Having in mind its decomposition during alcohol fermentation, there is very small possibility of its occurrence in wine.

Trichothecenes include about 100 mycotoxins produced by *Fusarium.* These toxins have very wide spectrum of biological effects: phytotoxic, insecticide, fungicide, antiviral and cytotoxic, and toxicity is demonstrated by inhibition of protein biosynthesis in cells of mammals. Poisoning with trichothecens provokes damage of bone marrow, small intestine, lymph system, heart muscle etc.

It could be said that the presence of these toxins on grapes, in must and wine has not been systematically analised. *Trichothecium roseum* is another mould that causes bitter rot on apples, while on grapes, it appears as a secondary infection, after *Botrytis cinerea*. It is able to produce mycotoxins trichothecenes, trichotecolon, and rosenon (F l e s c h et al., 1986). Already in quantity of 2,1 mg/l trichothecene can inhibit alcohol fermentation during which it remains unchanged (S c h w e n k and A l t m a y e r, 1985).

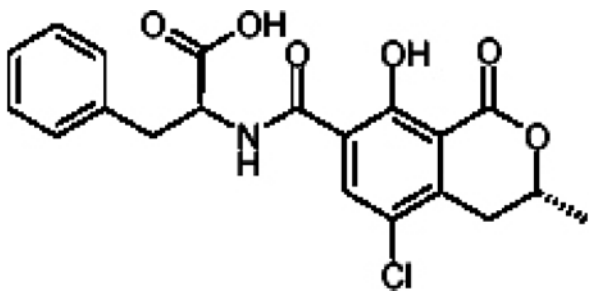


Patulin

OCHRATOXINS

Ochratoxins isolated in South Africa in 1965. were cyclic penthadicetides, i.e. derivatives of dihydroxyisocumarine tied to L-phenylalanine. Apart from ochratoxin A (OTA), which is the most important due to its toxicity, there is ochratoxin B, derivative of ochratoxin A that does not contain chlorine, as well as ochratoxin C which is ethylester of ochratoxin A. Bruto formula OTA is $C_{20}H_{18}ClNO_6$, molecul substance 403,8, chemical term (7-(L-β-phenyl-alanyl-carbonil)-carboxyl-5-chloro-8-xydroxi -3,4-dihydro-3R-methylisocumarin).

Ochratoxin A is produced by *Aspergillus* and *Penicillium* species of mould. Production of this mycotoxin was initially established with species *Aspergillus ochraceus*, later or also with *A. melleus*, *A. muricatus*, *A. petrakii*, *A. sclerotio-*



Structure formula of OTA

rum and *A. sulphureus*, *A. albertensis* and *A. alliaceus* (V a r g a et al., 2001). *A. glaucus*, *A. sydowii* and *A. repens* have also been identified as OTA producers, by the same authors. Furthermore, it has been established that OTA is a metabolite of certain species of *Nigri* section such as *A. niger*, *A. carbonarius*, *A. awamori* and *A. foetidus*. Some species of *Penicillium* also produce OTA, of which the best known are *P. verrucosum* and *P. veridicatum*. The stated moulds have been isolated from various cereals and their products, and can be found in coffee, meat, cheeses, beer, fruit juices, grape juice and wine.

The tests have confirmed that OTA has nephrotoxic and hepatotoxic effect causing kidney and liver cancer. OTA is also connected to the already known Balkan endemic nephrophathy and appearance of tumors in human urinary tract. This mycotoxin has genotoxic, immunotoxic, teratogenous and neurotoxic effect. International Agency for Cancer Research (IARC) has placed OTA into B_2 group, i.e. among substances potentially carcinogenic for humans. Taking into consideration that, according to the evaluations of Codex Alimentarius Commission (1998) about 15% of total daily OTA quantity enters organism through wine, EU Regulations Commission (EC 123/2005) has limited maximum OTA content in wine and grape juice to 2,0 ng/ml.

Ochratoxin A on grapes, in must and wine in the first 20 samples of sultanas from Retail network were analysed in Great Britain (M a f f, 1997) and 88% of the samples contained OTA ranging from 0,2 to 53,6 μg/kg. In France, a research was carried out with 373 samples of grape cultivars Carignan, Syrah, Suvignon and Muscat with regard to the appearance of mould after bunch closure, during veraison and during harvest (R o u s s e a u, 2001). Mould contamination of grapes was growing with the development of grapes, and during veraison only 10% of mould produced OTA, and 47% in full ripeness. Out of the identified OTA that produced moulds, 96% belonged to *Aspergillus* species, of which 95% belonged to *A. carbonarius* and 1% to *A. niger*, while about 4% was of *Penicillium* species.

Z i m m e r l i and D i c k (1995) were the first ones to report the existence of OTA in wine and the found quantities ranged from 10—20 ng/l. In their next paper published in 1996, the authors reported the results of more detailed research on OTA in wine. They analysed 118 table wines and 15 special wines sampled from Swiss retail network and found OTA content of about 3 to 388 ng/l, of which the average value for five dessert wines was 337 ng/l.

From the obtained results, it can be concluded that bigger contamination was found in red wines, particularly those originating from South Europe and North Africa. Mayerus and Ottender (1996) analysed 114 wines and found median of OTA concentration of 7 ng/l for white and 200 ng/l for red wine. The highest content of 1850 ng/l was found in red wine from Algeria which is in agreement with the opinion of former researchers that more frequent appearances of OTA and higher concentrations are typical for wines from Mediterranean basin. In Great Britain 10 samples of red wine have been analysed (Maff, 1997) and OTA concentrations were more than 20 ng/l in all samples, the highest level of concentration being 1100 ng/l.

According to the data from literature, mean OTA values in red wines ranged from 0,039 μg/l (Zimmerli and Dick, 1996) to 1802 μg/l (Cerruti et. al., 2000) and were very close to those in rose wines with range from 0,025 μg/l (Zimmerli and Dick, 1996) to 1348 μg/l (Cerruti et al., 2000). These average values are somewhat higher than those found for white wines that ranged from 0,011 μg/l (Zimmerli and Dick, 1996) to 0,535 μg/l (Visconti et al., 1999). Mayerus et al. (2000) are of opinion that different OTA concentrations in white, rose and red wines are caused by the method used in the production of these wines.

Domijan and Perajica (2005) analysed OTA content in 7 white and 7 red wines produced in Croatia. In red wines, OTA level ranged from 12—47 ng/l and in white wines, from 15—22 ng/l. Three white wines from the north (continental Croatia) did not contain OTA, while all those originating from the southern Croatia did contain OTA.

As for OTA content in must, i. e. grape juice, Zimmerli and Dick (1996) analysed 8 samples of red and 3 samples of white commercial must and established median of OTA content of 116 ng/l for all samples, while for the samples of red juice only, it was 235 ng/l. Mayers and Ottender (1996) analysed 20 samples of grape juice and established median of 1800 ng/l for red grape juice, while the highest content was 4700 ng/l. Abrunhosa et al. (2005), in eight independent experiments, after crushing of grapes, found OTA content in juice that was 59% ± 14 from the total concentration existing on grapes.

MEASURES FOR PREVENTING OTA APPEARANCE IN WINE AND POSSIBILITES OF ITS REMOVAL

If measures for prevention of OTA appearance, or its appearance in wine, are taken into consideration, they are primarily related to the production of grapes and are carried out all the way from planting of vineyard to harvest. As for wine production, one should know which operations in primary processing can contribute to wine contamination with this mycotoxin, as well as which treatments can reduce its content in wine.

When more important factors significant for OTA occurrence on grapes are considered, it is necessary to identify the types and species of mould existing in particular wineyard region. Moreover, information on microclimate

and technology of vine cultivation are also very important. Vine growers should be informed about the risk of appearance of moulds and mycotoxins on grapes, and measures of prevention in vinegrowing. Apart from avoiding the terrain with higher relative humidity for planting vineyard, a direct contact of grape clusters with soil should also be avoided, and a good control of pests and vine diseases provided. Great help in prevention of OTA can be a choice of particular clones within some cultivars which are more adaptable under given ecological conditions and less susceptible to mould development. Apart from suitable vine protection programme it is also necessary to pay attention to leaf substance — grapes relation which is closely related to the application of nitrogen fertilizers, since they can stimulate excessive growth of vine. During ripening of grapes, works in vineyard should be avoided or brought to a minimum, in order to prevent moulds from the soil to reach the grapes. In the case of irrigation, it should be done evenly, in order to avoid the breaking of berries and mould development. Marc, which is a secondary product of wine production from grapes, should not be used for fertilizing vineyard if it is contaminated with OTA producing moulds. Defoliation in grape zone during the ripening phase enables better ventilation which reduces the risk of mould appearance. Necessary measures should be taken to prevent grape berry damage by insects (grape moth, wasps), various diseases and pests. If necessary, the registred programmes of protection against mould should be applied by using corresponding management in order to avoid the occurrence of resistence in mould.

If grapes are moderately contaminated with toxicogene moulds, they should be removed before or during harvest. The existence of inspection line is useful in winery where the grapes are placed upon reception, and damaged and mouldy bunches removed. Transportation of grapes to winery, after the harvest, should be carried out in the shortest time possible. Vessels (containers) used for transportation of grapes should be properly cleaned after each use.

Grapes significantly contaminated with mould cannot be used for production of rectified concentrated must (RTK) or wine for human consumption, but only for wine used for distillation.

In case of OTA contaminated grapes, certain operations and processes in technological procedure of grape processing are somewhat modified, or completely eliminated. So, for example, thermic treatment of marc and its prolonged maceration should be avoided, and straining of the same carried out at smaller pressures with compulsory avoiding of strainers with continuous work. In case of OTA contaminated red grapes, it is necessary to appraise possibility of producing rose wine. Application of pectolytic enzymes should be avoided as well, since maceration action increases must yield, spreads extraction of phenol compounds from skins, but also increases OTA content in wine. In production of white wines, purification of must by filtering, centrifuging or flotation is recommended. In the cases where there is a risk of OTA contamination, measuring of its content in must is recommended, as well as the treatment with the lowest effective doses for its removal without lossing aromatic substances and phenol compounds. Alcohol fermentation should last as long as possible using yeast with good adsorption characteristics in regard to OTA.

Apart from wine yeast, partial reduction of OTA can be also achieved by means of suitable preparation of lactic acid bacteria which displays good adsorption with regard to OTA. After alcohol fermentation, aging of wine on yeast reduces OTA level, but it is necessary to have in mind the possible influence of this on wine quality. Organic and inorganic clarification substances have different adsorption with regard to OTA. Some preparations based on cellulose, as well as on silica gel combined with gelatin, are capable of partial reduction of OTA content in wine, but active oenology coal, by itself or in combination with other means, displayed the best efficiency in that respect.

A b r u n h o s a et al. (2005) found that OTA content diminishes during alcohol fermentation, and if malolactic fermentation is carried out as well, OTA content in wine is about 32% in relation to the quantity found in grapes. Considerable quantity of OTA, 50% ± 10,3, was found in lees after completion of alcohol fermentation. Addition of OTA values in wine and lees represented approximately total OTA quantity found in grapes. After separating from lees, OTA quantity was only about 11% of its value in grapes, while level of OTA in lees was 80%. In this research, malolactic fermentation caused reduction of OTA to about 3% of the quantity found in grapes. Wine settling agents can make a contribution to further reduction of OTA content, but application of effective doses is often limited by their adverse influence on wine colour and aroma. According to the above stated authors, some of the commercial enzymes are also able to reduce OTA level in wine having hydrolytic influence on it, which requires confirmation in further research.

S i l v a et al. (2007) have carried out extensive research on possibilities of OTA reduction in wine by means of various agents for treating wine (active coal, active coal combined with K-caseinate or silica gel, PVPP, cellulose, gluten and peas protein) and yeast, as well as preparations made out of yeast cell walls and hulls. Application of agent called ATOS (active coal and K-caseinate) in quantity 0,2 g/l reduced OTA content by 55%, while treatment with 0,5 g/l of this agent reduced OTA content by as much as 90% in relation to the initial content in wine. However, using up to 0,2 g/l of ATOS does not have more significant influence on colour intensity in red wines, while at higher doses wine gets noticeably colourless, which depends on type of active coal as well. Out of seven used active coal preparations (10 and 50 g/hl) two displayed very weak ability of OTA absorption, while with the others, using 50 g/hl for 24 hours, reduction of OTA ranged even up to 90%. As for using PVPP, cellulose, gluten and peas protein, the experiments show that PVPP (40 and 80 g/hl) and cellulose (10 and 30 g/hl) reduce OTA content by approximately the same per cent (from 28—42%) when remaining in contact with wine for 48 hours. Gluten and peas protein showed approximately the same effect, a reduction of OTA content in wine by 39 — 45% after 24 hour application. As for yeast, dried active, inactivated, cell walls and yeast cell hulls, their effectiveness in reducing OTA in wine differs. In all stated treatments, contact of preparation and wine lasted for 8 days, and doses recommended by the producer have been applied. Preparation based on yeast cell walls was not used in the quantity of 100 g/hl and it did not give satisfactory results, while preparation based on yeast cell hulls reduced OTA content in wine by 40%. Inacti-

vated yeast displayed poor activity in relation to OTA in wine, being applied in quantities of 20 and 40 g/hl it reduced OTA content by 25,8 and 26,3%. Preparation microsorb, based on yeast *Sacch. cerevisiae*, applied in quantity of 25 g/hl with 60 hour contact, reduced OTA by 77%, quantity of 50 g/hl reduced it by 81%, while quantity of 100 g/hl achieved OTA reduction by as much as 90%, but the intensity of wine colour was also reduced. Very strong point of this preparation is that it is highly effective in a very short period of time (60 hours).

The highest reduction of OTA content by yeasts and preparations based on their by — products has been achieved when contact with wine lasted for eight days at 20°C. Reduction ranged from 40—50% in cases when 100 g/hl of yeast cell walls were used, 40 g/hl yeast cell hulls, 200 g/hl active dried yeast and 10 g/l yeast cream were applied.

CONCLUSION

During processing of grapes and vinification, a considerable reduction of OTA occurs, and purification of must and fermentation processes were the most effective in that respect. Also, some wine clarification agents can be of great help in that sense, but they should be applied exclusively in concentrations that do not violate sensory characteristics of wine.

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МИКОТОКСИНИ У ВИНУ СА ПОСЕБНИМ ОСВРТОМ НА ОХРАТОКСИН А

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Резиме

Квалитет вина је комплексан, вишеслојни појам састављен од већег броја чинилаца попут сензорских карактеристика, хемијског састава, законодавно-правног, тржишно-потрошачког, при чему хигијенско-токсиколошки има посебан значај с обзиром на све израженије захтеве за здравствено безбедном храном. У раду су приказани резултати досадашњих истраживања микотоксина у вину (афлатоксини, трихотецен, патулин), при чему је посебна пажња посвећена најчешће присутном охратоксину А на грожђу, у шири и вину и утицај појединих технолошких операција и процеса у току производње вина на његов садржај. С обзиром на његову високу токсичност Европска Унија ЕГ регулативом 123/2005. свела је највише дозвољену количину охратоксина А на 2 μg/l.

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FREQUENCY OF *ASPERGILLUS FUMIGATUS* FRES. — A TOXIGENIC AND ALLERGENIC FUNGAL SPECIES IN MILKING COWS FEEDS THROUGHOUT ONE RESEARCH YEAR

ABSTRACT: Fungal contamination of 92 feed samples used for milking cows feeding during one research year (in all seasons) in Vojvodina was examined. A special attention was paid to the frequency of *A. fumigatus*, a fungal species harmful to human and animals. As it was found out all feed samples in summer and spring were contaminated with fungi and about 95% in autumn and winter period. Aspergilli occured as contaminants in all seasons. About 63% of feed samples in summer, 67% in autumn, 89% in winter and 48% in spring were contaminated with them. *A. fumigatus* was constantly present in feeds. It was isolated from 19% of feed samples investigated in summer, 33% in autumn, even 61% in winter and from 4% in spring.

KEY WORDS: Feed, fungal contamination, *A. fumigatus*

INTRODUCTION

Fungi are world-wide distributed microorganisms which can exist in all climate regions. They can be found on decaying soils and plant materials (Fink-Gremmels, 2005; Đukić et al., 2007), in air (Đukić et al., 2008; Matković et al., 2008), water (Sallenave-Namont et al., 2000; Grovel et al., 2003), in feed (Adamović et al., 2005; Bočarov-Stančić et al., 2005; D'Mello, 2002; Škrinjar et al., 2008a, 2008b) and food (Bagi et al., 2005; Janković et al., 2006; Škrinjar, 2005; Škrinjar et al., 2007; Šarić and Škrinjar, 2008) as well as in raw materials used in feed and food technologies for their processing and production of final products and by-products (Škrinjar, 2008). Presence of toxigenic and pathogenic fungi is of a high significance.

Fungal contamination of cereal grains, oil-seed meals and forages may present a major animal and human health risks throughout the world, especially in the humid climate regions (D'Mello, 2002). The risks reflect in the ability of numerous fungal species to produce harmful metabolites — mycotoxins (Samson and van Renen-Hoekstra, 1988).

Contamination of forages and cereals with mycotoxins frequently occurs in the field following infections of plants with pathogenic fungi or with symbiotic endophytes (D'Mello, 2002). The possibility of contamination may continue during the processing and storage of harvested products and feed (Adamović et al., 2005; Bočarov-Stančić et al., 2005; D'Mello, 2002). A degree of fungal and mycotoxin contamination depend significantly on environmental conditions, such as moisture content/humidity and ambient temperature.

Classical representatives of plant pathogenic species ("field fungi") belonged to the genera *Fusarium, Claviceps, Alternaria* and some other genera from the *Hyphomycetes Dematiaceous* group, while *Aspergillus* and *Penicillium* exemplify storage organisms.

More than 200 species are classified into the genus *Aspergillus*. Many of them are harmful to humans and animals. High incidents of *Aspergillus* mycotoxins are noticed in warm and humid regions, but they often occur in temperate zones, too.

According to the data (Dhand et al., 1998; Banerjee et al., 2000) *Aspergillus* species dominate all other fungi with respect to mycotoxin production in cereals and oilseeds in some regions.

Various members of the genus *Aspergillus* are known as carriers of human and animal diseases. *Aspergillus fumigatus* is a fungal species associated with over 80% of all human syndroms caused by aspergilli (Banerjee et al., 2000). These diseases range from being colonization of the lung to life threatening diseases such as allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis.

In addition to the upper airways, fungal infection of the mammary gland and the uterus during parturitions may have resulted due to their exposure to the external invasion of *A. fumigatus* conidia (Fink-Gremmels, 2005).

A. fumigatus was described as an etiologic agent identified in most of the aspergilli related human diseases by many other authors (Khoo and Denning, 1994; Oren and Goldstein, 2002; Maschmeyer et al., 2007; Pauel et al., 2008).

Sarfati et al. (1996) reported that conidia of *A. fumigatus* are in correlation with the airway hypersensitivity of horses as well as with bronchitis, mastitis and incidental abortion in cattle. In laboratory rats a local pulmonary response following intrasanal application of different doses of *A. fumigatus* conidia evaluated on 7 and 21 days of the experiment was described by Živanović et al. (2007).

A. fumigatus is a producer of a variety of termorgenic mycotoxins, a group of indole derivates, such as verruculogen, fumitremorgens and penitrem A. These toxic metabolites induce tremor and other symptoms of neurotoxity in laboratory animals and livestock (…).

In addition, *A. fumigatus* produces a toxic metabolite gliotoxin and other epipolythioidiopiperazines. Gliotoxin exert different biological effects. For example, it induces a specific calcium release from intact liver mitochondria (Salvi et al., 2004) and apoptotic cell death in numerous cell types. Gliotoxin has been found to be a strong immunosupresive agent, too (Watanabe et al., 2003).

In this paper the results of the occurence of *Aspergillus* species with a special attention paid to *Aspergillus fumigatus* frequency in feeds for milking cows feeding throughout one research year are presented.

MATERIAL AND METHODS

Mycological contamination of different feed samples (92) for milking cows feeding during one year in Vojvodina was examined in this work. Samples were taken from four farms throughout all seasons. Type and number of feed samples are given in Table 1.

Tab. 1 — Type and number of feed samples used for feeding of milking cows throughout one research year

Type of feed	Number of feed samples			
	Summer	Autumn	Winter	Spring
Hay	4	4	4	3
Dried lucerne	3	4	4	4
Concentrate	5	4	4	4
Sunflower meal	1	— [a]	—	—
Dried corn silage	—	3	4	2
Fresh corn silage	3	—	—	2
Corn grain silage	—	—	2	3
Pelleted malt spent grains	2	3	2	1
Fresh rape leaf	—	1	—	—
Pelleted sugar beet pulp	1	3	2	4
Fresh sugar beet pulp	1	1	1	1
Fresh rape-seed leaf	—	—	—	2
Total	20	23	23	26

[a] not used

Mycological investigation. Determination of total viable count of moulds per 1 g of each sample was done as well as the isolation and identification of all fungal genera. Their share in isolated mycopopulations, with a special attention paid to the presence and frequency of *Aspergillus fumigatus*, a fungal species with toxigenic and allergic properties, was examined, too.

Total viable counts of moulds per 1g of sample were determined by standard Koch' s method. Sabouraud maltose agar (SMA) with streptomycin (0.01—0.02%) was used as an isolation medium. Incubation was carried out at 25°C for 7 days and the identification of fungal genera and *A. fumigatus* according to Samson and van Reenen-Hoekstra (1988).

RESULTS AND DISCUSSION

Summer. All of the feed samples tested in summer period were contaminated with moulds (Fig. 1) and the number ranged from 10.0 (dried corn silage) to 2.8 x 10^7 /g (pelleted malt spent grains). It was found that 87% of the samples were contaminated with species from the genus *Penicillium*, 63% with *Aspergillus* spp., 56% *Mucor* spp., 50% *Cladosporium* spp., 12% with *Fusarium* and *Alternaria* species. About 56% of feed samples were spoiled with fungal species which belonged to the other genera.

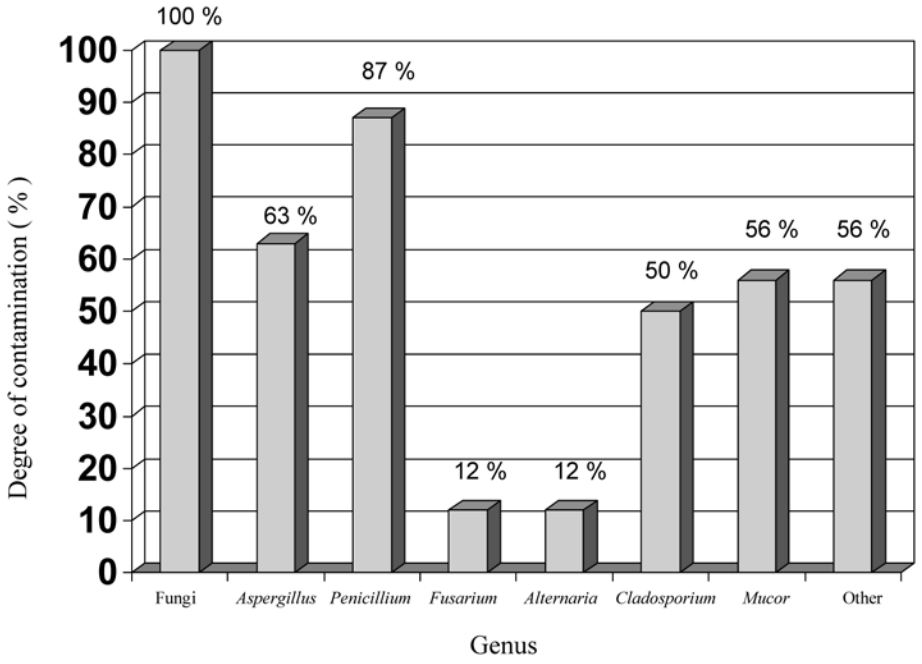

Fig. 1 — Contamination degree of feed samples with certain fungal genera in the summer

A. fumigatus was isolated from 19% of the samples (Fig. 2), including 30% of those contaminated with other species from the genus *Aspergillus. A. fumigatus* was found as a contaminant of concentrate (2 samples) and dried corn silage (1 sample).

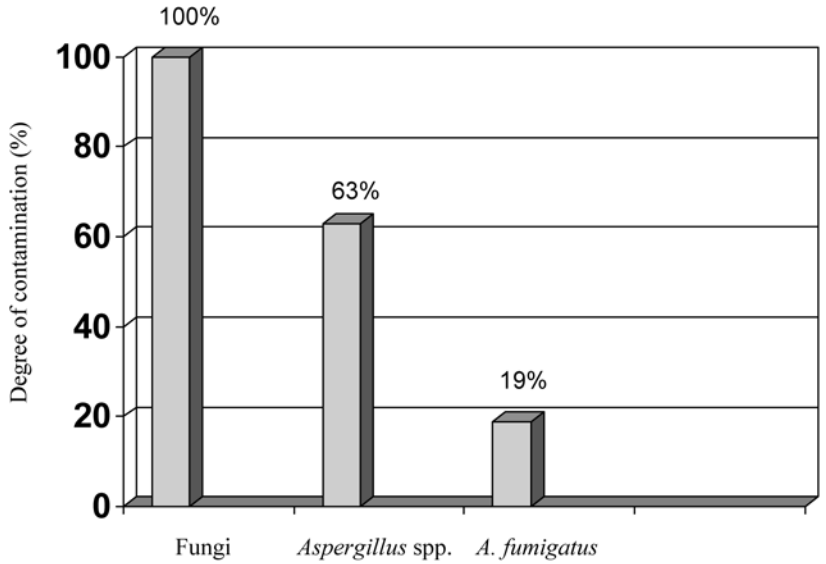

Fig. 2 — Contamination degree of feed samples with *A. fumigatus* in the summer

Autumn. In this season a higher frequency of *A. fumigatus* of feed mycopopulations was noticed (Fig. 3). This fungal species was isolated from six different feed samples (33% of total contaminated) (hay — 2 samples, dried corn silage — 1, pelleted malt spent grains — 1, pelleted sugar beet pulp — 2), which were spoiled with fungi. It is necessary to point out that even 50% of samples infected with *Aspergillus* spp. were contaminated with *A. fumigatus* at the same time.

As it was established, about 95% of feed samples (18 of 19 total examined) contained moulds at various degree (from 70.0 — pelleted sugar beet pulp to 4.0×10^5/g — pelleted malt spent grains).

Winter. Total viable count of moulds in winter research period varied between 10.0 (corn grain silage) and 2.1×10^7/g (dried corn silage). Results of fungal contamination of feeds were approximately in conformity with those determined in autumn.

A high incidence of *Aspergillus* spp. frequency was found. Even 89% of total contaminated feed samples were infected with species of genus *Aspergillus* (Fig 5). These results are not in accordance with a few data reported on *Aspergillus* spp. distribution, especially in climate regions with high temperature and humidity (D h a n d et al., 1998; B a n e r j e e et al., 2000). At the same time, a decrease of *A. fumigatus* frequency was noticed. The species was isolated from 11 samples (61% of total contaminated — Fig. 6) (dried lucerne — 3, concentrate — 3, dried corn silage — 3, pelleted sugar beet pulp — 1, pelleted malt spent grains — 1).

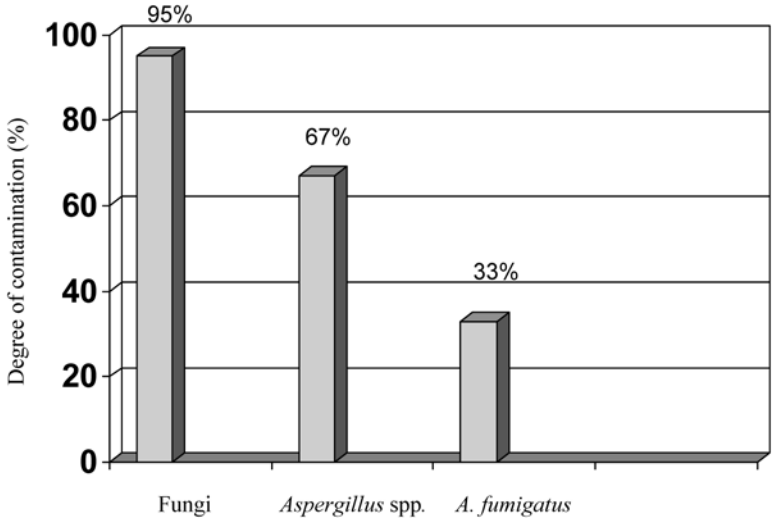

Fig. 3 — Contamination degree of feed samples with species *A. fumigatus* in autumn

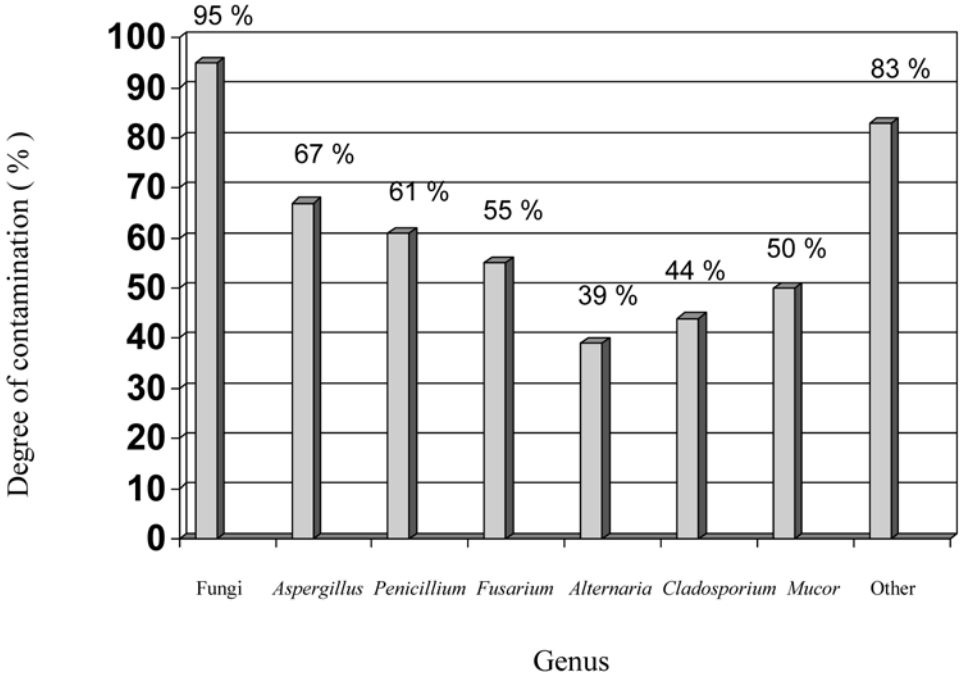

Fig. 4 — Contamination degree of feed samples with certain fungal genera in autumn

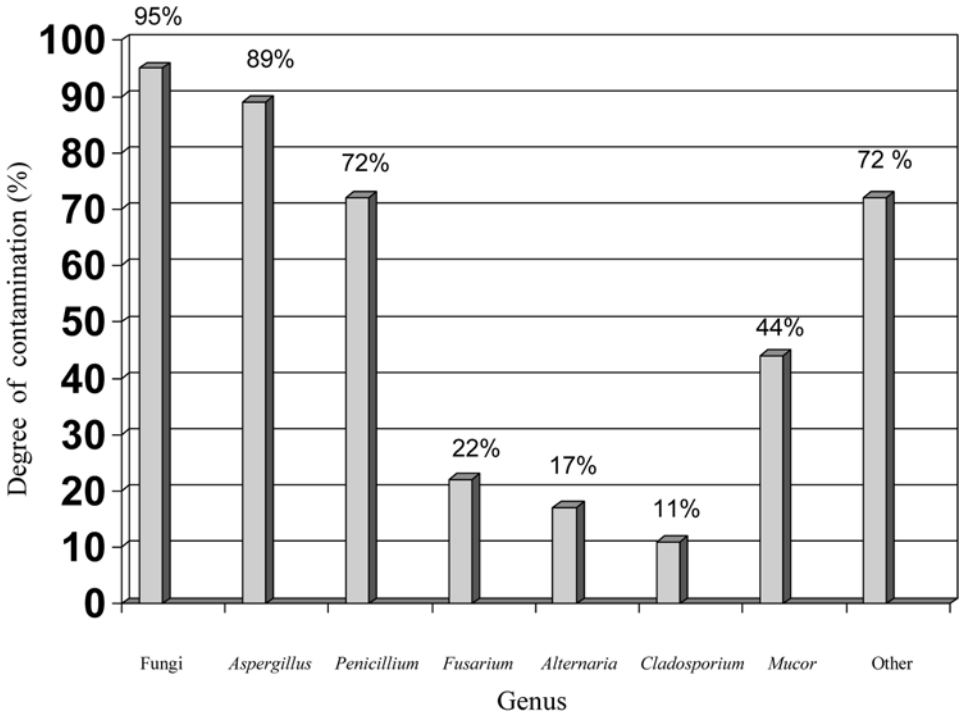

Fig. 5 — Contamination degree of feed samples with certain fungal genera in winter

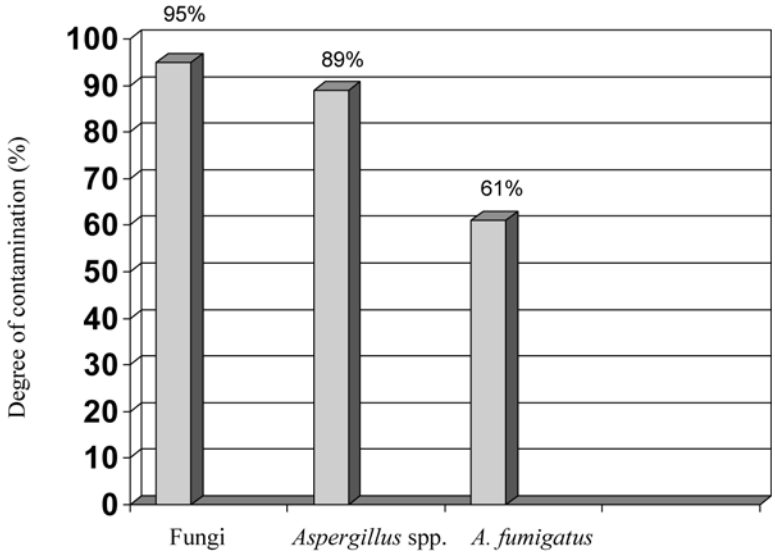

Fig. 6 — Contamination degree of feed samples with species *A. fumigatus* in winter

Spring. All feed samples were contaminated with fungi again (Fig. 7). Their number varied from 20.0 (pelleted malt spent grains, fresh sugar beet pulp) to 6.1 x 10^5 in 1g (dried lucerne).

The most frequent in mycopopulations were found to be *Penicillium* species which were isolated from 61% of the examined samples and *Aspergillus* spp. isolated from about 48% of them (Fig. 8). Only one feed sample (concentrate) was contaminated with *A. fumigatus* in spring period.

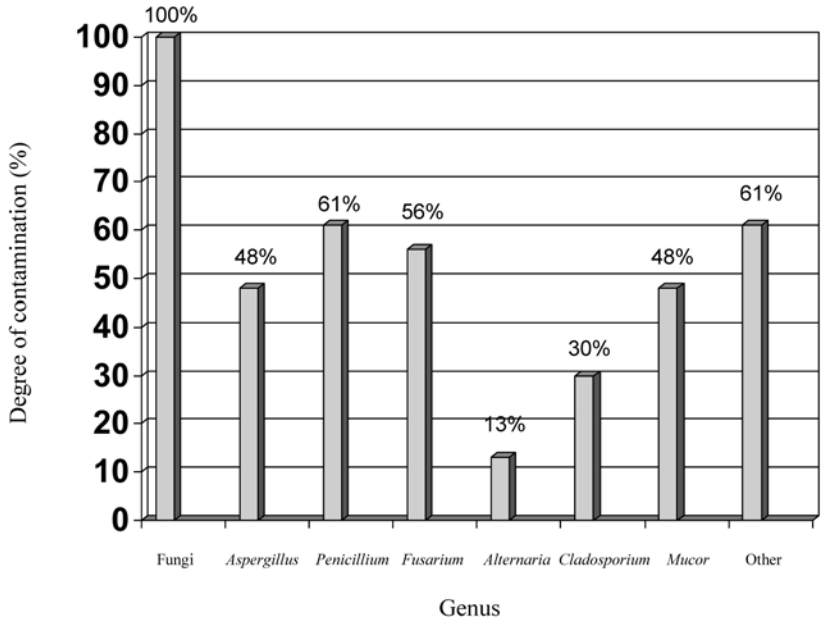

Fig. 7 — Contamination degree of feed samples with certain fungal genera in spring

Investigations of the frequency of *Aspergillus* species, especially of a toxigenic and allergenic fungal species *A. fumigatus* indicate that the highest incidence of feed contamination with *Aspergillus* spp. was noticed in winter research period. In this season even 89% of samples were contaminated with aspergilli (Fig. 9). The lowest contamination degree (48%) was determined in spring.

Comparing a frequency of *A. fumigatus* in feed samples throughout one research year (in all seasons) it can be seen that the highest contamination caused by *A. fumigatus* was observed in winter period, too.

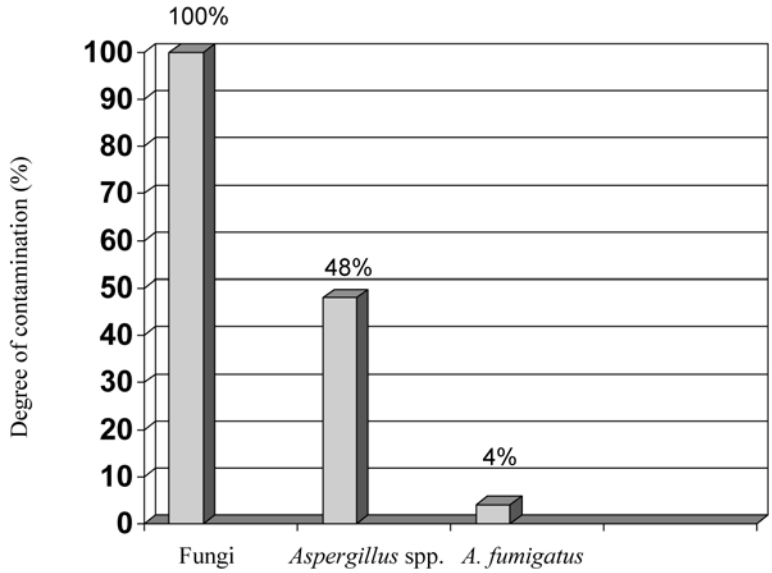

Fig. 8 — Contamination degree of feed samples with species *A. fumigatus* in spring

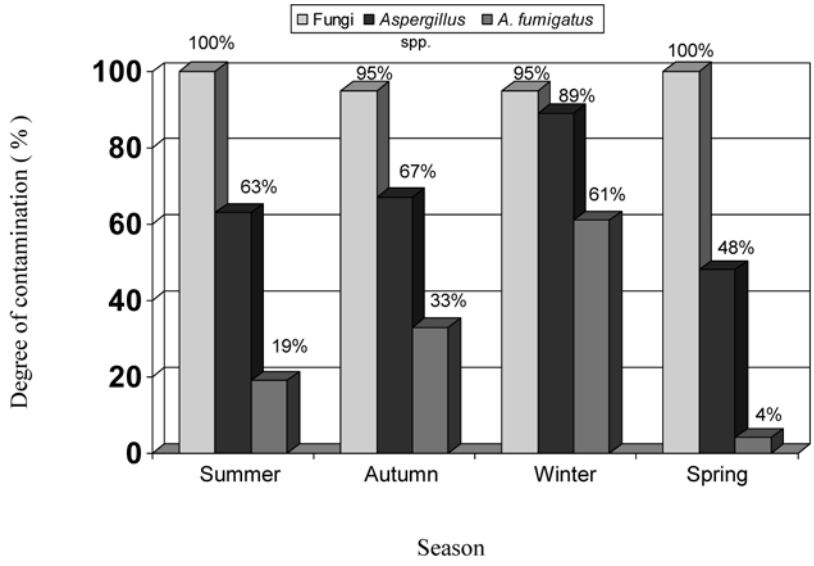

Fig. 9 — Contamination degree of feed samples with species *A. fumigatus* during one experimental year

CONCLUSION

Aspergillus species were constantly present in milking cows feeds throughout one research year (in all seasons). The highest degree of contamination (89%) with aspergillis was observed in winter period and the lowest (48%) in spring.

A. fumigatus was found as a contaminant of different types of feeds. About 19% of samples in summer were found to be contaminated with it, 33% in autumn, 61% in winter and only 4% in spring research period.

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ФРЕКВЕНЦИЈА РАСПРОСТРАЊЕЊА *ASPERGILLUS FUMIGATUS* FRES. — ТОКСИГЕНЕ И АЛЕРГЕНЕ ФУНГАЛНЕ ВРСТЕ У ХРАНИ ЗА ИСХРАНУ МУЗНЕ СТОКЕ ТОКОМ ЈЕДНЕ ИСТРАЖИВАЧКЕ ГОДИНЕ

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Резиме

Испитана је фунгална контаминација 92 узорка хране коришћене за исхрану музне стоке током једне године (у сва годишња доба) на подручју Војводине. Посебна пажња посвећена је распрострањењу *A. fumigatus*, фунгалне врсте штетне за људско здравље и здравље животиња. Установљено је да су сви узорци хране у току лета и пролећа били контаминирани гљивицама, као и око 95% у јесењем и зимском периоду. Аспергили су запажени као контаминенти у свим годишњим добима. Њима је контаминирано око 63% хране у летњем периоду, 67% у јесењем, 89% у зимском и 48% у пролећном. *A. fumigatus* био је константно присутан у храни. Изолован је из око 19% узорака хране у току лета, 33% у јесењем периоду, чак из 61% узорака у зимском и из 4% узорака у пролећном периоду.

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INFLUENCE OF PELLETING ON MICROBIOLOGICAL AND MYCOTOXICAL CORRECTNESS OF FEED MIXTURES WITH BENTONITE SUPPLEMENT

ABSTRACT: Influence of pelleting calf feed mixtures supplemented with bentoni on microbiological and mycotoxicological properties was investigated. Microbiological and mycotoxicological quality was investigated at the production day (day 0) and after 45 days of storage. Total count of microorganisms in the pelleted mixture, at the day 0 (280.000/g), was several times lower than in the powdered mixture (2.000.000/g). Similar results were obtained at day 45 when the total number of microorganisms in the pelleted mixture was 270.000/g and 1.800.000/g in the powdered mixture. Number of yeasts and molds at the production day in the pelleted mixture was 650/g, and in the powdered mixture it was 27.000/g. Similar results were obtained 45 days later when the number of yeasts and molds in the pelleted mixture was 540/g, and 16.000/g in the powdered mixture. There were 6 species identified in the pelleted mixture, and 9 species in the powdered mixture at the day of production. Similar mold species ratio in the pelleted (11) and powdered mixture (13) was found at day 45. In the examined samples representatives of *Fusarium* genus — *F. subglutinans* i *F. verticillioides* dominated. Number of sultite-redzcing clostridia in the mixtures, in both observed periods, was similar (below 1000/g of sample). By mycotoxicological analysis of mixtures at the production day, only trichotecene (T-2 toxin) presence was found in amount of 0,337 mg/kg. The applied technological procedure of pelleting with bentonite supplement, had positive influence on the improvement of microbiological and toxicological properties of mixture.

KEY WORDS: bentonite, calves, feed mixtures, micoorganisms, mycotoxins, pelleting

INTRODUCTION

Safe food production is an imperative for human food and animal feed, producers, today. Therefore, technological and technical procedures which contribute to the reduction of food contamination are becoming more and more important. In animal feed production, mixture pelleting is one of such proce-

dures. Positive effects of pelleting are: decrease of mixture decomposition, reduction of total number of microorganisms, increase of volume mass, decrease of dustiness, possible use of finely grinded feedstuffs, increase of manipulation possibilities (Đ o r đ e v i ć and D i n i ć, 2007; S r e t e n o v i ć Ljiljana at al., 1995). As a consequence of exposing the mixture to the influence of vapour, pressure and temperature, nutrients are being chemically transformed, and thereby digestibility of amilose, hemicellulose, cellulose and pentosan is increased (S t o j a n o v i ć, 2008, G r u b i ć et al., 1995). Due to increased temperature (between 70 i 80°C) some antinutritive ingredients of feedstuffs and mixtures decompose. Feed mixture pelleting has a positive effect on production results (daily gain, milk production), their better consumption and utilization. Pelleting presents a thermoplastic forming process when homogenized particles of powdered feed are pressed through die perforations in order to improve pellet quality (lasting and grinding durability). During feed mixture processing, different binding substances are used, among which Ca-lignosulphonate, Na and Ca-bentonite. Bentonite is a colloid clay of volcanic origin in form of hydratated aluminium-silicate composed of mineral montmorilonite (50—90%). Bentonite composition may vary, but most of the different bentonite types consist of replacable Na+, K+, Ca], Mg] ions, and according to the ions present they are named as sodium bentonite, potassium bentonite, calcium bentonite or magnesium bentonite. Bentonite has extremely large covering surface (1 g of bentonite covers the surface of 700—800 m^2). Chemical composition of bentonite varies depending on the deposition place and most often contains 46—58% SiO_2, 12—22% Al_2O_3, 0,20—0,40% K_2O, 0,04—0,08% Na_2O, 1,70—3,50% MgO, 3,30—5,90%, CaO, 3,50—4,70% Fe_2O_3. Burning loss ammounts to 12—17%. Due to amphotheric characteristics (accepts and releases hydrogen ions) it is used as supplement for rumen pH regulation in cattle (A d a m o v i ć et al., 2004; M u r r a y et al., 1990). Bentonite binds aflatoxins (B_1, B_2, G_1 i G_2) in fodder and decreases the presence of aflatoxine M_1 residues in milk (by 60 to 90%). However, its possibility to adsorb zearalenone and ochrataxin is limited (P a s h a i sar., 2008). Bentonite inclusion in cow rations contributed to the reduction of milk contamination with ^{137}Cs and ^{134}Cs from 50% to 80%. Bentonite adsorbs excesive NH_3 from rumen liquid when NH_3 concentration is high, and releases NH_3 when its concentration is low. This provides more efficient nitrogen utilization from ammonia for microbiological protein synthesis. Consequently, the resorption of NH_3 into blood, liver load and energy consumption for urea synthesis are decreased. Due to bentonite possibility to bind water, its volume increases as well as the digest volume in digestive tract. The enlargement of digest volume to the decrease of its passage speed through digestive organs, and thus provides longer activity of digestive enzymes and nutrien digestibility increase. Bentonite decreases Cu solubility in rumen and its content in liver, which can be useful for treating chronical Cu intoxications in animals. Disadvantage of bentonite, beside its affinity to bind certain minerals, is also an affinity to bind vitamines (H u w i g et al., 2001).

The goal of this investigation was to determine the influence of pelleting procedure of calves mixtures supplemented with bentonite on microbiological and mycotoxicological properties of mixtures.

MATERIAL AND METHODS

The investigated mixtures were produced in the Feed Mixture Industry Padinska Skela. Components were mixed with horizontal mixer (Buhler) with 3000 t capacity. Mixture pelleting was done using the press of the same manufacturer. Pellet diameter was 4 mm, and lenght 4 to 6 mm. Mixture composition is shown in Table 1. Bentonite used in the experiment was derived by a special technological procedure (impurity separation, drying, crushing and grinding) at the Institute for Technology of Nuclear and Other Raw Materials, Belgrade. Bentonite contained: 48,37% SiO_2; 22,39% $A1_2O_3$; 0,40% K_2O; 0,07% Na_2O; 1,81% MgO; 5,86% CaO; 4,73% Fe_2O_3; and 0,34% TiO_2. Size of particles was below 50 mm.

After feed mixture production, samples for microbiological and mycotoxicological analysis were taken (day 0). The mixture samples were kept in nylon bags during 45 days (period november-december), 20 cm above the floor, in ventilated, semi-dark and dry room. Average room temperature was 18°C.

Tab. 1 — Powdered and pelleted mixture composition, %

Component	% in mixture
Corn, ground	34,30
Barley, ground	10,00
Soybean, full fat	22,50
Sunflower meal, 33% UP	10,50
Wheat bran	15,00
Lucerna flour	3,00
Limestone	1,20
Dicalcium-phosphate	0,40
Salt	0,60
Vitamine and mineral premix	1,00
Bentonite	1,50
Total	100,00

Microbiological investigations were performed according to the *Regulations on maximal quantity of harmful materials and ingredients in fodder* (Sl. list SFRJ No. 2/90). Total count of bacteria, molds and yeasts as well as identification of pathogenic microorganisms (bacteria of fecal origin, *Salmonella* spp., sulfite reducing *Clostridium* spp.) was done in accordance to the method SFRJ No. 25/80.

Micotoxicological investigations. The presence of aflatoxin Bl (AFL Bl), ochratoxin A (OTA) and zearalenone (ZEA) was determined according to the standard method (Sl. list SFRJ No. 15/87), while diacetoxyscirpenol (DAS) and T-2 toxin were analyzed by applying the method of P e p e l j n j a k and B a b i ć (1991). Identification of potentially toxigenic fungi was done accor-

ding to Domsh et al. (1980) and Samson and van Reenen-Hoekstra (1988).

RESULTS AND DISCUSSION

Total count of microorganisms in the pelleted mixture at the production day (280.000/g) was several times lower than in the powdered mixture (2.000.000/g). Similar results were obtained at day 45 when the total number of microorganisms in the pelleted mixture was 270.000/g and 1.800.000/g in the powdered mixture. Number of yeasts and molds at the production day in the pelleted mixture was 650/g, and in the powdered mixture it was 27.000/g. Similar results were obtained 45 days later when number of yeasts and molds in the pelleted mixture was 540/g, and 16.000/g in the powdered mixture. There were 6 species identified in the pelleted mixture, and 9 species in the powdered mixture at the day of production. Similar mold species ratio in the pelleted (11) and powdered mixture (13) was found at the day 45. In the examined samples, representatives of *Fusarium* genus — *F. subglutinans* i *F. verticillioides* dominated. Number of sultite-reducing clostridia in the mixtures, in both measuring periods, was similar (below 1000/g per sample). Other pathogenic bacterial species were not determined (Table 2).

Tab. 2 — Microbiological properties of feed mixtures

Parameter	Powdered mixture		Pelleted mixture	
	Day 0	Day 45	Day 0	Day 45
Microorganism count/g	2.000.000	1.800.000	280.000	270.000
Yeast and mold count/g	27.000	16.000	650	540
Identified molds				
Absidia corymbifera	+	+		+
A cremonium fusidioides		+	+	
Acremonium sp.		+	+	+
Alternaria sp.		+	+	
Aspergillus flavus	+	+		+
Aspergillus fumigatus		+		+
Aspergillus niger	+	+	+	
Aspergillus versicolor	+			
Epicoccum purpurascens				+
Fusarium subglutinans	+	+	+	+
Fusarium verticillioides	+	+		+
Fusarium sp.	+		+	
Mucor sp.		+		+
Penicillium monoverticillata	+			
Penicillium sp.	+	+		
Rhizopus nigricans	+	+		+
Scopulariopsis brevicaulis		+		+
Pathogenic bacteria				
Salmonellae sp./50 g	0	0	0	0
Sulfite-reducing Clostridium/g	< 1000	< 1000	< 1000	< 1000
Coagulase positiv. Staph./50 g	0	0	0	0
Proteus sp./50 g	0	0	0	0
Escherichia coli/50 g	0	0	0	0

Among potentially toxigenic molds, it is important to emphasize the constant presence, of *A. flavus* (AFL B1) and *A. niger* (OTA) species in the basic powdered mixture (T j a m o s et al., 2004) as well as *Fusarium* spp. from section Liseola both at day 0 and day 45, *F. verticillioides* and *F. subglutinans*, potential moniliformine, beauvericine and fusiproliferine producers (L e v i ć, 2008), were also found in the pelleted mixture indicating viability of these molds under the pelleting conditions.

Inspite relatively great number of potential mycotoxin producers, only trechotecene (T-2 toxin) presence was determined in ammount of 0,337 mg/kg of mixture (Table 3) at the production day.

Tab. 3 — Presence of mycotoxins in feed mixtures

Parameter	Powdered mixture		Pelleted mixture	
	Day 0	Day 45	Day 0	Day 45
Aflatoxin B1	ND	ND	ND	ND
Zearalenone	ND	ND	ND	ND
Ochratoxin A	ND	ND	ND	ND
Trichotecenes (T-2)	0,337	ND	0,337	ND
Trichotecenes (DAS)	ND	ND	ND	ND

Legend: ND — not detected (< 0,0004 mg/kg AFLB1; < 0,037 ZEA; < 0,004 mg/kg OTA; < 0,04 DAS and T-2)

After 45 days of storage, mycotoxin presence was not detecte in the mixtures. This indicates that present mold species did not produce mycotoxins in quantities measurable by TLC detection methods under given conditions.

It can be concluded that the pelleting procedure of feed mixtures supplemented with bentonite at 1,5% level had positive effect on the improvement of microbiological and mycotoxicological properties of investigated mixtures.

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УТИЦАЈ ПЕЛЕТИРАЊА НА МИКРОБИОЛОШКУ И МИКОТОКСИКОЛОШКУ ИСПРАВНОСТ КРМНИХ СМЕША СА ДОДАТКОМ БЕНТОНИТА

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Резиме

У огледу је испитиван утицај пелетирања крмних смеша за телад са додатком бентонита на микробиолошку и микотоксиколошку исправност смеша. Брашнаста и пелетирана крмна смеша за телад су произведене по истој рецептури. Микробиолошка и микотоксиколошка исправност смеша испитана је на дан производње (0-ти дан) и после 45 дана лагеровања. Укупан број микроорганизама у пелетираној смеши, на дан производње (280.000/g) био је вишеструко мањи од броја у брашнастој смеши (2.000.000/g). Слично је било 45 дана касније, када је укупан број микроорганизама у пелетираној смеши износио 270.000/g, односно 1.800.000/g у брашнастој смеши. Број квасаца и плесни на дан производње у пелетираној смеши био је 650/g, а у брашнастој 27.000/g. Слични резултати утврђени су 45 дана касније, када је број квасаца и плесни у пелетираној смеши износио 540/g, а у брашнастој 16.000/g. У пелетираној смеши на дан производње идентификовано је 6 врста, а у брашнастој 9 врста плесни. Сличан однос врста плесни у пелетираној (11) и брашнастој (13) утврђен је и 45 дана касније. У испитаним узорцима су доминирали представници рода *Fusarium — F. subglutinans* и *F. verticillioides*. Број сулфиторедукујућих клостридија у смешама, у оба термина контроле, био је сличан, односно испод 1000/g узорка. Остале врсте патогених бактерија нису идентификоване. Микотоксиколошком анализом смеша на дан производње утврђено је једино присуство трихотецена (Т-2 токсин) у количини од 0,337 mg/kg смеше. Примењени технолошки поступак пелетирања, уз додатак бентонита као везивног средства, имао је позитиван утицај на побољшање микробиолошке и токсиколошке исправности испитиваних крмних смеша.

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INFLUENCE OF *ALLIUM AMPELOPRASUM* L. AND *ALLIUM CEPA* L. ESSENTIAL OILS ON THE GROWTH OF SOME YEASTS AND MOULDS

ABSTRACT: Essential oils extracted from spices, as natural antimicrobial agents, attract particular attention due to their possible role in food protection from microorganisms, and their nontoxicity, in contrast to the synthetic preservatives. In this work, inhibitory effect of *Allium ampeloprasum* and two onions (*Allium cepa*), Junski srebrnjak and Kupusinski jabučar, essential oils in different concentrations (1, 4, 7 and 10%) on three yeasts (*Saccharomyces cerevisiae, Candida tropicalis* and *Rhodotorula* sp.) and three moulds (*Aspergillus tamarii, Penicillium griseofulvum* and *Eurotium amstelodami*) was investigated. All three essential oils showed the strongest inhibitory effect against *S. cerevisiae* in concentration of only 1%. Among onions, Kupusinski jabučar essential oil had stronger influence to *C. tropicalis*, while *Allium ampeloprasum* essential oil did not show any influence on this yeast. *Rhodotorula* sp. was influenced only by *Allium ampeloprasum* essential oil. The strongest inhibitory effect on *A. tamarii* showed Kupusinski jabučar (57% of inhibition, in concentration of 10%), while on *P. griseofulvum*, the strongest influence showed *Allium ampeloprasum* essential oil (78.3% of inhibition, in concentration of 10%). Junski srebrnjak and Kupusinski jabučar essential oils, in concentrations of 7 and 10% respectively, completely inhibited the growth of *E. amstelodami*.

KEY WORDS: *Allium* essential oils, antimicrobial activity, moulds, yeasts

INTRODUCTION

Fungi, as a large group of microorganisms, are common food contaminents and the cause of food deterioration. Besides, they are responsible for unpleasant odour and production of very toxic secondary metabolities — mycotoxins and alergen compounds. The most frequently isolated species of filamentous fungi from food belong to the genus *Aspergillus, Penicillium, Cladosporium, Mucor, Rhizopus, Fusarium, Alternaria, Eurotium, Emericella* etc. (Jay et al., 2005; Montville and Matthews, 2005; Škrinjar et al., 2005; Stanković et al., 2007) and yeasts to the genus *Saccharomyces*, *Rhodotorula, Candida, Debaromyces, Hansenula, Kloeckera, Lodderomyces,*

Torulopsis etc. (Davis, 2003; Jay et al., 2005; Montville and Matthews, 2005). An attempt to decrease the growth and evolution of fungi, as well as the synthesis of toxic metabolites, is achieved by the addition of synthetic preservatives. In recent years, there has been an increasing interest for the application of essential oils, obtained from spices and other herbs, as alternative antimicrobial agents in food for human nutrition. Also, the advantage of spice extracts is that they do not contain microorganisms which contaminate natural spices (Karan et al., 2005).

Garlic (*Allium sativum*) and onion (*Allium cepa*) are widely used as culinary ingredients and in food industry for taste improvement. Garlic has also been known for centuries as a spice with a very wide range of application, but also as a herb with important healing features (Harris et al., 2001). According to morphology and flavour, *Allium ampeloprasum* is very similar to garlic. It is mostly called as great-headed garlic, elephant garlic or pearl onion. The main active antifungal agents from garlic and onion essential oils are the degradation products of allicin, including diallyl trisulphide (DATS), diallyl disulphide (DADS), diallyl sulphide (DAS), which have greater antifungal effect than allicin (Tansey and Appleton, 1975; Corzo-Martinez et al., 2007), fistulosin (octadecyl 3-hydroxyindole), that has been isolated from welsh onion (Phay et al., 1999), antifungal proteins and peptides (allicepin, novel, isolated from onion bulb) (Lam et al., 2000; Wang and Ng, 2001; Wang and Ng, 2004), steroid saponins (eruboside-B, isolated from garlic bulb) (Matsuura et al., 1988). Garlic and onion germs are especially rich in essential oils (Stanković and Nikolić, 2002; Corzo-Martinez et al., 2007).

Content and composition of these active compounds, as well as their antimicrobial activity, are influenced by variety, area, climatic and storage conditions (Russo et al. 1998; Ceylan and Fung, 2004). For these reasons, the aim of this work was to investigate the antimicrobial activity of *Allium* plants esential oils from the region of Vojvodina against some food contaminating fungi.

MATERIAL AND METHODS

Allium plants. *Allium ampeloprasum* and two types of onion (*Allium cepa*), Junski srebrnjak and Kupusinski jabučar, grown at the Institute of Field and Vegetable Crops, Novi Sad, Serbia, were used for obtaining essential oil.

Test microorganisms. Three yeasts: *Rhodotorula* sp. (isolated from air), *Candida tropicalis* (clinical isolate), *Saccharomyces cerevisiae* 112 Hefebank Weinhenstephan, and three moulds: *Aspergillus tamarii, Penicillium griseofulvum* and *Eurotium amstelodami* (isolated from spices) were used for antimicrobial investigations. Microorganisms were maintained on Sabouraud maltose agar slants (SMA), at temperature of 4°C. For this experiment, 48 hours and 7 days old cultures of yeasts and moulds were used, respectively.

Essential oil extraction. Essential oils were extracted by steam distillation. They were chopped in small pieces and mashed with domestic blender (Braun Minipimer MR 400). Sample was transferred into a 2 l flask and mixed with distilled water (1:1 ratio), and the Clevenger apparatus was installed. The

system was heated during 3 h and the essential oil was collected in petroleum ether layer, in the oil separator tube. When the extraction time ran out, the petroleum ether layer was collected in a centrifuge tube and left at room temperature to evaporate the solvent. The tube, containing essential oil, was sealed with rubber stopper and stored in refrigerator.

Assessment of yeast growth inhibition. The assay was carried out by disc-diffusion test (L e b o f f e and P i e r c e, 2005). The tested concentrations of essential oils were 1, 4, 7 and 10%. One mililiter of inoculum (10^5 cfu/ml) was added to 10 ml SMA, which was consequently placed in 0 9 cm Petri dishes. Sterile 5 mm paper discs, after gelling, were placed in the centre of agar medium and soaked with 10ml prepared concentrations of ethanol-water solution of essential oils. Dishes were incubated at 25°C for 48—72 h, and the zones of inhibition showing the presence of antimicrobial activity were measured. All tests, including the control test (ethanol-water solution), were done in triplicates.

Assessment of mould growth inhibition. For each isolate, a conidial spore suspension (10^6/ml) was prepared in medium which contained 0.5% Tween 80 and 0.5% agar in distilled water (N i e l s e n and R i o s, 2000). Inoculation was performed with 1 ml of spore suspension (10^3 spores/ml) in the standard Petri dishes containing centred SMA medium. Steril disc (5 mm) was placed in the centre of every dish cover, and 10 ml of essential oil in concentrations of 1, 4, 7 and 10% were added. Then, the plates were closed with parafilm and left to incubate at 25°C for up to 7 days. The colony diameters were measured every day.

RESULTS

Antimicrobial activity of essential oils against yeasts

From Tab. 1, it can be seen that essential oil from *Allium ampeloprasum*, and the lowest concentration, had inhibitory influence only on *S. cerevisiae*. The zone of inhibition was 10 mm.

The increased concentration of essential oil did not show any influence on *C. tropicalis*, whereas the growth of *Rhodotorula* sp. and *S. cerevisiae* was completely inhibited.

Tab. 1 — Influence of *Allium ampeloprasum* essential oil on yeast growth

concentrations / yeasts	1%	4%	7%	10%
Rhodotorula sp.	—	ng	ng	ng
C. tropicalis	—	—	—	—
S. cerevisiae	10	ng	ng	ng

zone of inhibition is expressed in mm; — no activity; ng — no growth

Essential oil of onion (*Allium cepa*) Junski srebrnjak did not show inhibitory effect on *Rhodotorula* sp. (Tab. 2). Concentrations of 1 and 4% inhi-

bited the growth of other two yeasts, *C. tropicalis* and *S. cerevisiae*, with inhibition zones of 13 and 14 mm, and 14 and 16 mm, respectively, and complete inhibition at concentration of 7%.

Tab. 2 — Influence of onion (*Allium cepa*) Junski srebrnjak essential oil on yeast growth

concentrations / yeasts	1%	4%	7%	10%
Rhodotorula sp.	—	—	—	—
C. tropicalis	13	14	ng	ng
S. cerevisiae	14	16	ng	ng

zone of inhibition is expressed in mm; — no activity; ng — no growth

Data displayed in Tab. 3 show strong influence of 1% of onion (*Allium cepa*) Kupusinski jabučar essential oil on the growth of *S. cerevisiae* (21 mm inhibitory zone), weaker influence on *C. tropicalis* (10 mm inhibitory zone), and capability to completely stop their growth at concentration of 4%. *Rhodotorula* sp. in this case did not show any sensitivity.

Tab. 3 — Influence of onion (*Allium cepa*) Kupusinski jabučar essential oil on yeast growth

concentrations / yeasts	1%	4%	7%	10%
Rhodotorula sp.	—	—	—	—
C. tropicalis	10	ng	ng	ng
S. cerevisiae	21	ng	ng	ng

zone of inhibition is expressed in mm; — no activity; ng — no growth

Antimicrobial activity of essential oils against moulds

Allium ampeloprasum essential oil in concentration of 1% showed low inhibition of the growth of tested moulds (Tab. 4). The growth of *A. tamarii* (8.9%) was weakly inhibited in concentration of 4%, while *E. amstelodami* and *P. griseofulvum* showed higher sensitivity (23.2 and 18.7%). To achieve a similar effect on *A. tamarii*, 10% of essential oil was needed. At this level, the growth of *P. griseofulvum* was significantly reduced by 78.3%, and of *E. amstelodami* by 61.6%.

Tab. 4 — Inhibition of mould growth (%) influenced by *Allium ampeloprasum* essential oil

concentrations / yeasts	1%	4%	7%	10%
A. tamarii	43	8.9	14.8	23.2
P. griseofulvum	8.7	18.7	34.8	78.3
E. amstelodami	8.1	23.2	54.0	61.6

Results obtained for (*Allium cepa*) Junski srebrnjak (Tab. 5) indicate that *E. amslelodami* was very sensitive mould, with higher growth reduction in comparison to other two moulds, which could be seen with already 1% of oil, and complete inhibition was at the level of 7% of essential oil. The growth of *A. tamarii* was equivalent to the control sample under influence of 1% of oil, with 0.9% of inhibition. Generally, essential oil of Junski srebrnjak was the least active against *A tamarii*.

Tab. 5 — Inhibition of mould growth (%) influenced by onion (*Allium cepa*) Junski srebrnjak essential oil

concentrations / yeasts	1%	4%	7%	10%
A. tamarii	0.9	6.5	9.3	20.0
P. griseofulvum	2.2	6.5	14.5	19.6
E. amstelodami	29.7	40.5	100	100

1% essential oil of onion (*Allium cepa*) Kupusinski jabučar had less than 10% inhibitory effect on *A. tamarii* and *P. griseofulvum*. High concentrations (7 and 10%) lowered the growth of these moulds by 18.5 and 57% (*A. tamarii*) and 21.7% (*P. griseofulvum*). *E. amstelodami* was completely inhibited with concentration of 10% (Tab. 6).

Tab. 6 — Inhibition of mould growth (%) influenced by onion (*Allium cepa*) Kupusinski jabučar essential oil

concentrations / yeasts	1%	4%	7%	10%
A. tamarii	8.3	15.7	18.5	57.0
P. griseofulvum	5.7	12.2	21.7	21.7
E. amstelodami	27.0	46.0	67.6	100

Under impact of *Allium ampeloprasum* essential oil (Figure 1), of three investigated moulds, only the growth of *E. herbariorum* at lowest concentration of essential oil (1%) was delayed for two days, in comparison to the control. The growth of *A. tamarii* at concentration of 4% was delayed for two and three days, when used at higher concentrations. The absence of *P. griseofulvum* for three and six days, and the absence of *E. herbariorum* for five and six days in the presence of 7 and 10% of oil, indicates their higher sensitivity. With concentrations over 4%, stronger inhibitory effect on the growth rate of all investigated moulds can be noticed. An increase in the quantity of oil lengthens the time needed for mould colonies to reach the size of control sample colonies (more than 7 days).

Among onions, (*Allium cepa*) Kupusinski jabučar showed stronger inhibitory effect on the germination and growth rate of *A. tamarii* and *E. herbariorum* than of *P. griseofulvum* (Figure 2). *P. griseofulvum* was already noticed in the first two days, at all applied concentrations, while *A. tamarii* was

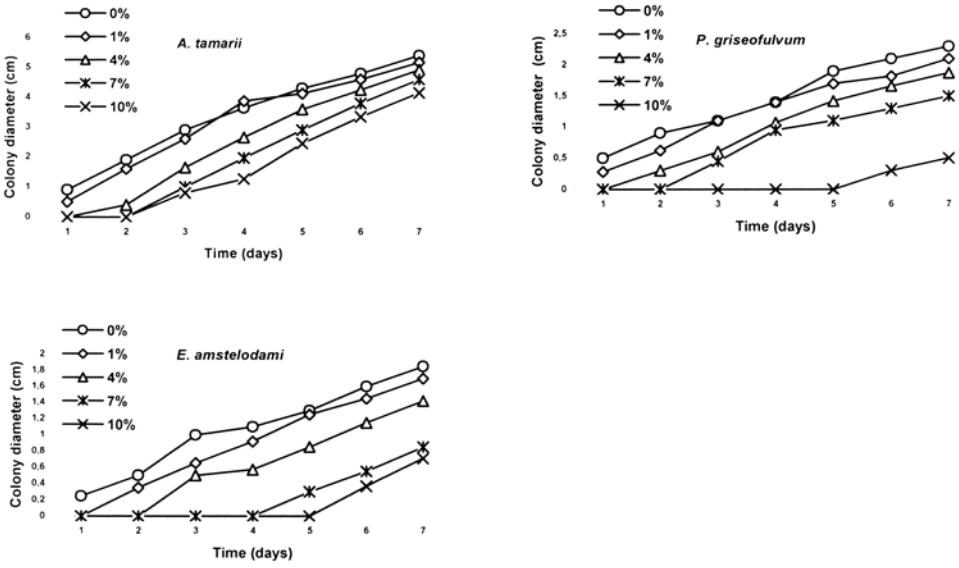

Fig. 1. Effect of essential oil of *Allium ampeloprasum* on the growth of moulds

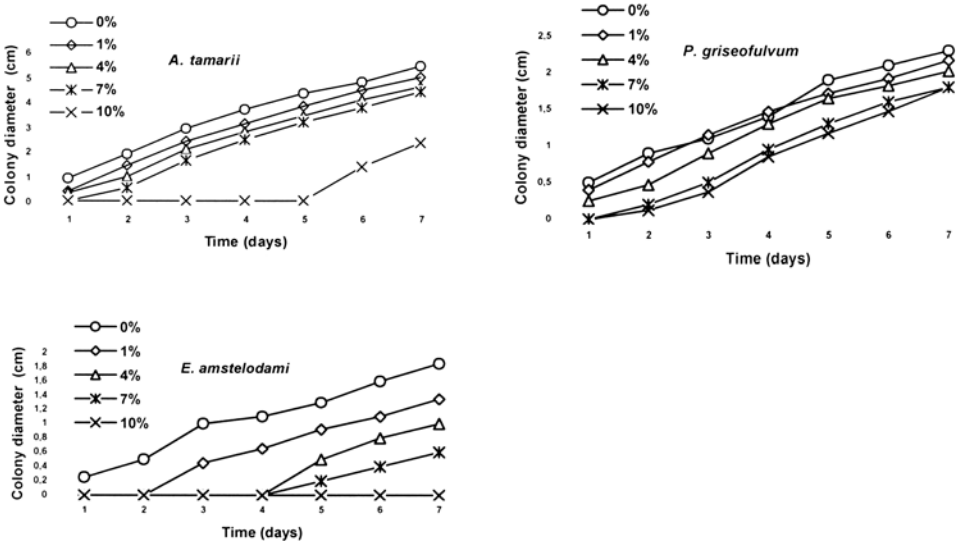

Fig. 2. Effect of essential oil of onion (*Allium cepa*) Kupusinski jabučar on the growth of moulds

noticed at concentration of up to 7%, and the growth was suppressed until the sixth day at the highest concentration. The most inhibited mould, with the lowest rate of development was *E. amstelodami*. Although *A. tamarii* and *P. griseofulvum* both showed at the concentration of up to 7%, stronger influence of essential oil was noticed on *A. tamarii*.

DISCUSSION

These investigations showed that *Allium* essential oils have the potential to inhibit the growth of yeasts and moulds. Essential oil of *Allium ampeloprasum* showed inhibitory effect on *Rhodotorula* sp. and *S. cerevisiae*, while *C. tropicalis* did not show any sensitivity. Essential oils of onion (*Allium cepa*) Junski srebrnjak, and particularly Kupusinski jabučar were more effective against *C. tropicalis* and *S. cerevisiae*. However, the latest two oils did not show any effect on *Rhodotorula* sp. All three oil samples had the strongest influence against *S. cerevisiae*. Essental oil of Kupusinski jabučar showed the strongest inhibitory activity against the investigated yeasts.

Conner and Beuchat (1984) pointed out the higher sensitivity of yeasts (*Candida lypolitica, Debaromyces hansenii, Hansenula anomala, Kloeckera apiculata, Lodderomyces elonginosporus, Rhodotorula rubra, S. cerevisiae* and *Torulopsis glabrata*) towards the influence of garlic, in comparison to onion. The onion essential oil inhibited only the growth of *S. cerevisiae*. The results of Dimić et al. (2008) showed good antimicrobial activity of onion essential oil against yeasts.

Among the tested moulds in this study, *Allium ampeloprasum* essential oil showed the strongest inhibitory effect on *P. griseofulvum. A. tamarii* turned out to be the most resistant mould towards the influence of essential oils. The highest inhibitory effect on *A. tamarii* was showen by the essential oil of Kupusinski jabučar. The most sensitive was *E. amstelodami*. The growth of this mould was completely stopped at concentration of 7% (Junski srebrnjak) and 10% (Kupusinski jabučar).

Yin and Tsao (1999) investigated the antifungal effect of seven herbs from *Allium* family. According to their results, garlic showed the highest antifungal activity against three *Aspergillus* species investigated. Fistulosin, antifungal compound isolated from Velsh onion, had expressed antifungal activity against few mould species, especially *P. roqueforti* and *A. oryzae* (Phay et al., 1999). Hsieh et al. (2001) noticed high sensitivity of *A. niger* towards combined extract of cornelberry, cinnamon and oriental onion (1:6:6, vol/vol/vol). Benkebala (2004) confirmed the inhibitory effect of onion and garlic on *A. niger, P. cyclоprium* and *F. oxisporum*. Hitikoto et al. (1980) and Hasan and Mahmoud (1993) showed that essential oils of caraway, clove, onion and garlic can influence the synthesis of sterigmatocystine and aflatoxins, toxic metabolits of *Aspergillus* species. Dimić et al. (2008) point out the antifungal activity of onion essential oil against *P. commune, P. aurantiogriseum, P. griseofulvum, P. corylophilum* and *A. ochraceus*. Investigations about the influence of different spice extracts on the growth of fungi showed that besides limiting the growth, they also cause changes in the morphology of colonies (Dimić et al., 2007a, 2007b), as well as changes on cellular level (destruction of fungal cells, decreasing the oxygen uptake, reducing the cellular growth, inhibiting the synthesis of lipids, proteins and nucleic acids, changing the lipid profile of the cell membrane and inhibiting the synthesis of the fungal cell wall) (Tansey and Appleton, 1975; Adetumbi et al.,

1986; Ghannoum, 1988; Gupta and Porter, 2001; Rassoli et al., 2006, Corzo-Martinez et al., 2007).

CONCLUSION

Results obtained in this investigation point out that essential oils of investigated *Allium* plants could be useful in controlling the development of yeasts and moulds in different foods, acting directly against microorganisms of food deterioration, or with surroundings, in case of packaged foods.

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ИНХИБИТОРНИ УТИЦАЈ ЕТАРСКИХ УЉА *ALLIUM AMPELOPRASUM* L. И *ALLIUM CEPA* L. НА РАСТ НЕКИХ КВАСАЦА И ПЛЕСНИ

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Резиме

Етарска уља екстрахована из зачина као природни антимикробни агенси привлаче посебну пажњу због улоге коју би могла имати у заштити хране од микроорганизама и за разлику од синтетичких конзерванаса нису токсична. У раду је испитиван инхибиторни утицај различитих концентрација (1, 4, 7 и 10%) етарских уља *Allium ampeloprasum* и две сорте црног лука (*Allium cepa*) Јунски сребрњак и Купусински јабучар на три квасца (*Saccharomyces cerevisiae, Candida tropicalis* и *Rhodotorula* sp.) и три плесни (*Aspergillus tamarii, Penicillium griseofulvum* и *Eurotium amstelodami*). Сва три етарска уља имала су најјачи инхибиторни ефекат према *S. cerevisiae* већ при концентрацији од 1%. Од црних лукова, етарско уље (*Allium cepa*) Купусински јабучар је јаче деловало на *C. tropicalis*, док етарско уље *Allium ampeloprasum* није показало ефекат према овом квасцу. На *Rhodotorula* sp. једино је деловало етарско уље *Allium ampeloprasum*. Најјачи инхибиторни ефекат према *A. tamarii* је показало етарско уље (*Allium sera*) Купусински јабучар (57% инхибиције при концентрацији од 10%), а према *P. griseofulvum* етарско уље *Allium ampeloprasum* (78.3% при концентрацији од 10%). Раст *E. amstelodami* је потпуно инхибиран при 7 и 10% етарских уља (*Allium cepa*) Јунски сребрњак и Купусински јабучар, респективно.

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EFFECT OF MINT (*MENTHA PIPERITA* L.) AND CARAWAY (*CARUM CARVI* L.) ON THE GROWTH OF SOME TOXIGENIC *ASPERGILLUS* SPECIES AND AFLATOXIN B1 PRODUCTION

ABSTRACT: An inhibitory effect of various concentrations (0.0, 0.5, 1.0, 1.5 and 2,0%) of mint (*Mentha piperita* L.) and caraway (*Carvum carvi* L.) on the growth of *A. fumigatus, A. flavus* and *A. ochraceus* was examined during 10 days of cultivation in YES medium at temperature of 25°C.

Mint showed stronger inhibitory effect than caraway. Total dry weight (g/l) after 10 days of the growth of *A. fumigatus* in YES medium with 0.5% of mint decreased by about 95%, *A. flavus* by 97% and *A. ochraceus* by about 82%. Addition of higher concentrations of mint (1.0, 1.5 and 2.0%) reduced the growth of all tested species. It was poor and hardly visible. pH values of the media increased with the increase of mint concentrations.

A. fumigatus showed the highest sensitivity towards caraway and *A. flavus* the lowest. Total dry weight (g/l) after 10 days of growth of *A. fumigatus* in medium with 0.5% of caraway decreased by about 72% in comparison to the control. In media with higher concentrations of caraway, its growth was found to be very poor. Concentration of 1.0% of caraway reduced *A. flavus* growth by 15% and of 1.5% by 92%, in regard to the control. In medium with 2.0% of caraway the growth of *A. flavus* was observed as poor and hardly visible.

The growth of *A. ochraceus* in medium with 0.5% of caraway decreased by about 85% comparing with control and further decrease was noticed by the increase of concentrations. In medium with 1.5% of caraway a reduction of about 95% of growth was found and under 2.0% of caraway it was poor.

pH of the media also increased with the increase of caraway concentrations.

Applied concentrations of mint and caraway inhibited completely the production of AB1 by *A. flavus.*

INTRODUCTION

Spices and herbs are produced from botanically diverse plants grown in a wide variety of soils and climates. Depending on the plant, different parts of

them may be used, such as: bulbs, roots, rhizomes, stems, leaves, bark, berries, buds, arils and seeds. In some cases, the whole plant is used.

Spices and herbs exhibit antimicrobial and antifungal activities because of their content of essential oils (Burt, 2004; Mayachiew and Devahastin, 2008; Ponce et al., 2008).

Mint (*Mentha piperita* L.), commonly called peppermint, is a well-known herbal remedy used for a variety of symptoms and diseases. In the popular medicine, it is used to treat nausea, flatulence, vomiting, indigestion, stomach cramps, menstrual cramps and parasitosis (Fonseka-Krueal and Fernandez, 2003). It is also recognized for its carminative, stimulant, antispasmodic, antiseptic, anti-inflammatory, antibacterial and antifungal activities (Guedón and Pasquier, 1994; Sean et al., 2004). Among the identified compounds some had already been reported as having antimicrobial activity, including 1,8-cineole, limonene, linalool and menthol (Mazzanti et al. 1998; Iscan et al., 2002).

Caraway (*Carum carvi* L.), with its pleasant aroma, is used to spice different foods. The finally chopped leaves are used in the preparation of soups, and seeds in bakery and confectionary industry. Also, it plays an important role in the flavouring of alcoholic beverages.

The dried ripe fruits of *Carum carvi* are used in folk medicine as a carminative, since it is effective against spasmodic gastrointestinal complaints, flatulence, irritable stomach, indigestion, lack of appetite, and dyspepsia in adults (Lemon, 2002; Thompson Coon and Ernst, 2002; Holtman et al., 2003; Madish et al., 2004).

Because of its aromatic properties, caraway essential oils are used in mouthwashes, toothpastes, soaps and perfumes.

Many data (Soliman and Badea, 2002; Dimić et al., 2007; Karalić, 2008) indicate its antimicrobial and antifungal activities.

The aim of this study was to investigate the effect of various concentrations of mint and caraway on the growth of three mycotoxigenic *Aspergillus* species (*A. fumigatus, A. flavus* and *A. ochraceus*) and production of aflatoxin B1 by *A. flavus*.

MATERIAL AND METHODS

Effect of various concentrations of caraway and mint on the growth of three toxigenic species of genus *Aspergillus* (*A. fumigatus, A. flavus and A. ochraceus*) was investigated. An inhibition of aflatoxin B1 (AB1) production during the growth of *A. flavus* by spices was examined, too.

Microorganisms. *A. fumigatus* Fres., *Aspergillus flavus* Link and *A. ochraceus* Wilhelm were isolated from some spices and then maintained on Sabouraud maltose agar (SMA) slants at 4°C.

Spices. Mint (*Mentha piperita* L.) and caraway (*Carum carvi* L.) were taken from the market. Before the experiments started, examination of a possible contamination of spices with AB1 was done.

Spices. Yeast extract sucrose (YES) medium (yeast extract — 20g, sucrose — 150g, distilled water — 1000 ml) was used for the investigation of the

fungal growth and AB1 production. Various concentrations (0.5, 1.0, 1.5 and 2.0%) of dried and sterilized spices were added to the basal medium.

Culture conditions. Erlenmayer flasks (300 ml) with YES medium (100 ml) were inoculated with 10 ml of inoculum and then incubated on rotary shaker for 10 days at 25°C. The cultures were grown on SMA slants for 7 days at 25°C. Conidia were harvested from slants with 10 ml of sterilized distilled water. The final conidial suspension was adjusted to an approximate conidia concentration of 10^7/ml.

All experiments were performed in duplicate.

Analyses. Total dry weight (g/l), pH values and residue of AB1 after 10 days of the growth were determined.

Total dry weight determination: the obtained biomass was separated from the medium by filtration through a filter paper, followed by drying at 105°C for 24 h. The yield of obtained biomass was calculated and expressed as total dry weight (g/l) (Šarić, 2007).

pH values of YES medium (initial — 5.50 and at the end of experiments) were measured with an Iskra MA 5730 pH-meter.

Qualitative and quantitative determination of AB1 was carried out by using comparatively two methods: a) thin-layer chromatography (TLC) and b) direct enzyme-linked immunosorbent assay (CD-ELISA).

a) TLC method (according to O.A.O.C. Methods, 1990): 25 ml of sample was extracted with 100 ml of a mixture of acetonitril and tap water (9:1), agitated on a shaker for 30 min. and then filtered; 50 ml of filtrate was extracted with 2x25 ml of n-hexane. Concentrations of AB1 were estimated visually, comparing them with a standard of pure AB1 from *Aspergillus flavus* supplied by Fluka Biochemika 294889, Switzerland.

b) Direct enzyme-linked immunosorbent assay (CD-ELISA) was done using Neogen Veratox® testing kits. Free mycotoxin in the samples and controls are allowed to compete with enzyme labeled mycotoxin (conjugate) for the antibody binding sites. After washig, the substrate is added, which reacts with the bound conjugate to produce blue colour. More blue colour means less mycotoxin. The test is read in a microwell reader (Thermolabsystem, Thermo, Finland) to optical densities. The optical densities of the controls from the standard curve and the sample optical densities are plotted against the curve to calculate the exact concentration of mycotoxin.

RESULTS AND DISCUSSION

Mycological investigation.

Mint. Results given in Tables 1, 2 and 3 point to an extremely high effect of mint concentrations at 0.5, 1.0, 1.5 and 2.0% on the growth of *A. fumigatus, A. flavus* and *A. ochraceus*.

After 10 days cultivation of *A. fumigatus* in YES medium with addition of 0.5% of mint, and at temperature of 25°C, a decrease of about 95% of the content of total dry weight comparing to the control (medium with 0.5% of

mint — 0.14 g/l, control — 2.57 g/l) was found out (Table 1). Visually, a poor, loose growth was observed. Colour of the medium was reddish.

In samples with 1.0, 1.5 and 2.0% of mint at the end of experiment, the growth of *A. fumigatus* was reduced, and hardly visible. Media were yellowish and hazy. pH values increased with the increase of mint concentrations (0.5% — 6.86, 2.0% — 7.91).

Table. 1. Effect of various concentrations of mint on the growth of *A. fumigatus* in YES medium

Concentration %	Total dry weight $g.l^{-1}$	pH value of YES medium after the growth
0.0	2.57	5.53
0.5	0.14	6.86
1.0	+	7.40
1.5	+	7.80
2.0	+	7.91

+ growth hardly visible

A. flavus showed higher sensitivity towards mint concentrations (Table 2). After the experiment, total dry weight (g/l) in control was 2.86. The growth was reduced by about 97% in medium with 0.5% of mint (0.01 g/l). A pelleted growth was observed, medium was hazy and yellowish. By the further increase of concentrations the growth was minimized. Media were bright brown (1.0% of mint) and brown (1.5 and 2.0% of mint) coloured. Small changes in pH values were noticed (medium with 0.5% of mint — 6.34 and with 2.0 — 7.02).

Table. 2. Effect of various concentrations of mint on the growth of *A. flavus* in YES medium

Concentration %	Total dry weight $g.l^{-1}$	pH value of YES medium after the growth
0.0	2.86	6.42
0.5	0.01	6.34
1.0	+	6.57
1.5	+	6.93
2.0	+	7.02

+ growth hardly visible

Total dry weight in YES medium with 0.5% of mint after 10 days cultivation of *A. ochraceus* was decreased by about 82% in regard to the control (Table 3). A loose growth was observed. Medium was yellowish and clear.

Mint concentrations of 1.0, 1.5 and 2.0% affected significantly the growth of *A. ochraceus.* It was hardly visible. At the end of the cultivation, medium containing 1.0% of mint was hazy and greenish-brown, with 1.5% reddish--brown, and those with 2.0% of mint were brown. pH values varied slightly.

Table. 3. Effect of various concentrations of mint on the growth of *A. ochraceus* in YES medium

Concentration %	Total dry weight $g.l^{-1}$	pH value of YES medium after the growth
0.0	4.47	6.59
0.5	0.81	4.83
1.0	+	6.73
1.5	+	6.53
2.0	+	5.51

+ growth hardly visible

Caraway. Results shown in Tables 4, 5 and 6 point to slower antifungal activity against fungal species tested in comparison with mint.

The most sensitive was found to be *A. fumigatus.* It was established that the growth of *A. fumigatus* in YES medium with 0.5% of caraway was inhibited about 72% (total dry weight — 0.73 g/l) in regard to control (total dry weight — 2.58 g/l) (Table 4). A loose growth was observed and colour of medium changed from yellow (at the beginning of cultivation) to bright brown (at the end of cultivation). Medium was hazy.

Concentrations of caraway at 1.0, 1.5 and 2.0% reduced the growth of *A. fumigatus* significantly (growth hardly visible). Media were clear and bright yellow coloured, at the beginning of experiment and intensive yellow (medium with 1.0 and 1.5% of caraway) or yellowish-brown (medium with 2.0% of caraway) at the end of experiment. pH values of media increased constantly from 5.53 (control) to 7.79 (medium with 2.0% of caraway).

Table. 4. Effect of various concentrations of caraway on the growth of *A. fumigatus* in YES medium

Concentration %	Total dry weight $g.l^{-1}$	pH value of YES medium after the growth
0.0	2.57	5.53
0.5	0.73	6.19
1.0	+	7.34
1.5	+	7.48
2.0	+	7.79

+ growth hardly visible

In further experiments, it was found out that *A. flavus* showed higher resistance against caraway. After 10 days cultivation of *A. Flavus*, in medium containing 0.5% of caraway, a decrease of about 15% of the growth was observed (Table 5). At the beginning of cultivation, a pelleted growth of *A. flavus* was noticed and medium was clear and in yellow-green shades. At the end of cultivation colour of medium changed into yellow. The broth was hazy.

With the increase of caraway concentrations, the growth of *A. flavus* was reduced. At the end of experiment, in medium with 1.5% of caraway, the total dry weight (g/l) was only 0.23 (control — 2.86 g/l). In medium with 2.0% of caraway a hardly visible growth was observed.

Growing in YES medium with 1.0% of caraway *A. flavus* produced pellets. Medium was slightly hazy and yellow-green. During the growth, colour changed into bright brown shades. At the end of cultivation, medium was hazy, as well as those with the addition of 1.5 and 2.0% of caraway. At higher concentrations colour of media varied from bright brown to dark brown. pH of these media increased with the increase of caraway concentrations (control — 6.42, medium with 2.0% of caraway — 7.74).

Table. 5. Effect of various concentrations of caraway on the growth of *A. flavus* in YES medium

Concentration %	Total dry weight $g.l^{-1}$	pH value of YES medium after the growth
0.0	2.86	6.42
0.5	2.43	7.31
1.0	1.32	7.58
1.5	0.23	7.66
2.0	+	7.74

+ growth hardly visible

A significant inhibitory effect of caraway concentrations on the growth of *A. ochraceus* was established. The concentration of only 0.5% reduced total dry weight by about 85% (0.65 g/l) in regard to control (4.47 g/l) (Table 6). Application of higher concentrations of caraway reduced more the growth of *A. Ochraceus*, si in the medium with 2.0% of caraway it was very poor.

Colour of YES media with caraway added varied from yellow (0.5, 1.0 and 2.5% of caraway) to intensive yellow (2.0% of caraway). The media were clear (0.5% of caraway) to slightly hazy (1.0% of caraway) or hazy (1.5 and 2.0% of caraway) at the beginning of the experiment. At the end of the growth, the colour varied from yellowish (0.5% of caraway), greenish-brown (1.0% of caraway), reddish-brown (1.5%) to brownish shades (2.0% of caraway). pH values increased with the increase of caraway concentrations (control — 6.59, medium with 2.0% of caraway — 7.75).

Many authors examined an antifungal activity of caraway, too. Soliman and Badea (2002) investigating the effect of caraway and some other spices on the growth of a few *Aspergillus* species (*A. flavus, A. parasiticus, A. ochraceus*) established an inhibition against them.

Karalić (2008) investigated the effect of various concentrations (0.07, 0.1, 0.5, 1.0 and 2.0%) of caraway exstracts on the growth of *A. flavus, A. sydowii, Eurotium herbariorum, Penicillium aurantiogriseum* and *P. corylophilum.* According to this research, the growth of *A. sydowii, E. herbariorum* and *P. aurantiogriseum* was inhibited completely (100%) by 0.5% of caraway extract, 88% of *A. flavus* and 73% of *P. Corylophilum.*

Table. 6. Effect of various concentrations of caraway on the growth of *A. ochraceus* in YES medium

Concentration %	Total dry weight $g.l^{-1}$	pH value of YES medium after the growth
0.0	4.47	6.59
0.5	0.65	7.00
1.0	0.25	7.04
1.5	0.21	8.12
2.0	+	7.75

+ growth hardly visible

Aflatoxicological analyses. After 10 days growth of *A. flavus* in YES medium with various concentrations (0.5, 1.0, 1.5 and 2.0%) of mint and/or caraway at temperature of 25°C, production of AB1 was inhibited. AB1 was detected only in control medium. Concentrations of AB1 determined by TLC method was 12.20 μg/l and 16.4 μg/l by DC-ELISA test.

CONCLUSION

All concentrations of mint and caraway showed a high inhibitory effect against fungal species tested (*A. flavus, A. fumigatus, A. ochraceus*). Mint had stronger effect than caraway. No AB1 was found in media with mint and/or caraway concentrations added.

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УТИЦАЈ МЕНТЕ (*MENTHA PIPERITA* L.) И КИМА (*CARUM CARVI* L.) НА РАСТ НЕКИХ ТОКСИГЕНИХ *ASPERGILLUS* ВРСТА И СТВАРАЊЕ АФЛАТОКСИНА Б1

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Резиме

Испитан је инхибиторни утицај различитих концентрација (0,0; 0,5; 1,0; 1,5 и 2.0%) менте (*Mentha piperita* L.) и кима (*Carvum carvi* L.) на раст *A. fumigatus, A. flavus* и *A. ochraceus* током 10 дана гајења у YES подлози и температури од 25°C.

Мента је показала већи инхибиторни ефекат од кима. Укупан садржај суве материје (g/l) након 10 дана раста *A. fumigatus* у YES подлози са 0.5% менте смањен је за 95%, *A. flavus* за 97%, а *A. ochraceus* за 82%. Уз додатак виших концентрација менте (1,0; 1,5 и 2,0%) раст у подлози био је једва видљив у свим случајевима. pH вредност подлога повећавала се са повећањем концентрација менте.

Највећу осетљивост према киму показао је *A. fumigatus*, а најмању *A. flavus*. Укупан садржај суве материје (g/l) након 10 дана раста *A. fumigatus* у подлози са 0,5% кима смањен је за 72% у односу на контролу, док је у подлогама са већим концентрацијама кима забележен веома скроман и једва видљив раст. Концентрација кима од 0,5% смањила је раст *A. flavus* за 15% у односу на контролу, а концентрација од 1,5% за 92%. Раст *A. flavus* у подлози са 2,0% кима био је веома редукован.

Раст *A. ochraceus* смањен је у присуству кима у концентрацији од 0,5% за 85% у односу на контролу и даље се смањивао са повећањем концентрација. У подлози са 1,5% кима забележено је смањење раста за 95%, док је у подлози са 2,0% кима раст био веома скроман, једва видљив.

pH вредности подлога такође су се повећавале са повећањем концентрација кима током раста испитиваних врста плесни.

Примењене концентрације менте и кима потпуно су инхибирале стварање АБ1 помоћу *A. flavus*.

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SUSCEPTIBILITY LEVEL OF CUCUMBER DOWNY MILDEW (*PSEUDOPERONOSPORA CUBENSIS*) TO METALAXYL

ABSTRACT: Level of susceptibility of *Pseudoperonospora cubensis* isolate from Ratkovo to metalaxyl in concentrations 50, 100, 200, 400 and 800 μg/ml was investigated. The trials were conducted on cotyledon and fully developed young leaves using cucumber cultivar Haroš. Reduced level of susceptibility was detected in metalaxyl concentrations of 50, 100 and 200 μg/ml because the intensity of sporulation in these treatments was on the same level as in control. Sporulation was also observed on developed leaves treated with metalaxyl in concentrations of 400 and 800 μg/ml.

KEY WORDS: cucumber downy mildew, level of susceptibility to fungicides, metalaxyl

INTRODUCTION

Downy mildew, caused by *Pseudoperonospora cubensis* (Berk. & Curtis) Rostovzev, is a widespread disease of greenhouse and field-grown cucumber plants and it can lead to a significant yield loss (Yang et al., 2008; Ko et al., 2008). In Serbian agroecological conditions downy mildew occurs every year in epiphytotic proportion (Balaž et al., 2006). The heaviest losses occur regularly on pickling cucumber in postharvest production.

First symptoms of downy mildew in vegetation can be detected on older leaves, usually during the end of June and the beginning of July. These leaves have chlorotic spots which in a short time become necrotic. Reproductive organs are being formed on the lower side of leaves. The optimal temperature for sporulation of this parasite is around 15°C. Fruits are not directly attacked, but due to leaf necrosis they become smaller and of poor quality.

Although differences in susceptibility to downy mildew are registered among genotypes, the basic control measure of cucumber downy mildew is still the use of fungicides. A large number of sprays during vegetation increase

the risk of parasite resistance to systemic fungicides. Detection of resistance may limit the use of fungicides to which resistance appeared, decrease the number of treatments, and in that way it may contribute to the safe-food production.

One of the widespread used fungicide active ingredients against downy mildews of different cultivated plants is metalaxyl. This is a systemic fungicide which inhibits protein synthesis in the pathogen cells. According to the literature data, a frequent use of metalaxyl worldwide caused a resistance of many downy mildew pathogens (Daayf et al., 2001; Molinero-Ruiz et al., 2005; Urban and Lebeda, 2006). The aim of this research is detection of resistance of *P. cubensis* population to metalaxyl in our country. These investigations will contribute to more effective cucumber downy mildew control which will result in decrease of number of treatments and in production of healthy and safe cucumber fruits for human consumption.

MATERIAL AND METHODS

Laboratory experiments for determination of sensitivity level of *P. cubensis* to metalaxyl were conducted from July to October, 2008 at the Faculty of Agriculture, Novi Sad, at the Department for Plant and Environmental Protection, according to the method described by Urban and Lebeda (2006). Cotyledons and fully developed young leaves of cucumber.

Preparation of inoculum for artificial inoculations

In these trials, *P. cubensis* isolate from Ratkovo locality was used. The infected leaves of cultivar Haroš were collected and brought in the laboratory in portable refrigerator. The leaves were washed and placed for incubation on a wet filter paper in Petri dishes for 2 days. After this period, zoosporangia which formed on leaves were shaken off in container with distilled water, and suspension of zoospores was prepared. Total volume of suspension was 7,2 ml and 9,6 ml for inoculation of cotyledons and fully developed leaves, respectively. In both cases, concentration of suspension was measured with hemocytometer, and it was set up to 10^5 zoospores/ml.

Preparation of plants for artificial inoculations

Cucumber seed of cultivar Haroš was used for cucumber cotyledon production. Seeds were sown in plastic pots (volume 500 cm^3) in 50 replications. The substrate was a mixture of 3 parts of soil and 1 part of sand. 2 to 4 seeds were sown in every pot and plants were grown in the greenhouse at temperature of 15°C during night and 25°C during day. Cotyledons were used for inoculations when they were approximately 3 cm in diameter. Fully developed leaves were around 7 cm in diameter.

Concentrations of metalaxyl

Solutions of pure, technical metalaxyl in methanol were prepared for determination of *P. cubensis* sensitivity and tested concentrations were: 50, 100, 200, 400 and 800 μg/ml.

Cotyledons and fully developed young leaves were sprayed with metalaxyl in noted concentrations one hour prior to artificial inoculations with *P. cubensis* spore suspension.

Cucumber artificial inoculations with *P. cubensis*

After the treatment with metalaxyl, cotyledons, as well as fully developed leaves were placed with adaxial side down in Petri dishes containing double layer of wet filter paper. The trial was set up in five and four replications regarding cotyledons and developed leaves, respectively.

P. cubensis spore suspension was sprayed on tested leaves. After the inoculation, leaves were incubated in the climate chamber at temperature of 15°C with photoperiod of 12 hours. Under these conditions, sporulation was expected after 6—10 days when the first evaluation was also performed.

Evaluation of sporulation intensity and data analysis

Sporulation intensity was evaluated on the basis of visual observation of cotyledons under the magnifying glass at 45 X magnification. The scale for evaluation was 0 to 4:

0 — no sporulation,
1 — sporulation on less than 25% of the leaf area,
2 — sporulation on 25—50% of the leaf area,
3 — sporulation on 50—75% of the leaf area,
4 — sporulation on over 75% of the leaf area.

Average value of sporulation intensity was calculated on the basis of sporulation intensity in each replication. Data were statistically processed using software Costat which performed Duncan's Multiple Range Test.

RESULTS

The first screening was performed 6 days after the inoculation. However, the first signs of sporulation were detected after ten and seven days at cotyledons and fully developed leaves, respectively and then the evaluation of sporulation intensity was performed. After this, intensity of sporulation was estimated on a daily basis. Final assessment of the trial was performed after 13 and 9 days, with tests on cotyledons and fully developed leaves respectively (Tables 1 and 2).

Tab. 1 — Sporulation intensity of *P. cubensis* on cucumber cotyledon leaves treated with metalaxyl

Metalaxyl concentrations mg/ml	Sporulation intensity Replications					Aver	Rank
	I	II	III	IV	V		
0 (control)	1	1	1	3	2	1,6	a
50	2	0	0	1	1	0,8	abc
100	2	1	1	1	0	1,0	ab
200	0	0	1	0	1	0,4	bc
400	0	0	0	0	0	0	c
800	0	0	0	0	0	0	c

LSD 0,05 = 0,81

Tab. 2 — Sporulation intensity of *P. cubensis* on developed cucumber leaves treated with metalaxyl

Metalaxyl concentrations mg/ml	Sporulation intensity Replications				Aver	Rank
	I	II	III	IV		
0 (control)	2	4	2	3	2,75	a
50	1	2	3	1	1,75	ab
100	2	1	2	1	1,5	ab
200	0	1	1	3	1,25	ab
400	0	2	0	0	0,5	b
800	0	0	1	2	0,75	b

LSD = 1,44

As expected, the maximum sporulation was determined in control, with tests on both cotyledons and fully grown leaves.

In cotyledons test, sporulation intensity in treatments with metalaxyl in concentrations of 50 and 100 μg/ml, was statistically on the same level with control. In tests on fully developed young leaves, sporulation intensity in control was also on the same level with metalaxyl treatments in concentrations of 50 and 100 μg/ml. However, in latter decreased sensitivity was also determined in concentration of 200 μg/ml.

Treatments with metalaxyl in concentrations of 400 and 800 μg/ml on cotyledon leaves completely disabled sporulation of the pathogen, while in tests on developed leaves sporulation was signicifinantly lower than the one in control.

DISCUSSION

Cucumber downy mildew, in our conditions, presents very destructive disease against which a large number of fungicide treatments are performed every year. Since cucumber harvest is continuous during vegetation, it is necessary to undertake integral measures of protection in order to reduce the number of fungicide treatments (B a l a ž et al., 2006). Required treatments

should be carried out using effective fungicides and it is essential to utilize high quality application technique (B a l a ž et al., 2007). Due to a large number of treatments and high reproductive capacity of *P. cubensis*, a risk of decreased sensitivity, i.e. resistance to systemic fungicides is always present. This especially includes fungicides which have relatively narrow mode of action on pathogen's metabolism.

Results on decreased sensitivity of *P. cubensis* have been published by U r b a n and L e b e d a (2006), who determined that fungicides fosetyl Al and metalaxyl are less effective in cucumber downy mildew control, while propamocarb is still highly efficient.

Similar results were gained in our research, in which it was determined that even after the treatment with metalaxyl in concentration of 200 μg/ml (which represents the amount of active ingredient which is used in practice, U r b a n and L e b e d a, 2006) parasite still sporulates on developed leaves. Based on these experiments, it can be concluded that metalaxyl, in given concentration is not effective enough agaist *P. cubensis* population from Ratkovo. Also, alarming fact is that parasite sporulated with low intensity on developed leaves, even after the treatments with metalaxyl in concentrations of 400 and 800 μg/ml.

For more complete insight in sensitivity level of *P. cubensis* to metalaxyl, it is necessary to test more isolates from a large number of localities. Also, these trials should include other active ingredients such as fosetyl Al and azoxystrobin, which are widely used in our conditions and for which reduced efficacy was also detected in some other countries (B r o w n et al., 2004; H a n et al., 2005).

These investigations are essential for healthy and safe human consumption of cucumber fruits and also for the possibility of exporting these products into the countries of European Union.

CONCLUSION

In this research, reduction in sensitivity of *Pseudoperonospora cubensis* isolates from Ratkovo to metalaxyl has been determined. In order to produce cucumber which is healthy and safe for human diet, as well as to achieve higher fungicide efficacy in cucumber powdery mildew control, it is necessary to adapt integral measures for disease control and to use highly effective fungicides with favorable ecotoxicological characteristics and short waiting period.

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НИВО ОСЕТЉИВОСТИ ПРОУЗРОКОВАЧА ПЛАМЕЊАЧЕ КРАСТАВЦА (*PSEUDOPERONOSPORA CUBENSIS*) ПРЕМА МЕТАЛАКСИЛУ

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Резиме

Пламењача краставца у нашим условима представља изузетно деструктивно обољење, против којег се сваке године предузме већи број хемијских третмана. Пошто је берба краставца током вегетације континуирана, неопходно је предузимање интегралних мера заштите у циљу смањења броја хемијских третмана. Неопходне хемијске третмане је потребно извести са ефикасним фунгицидима и потребно је водити рачуна о квалитетној техници апликације. Услед великог броја третмана и велике репродуктивне способности паразита постоји ризик од појаве смањене осетљивости, односно резистенције *Pseudoperonospora cubensis* нарочито према системичним фунгицидима који имају релативно узак спектар деловања на метаболизам паразита. У раду је испитан ниво осетљивости проузроковача пламењаче краставца *P. cubensis*, изолата из Раткова, према металаксилу примењеном у концентрацијама 50, 100, 200, 400 и 800 μg/ml. Тест је изведен на котиледоним и развијеним листовима краставца сорте Харош. Утврђена је смањена осетљивост металаксила у концентрацијама од 50, 100 и 200 μg/ml. Спорулација је на развијеним листовима утврђена и у концентрацијама 400 и 800 μg/ml.

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REPRODUCTIVE STRATEGIES OF FIELD *PHYTOPHTHORA INFESTANS* POPULATIONS ON POTATO AND TOMATO IN SOME REGIONS OF RUSSIA

ABSTRACT: The occurrence of zoosporangia and oospores is analyzed for 88 field populations of *Phytophthora infestans* (Mont.) de Bary in Moscow region and other regions of Russia from 1997 to 2006. For estimation of zoosporangia and oospore frequencies in the populations, the indexes IZ (for zoosporangia) and IO (for oospores) are suggested. The combinations of these indexes enabled a reveal of 4 strategies for *P. infestans* reproduction, which were found in the field populations of the pathogen. These strategies were more numerous in *P. infestans* field populations on tomato than on potato (4 versus 2) in spite of the fact that potato populations in Moscow region strongly predominated. It was connected with rareness or absence of oospores in potato and their presence in tomato, especially in fruit of many populations.

The knowledge about strategies of reproduction can be applied for verification of efficiency of new methods for crop protection against late blight, and for investigations of *P. infestans* populations.

KEY WORDS: *Phytophthora infestans*, late blight, oospores, zoosporangia, field estimation

INTRODUCTION

Phytophthora infestans is a dangerous parasite of potato and tomato. The losses of these crops caused by late blight are very high. Much is known about biology, ecology and genetics of *P. infestans* (Brasier, 1983; Shaw, 1987; Shattock et al., 1987; Dyakov, 1992). Effective methods are used (Kadish & Cohen, 1988) for estimation of fitness and aggressiveness of *P. infestans* isolates under the laboratory conditions. The approaches for estimation of late blight in the field are also numerous (Popkova, 1972; Dorozhkin et al., 1976).

The current situation with *P. infestans* investigation is that the laboratory and field studies of this parasite are mostly separated from each other. For instance, in Russia and some other countries, the occurrence of oospores (source

of primary infection), as well as the intensity of formation of zoosporangia (source of secondary infection), are often excluded from field estimations at crop protection measures. On the other hand, the direct results of field observation on late blight severity are generally not closely connected with the investigations of biology of its agent.

Our purpose is to suggest the approach which enables a reveal of the ratio of structures for asexual and sexual reproduction (zoosporangia and oospores) in the field *P. infestans* populations. Thus, we would be able to clearly understand the strategies of reproduction of certain *P. infestans* populations and use adequate methods of protection against them.

MATERIAL

From 1997 to 2006, the samples from 88 field populations from different regions of Russia (Moscow; Moscow, Leningrad, Novgorod, Yaroslavl, Tula, Bryansk, Kaluga, Tambov, Ryazan, and Tomsk regions; as well as from Mordovia, Northern Caucasus, Stavropol Territory) were studied. The samples were blighted leaflets of Russian potato cultivars Nevsky, Lugovskoy, Lorkh, Syneglazka (blue-eyed potatoes), and Dutch cultivar Sante, as well as some other cultivars and their mixtures of undetermined status; the blighted leaflets and fruits of tomato cultivars Ottawa, Yamal, Beliy Naliv, etc. The number of samples per population varied from 10 to 50 depending on the size of a plot.

METHODS

Determination of presence of oospores and zoosporangia in the blighted samples. The blighted leaflets were placed in moist chambers (Petri dishes with moist filter paper). After one day of incubation the freshly formed zoosporangia were transferred to Petri dishes with oatmeal agar for collection of *P. infestans* isolates. Then, the leaflets were boiled in 96% ethyl alcohol for 3 min, for chlorophyll removal and exposed to 10% water solution of bleaching chlorine for 1 hour. The blighted tomato fruits were also put in moist chambers and the *P. infestans* isolates were collected from them in the same way. After this the fruits were incubated in the moist chambers until their rotting. Decolorized samples were investigated under the light microscope, and oospores and empty zoosporangia (as their content was destroyed; Fig. 1, 2) were looked for.

If the presence of oospores or zoosporangia was not more than 50 per field of vision (1 mm^2), from 51 to 250, and more than 250, it was assessed as rare, moderate, and frequent, respectively.

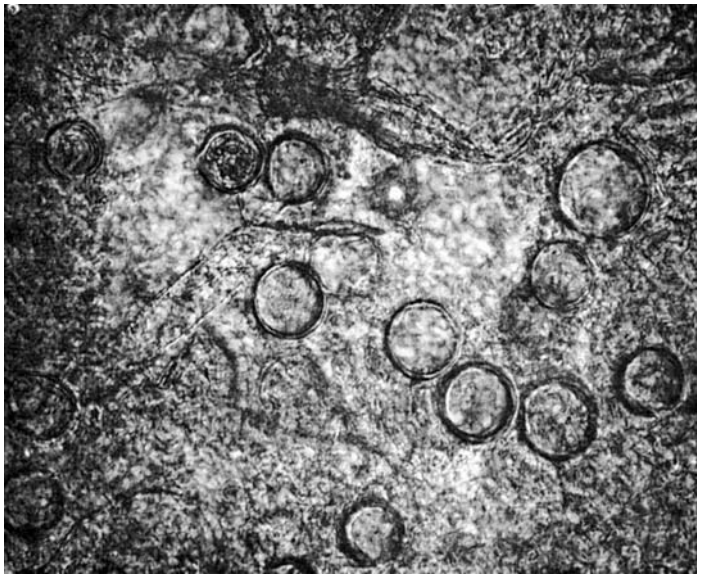

Fig. 1 — Numerous oospores of *P. infestans* in tomato fruits

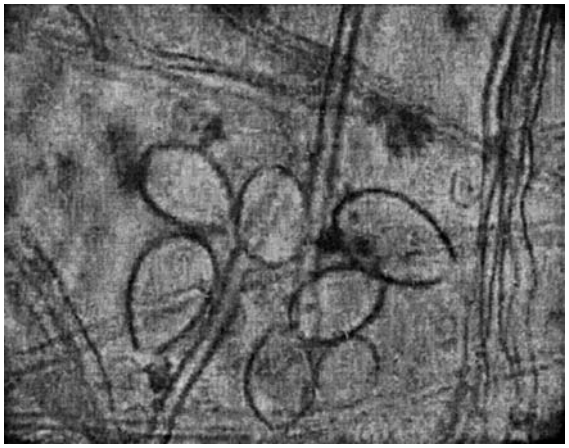

Fig. 2 — Empty zoosporangia of *P. infestans* in potato leaf after boiling in ethanol solution and exposure to solution of bleaching chlorine.

The indexes of presence of oospores (IO) and zoosporangia (IZ) were calculated as follows:

$10 = 0.05 \cdot RO + 0.5 \cdot MO + FO$, where:
RO — presence of samples with rare oospores, %
MO — presence of samples with moderate oospores, %
FO — presence of samples with frequent oospores, %;

IZ = 0.05 . RS + 0.5 . MS + FS, where:
RS — presence of samples with rare zoosporangia, %
MS — presence of samples with moderate zoosporangia, %
FS — presence of samples with frequent zoosporangia, %.

Thus, this method allows offers qualitative estimation of occurrence of oospores and zoosporangia in the same blighted samples.

Determination reproduction strategies. Different distribution of indexes of presence of oospores and zoosporangia gave an outline of four possible strategies of *P. infestans* reproduction. Four strategies and 20 substrategies were finally revealed (Table 1).

Tab. 1 — Strategies and substrategies of reproduction of field *P. infestans* populations

Strategy	Substrategy	Occurrence of zoosporangia		Occurrence of oospores	
		IZ	Characteristic	IO	Characteristic
		0.1—20.0	**Rare**	**0—10.0**	**Limited**
W (Weak populations)	W1			0	absent
	W2			0.1—3.0	negligible
	W3			3.1—10.0	slight
		20.1—60.0	**Quite frequent**	**0—10.0**	**Limited**
A (Asexual populations)	A1	20.1—40.0	moderate	0	absent
	A2			0.1—3.0	negligible
	A3			3.1—10.0	slight
	A4	40.1—60.0	frequent	0	absent
	A5			0.1—3.0	negligible
	A6			3.1—10.0	slight
	A7	60.1—100	very frequent	0	absent
	A8			0.1—3.0	negligible
	A9			3.1—10.0	slight
		0.1—20.0	**Rare**	**10.1—100**	**Quite frequent**
S (Sexual populations)	S1			10.1—25.0	moderate
	S2			10.1—100	frequent
		20.1—60.0	**Quite frequent**	**10.1—100**	**Quite frequent**
B (Both asexual and sexual populations)	B1	20.1—40.0	moderate	10.1—25.0	moderate
	B2			10.1—100	frequent
	B3	40.1—60.0	frequent	10.1—25.0	moderate
	B4			10.1—100	frequent
	B5	60.1—100	very frequent	10.1—25.0	moderate
	B6			10.1—100	frequent

A certain strategy reflects the ratio of oospore and zoosporangia presence in the field *P. infestans* population and its fitness. The higher is the number of zoosporangia in population, the higher is its fitness.

RESULTS

Strategies of reproduction of *P. infestans* on potato and tomato in Moscow region. The analysis of 88 *P. infestans* populations revealed all four possible strategies for their reproduction (Table 2).

Tab. 2 — Number of *P. infestans* Russian populations with a certain strategy/substrategy of reproduction on potato leaves, as well as on leaves and fruits of tomato from 1997 to 2006

Strategy of reproduction	Substrategy of reproduction	Number of *P. infestans* populations on		
		potato leaves	tomato leaves	tomato fruits
		9	**2**	**2**
W (Weak populations)	W1	2	0	0
	W2	5	2	1
	W3	2	0	1
		46	**12**	**10**
A (Asexual populations)	A1	8	4	1
	A2	8	3	0
	A3	0	0	6
	A4	9	4	1
	A5	8	0	1
	A6	3	0	0
	A7	5	1	1
	A8	4	0	0
	A9	1	0	0
		0	**0**	**2**
S (Sexual populations)	S1	0	0	0
	S2	0	0	2
		0	**2**	**3**
B (Both asexual and sexual populations)	B1	0	1	2
	B2	0	0	0
	B3	0	0	1
	B4	0	0	0
	B5	0	1	0
	B6	0	0	0
General number of populations		55	16	17
General number of strategies		**2**	**3**	**4**
General number of substrategies		11	7	10
Index of Shannon (for substrategies)		3.23	1.85	2.94

Strategy A (asexual type of reproduction) predominated in populations with different origination. Strategy W (weak type of population producing just a few zoosporangia and/or oospores) was also quite common in different populations, but occurred much more rarely. However, strategies S (sexual type) and B (both sexual and asexual types of reproduction in population) were re-

vealed only in several *P. infestans* populations isolated from tomato fruits (Table 2).

The calculation of general number of reproduction substrategies and corresponding Index of Shannon in all host plants has indicated that the diversity of reproduction substrategies was higher in *P. infestans* populations from tomato fruits and potato leaves than in *P. infestans* populations from tomato leaves (Table 2).

The testing determination of reproduction strategies. In 1997 for 20 field populations, the average means of oospore number per sample were calculated (Smirnov, Elansky, 1999). For these populations, IO was also determined and correlation between average means of oospore presence and IO means were calculated. The mean of correlation coefficient was 0.93 ± 0.03. Thus, the correlation between them approaches identity and IO values reflect oospore presence in field *P. infestans* populations very well.

DISCUSSION

In the previous investigations (Smirnov & Elansky, 1999; Smirnov & Kuznetsov, 2001; Smirnov et al., 2008) it was demonstrated that the field *P. infestans* populations may be very different from each other in occurrence of oospores and zoosporangia, so the idea about different strategies of reproduction was suggested. However, no clear quantitative assessment was made. Our current method of determination of strategies for reproduction provided principally new reliable results. Their authenticity was supported by the cluster and correlation analyses.

Previously, the ranked approach was applied by Flier et al. (2001) for the estimation of oospore formation in the Toluca Valley, Mexico.

The method used for detection and calculation of oospores and zoosporangia in the samples does not imply very high level of exactness. It provides true qualitative estimation. Majority of detected oospores were formed under the field conditions. For zoosporangia, our observations are not able to reveal all dynamics of their formation *in vivo* (and we did not investigate it in our study); it is only possible to assess the potential of their formation under the favorable conditions of moist chambers. IZ value reflects status of *P. infestans* mycelium in lesion very well.

The novelty of our method lies the joint analysis of oospore and zoosporangia occurrence with each of these features expressed by one figure. The count of oospore number per certain lesion area used in many previous investigations was always somewhat distorted by deviation from the average value. This complicates analysis of the obtained results. Per cent of samples with oospores is an unambiguous value, yet it does not guaranty a precise estimation because of different oospore occurrence in the samples. Our method excludes all aforementioned problems.

The suggested equations for the calculation of IO and IZ are analogous to the equation of intensity of disease development (Dorozhkin et al., 1976).

In the tomato populations, more strategies were found than in the potato populations. A few populations were similar to Mexican population on potato

in Toluca Valley — so called 'small Mexico' (Smirnov, Kuznetsov, 2001; Smirnov et al., 2008). It is probably not connected with genotypic differences between the populations on potato and tomato, since on these crops both the same and different strains can occur (Elansky et al., 1999). The formation of oospores in fruits and seeds of tomato was previously proved in Israel (Rubin et al., 2001). Under conditions of Moscow region, the tomato (especially fruits) is a suitable substrate for *P. infestans* suitable for formation of both oospores and zoosporangia, especially in the depressive seasons when such formation is difficult in potato. It is highly probable that tomato populations with S and B strategies can increase the pathogenicity (Goodwin et al., 1995), and under the conditions of Moscow region it might be dangerous for both primary (strategies S and B) and secondary (strategies A and B) infection of potato, though the infection dissemination from tomato to potato is not rapid. In potato, the populations with such strategies are rare or absent.

Interconnection between the occurrence of oospores and zoosporangia was not detected. This indicates that their formation is regulated by different factors.

Very important practical aspect in plant pathology is the estimation of fitness (aggressiveness) of *P. infestans* strains. For its estimation, it was necessary to isolate the strains as pure cultures, inoculate leaflets or tuber discs, and determine the infection frequency (IF), lesion area (LA) and sporulation capacity (SC). The compositive fitness index CFI = IF x LA x SC allows estimation of the fitness (aggressiveness) of a certain *P. infestans* strain (Kadish & Cohen, 1988). Thus, our approach can be adapted to this.

The aforementioned method was not applied in the field as it was difficult to estimate the sporulation capacity. The application of IZ solves this problem. The spread of disease (S) corresponds with the infection frequency (IF), intensiveness of disease (I) — sporulation capacity (SC). Thus, by combining new and well known approaches, it is possible to obtain the estimation of aggressiveness of the field *P. infestans* population. Laboratory estimation of aggressiveness of isolates collected from the latter would not provide reliable result as many properties of *P. infestans* can be essentially changed after cultivation on the agar media and re-isolation.

P. infestans fitness would be expressed differently under laboratory and field conditions. Under the laboratory (as a rule optimal) conditions all components (LA, SC, and IF) can essentially influence CFI, but under field conditions it is not always true. IZ better indicates fitness of the field *P. infestans* population and its alterations than S and I. In the laboratory experiments such effects do not play an important role.

The knowledge of strategy distribution will help understand the biology of *P. infestans* much better and increase the efficiency of protective measures against late blight. In ecology, sexual and asexual reproductions represent opposite strategies of survival (Brasier, 1983; Dyakov, 1992; Cohen et al., 2000). Hence, our approach can be used for development of models connected with DSS (Decision Support Systems) directed at proper application of protective measures against late blight.

Also, it is possible to apply our method to the issues which are usually investigated by means of traditional approaches to population biology. First of all, it is the comparison of *P. infestans* strains on potato and tomato, as well as the interconnection between occurrence of zoosporangia, oospores, and mating types.

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РЕПРОДУКТИВНЕ СТРАТЕГИЈЕ ПОПУЛАЦИЈЕ *PHYTOPHTHORA INFESTANS* КОД ПРОИЗВОДЊЕ КРОМПИРА И ПАРАДАЈЗА НА ОТВОРЕНОМ У НЕКИМ РЕГИОНИМА РУСИЈЕ

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Резиме

Испитивано је присуство зооспорангија и ооспора у 88 популација *Phytophthora infestans* (Mont.) de Bary у призводњи на отвореном у московском региону као и у другим регионима Русије у периоду од 1997. до 2006. За процену учесталости зооспорангија и ооспора у популацији предложени су индекси ИЗ (за зооспорангије) и ИО (за ооспоре). Комбинацијом ових индекса откривено је постојање 4 репродуктивне стратегије у њивским популацијама овог патогена. Ове стратегије су биле заступљеније у њивским популацијама *P. infestans* на парадајзу (4 према 2) упркос чињеници да је популација била доминантна на кромпиру у региону Москве. Ово је највероватније последица одсуства или незнатног присуства ооспора у кромпиру и њиховог присуства у парадајзу, нарочито плоду, код великог броја популација.

Познавање репродуктивне стратегије може се користити за утврђивање ефикасности нових метода заштите против пламењаче, као и за испитивање популације *P. infestans*.

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MORPHOLOGICAL AND PATHOGENIC CHARACTERISTICS OF *COLLETOTRICHUM TRIFOLII* BAIN ET ESSARY, THE ALFALFA ANTHRACNOSE INDUCER

ABSTRACT: During summer and autumn, easily recognizable diseased plants can be detected in alfalfa fields. Diseased plants have characteristically curved apical part, commonly known as "shepard's hook". Using the standard mycological methods, several fungi isolates were obtained. Four of these isolates were used for further study. Two standard mycological methods were used for determination of pathogenicity of *Colletotrichum* spp. isolates: with and without damaging of the stems. During these studies, it was determined that all of the isolates were causing the symptoms of alfalfa anthracnose. All of the selected isolates form germinative bodies — acervulae — on mycelium. Acervulae can be grouped or scattered across the colony area. Their size in the culture was 100—250 mm. Conidia were short, cylindrical, relatively broad, with both end obtuse 7,85 x 3,85 mm. Appressoria were ovate to obovoid, pale to medium brown 7,5—16,5 x 5,5—8,9 μm.

KEY WORDS: *Colletotrichum trifolii* Bain et Essary, alfalfa, morphology, pathogenicity

INTRODUCTION

Colletotrichum trifolii Bain et Essary, inducer of alfalfa anthracnose, is widespread in many areas in Central Serbia and Vojvodina. Considering that and, also, the damage it can cause, anthracnose is economically very important disease (Robotić i sar., 1983; Robotić i Klokočar-Šmit, 1983). The damage caused by this pathogen agent can be seen trough decreased quantity and quality of green mass from 10 to 70%, depending on the alfalfa cultivar, pathogen species and edaphic factors (Stuteville and Erwin, 1990).

The effect of this disease is a decrease of the alfalfa field duration. Anthracnose decreases vigor of the individual plants and thins out the plant popu-

lation. During summer and autumn, the diseased plants start to appear in the alfalfa fields. These diseased plants have characteristic appearance. Several stems of the plant have yellow to silverfish color and start to show the signs of wilting. The apical part of the plants is curved down, forming the so-called "shepard's hook". These symptoms correspond with symptoms described in the other countries by various authors (Ostazeski et al., 1969; Devine et al., 1971). In our country, authors that have studied this fungus are Robotić i sar. (1983a); Milijić i sar. (1986); Vasić i sar. (2005).

Considering the damage that anthracnose induces in the alfalfa fields in our country and considering the fact that this disease was not studied in detail, this study was performed. The aim of this study was to collect the isolates of *Colletotrichum* spp. in Serbia and to study its pathogenicity and morphological characteristics. A detailed study of alfalfa anthracnose inducer enables us to understand pathologic processes and possibilities for eradication of this pathogen agent.

MATHERIAL AND METHODS

Using standard mycological methods, several fungi isolates were obtained. For the further study, four of these isolates were selected, while the fifth isolate CBS 158.83 was imported from the Netherlands. These five selected *Colletotrichum* spp. isolates (Luc-7, Luc-17, Luc-27, Luc-33 and CBS 158.83) were further tested by using two different methods. Alfalfa cultivar K-28 was used for this experiment. The plants were sampled from fields and transplanted into vessels. Two methods of inoculation were used. The first method (A) is based on inoculation of the damaged alfalfa plants by applying fragments of *Colletotrichum* spp. colonies of the studied isolates. The second method (B) is based on inoculation of the undamaged alfalfa plants by applying colonies fragments, too. The marks used for the evaluation of the plants range from 1 to 5 (Ostazeski et al., 1969).

The macroscopic and microscopic morphological characteristics of the selected isolates were studied. The basic characteristics of the colonies were considered and described for macroscopic study. The morphological traits of the five selected *Colletotrichum* spp. isolates (Luc-7, Luc-17, Luc-27, Luc-33 and CBS158.83) were studied on the nutrient medium using the method of Baxter et al. (1983). The morphological traits of appressoria of the studied isolates were determined using a modified method after Hawksworth (1974). The presence or absence of sets in the culture was determined using the method by Smith and Black (1990). Also, the possibility of forming teleomorphic state in the isolates was studied according to Baxter et al. (1983).

RESULTS

Symptoms

During a three year period, in summer and autumn, it has been determined that diseased plants appear in the alfalfa fields. These plants have characteristic appearance. Several stems of one plant or shrub have yellow or silvery color and start to wilt. The diseased plants have characteristic curved apical parts, which form the so-called "shepard's hook".

Fig. 1 — "The shepard's hook" and the symptoms on the root

At the lower parts of the diseased plant, the most often at the lower third, light to dark brown spots with black borders can be seen. Mycelium of the parasite grows right down trough the stem, and when the stem withers, the so-called "crown anthracnose" starts to show. The infection of the plant and root crown is characterized by dry rotting that stains the infected tissue in blue-black color (Fig. 1.).

Pathogenicity assessment

All of the studied isolates have caused the symptoms of alfalfa anthracnose on the plants that were treated using both quoted methods (A and B). The control group of plants did not develop these symptoms, since they were inoculated with the substrate that did not contain mycelium. However, the intensity of the symptoms was different between the used methods after 15 days. The intensity of the symptoms in the first method (A) was somewhat stronger than the intensity of the symptoms in the second method (B). This can be explained by the fact that conidia have needed some time to start germinating, to form the appressoria, to penetrate cuticle and to infect the plant. Thirty days after the infection, there weren't any significant differences in the expression of the symptoms between the quoted inoculation methods (tab. 1).

Tab. 1 — The pathogenicity of the five *Colletotrichum* spp. isolates on the alfalfa, assessed by using two different inoculation methods.

Isolates	Method A		Method B	
	After 15 days	After 30 days	After 15 days	After 30 days
Luc-7	+	++	+	++
Luc-17	++	+++	+	+++
Luc-27	++	+++	+	+++
Luc-33	++	+++	+	++
CBS158.83	++	+++	++	+++
Control	—	—	—	—

LEGEND: — no lesions (class 1),
+ lesions are present on the stem, but there are no withered stems (class 2),
++ numerous lesions on the stem, stem starts to wilt (class 3),
+++ stems are withered and dry (class 4).

Morphological characteristics

On the very first day, on the KDA medium, studied isolates Luc-7, Luc-17, Luc-27 and Luc-33 form white mycelia 3—4 mm in diameter. When the colonies reach 40—55 mm in diameter, the middle part of the colony starts to turn darker and gains green or olive green coloration, while the colony border still keeps white coloration.

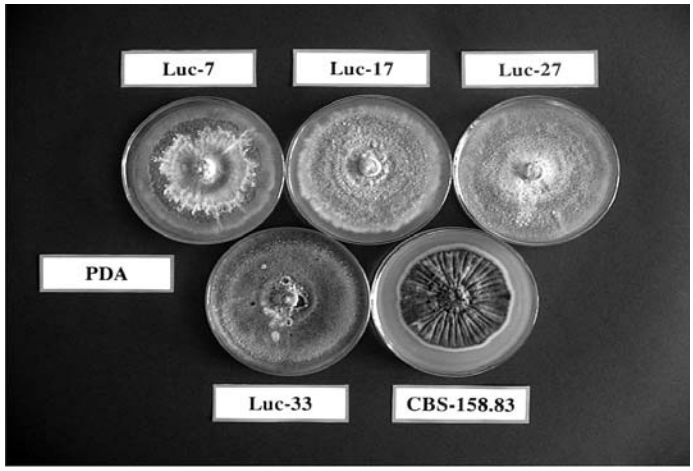

Fig. 2 — The appearance of the colonies on the KDA nutritive medium

Also, as it ages, the colony develops uniformly. It gains olive green to gray coloration (Fig. 2), on the entire surface. Also, it creates acervulae and pink spore mass. The studied *Colletotrichum* spp. isolates formed germinative bodies — acervulae. Acervulae were small in size, misshapen or round, pale to dark brown, almost black in color and stromatic. Acervulae that were formed in these cultures were 100—250 mm in diameter. It was also determined that

the isolates Luc-17 and CBS 158.83 formed sets in the conidiomates. Sets were light to dark brown in color, usualy straight, with smooth or lightly wrinkled surface, often wider in the basis and more pointed and somewhat darker at the top. Sets were septated with 1—3 septs, and 45,5—65,45 x 3,2—5 mm in size (Fig. 3).

The average colony size was 7,85 x 3,87 μm. Insignificant differences between isolates originating from different part of Serbia were determined, while CBS 158.83 isolate has formed significantly larger conidia. Based on the quoted morphological criteria, all of the studied isolates were positioned into isolates with dominant cylindrical conidia, smooth at both ends (Fig. 4).

During germination, conidia undergo certain morphological changes. First, the conidia swell up, after which they often lose

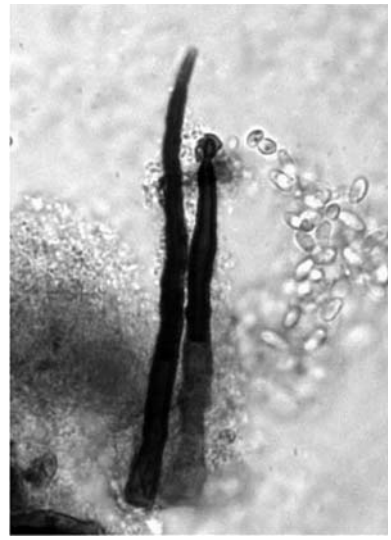

Fig. 3 — Appearance of the sets in the isolate Luc-17 and CBS 158.83

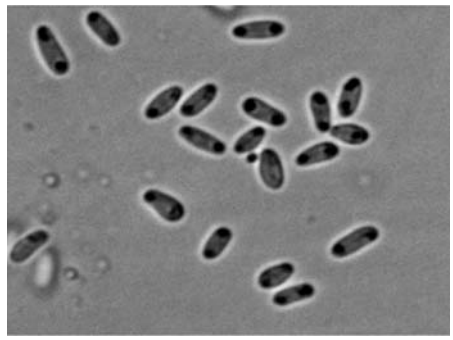

Fig. 4 — Conidia appearances of the studied *C. trifolii* isolate Luc-27

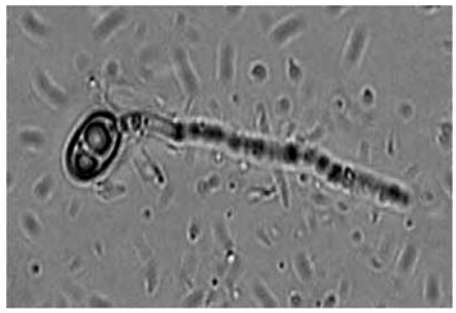

Fig. 5 — Conidia of isolate Luc-7 starts to germinate, while septa are not formed

their granular content and become more transparent, while in their equatorial part there is no development of the septum (Fig. 5).

On the apical parts of the germinated initial hyphae or their branches, appressoria or secondary conidia can be often formed. Formed appressoria were light brown or hyalic at first, but, in time, oil globules are formed, and their outer walls become thicker, after which they become dark brown in color. Average dimensions of the appressoria were 7,5—16,5 x 5,5—8,9 μm (Fig. 6).

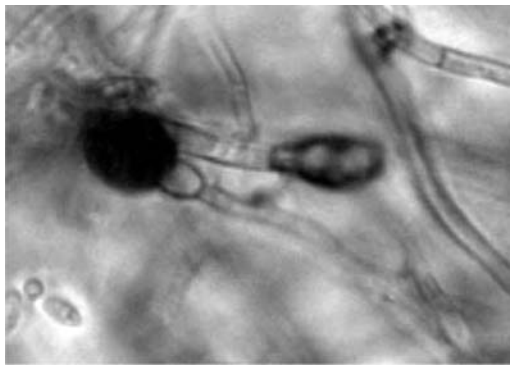

Fig. 6 — Appressoria of the isolate Luc-27, formed in the culture

All of the studied *Colletotrichum* spp. isolates obtained from Central Serbia and Vojvodina, as well as the control isolate CBS 158.83 obtained from the Netherlands did not form perithecia.

DISCUSSION

After the study of pathogenicity of the *C. trifolii* isolates obtained from Serbia it was determined that there had been expression of the symptoms of anthracnose in the inoculated plants. Baxter et al. (1983) point that *Colletotrichum trifolii*, when cultivated on KDA medium, forms acervulae. The same is with our results. According to Baxter et al. (1983), Stutveille and Erwin (1990), conidia are cylindrical, rounded at both ends, 11—15 x 3—5 μm in size, which corresponds to our results. Graham et al. (1976) point that phytopatogen fungus *C. trifolii* rarely forms sets in the culture. The similar results were achieved in our study. Studies of Bailey et al. (1992) and Baxter et al. (1983) point that phytopatogen fungus of *Colletotrichum* spp. genus has the ability to form appressoria when infectious hyphae touches solid surface. This fact also corresponds to our results. Baxter et al. (1983) point that teleomorphic state was never found in association with anamorphic state in the culture.

On the basis of the symptoms found in fields, and pathogenic and morphologic traits of the five selected isolates of the studied fungus, it has been determined that they are similar to *Colletotrichum trifolii* Bain et Essary spe-

cies. According to Sutton (1992), the morphology is the base for differentiation of the species of *Colletotrichum* genus. However, according to this author, it is necessary to use molecular methods to create a reliable system for identification of species of this genus. Identification of the obtained *Colletotrichum* spp. isolates using somewhat modified Cano et al. (2004) method has shown that the isolates Luc-7, Luc. 17, Luc. 27 and Luc-33 are indeed *C. trifolii*.

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МОРФОЛОШКЕ И ПАТОГЕНЕ ОДЛИКЕ *COLLETOTRICHUM TRIFOLII* BAIN ET ESSARY, ПРОУЗРОКОВАЧА АНТРАКНОЗЕ ЛУЦЕРКЕ

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Резиме

У току лета и јесени у луцеришту су запажене оболеле биљке лако препознатљиве по боји и изгледу. По неколико стабала на једној биљци добијају сламасто жуту до седефасту боју и вену. Оболеле биљке имају карактеристичан врх повијен надоле, у виду тзв. "пастирске куке". Из заражених биљака луцерке, стандардним миколошким поступцима, добијено је више изолата гљиве, од којих су за даља проучавања одабрана четири. За проверу патогености испитиваних изолата *Colletotrichum* spp. коришћене су две стандардне миколошке методе: са повређивањем стабљика луцерке и без повређивања стабљика. Током ових проучавања утврђено је да су сви проучавани изолати проузроковали симптоме карактеристичне за антракнозу луцерке. Сви одабрани изолати на мицелији образују плодоносна тела — ацервуле, које у оквиру колонија могу бити груписане у једном делу или разбацане по целој површини колонија. Њихова величина у култури је износила 100—250 μm. У ацервулама се формирају многобројне једноћелијске конидије које су издуженог цилиндричног облика, заобљене на крајевима, величине 7,85 x 3,85 μm. После клијања конидија сва четири проучавана изолата формирају апресорије, чије су димензије у просеку 7,5—16,5 x 5,5—8,9 μm, што је једна од главних карактеристика фитопатогених гљива из рода *Colletotrichum*.

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BIPOLARIS SOROKINIANA (TELEOMORPH *COCHLIOBOLUS SATIVUS*) — CAUSER OF BARLEY LEAF LESIONS AND ROOT ROT IN MACEDONIA

ABSTRACT: Diseased barley plants (*Hordeum vulgare*), were noticed in the area of Kumanovo, Bitola, Probistip, Skopje and Kocani, at the beginning of March, 2006. Our investigations were carried out in the period from 2006 to 2009. The plants were highly diseased, probably in the stage of germination, dwarfed with necrotic leaves and with poorly developed root. A rotten root collar was noticed notice in some plants, which could be easily pulled out from the soil. Plants infected in a later developing stage became yellow from the top of the leaf, and many brown-olive, oval shape lesions were noticed. Conidia of *Bipolaris sorokiniana* (Sacc.) Shoen., were isolated from symptomatic lesions. Pseudothecia with asci and ascospores from teleomorph *Cochliobolus sativus*, were found on the barley straw in the same field the previous year.

KEY WORDS: ascuses, ascospores, conidia, *Hordeum vulgare, Cochliobolus sativus*, pseudothecia

INTRODUCTION

Barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) are the most important crops in the Republic of Macedonia, and are cultivated in over 88735,55 ha (wheat) and 32864,52 ha (barley) (National Statistics of Macedonia, 2007).

Barley and wheat, like all other crops, are host plants for many different pathogens, causal agents of diseases.

Cochliobolus sativus (Ito and kurib.) Drechsl. ex Dastur *Bipolaris sorokiniana* (Sacc in sorok.), the causal agent of spot blotch of barley (*Hordeum vulgare*) is a common foliar pathogen worldwide, but is particularly aggressive under conditions of high relative humidity and temperature associated with imbalanced soil fertility (Duveiller and Altamirano, 2000). It has a

wide host range and it is economically important for wheat (M a t h r e, 1982, N u t t e r et al., 1985).

Spot blotch of barley caused by *C. sativus* (Ito and Kurib.) Drechsl. ex Dastur *Bipolaris sorokiniana* (Sacc in sorok.) Shoem. *Helminthosporium sativum* Pamm, King and Bakke is responsible for low yield and poor quality in many parts of the world (P i e n i n g et al., 1976; N u t t e r et al., 1985). In susceptible barley cultivars, average yield losses of 16%—33% have been reported (C l a r k, 1979). The development of barley genotypes resistant, or tolerant, to spot blotch is considered to be the most economic way for controlling this disease.

Symptoms in barley

Spots (lesions) that are chocolate brown-to-black appear near the soil line or at the base of the sheaths that cover the seedling leaves. Infections may progress until the seedlings turn yellow and die, either before or after the emergence, thus reducing the stand. The latter case is more frequent. Affected seedlings may be dwarfed and have dark green leaves. Diseased barley seedlings commonly have weakened, dark brown rotted crowns and roots. When seedling infections are severe, plants may be dwarfed, the heads may not emerge completely, and the kernels are poorly filled (I v a n o v i c, 2001).

Barley plants that avoid serious seedling infection usually appear normal until about heading time, when the characteristic leaf lesions, of various sizes and shapes appear on the lower leaves after warm, moist weather. The center of each lesion is dark brown with gradual change into green colour at the edge of the leaf. Many spots are oblong or lens-shaped, with centers lighter brown than the edges. Where numerous, the lesions may merge, thus producing large irregular blotches. Heavily infected leaves dry out and die prematurely.

The centers of older lesions on both living and dead leaves have an olive--green cast caused by fungus growth and an abundant production of summer spores (conidia). Spot blotch starts on the older leaves and sheaths, spreading upward to the younger leaves. The lesions never have the netted appearance characteristic of net blotch.

A spot blotch is a very important disease in Macedonia but before this study no other studies conducted about the diversity of the *Cochliobolus sativus* populations in the barley fields have been carried out.

In this study the presence and distribution of *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*) from different regions in Macedonia, and the possible ways of control and prevention were investigated.

MATERIAL AND METHODS

Host plants

Barley fields in Kumanovo, Bitola, Probistip, Skopje and Kocani areas, were used for this observation. Initial symptoms of common root rot in barley

on young seedlings from inoculum carried on the seed, or from infections originating from soilborne conidia near the seedling, were observed. Dark brown lesions appear on the outer coleoptile tissue and/or on the leaf base (Figure 1). Lesions may coalesce into long areas of necrotic brown tissue. In extreme cases, the entire seedlings may die. In most cases, however, the seedling will survive but the growth of the developing plant may be stunted (Figure 2).

Fig. 1 — Dark lesions on the coleoptile

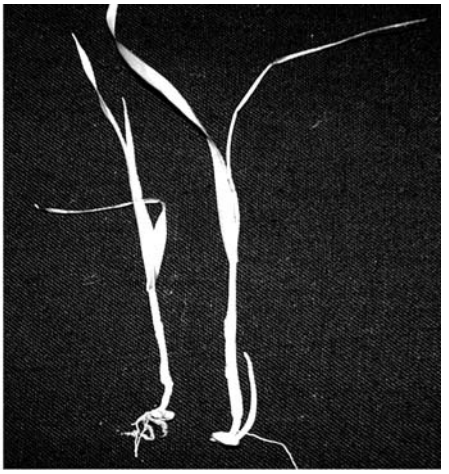

Fig. 2 — Stunting of seedlings infected and leaf bases caused by *C. sativus* by the common root rot organism

Dark brown-to-black spots may appear on the glumes and kernels. The black point at the germ (embryo) end of a kernel is a common symptom (Figure 3). Kernels infected early are shriveled and lightweight. Barley seeds, healthy and infected, were put on filter paper for observation (Figure 4).

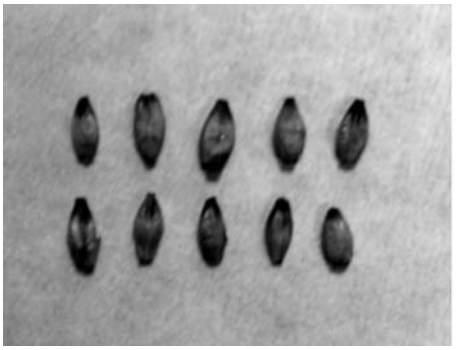

Fig. 3 — Black point of barley seeds

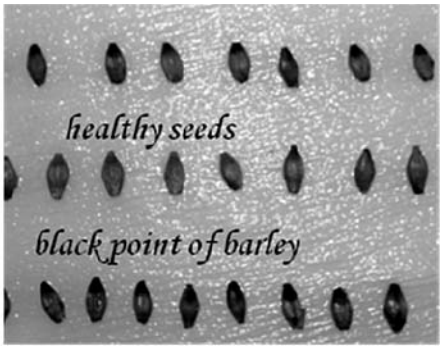

Fig. 4 — Barley seeds on filter paper

In this observation analysis of wheat straw from the previous year collected from the same field, was also performed (Figure 5).

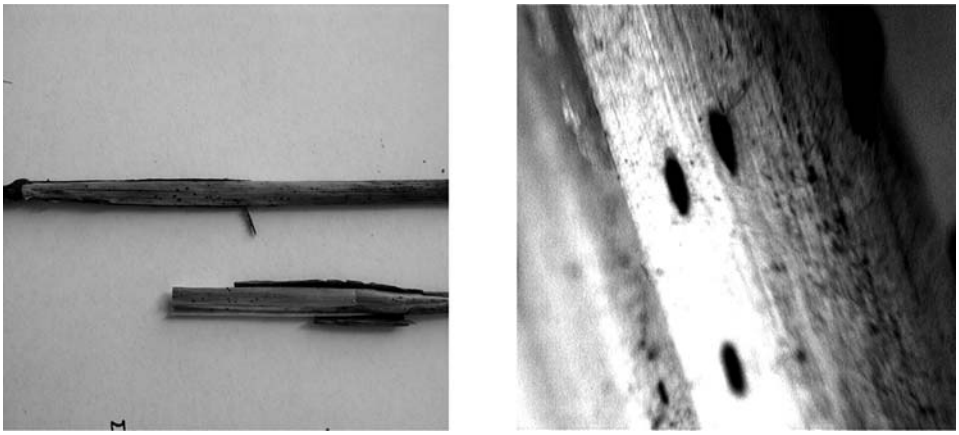

Fig. 5 — Barley straw with presence of pseudothecia from teleomorph *C. sativus*

Fungal isolates

During the seasons 2006, 2007, 2008 and 2009, the isolates from *Cochliobolus sativus* were obtained from barley leaves showing spot blotch symptoms in different locations of Macedonia.

Isolates from plant tissue. In order to isolate the pathogen from infected tissues on various media, it is best to wash the tissue well with running water for several minutes and up to 1 or 2 hours. After that, leaf surfaces were sterilized in 0.1% aqueous solution of mercuric chloride, rinsed twice with distilled water and dried between filter papers. The sterilized leaves were cut into small pieces 5 mm long and transferred to Petri dishes containing potato dextrose agar medium (PDA).

Isolates from infected barley seeds. The seeds were sterilized in the 0.1% aqueous solution of mercuric chloride, rinsed twice with distilled water and dried between filter papers. After that, the seeds were cut lengthwise and transferred to Petri dishes containing potato dextrose agar medium (PDA).

RESULTS

The anamorph stage of *Cochiobolus sativus* was easily isolated from symptomatic tissue placed under moist conditions. The mycelium of *Bipolaris sorokiniana* had an olive colour, and when older, it had dark or black colour (Figure 6).

More often, we found individual or group olive-brown conidiophores, and large brown phragmoid (cross walls only in one direction) conidia borne laterally and terminally on the conidiophore. Conidia were formed quickly and were quite evident by their characteristic dark brown oval shape with thick cross walls (Figure 7). They usually appeared within a few days at a room temperature.

Fig. 6 — Olive micelia on nutrient agar

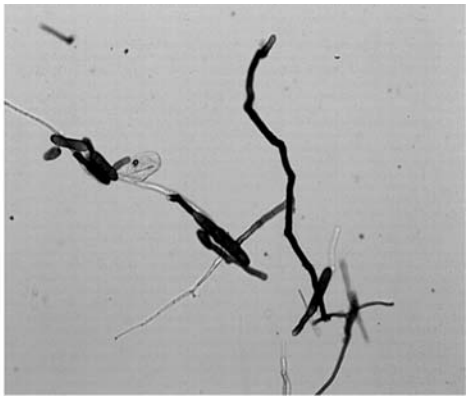

Fig. 7 — Conidiophores and conidia of common root rot pathogen, *C. sativus*

In microscopic observations, 3—7 septata conida, from conidialen stadium of *Cochiobolus sativus, Drechslera sorokiniana* (Syn. Bipolaris sonokiniana) were found. In more cases, conidia were with 5 to 6 septa, cylindrical with oval point, with dimensions of 5—6 mm in width and 120—140 mm in length, and had olivaceous colour (Figure 8).

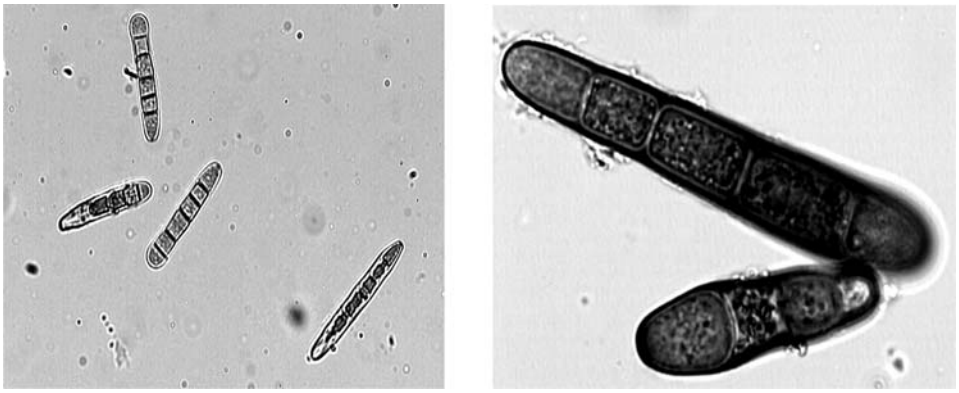

Fig. 8 — Conidii from *Drechslera sorokiniana*

Leaves from filter paper, at temperature of 25°C, after 5 days showed the presence of septic, granulated mycelia with olive color (Figure 9). On the plant tissue the presence of conidiophores and conidia of *Bipolaris sorokiniana* was also observed (Figure 10).

Pseudothecia with asci and ascospores from teleomorph *Cochliobolus sativus*, Pammel; King & Bakke, were found on barley straw from the previous year, on some fields (Figure 11 and 12).

Ascospores are usually slight, septic and discoloured with the dimensions of 6x320 mm and with 6 or 7 septate (Figure 13).

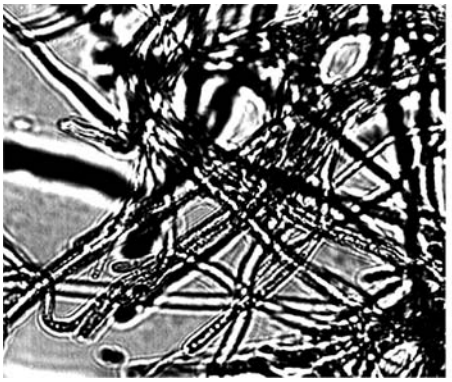

Fig. 9 — Mycelium on PDA

Fig. 10 — Conidiophores and conidia from *Cochliobolus sativus*

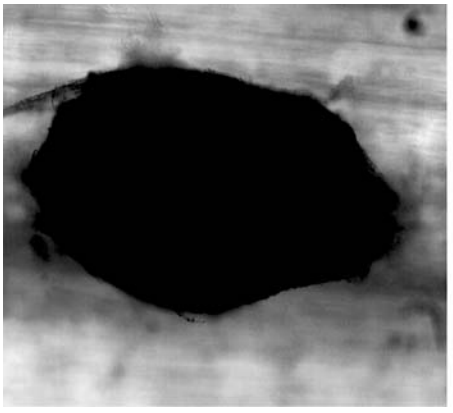

Fig. 11 — Pseudothecia

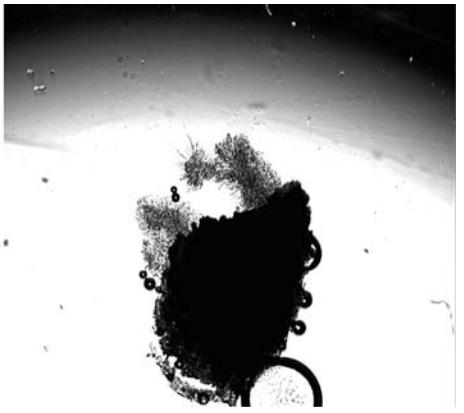

Fig. 12 — Cracking of pseudothecia and releasing of ascospores

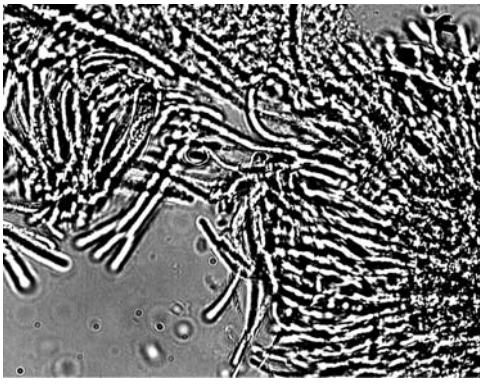

Fig. 13 — Ascospores

DISCUSSION

Cochliobolus sativus (Ito and kurib.) Drechsl. ex Dastur *Bipolaris sorokiniana* (Sacc in sorok.), the causal agent of spot blotch of barley (*Hordeum vulgare*) is a common foliar pathogen worldwide. It has a wide host range and it is economically important for wheat (Marthre, 1982, Nutter et al., 1985).

Diseased barley plants (*Hordeum vulgare*), were noticed in the areas of Kumanovo, Bitola, Probistip, Skopje and Kochani at the beginning of March, 2006. The plants were highly infected, probably in the stage of germination, dwarfed with necrotic leaves and with poorly developed root. A root rot collar was noticed in some plants, which could be easily pulled out from the soil. Plants infected in a latter developing stage became yellow from the top of the leaf and many brown-olive oval shape lesions were noticed. Conidia from *B. sonokiniana* were isolated from symptomatic lesions.

Pseudothecia with asci and ascospores from teleomorph Pammel; King & Bakke were found on the barley straw from the previous year, in some fields.

Our results indicate the recesseity of monitoring and controlling the barley plants in order to prevent the infections of *Cochiobolus sativus*, which can occur in all stages, from early spring, before germination, untill the end of the vegetation.

In all observed barley fields, the symptoms of spot blotch of barley, were present in the earliest phase of development, thus leading to a conclusion that the disease came from the seed material or soil in which pathogen had spent the winter.

Damages in the barley production during the mentioned years (from 2006 to 2009) were estimated to range from 30% tp 70% of yield losses.

Control and prevention

To reduce the infection in the barley fields, the most important practice is to use healthy and certified seed material. Before use, seeds must be treated with fungicide. If the infection already exists, as it was the case with our investigation in 2007, then it is necessary to treat the seeds with fungicide "propiconazol" in dosage of 0,5 l/ha, twice per season. With constant monitoring of plants, along with treated seeds of barley, the reduction in percentage of the infected grains could be detected next year.

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BIPOLARIS SOROKINIANA (TELEOMORPH *COCHIOBOLUS SATIVUS*) — УЗРОК ОШТЕЋЕЊА И ПЕГАВОСТИ ЛИСТА И ТРУЛЕЖИ КОРЕНА ЈЕЧМА У МАКЕДОНИЈИ

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Резиме

У првој недељи месеца марта 2006. године регистрована је болест јечма у околини Куманова, Битоља, Пробиштипа, Скопља и Кочана. Теренска и лабораторијска испитивања зараженог материјала су урађена у периоду од 2006. до 2009. године. Анализом је утврђено да су биљке највероватније биле заражене још у фази клијања. Констатоване су њихова закржљалост, пожутелост листова и слаба развијеност корена. Код неких биљака је забележена појава трулежи приземног дела стабла као и њихова слаба веза са подлогом. Код биљака које су касније заражене некроза се појавила прво на листовима и манифестована је преко многобројних кафено маслинастих пега неправилног елипсоидног облика. Из симтоматичних пега су издвојене конидије фитопатогене гљиве *Bipolaris sorokiniana* (Sacc.) Shoen.

Псеудотеци је са аскусима и аскоспорима телеморфног стадијума *Cochliobolus sativus* Pammel; King & Bakke, пронађене су у остацима сламе јечма од претходне године са исте парцеле у близини парцеле са зараженим биљкама.

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GIBBERELLA FUJIKUROI (SAWADA) WOLLENWEBER, THE NEW PARASITICAL FUNGUS ON RICE IN THE REPUBLIC OF MACEDONIA

ABSTRACT: The recent observation of *Gibberella fujikuroi* (Sawada) Wollenworth (teleomorph) (anamorf: *Fusarium moniliforme* Sheld.) *Fusarium fujikuroi* Nirenberg (anamorph), *F. moniliforme* J. Sheld. (synonym), the causal agent of Bakanae disease in rice fields, provides an opportunity to observe the characteristics of the pathogen and the possibility of prevention in Kocani area. Plant material with *Gibberella fujikuroi* symptoms was collected from rice (*Oryza sativa*) over the period of 3 years (from 2006 to 2008). Within this study, the presence and damage caused by this pathogenic fungus were confirmed. The objective of this study was a continuous field observation of symptoms in order to establish the percentage of infection and use of laboratory methods for proper protection.

KEY WORDS: Bakanae, *Gibberella fujikuroi*, Kocani, rice fields

INTRODUCTION

Rice (*Oryza sativa* L.) is an important crop in the Republic of Macedonia, and is cultivated in over 2991,55 ha, from which 2840,60 ha are located in the area of Kočani (National Statistics of Macedonia, 2007). Among various diseases, which effect the crop production, Bakanae is the leading one.

The name Bakanae means "bad" or "naughty" seedlings in Japanese, referring to the elongation symptoms specific for the disease, and caused by gibberellin production of the pathogen upon infection of the host. The causal agent of Bakanae disease of rice is fungus (*Gibberella fujikuroi*, Sawada, Wollenworth — teleomorph, (anamorf: *Fusarium moniliforme* Sheld.), *Fusarium fujikuroi* Nirenberg (anamorph), *F. moniliforme* J. Sheld. synonym). The fungus produces gibberellins and other secondary metabolites such as carotenoids, bikaverin and fusarin, which directly affect the rice growth.

Bakanae is a monocyclic disease and the pathogen is often spread predominantly with infected seeds, although infected crop residue from the previous season may also serve as a source of inoculum.

Bakanae is traditionally associated with rice, but water grass plants such as *Echinochloa* spp., with classic symptoms of Bakanae were also observed in California in 2002 (C a r t e r, L. A., 2008). Although the Bakanae disease usually causes dieback or sterility of rice, mycotoxin contamination also presents a concern since the pathogen is seed-borne.

The teleomorph, *Gibberella fujikuroi*, has been reported on rice in China, Japan and Taiwan, (S u n, 1975; S u n g and S n y d e r, 1977; W a t a n a b e and U m e h a r a, 1977). Ascospores are reported as the primary source of inoculum for Bakanae in Taiwan (S u n, 1975).

Disease Symptoms

Symptoms vary depending on the strain, inoculum levels and the presence of toxin. High inoculum density leads to seedling blight, stunting and chlorosis. Low inoculum levels result in etiolation symptoms due to gibberellin production.

Typical symptoms caused by *Gibberella fujikuroi* are usually characterized with:

— infected plants that are several inches taller than normal plants in seedbed and field;

— thin plants with yellowish green leaves and pale green flag leaves;

— dieback seedlings at early stage;

— reduced and dried of leaves at late infection;

— partially filled sterile or empty grains on surviving plant at maturity;

— infected seedlings with lesions on roots, that die before or after.

Moreover, there is no other disease with symptoms similar the those of the Bakanae disease of rice.

Characteristics of Gibberella fujikuroi

The pathogen sexually produces ascospores that are formed within a sac known as ascus. Asci are contained in the fruiting bodies called ascocarps which are referred to as perithecia. The perithecia are dark blue and measure 250—330 x 220—280 μm. They are spherical to oval and somewhat roughened outside. The asci are cylindrical, piston-shaped, flattened above, and their dimensions are 90—102 x 7—9 μm. They are 4, 6 but seldom 8-spored. The spores are one-septate and about 15 x 5.2 μm. They are occasionally larger, measuring 27—45 x 6—7 μm.

The anamorph produces gibberellin and fusaric acid. Biological studies of the two substances showed that fusaric acid causes stunting and giberrellin causes elongation.

Hyphae are branched and septated. The fungus has micro and macroconidiophores bearing micro and macroconidia, respectively. The microconidiophores are single, lateral, and subulate phialides. They are formed from aerial hyphae. The microconidia are, more or less, agglutinated in chains and remain

joined or cut off in false heads. They are later scattered in clear yellowish to rosy white aerial mycelia as a dull, colorless powder. They are 1—2 celled and fusiform oval.

The macroconidiophores have basal cells with 2—3 apical phialides, which produce macroconidia. The macroconidia are delicate, slightly sickle--shaped or almost straight. They narrow at both ends and are occasionally somewhat bent into a hook at the apex and distinctly or slightly foot-celled at the base.

The sclerotia are 80 x 100 μm. They are dark blue and spherical. The stroma are more or less plectenchymatous and yellowish, brownish, or violet.

MATERIAL AND METHODS

Collection of samples

Field analyses of plant material

Plant material with symptoms symilar to that caused by *Gibberella fujikuroi*, was collected from rice (*Oryza sativa*) during the period of 3 years (2006—2008). Each year, collections were made throughout the whole rice growing season. The seedlings with early symptoms of Bakanae disease, particularly chlorosis and elongation, were collected from rice fields in Kočani areas. Later in the season, plants showed visible symptoms in the field (Figure 1). During this period, 10 different fields were observed, and 5 infected sam-

Fig. 1 — Visible symptoms in the field caused by *Gibberella fujikuroi*

Fig. 2 — Abnormal oblong stem and leaves

ples, were collected. Symptomatic plants have thin stem, 30 cm higher than normal, and leaves are usually yellowish green and elongated in comparison with normal leaves (Figure 2).

Isolation of the pathogen

Isolation of the pathogen was made from the stem and leaves of the symptomatic plants collected from the rice fields in Kočani areas. They were cut into 1 cm^2 pieces, sterilized with 0.1% aqueous solution of mercuric chloride, rinsed twice with distilled water and then placed on potato dextrose agar medium (PDA). The plates were incubated at 27 ± 2°C for 5 to 7 days. After the development, isolated fungi were identified according to Nelson et al. (1983).

Pathogenicity testing

All collected isolates were screened for ability to induce simptoms of Bakanae on rice seedlings. Isolates grew on the potato dextrose agar for 10 to 15 days, until the mycelia clearly emerged. When mycelia fullfiled Petri dishes, one small piece of mycelia with agar was cut and used to make a suspension with sterile distilled water. Mycelium was scarped with a sterile glass slide. Ten seeds of different rice cultivars (San Andrea and Monticelli), were soaked in 25 ml of suspension for 1 h at 25°C. The control seeds were soaked only in sterile distilled water (Figure 3). The experiment was conducted during May in

Fig. 3 — Test of pathogenicity — control with sterile water and infected plant with suspension from *Gibberella fujikuroi*

an open environment and data for Bakanae symptoms, expressed in percentage of plant infection were evaluated 60 days after sowing.

RESULTS

Morphology

After field analysis, a laboratory testing for detection and identification of rice pathogen was conducted. Thirty morphologically consistent isolates gained from the plant material with typical Bakanae symptoms were analysed. All isolates on potato dextrose agar (PDA), established the same mycelia. Clear culture had mycelia with white color on the top of Petri dishes and the mycelia was redish to brown coloured on the underside. In our case, all Petri dishes were with lilac colour on the underside (Figure 4).

The fungus has micro and macro conidiophores bearing micro and macro conidia, respectively (Figure 5). Microconidiophores are single, lateral and formed from hyphae, while macroconidiophores consist of a basal cell bearing 2—3 apical phialides which produce macroconidia. Macroconidia are multi--celled (3 to 7 septate), slightly curved or bent at pointed ends, typically canoe-shaped and measure 25—60 x 2.5—4 μm. Microconidia are one-celled, ovoid or oblong, borne singly in chains or false head on laterally borne conidiophores and measure 5—12 x 1.5—2.5 μm. Some conidia are intermediate, with two or three cells, oblong or slightly curved.

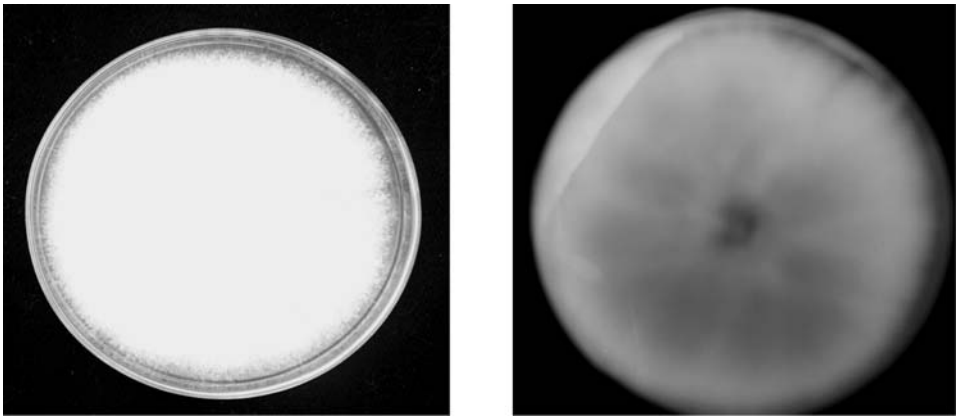

Fig. 4 — Mycelia from parasitic fungus *Gibberella fujikuroi*

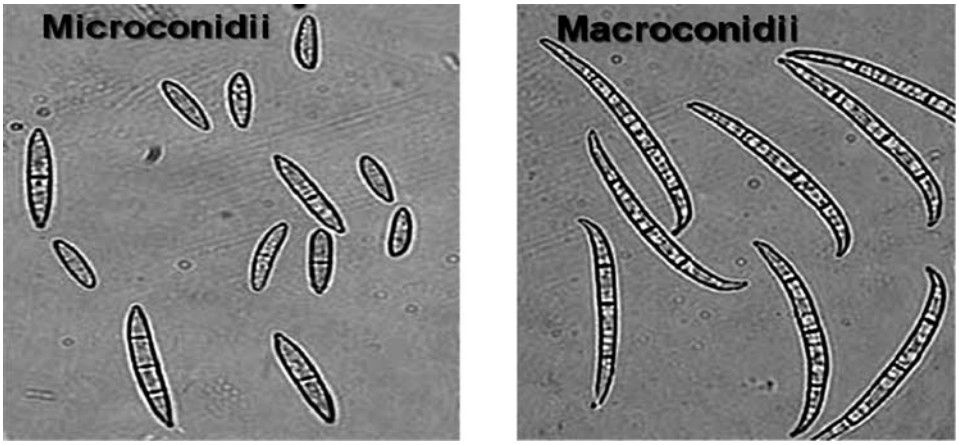

Fig. 5 — Micro and macroconidii from *Gibberella fujikuroi*

The results from pathogenicity testing showed visible symptoms on inoculated plants. The ten seeds of different rice cultivars (San Andrea and Monticelli), which were soaked in 25 ml of suspension for 1 h at 25°C, after 60 days showed visible symptoms in comparison with the control seeds, which were soaked only in sterile distilled water.

Symptomatic plants have stem 30 cm higher than normal, and leaves are usually chlorotic and elongated when compared to the normal ones, with angle position of 90°C.

DISCUSSION

The teleomorph, *Gibberella fujikuroi*, has been reported on rice in China, Japan and Taiwan (Sun, 1975; Sung and Snyder 1977; Watanabe

and Umehara 1977). Ascospores are reported as the primary source of inoculum for Bakanae in Taiwan (Sun, 1975).

Bakanae is a serious disease endangering rice fields in the Republic of Macedonia, and with this observation, the presence and damage caused by this pathogenic fungus were confirmed.

The seeds are usually infected during the flowering stage of the crop. Severely infected seeds are discolored because of the pathogen conidia. Seed infections occur via airborne ascospores and lead to conidia that contaminate the seed during harvesting. Discoloured seeds give rise to stunted seedlings, whereas the infected seeds without discolouration produce seedlings with typical Bakanae symptoms. Infections may also take place with spores and mycelium, which are left in the water used for seed soaking.

The fungus infects plants through roots or crowns. It later becomes systemic, i.e. it grows within the plant and systemically infect the panicle. The microconidia and mycelium of the pathogen are found to be concentrated in the vascular bundles, particularly large pitted and xylem vessels.

Sine the production of gibberilin has not been investigated in Macedonia, our future observations are planned to focus on gibberellin production by the pathogen upon infection of the host.

Control and prevention

Clean seeds should be used to minimize the occurrence of the disease. Salt water can be used to separate lightweight and thereby reduce seedborne inoculum. In our observation, we did not treat our seeds, but the already published studies proved that seed treatments using fungicides such as thiram, thiophanate-methyl, or benomyl are effective before planting. Benomyl or benomyl-t applied at dose of 1—2% (seed weight basis) should be used for dry seed coating. However, rapid increase of resistance against benomyl and carbendazim has been reported which may be caused by their successive applications. Triflumizole, propiconazole and prochloraz were found to be effective against strains that are resistant to benomyl and the combination of thiram and benomyl.

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GIBBERELLA FUJIKUROI (SAWADA) WOLLENWEBER, НОВА ПАРАЗИТНА ГЉИВА НА ПИРИНЧУ У РЕПУБЛИЦИ МАКЕДОНИЈИ

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Резиме

У ова истраживања били су укључени узрочници Бакане болести на пиринчаним пољима око Кочана, са циљем да се испитају карактеристике патогена *Gibberella fujikuroi*, као и могућност заштите пиринча. У трогодишњем периоду, од 2006. до 2008. године, биљни материјал са симптомима *Gibberella fujikuroi* био је прегледан и колекциониран са пиринча (*Oryza sativa*). Овим детаљним испитивањима потврђени су присуство и штета прузрокована овом патогеном гљивом. Циљ овог истраживања је био да се континуирано прате симптоми болести са поља да се утврди проценат инфекције, а такође преко лабораторијских метода омогући одговарајућа заштита.

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RESISTANCE TO *PUCCINIA TRITICINA* AT DIFFERENT STAGES OF WHEAT

ABSTRACT: Ten wheat genotypes were tested for resistance characteristics to *Puccinia triticina*. Infection intensity in the field was evaluated at different growth stages, and time of spike appearance and leaf senescence were recorded. At seedling stage, under the controlled conditions of greenhouse, latency period, infection frequency and reaction type were determined. Resistance characteristics at different wheat growth stages were strongly correlated. Correlation coefficient between LP x RT x IF and AUDPC values, was 0.828. The highest coefficients of correlation between particular resistance characteristics and maximal intensity in the field were determined with the last evaluation in the field (0.665, 0.476 and 0.834). Time of spike appearance was very variable for different genotypes, whereas leaf senescence was recorded concomitantly for near all genotypes. The exception was Rusalka, as the most resistant in the field. All genotypes included in this three-year long experiment expressed stability with respect to infection intensity at different growth stages. Genotype Timson showed the highest level of resistance according to all tested characteristics, while genotype Pkb krupna showed the lowest.

KEY WORDS: *Puccinia triticina*, resistance, wheat genotypes

INTRODUCTION

Leaf rust, as an important wheat disease (*Puccinia triticina*), was described in 1797, and the cycle of disease development was described in 1927. Nowadays, it is still one of the major parasites worldwide. In our country, average yield losses due to this disease were about 38%, and considerably smaller damage was recorded on domestic wheat genotypes (Jerković and Putnik-Delić, 2004, 2005).

Consequences of pathogen attack were reduction of photosynthesis and collapse of metabolism. At the beginning of plant development, infection reduces resistance to low temperatures or reduces stalk and seed number (Putnik-Delić, 2008). The most frequent consequence of infection is poor seed quality (Jerković, 1995). Susceptibility of commercial varieties is the result of growing the same varieties for long time on very large surfaces. On the

other hand, wheat breeding does not always follow the dynamics of pathogen changes (Putnik-Delić, 2006).

Species from genus *Puccinia* have huge reproductive potential. Therefore, initial inoculums originating from different sources are always present.

Wheat breeding for resistance to parasites could be improved with the help of seedling test. Experimental data suggest that it is possible to select for sources of resistance already at seedling stage.

The most exploited method for evaluation of wheat resistance at seedling stage to *Puccinia triticina* is according to the reaction type. This feature is dependent on temperature, and at seedling stage optimal temperature is 15—20°C. Even then, some results are not correlated with infection efficiency and reaction type in the field. Low infection efficiency and prolonged latent period are sufficient to reduce the development of pathogen and make non-economic application of the other protection measures (Jerković, 1992). Low infection efficiency is the most discussible parameter whose better explanation is necessary in order to fully understand the process of overcoming the genes for resistance (Niks et al., 2000).

Environmental conditions have the highest influence on latent period (Broers and Wallenburg, 1989). The other characteristics, such as infection efficiency, are of lower variability. Prolonged latent period is the last characteristic before resistance overcoming. In the field, reliable criteria for incomplete resistance are time and intensity of the beginning of infection, and AUDPC value (Putnik and Jerković, 2002). The first infection, according to the modified Cobb scale (from 5 and higher, Peterson et al. (1948)), under our experimental conditions appear almost in the third (wheat in phase > 70), and sometimes in the second decade of May, at blooming (phase 60—69 according to Zadoks et al., 1974).

Parasite has the possibility of continuous and fast adaptation, so it can multiply on each variety after shorter or longer period of time. Process of changes in the pathogen population infecting particular genotype is concomitant with the breeding process (Parlevliet, 1986). Resistance which exists more than ten years could be called durable (Roelfs et al., 1992).

Rubiales and Niks (2000), revealed that the resistance to *Puccinia triticina* could survive longer than usual. One of the significant strategies is incorporation of more major resistant genes into new cultivar (Staletić et al., 2005). Combinations of Lr genes could lengthen wheat resistance for few years more (Kolmer, 2001).

The aim of this study was to choose the values or relations of resistance characteristics at seedling stage in the greenhouse which can be used to predict intensity of infection in the field.

MATERIAL AND METHODS

Ten wheat genotypes were tested for three resistance characteristics in the greenhouse. Latency period (LP), reaction type (RT) and infection efficiency (IF) at seedling stage were evaluated. In the field, time of spikes appearance

and leaf senescence were marked, and infection intensity was evaluated at three time points.

The resistance characteristics of seedlings were analysed at 20°C in greenhouse during winter period. Seven days old seedlings were inoculated by trashing spores (S t a k m a n, 1954). Incubation in high moisture lasted for 24 hours. The RT estimated after ten days from incubation was transformed as described before (0 = 6, 1 = 7, 2 = 8, 3 = 9, 4 = 10) (J e r k o v i ć and P u t n i k - D e l i ć, 2004). The latency period (LP) was observed visually six and seven days after the incubation and marked adequately (1 = pustule appeared on the first day, 0.8 = some of them on the second day, 0.7 = all after the second day), while the infection frequency (IF) was observed after 10 days. IF represents maximal number of pustules in the observed area (6 = less than 20 pustules in the middle of the first leaf, 7 = 20—30, 8 = 31—40, 9 = 41—50 and 10 = more than 50). The data obtained using the formula RT x IF x LP were correlated with maximal infection intensities in field, and AUDPC (Area Under the Disease Progress Curve) (B j a r k o and L i n e, 1988). The results of seedlings test in the greenhouse and at the adult stage in the field monitored during three years were presented in the paper. The infection intensities in the field were estimated according to the modified Cobb scale. The experiment was done in experimental fields of the Institute of Field and Vegetable Crops, Novi Sad. The correlation coefficients between the estimated parameters were calculated using the computer program STATISTICA 8.

RESULTS

Five varieties showed complete susceptibility at seedling stage. Those genotypes have high values of maximal infection intensity, too (Tab. 1). Time of spiking was very variable between the genotypes, and this trait had no influence on infection intensity. In two genotypes (Ana and Nizija) at seedling stage, only prolonged LP appeared as the last characteristic before the resistance was overcome. However, in contrast to Ana, Nizija is early-spiking and its maximal infection intensity was low (40). Pobeda showed lower values for two resistance characteristics at seedling stage and it was in accordance with its maximal infection intensity in the field. Varieties Rusalka and Timson expressed the lowest values of the tested characteristics.

Year 2003 was bad for both, parasite and wheat development (Fig. 1). Ana had the highest AUDPC values in 2002 with respect to the other experimental years and analysed genotypes. Correlated derived data in seedling stage and infection intensity under field conditions obtained high values (Tab. 2). Correlation coefficients between the greenhouse and field evaluations were the highest with the last evaluation in the field (0.665, 0.476, and 0.834). The highest correlation coefficients were found from 2004 data, because that year was near optimal for both host and fungus development. All genotypes expressed stability of resistance characteristics at different wheat growth stages.

Tab. 1 — Values of LP, IF and RT at seedling stage, maximal infection intensity in the field, time of spiking and leaf senescence for tested genotypes

Number	Genotype	LP	IF	RT	Max. infection intensity in the field	Date of spikes apperance		Leaf senescence in 2004 (number of estimation)
						may 2003	may 2004	
1. 207/439	Pkb-krupna	1	10	10	70	11.	11.	4.
2. 216/448	NS 55—25	1	10	10	60	8.	10.	4.
3. 166/398	Bankut 1205	1	10	10	60	12.	17.	4.
4. 178/410	Florida	1	10	10	70	13.	18.	4.
5. 169/401	Bezostaja 1	1	10	10	60	12.	16.	4.
6. 165/397	Ana	0.8	10	10	70	10.	10.	4.
7. 200/432	Nizija	0.8	10	10	40	8.	11.	4.
8. 58/166	Pobeda	0.8	9	10	50	10.	15.	4.
9. 223/455	Rusalka	0.8	8	9	15	7.	10.	3.
10. 234/466	Timson	0.7	6	6	15	8.	10.	4.

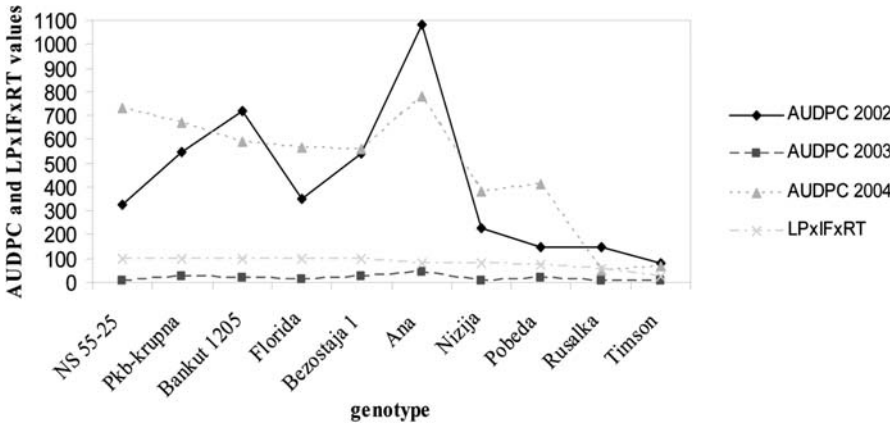

Fig. 1 — Resistance characteristics at adult and seedling stage for tested wheat genotypes

Tab. 2 — Correlation coefficients (r) based on data from field and greenhouse experiments, for tested wheat genotypes

Correlation coefficients (r)	Infection intensity in the field								AUD PC		
	2002		2003		2004						
	28. 05	07. 06.	09. 06.	10. 06.	28. 05.	02. 06.	09. 06.	16. 06.	2002	2003	2004
07. 06. 2002	0.833										
09. 06. 2003	0.672	0.678									
10. 06. 2003	0.701	0.685	0.542								
28. 05. 2004	0.721	0.718	0.519	0.780							
02. 06. 2004	0.617	0.691	0.511	0.427	0.840						
09. 06. 2004	0.785	0.797	0.584	0.520	0.723	0.882					
16. 06. 2004	0.750	0.866	0.556	0.728	0.703	0.702	0.889				
AUDPC 2002	0.897	0.715	0.846	0.674	0.753	0.690	0.767	0.672			
AUDPC 2003	0.826	0.758	0.801	0.728	0.717	0.549	0.589	0.659	0.870		
AUDPC 2004	0.797	0.857	0.572	0.607	0.761	0.857	0.977	0.952	0.743	0.662	
LP x IF x RT	0.571	0.665	0.469	0.476	0.427	0.594	0.832	0.834	0.498	0.385	0.828

DISCUSSION

Weather conditions in three experimental years were significantly different. This can explain variability in maximal infection intensities in the tested genotypes. Similar variations between experimental years in Serbian agroecological conditions were found by Jerković and Jevtić (1997). Besides the influence of the other fungi (Jerković and Putnik-Delić, 2005), the basic cause of pseudo resistance to *Puccinia triticina* is early leaf senescence, which could have happened with variety Timson, at the third evaluation time point (Jerković et al., 2004).

Genotypes with high correlation coefficients among all data are at the same time stress resistant. Some experiments suggest that the content of soluble sugars is higher in older leaves (Shiroya et al., 1961; Trippi, 1965; Egli et al., 1980; Thimann, 1980; Crafts-Brandner et al., 1984), and increased sugar concentration blocks photosynthesis (Lazan et al., 1983). Accumulation of soluble carbohydrates and salts in seedling stems may be an indicator of osmotic stress, but also an indicator of better tolerants to *Puccinia triticina* (Kerepesi and Galiba, 2000). The results of the cited experiments suggest that the reduction of *Puccinia triticina* could be related to speed of plant development, or to a seed with influence to *Puccinia triticina* development. This is especially the case with varieties that have small number of seeds per spike, especially those with early earning.

Breeding for prolonged LP, lower values of IF and smaller number of pustules lead to lower AUDPC values in wheat (Lal Ahmed and Singh, 2003). In general, when genotypes showed stability in infection intensity at different growth stages over the years, lower values of AUDPC were well correlated with resistance characteristics in seedlings.

According to Broers (1997), latent period, infection efficiency and reaction type are highly correlated with data of disease development in the field. Those results suggest that testing for one of these components in the greenhouse could result in varieties with high level of quantitative resistance. If inoculation in the glasshouse is uniform, monocyclic evaluation may be sufficiently reliable and at the same time, very convenient because of its simplicity.

Choosing the genotypes with same type of resistance determined according to the results obtained by only one evaluation in the field is unreliable. According to the maximal infection intensity one could select for pseudo resistance, through partial resistance or two mixed types of resistance. The most reliable data for choosing the source of resistance for breeding derive from experiments done under controlled conditions, at seedling stage, which is optimal for development of both parasite and the host.

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ОТПОРНОСТ ПРЕМА *PUCCINIA TRITICINA* У РАЗЛИЧИТИМ ФАЗАМА РАЗВОЈА ГЕНОТИПОВА ПШЕНИЦЕ

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Резиме

Десет генотипова пшенице је тестирано на поједине карактере отпорности према *Puccinia triticina*. Утврђени су интензитет заразе у пољским условима у различитим фазама развоја, време класања и сушења лисне масе, као и реакциони тип, латентни период и успешност инфекције у стадијуму сејанаца у контролисаним условима у стаклeнику. Највећи степен отпорности по свим тестираним карактерима испољио је генотип Тимсон, а најмањи генотип Пкб-крупна. Повезаност показатеља отпорности у различитим фазама развоја пшенице била је врло висока. Корелацијом карактера отпорности добијених у контролисаним условима LP x RT x IF и AUDPC вредности добијен је врло висок коефицијент (r = 0,828). Највиши коефицијент корелације карактера отпорности у стаклeнику утврђен је с последњим очитавањима у пољским условима (0.665, 0.476, 0.834). За разлику од времена класања, које се код испитиваних генотипова веома разликовало, сушење лисне масе је констатовано код свих генотипова приликом четвртог очитавања, изузев код Русалке где је установљено при трећем. Сви тестирани генотипови су испољили стабилност по питању интензитета заразе у појединим фазама развоја током три године огледа.

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GIBBERELLA INTERMEDIA THE PATHOGEN OF ST. JOHN'S WORT, CONEFLOWER AND MARSHMALLOW IN SERBIA

ABSTRACT: *Gibberella intermedia* (Kuhlmann) Samuels et al. (anamorf: *Fusarium proliferatum* /Matsushima/ Nirenberg) was isolated from seeds of St. John's wort, marshmallow, and coneflower, as well as from roots and stalks of marshmallow and roots of coneflower. These plants had symptoms of leaf chlorosis, malformation, withering and plant dwarfing and were collected from several localities in Serbia during five-year investigations of mycopopulations of the mentioned plants.

The morphological characteristics of the pathogen were described.

KEY WORDS: medicinal plants, St. John's wort, marshmallow, coneflower, *Gibberella intermedia*

INTRODUCTION

Medicinal plants are among economically most significant plants in Serbia. There are over 700 species of these plants on the territory of Serbia, contributing 19,65% of the total flora (Sarić, 1989). Out of 420 registered species, 279 are used commercially as medicinal and aromatic plants. In order to preserve medicinal plants in nature from overexploitation, they are grown as commercial crops in plantation. There is a long tradition of growing medicinal and aromatic herbs commercially such as mint, marshmallow, sage, St. John's wort, coneflower, etc. in Serbia. The commercial cultivation led to the occurrence and spread of the plant pathogens with epidemic distribution on some hosts (St. John's wort, marshmallow, and coneflower).

During the experimental research of mycopopulation of medicinal herbs, there was a total of 7 *Fusarium* species identified: 6 species on St. John's wort, 5 species on *Althea officinalis*, 3 on *E. angustifolia* and 5 on *E. purpu-*

rea (Pavlović et al., 2008). *F. proliferatum* /Matsushima/ Nirenberg was identified on all three hosts. This research was conducted to investigate basic morphological, cultural and pathogenic characteristics of *Gibberella intermedia* (Kuhlmann) Samuels et al. (anamorph: *Fusarium proliferatum* /Matsushima/ Nirenberg) isolates.

MATERIALS AND METHODS

Collection and preparation of the samples: The samples of diseased plants of St. John's wort, marshmallow and coneflowers were collected from the medicinal plant collection of the Institute in Pančevo vicinity, as well as from the cultivation fields of cooperatives in several localities of Serbia (Zrenjanin, Ruma, Inđija, Pančevo, Kačarevo) over the period 2002—2006. The plants showing the symptoms of withering, dwarfness, chlorosis and leaf shrivelling were collected monthly from March to November. Samples were washed with tap water, disinfected with 2% NaOCl for 30 seconds and washed again with sterile water.

The methods that were used: The standard procedure of Dhingra and Sinclair (1986) was used for isolation of fungi from the tissue of leaf petioles, stems, roots and seeds of diseased plants on carrot agar (CA) and carnation leaf agar (CLA). A modified method of Leslie (1991) was used to investigate teleomorph development. The fragments of diseased tissue were incubated on CA at 25±2°C. After 2—4 days, the existing mycelia were transferred on the 60 mm Petri dishes with potato dextrose agar (PDA) to obtain a pure culture.

The morphological characteristics of isolates were studied on potato dextrose agar (PDA), synthetic nutrition agar (SNA), carrot agar (CA) and carnation leaf agar (CLA), prepared according to directions supplied by Booth (1971), Muntanola-Cvetković (1982), Fisher et al. (1982), Leslie (1991), Leslie and Summerell (2006) and Lević, (2008).

The physiological characteristics (the growth of selected isolates) were tested on PDA at 25 and 30°C. The colony diameter was measured after 73 hours of incubation, as suggested by Burgess et al. (1994) and the results represented the mean of three replicates.

The identification of the pathogen was based on morphological and physiological characteristics of the isolates (appearance of aerial and substrate mycelia, morphology of macro- and micro conidia, the colony growth on PDA at 20°C, and pigmentation of the medium). The taxonomic keys of Booth (1971), Gerlach and Nirenberg (1982), Nelson et al. (1983), Burgess et al. (1994), Summerell et al. (2002), Leslie and Summerell (2006), Dugan (2006) and Lević (2008), were used for the identification of the obtained isolates.

The pathogenicity was confirmed by the modified method of Molt and Simone (1967). Four hundred seeds of each medicinal plant were sown in plastic pots with sterile sand. The pots were irrigated with 100 ml of conidial suspension, prepared from 7-day old culture grown on PDA. After 21 days the

seedlings were picked and washed in distilled water. The level of root necrosis was calculated according to the scale from 0 to 3 (0 — health seedling; 1 — root tip necroted; 2 — root and down part of stem necroted; 3 — whole seedling necroted).

RESULTS

Collection of isolates

Gibberella intermedia was isolated from seeds of all tested medicinal plants, as well as roots and stems of marshmallow, and roots of *Echinacea purpurea*. The pathogen caused symptoms of seedlings decay and root rot, which resulted in suppressed growth, chloroses and malformation of diseased plants. From around 200 isolates, 29 of them were selected for further investigations: 15 were obtained from seeds, 8 from roots, 3 from stalks and 3 from seedlings. From marshmallow, coneflower (*Echinacea purpurea* and *E. angustifolia*) and St. John's wort 10, 15 (10 + 5), and 4 isolates were obtained, respectively (Table 1).

Tab. 1 — The list of *Gibberella intermedia* isolates used in the study

Host	Isolates	Place of isolation	Locality
Althaea officinalis	S-4	stalk	Zrenjanin
	S-7	seed	Pančevo
	S-8	seed	Pančevo
	S-10	root	Zrenjanin
	S-13	root	Zrenjanin
	S-18	root	Zrenjanin
	S-20	seed	Pančevo
	S-30	root	Ruma
	S-35	root	Zrenjanin
	S-38	stalk	Zrenjanin
Echinacea purpurea	E-67	seedlings	Ruma
	E-44	seedlings	Ruma
	E-7	seed	Pančevo
	E-202	root	Inđija
	E-204	root	Inđija
	E-253	seed	Pančevo
	E-263	stalk	Ruma
	E-266	seed	Pančevo
	E-400	seed	Pančevo
	E-404	root	Inđija
Echinacea angustifolia	Ea-31	seedlings	Ruma
	Ea-34	seed	Pančevo
	Ea-77	seed	Pančevo
	Ea-78	seed	Pančevo
	Ea-19	seed	Pančevo
Hypericum perforatum	K-77	seed	Pančevo
	K-79	seed	Kačarevo
	K-80	seed	Kačarevo
	K-82	seed	Kačarevo

Morphological characteristics

Mycelium was abundant, woolly, whitish, and with age it became light to dark violet. Colour of mycelium and medium pigmentation varied upon the isolate (Fig. 1).

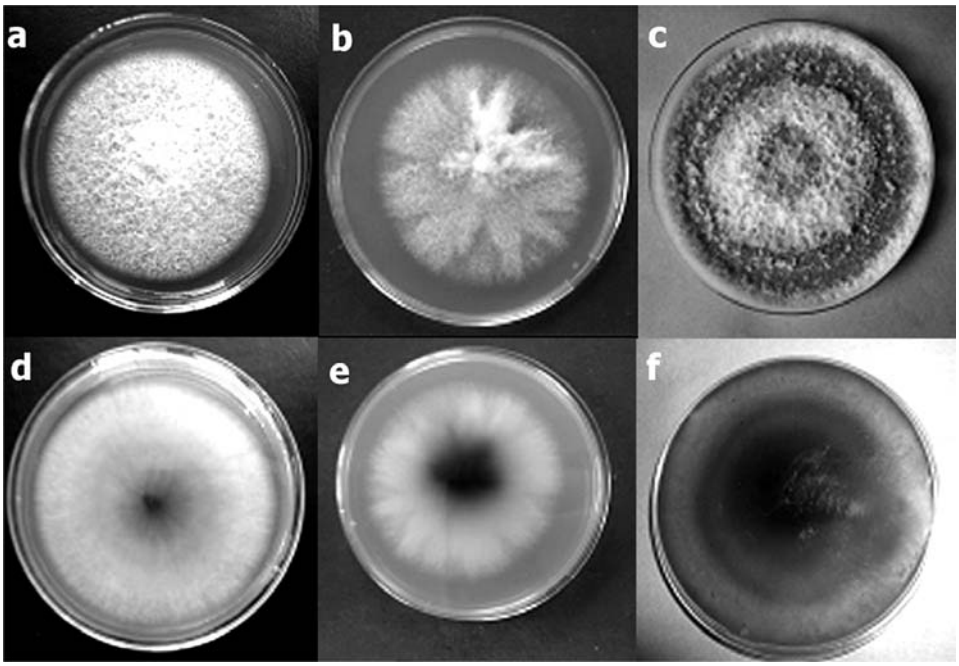

Fig. 1 — Appearance of aerial (upper row) and substrate mycelia (lower row) of *Gibberella intermedia* isolates E-404 (a, d), K-79 (b, e) and S-18 (c, f)

On infected germinating seeds, incubated on wet filter paper, mycelium was powdery, white, almost entirely covering the infected seeds. Such seeds did not germinate and they completely decayed. The filter paper under these seeds was coloured ink-blue. *Ascocarp* was perithecia, spherical or obovoid, black, 210—390 mm in diameter on CA. *Ascuses* were elongated, hyaline, with 4—8 ascospores, 66,5—88,0 x 8,50—14,20 mm. *Ascospores* were with 2—3 septa, straight or slightly narrowed by septa, hyaline, 15—17,5 x 5,0 mm. *Microconidia* were formed in chains or "false heads" on polyphialides and monophyalides. They were oval with rounded base, or rarely two-celled, hyaline, 3,0—19,5 x 2,0—5,0 mm. *Macroconidia* were formed on monophyalides on branched conidiophores in sporodochia, rarely on monophyalides on hyphae. The abundant pale orange sporodochia formed on CLA under combination of fluorescent and UV light. They were slender, falcate or straight, thin-walled, mostly with 3—5 septa, the basal cell pediculate, 16,5—73,0 x 2,5—5,0 mm (Figure 2). There were some variation in conidia dimensions depending on isolate and host. *Hlamydospores* were absent.

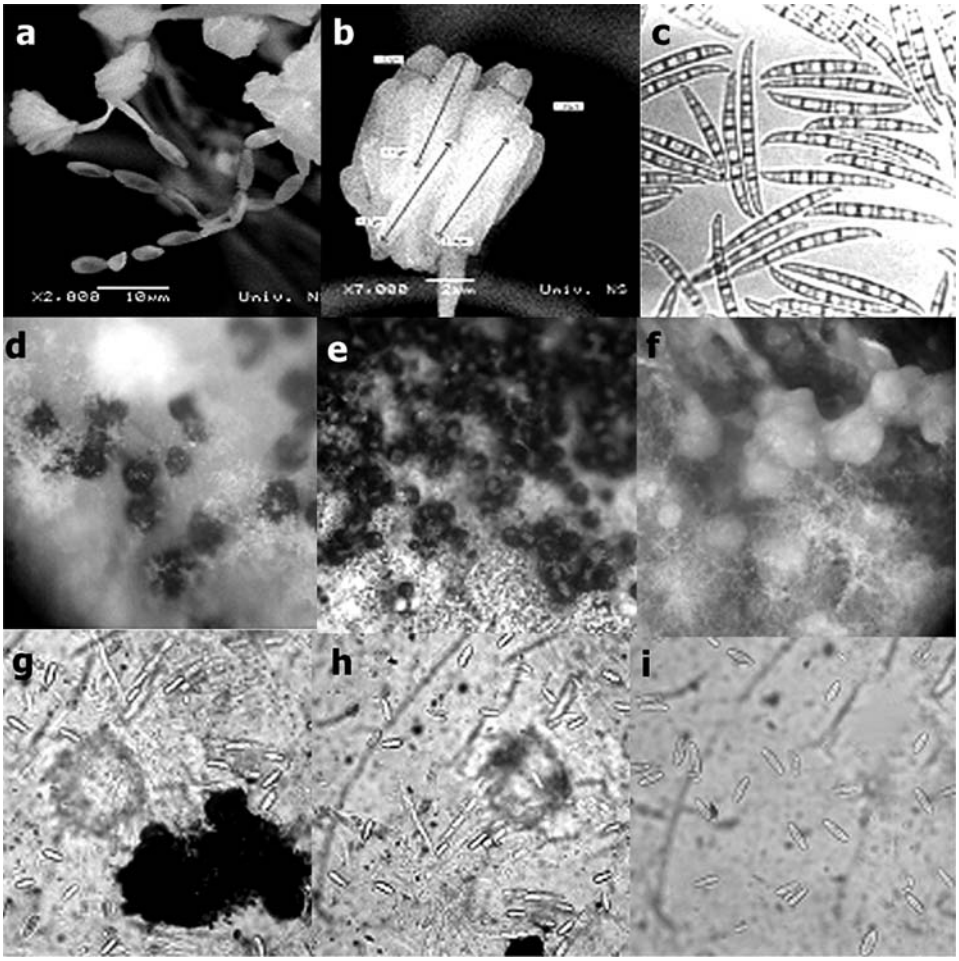

Fig. 2 — *Gibberella intermedia*: microconidia in long chains (a) and "false heads" (b) (SEM); macroconidia from sporodochia (c); perithecia of *Gibberella intermedia* obtained by crossing isolates (d, e); leaking fertile perithecia (f), ascuses (g, h) and ascospores (i)

Physiological characteristics

The uniform growth of isolates at 25 and 30°C was observed. However, the growth of the isolates from marshmallow was faster at 30°C (Table 2). There were no statistically significant differences in average values of growth of all isolates from the investigated medicinal plants at 25 and 30°C (34,1 mm and 34,5 mm, respectively) ($LSD_{0,05}$ = 3,24, $LSD_{0,01}$ = 5,95).

Tab. 2 — Radial growth of colonies of *G. intermedia* isolates from marshmallow, St. John's wort and coneflower on PDA at 25 and 30°C after three days (in mm)

Host	Isolates	Colony growth at	
		25°C	30°C
Althaea officinalis	S-4	32.7 d[y]	33.0 d
	S-7	35.7 bc	36.0 c
	S-8	31.0 e	35.7 c
	S-10	35.7 bc	37.7 b
	S-13	36.7 b	39.7 a
	S-18	35.0 c	35.7 c
	S-20	38.7 a	40.7 a
	S-30	34.7 c	37.0 bc
	S-35	32.0 de	37.0 bc
	S-38	37.0 b	39.7 a
	Average[z]	34.9 B	37.2 A
Hypericum perforatum	K-77	36.0 a	33.0 ab
	K-79	32.0 a	32.0 b
	K-80	36.7 a	34.0 a
	K-82	28.7 b	25.7 c
	Average	33.4 A	31.2 A
Echinacea purpurea	E-67	35.0 a	34.0 bcd
	E-44	32.0 c	34.7 ab
	E-7	34.0 ab	36.0 a
	E-202	35.0 a	36.0 a
	E-204	33.7 ab	32.3 ce
	E-253	35.0 a	35.3 ab
	E-263	32.7 bc	34.0 bcd
	E-266	34.7 a	34.0 bc
	E-400	35.0 a	36.3 a
	E-404	35.0 a	36.3 a
	Average	34.2 A	34.9 A
Echinacea angustifolia	Ea-31	34.0 a	34.3 abc
	Ea-34	34.0 a	34.0 bc
	Ea-77	33.0 a	33.3 c
	Ea-78	33.0 a	35.3 ab
	Ea-19	34.0 a	36.0 a
	Average	33.6 A	34.6 A

[y] Values in the columns designated by the same letter are not statistically significantly different on the basis of Duncan's test (P = 0,05).

[z] Average values of growth of all isolates under tested temperatures for the same host, designated by the same capital letter are not statistically significantly different on the basis of Duncan's test.

Pathogenicity of tested isolates

All isolates of *G. intermedia* caused root necrosis of seedlings. There were no significant differences between the isolates, but the isolates Ea-77 and Ea-78 showed lower pathogenicity in comparison with other isolates (Table 3).

Tab. 3 — Effect of different *G. intermedia* isolates on seedling root necrosis of tested medicinal plant species under laboratory conditions

Host	Isolates	Root necrosisz
Althaea officinalis	S-7	3,0 a
	S-10	2,0 a
	S-18	3,0 a
	S-30	2,0 a
	S-35	3,0 a
	check	0,0 b
Echinacea purpurea	E-7	2,0 a
	E-44	3,0 a
	E-204	2,0 a
	E-266	3,0 a
	E-400	2,0 a
	check	0,0 b
Echinacea angustifolia	Ea-19	2,7 a
	Ea-31	3,0 a
	Ea-34	3,0 a
	Ea-77	2,0 b
	Ea-78	1,0 c
	check	0,0 b
Hypericum perforatum	K-77	3,0 a
	K-79	2,0 a
	K-80	3,0 a
	K-82	2,0 a
	check	0,0 b

z Values in the columns designated by the same letter are not statistically significantly different on the basis of Duncan's test (P = 0,05).

DISCUSSION

Morphological, cultural and pathogenic characteristics of the tested isolates were described according to data for *G. intermedia* given in the literature (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson et al., 1983; Burgess et al., 1994; Leslie and Summerell, 2006). This species is known as a pathogen found on seeds of a number of cultivated plant species, such as wheat, corn, broomcorn, sugar beet, sunflower and soybean (Jovičević and Milošević, 1990; Summerell et al., 2002; Lević et al., 2003; Leslie and Summerell, 2006; Lević, 2008), and it causes a decrease of germination and germination energy, wilting and seedling decay, known as firing and melting of seedlings (Jasnić and Maširević, 2006). Anamorph of this species (*F. proliferatum*) was already identified on seeds and seedlings of coneflower (Pavlović et al., 2006, 2006a), and seeds, roots, and lower part of stalks of marshmallow (Pavlović and Stojanović, 2002, Pavlović et al., 2007).

The results of the conducted study, which was preferentially diagnostic in nature, will allow more comprehensive recognition of mycoflora of medicinal and aromatic plants in our conditions. These results will also enable more ef-

fective protection of medicinal and aromatic plants, i.e. improved and more profitable cultivation.

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GIBBERELLA INTERMEDIA ПАТОГЕН КАНТАРИОНА, ЕХИНАЦЕА И БЕЛОГ СЛЕЗА У СРБИЈИ

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Резиме

Gibberella intermedia (Kuhlmann) Samuels et al. (анаморф: *Fusarium proliferatum* /Matsushima/ Nirenberg) је констатована на белом слезу, ехинацеи и кантариону и то на семену, корену и стаблу, као и на расаду ехинацеа. Основни симптом заражених биљака *G. intermedia* су били: пропадање клијанаца, трулеж корена и кореновог врата, патуљавост, асиметричан пораст, увелост, хлороза и смежураност листова. Узорци зараженог биљног материјала били су пореклом из околине Панчева, Зрењанина, Банатског Новог Села, Инђије, Руме и Старе Пазове. У раду су дате морфолошке, физиолошке и патогене карактеристике изолата *G. intermedia*.

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MINERAL FERTILIZERS AS A GOVERNING FACTOR OF THE REGULATION OF THE NUMBER OF FUNGI IN SOIL

ABSTRACT: Over 2003—2005 period, a study was performed on the effect of different rates of NPK fertilizer of formulation 8:16:24 + 3% MgO (N_1 — 400 kg ha^{-1}; N_2 — 600 kg ha^{-1}; N_3 — 800 kg ha^{-1}; N_4 — 1000 kg ha^{-1}) on development of the soil fungi. The trial was set up in the experimental plum orchard established by Fruit Research Institute Čačak, and the laboratory of Department of Microbiology, Faculty of Agronomy Čačak. Unfertilized soil was used as the control soil. Each of the stated variants was carried out in three replications. The size of the basic plot was 68 m^2. The effect of the studied mineral fertilizer rates was determined three times over the growing season, the number of fungi being checked by the indirect rarefaction method on Chapek nutritive medium. The results of the study inferred that the application of mineral fertilizers brought about the decrease in the number of fungi. Of all studied variants, the one with the highest nitrogen rate (variant N_4) exhibited the strongest effect. The influence of the fertilizer was highest at the third sampling. Furthermore, the effect was highest in season 2003.

KEY WORDS: fungi, mineral fertilizers, soil

INTRODUCTION

Intensive development of modern technologies has resulted in the progress of society in general, but it brought about damaging consequences as well. In addition, the problem of protection of soil from the pollution and other antropogenic factors appears to be one of the major problem of our time. The importance of the productive capacity of soil is reflected in the fact that by 2020 meeting world food requirements will presuppose 50% increase in food production. For that purpose, by 2010, about 90 million hectares of the global acreage will have to be turned into arable land, which presupposes maintainance of the current soil fertility (www.ekoforum.org.yu, 2000; Pešaković, 2007).

The spread of human-produced harmflul substances is a major cause of soil pollution. On the one hand, human loss of control over these substances leads to soil degradation, whereas on the other hand, these substances return to humans through the food chain. Aimed at lowering the level of both air- and soil-pollution, the control over the emission of harmful substances has been greatly intensified over the recent period. This issue has not been duly addressed so far in the field of technology of agricultural production. Such shortages may result in serious economic and ecological consequences reflected in lowering of soil productivity, the loss of agro-biodiversity, the incidence of methemoglobinemia, cyanosis as well as mutagenic and teratogenic effects. Therefore, one of the goals of science is ensuring intensive environmental and human-friendly agricultural production, whereby the attention should be focused on proper application of different fertilizing modes. Rational and efficacious application of fertilizers (mineral ones in particular) in modern agriculture need to be based on a more complex approach to the issue, which presupposes prominent positioning of macrobiological investigations (M a n d i ć et al., 2004). The objective of this work, i.e. the investigation of the effects of different fertilizer rates on the development of fungi in soil has been determined in accordance with the fact that the change in population pressure of some systematic and physiological microrganism groups may serve as a parameter for establishing the most optimal criteria for agricultural nutrition.

MATERIAL AND METHODS

The investigations in question were performed over 2003—2005 period in the experimental plum orchard established by Fruit Research Institute Čačak and the laboratory of Department of Microbiology, Faculty of Agronomy. The soil type is alluvium, and trial system included random-block design in three replications. The size of the basic trial plot was 68 m^2.

The soil was acid in type, pH = 1nKCl = 5.9. It was poor in organic matter (2.65% humus) and rich in available phosphorous (AL-method: mg 100 g^{-1} of soil = 15.0 P_2O_5), whereas potassium content was moderate (AL--method: mg 100 g^{-1} of soil = 20.4 K_2O).

A plum fruit of cv Stanley was used as a test fruit.

All cultural practices, i.e fertilization, green and winter pruning, inter-raw soil cultivation, protection from diseases and pests were applied at establishing a training system.

The temperature and water data of the studied region are presented in Table 1.

Tab. 1 — Weather characteristics (Čačak Weather Bureau) and long-term means (LTM)

Period	Precipitation rates and mean air-temperatures in Čačak*								Total	Mean
		May	June	July	Aug.	Sept.	Oct.	Nov.		
2003	mm	62	51	69	6	34	77	27	326	
	°C	19.8	25.1	24.2	26.4	17.3	10.2	8.9		18.8
2004	mm	66	121	82	58	35	27	98	487	
	°C	16	21.7	23.5	22.8	18.1	14.3	6.2		17.5
2005	mm	72	84	100	66	91	23	83	519	
	°C	17.2	21	23.7	20.3	18.2	11.8	5.2		16.8
LTM (1965—1994)	mm	89	98	76	60	56	48	59	486	
	°C	16.2	19.5	20.9	20.5	16.9	11.8	5.8		15.9

* 5 km eastwards from the trial field, as the crow flies.

Over 2000—2002 period, the soil was treated with different rates of mineral fertilizer of type 8:16:24 + 3% MgO, i.e. variant N_1 — 400 kg/ha; variant N_2 — 600 kg/ha; variant N_3 — 800 kg/ha; N_4 — 1000 kg/ha; variant Ø — the control (untreated soil).

The samples subjected to microbiological analyses were collected three times over the growing period, i.e. May 12, Sept. 17 and Nov. 11 in seasons 2000—2002.

The number of fungi was determined by indirect dilution method on Chapek nutritive medium. The duration of incubation was 5 days (28°C).

The analyses were performed in three replications, whereby the number of fungi was calcultated on 1.0 of absolutely dry soil.

The data provided by this research were subjected to variance analysis method of threefactorial trial of 5 x 3 x 5 form (chemical x period x year). The testing of significant differences among individual and interaction environments was carried out by the LSD test.

RESEARCH RESULTS AND DISCUSSION

The experimental results of the average number of fungi in soil in the studied variants by years inferred that all studied factors exerted statistically high significance regarding the presence of this microorganism group in soil (Tab. 2). In addition, it is obvious that the interaction among the studied factors (A x B, A x C, and B x C) had marked influence on the development of this microorganism group.

Generally speaking, all fertilizer rates had a stimulating effect on the development of soil fungi, which was particularly evidenced in high-rate variants over all phases of the growing season in this fruit species. This trend was notably observed in the N_4 variant applied in mid- and final phases of the growing season, the growing of which was favoured by higher temperatures and moisture rates alike (Tab. 1), which is also inferred by other authors' results (J e m c e v and Đ u k i ć, 2000).

The incorporation of higher rates of mineral fertilizers into soil, acid ones in particular, and their long-term usage is depressing for the majority of microorganisms (Jemcev and Đukić, 2000; Pešaković, 2007). The occurrence of this may be due to the transformation of alluminium compounds into the soil solution which becomes toxic not only for microorganisms but also for cultivated fruit species. However, soil fungi which exhibit a steady enzyme system that enables them to inactivate even heavily degradable chemical compounds (Mandić, 2002) fare well even under such conditions, and this stimulating effect of higher mineral fertilizer rates is therefore anticipated. With regard to the predominance of fungi in acid soils, it is also suggested that their population number rises with more intensive application of the stated fertilizers. A large number of authors addressing this issue (Acosta-Martinez & Tabatabai, 2000; Solovova et al., 2001; Zhang, Wang, 2006; Pešaković et al., 2008) account for this rise in population density and activity of the majority of microorganisms in soil by limiting of the C:N relation and the intensification of the mineralizing processes therein, as well as by the re-distribution within the complex of microbial cenoses in favour of soil fungi.

Tab. 2 — The average number of fungi in soil (10^5/1,0 g of absolutely dry soil) as affected by the applied fertilizers (A), period of sampling (B) and the year of study (C) in a trial field planted with plum cv Stanley

Fertilizer (A)				N_1	N_2	N_3	N_4	Ø	$\overline{X}$
Year (C)	2003	Period (B)	I	4,500	5,335	8,165	9,670	3,835	6,301
			II	13,500	15,835	22,335	23,330	8,831	16,766
			III	6,170	7,835	11,170	23,555	3,500	10,446
		$\overline{X}$		8.057	9.668	13,890	18,852	5,389	11,171
	2004	Period (B)	I	2,830	3,670	5,335	6,165	2,335	4,067
			II	8,500	11,665	13,000	14,500	7,000	10,933
			III	5,333	7,665	9,330	12,830	3,665	7,765
		$\overline{X}$		5,554	7,667	9,222	11,165	4,333	7.588
	2005	Period (B)	I	2,670	4,335	4,665	5.000	2.330	3.800
			II	9,330	12,500	14,335	16.330	6.835	11.866
			III	4,000	4,165	5,000	6.335	3.335	4.567
		$\overline{X}$		5,333	7,000	8,000	9,222	4,167	6.744
	$\overline{X}$		I	3,333	4,447	6,055	6,945	2,833	4.723
			II	10,443	13,333	16,557	18,053	7,555	13.188
			III	5,168	6,555	8,500	14,240	3,500	7.593
$\overline{X}$				6.315	8,112	10,371	13,079	4,630	8,501
lsd									
lsd		A		B	C	AxB	AxC	BxC	AxBxC
0.05		1,831		1,418	1,418	3,171	3,171	2,456	5,492
0.01		2,425		1,878	1,878	4,200	4,200	3,254	7,275

The analysis of the interaction effect of the applied fertilizers and years of study suggests similar effects. Over the entire three-year period of study, N_4

variant (1000 kg/ha) exerted high effects, which could also be applied to N_3 variant (800 kg/ha), particularly in season 2003. Similarly, the most pronounced variation, in respect to the number of fungi was also evidenced in season 2003. The contrastive analysis of the obtained values with those of the control soil over the stated year inferred that all variants, except N_1, had quite an impact on the rise in the number of fungi.

The stated effects of the studied fertilizer were the most obvious in season 2003 when number of fungi was highest, whereupon the effect was observed to be gradually decreasing, which was particularly to evident at the end of the season 2005.

Generally speaking, the rise in fertilizer rate was accompanied by the rise in the number of soil fungi, which, by certain degree, may be considered positive. However, over-activation of fungi may be damaging, as the processes directed towards establishing of the disturbed balance lead to the weakening of physical, chemical and biological properties of soil (Mandić et al., 2004) and the incidence of toxic fungi (Milošević et al., 1993), whereby Penicillium species assume predominance (Mandić, 2002; Pešaković, 2007).

Besides causing undesirable effects on biosphere in general, and its constituent elements, inappropriate application of mineral fertilizers can influence agricultural production from the aspect of economy. In other words, the production of cultivated species can be markedly affected (Đukić et al., 2007a). Therefore, fertilizer rates exceeding 600 kg/ha may not only be unprofitable but may turn depressing on productivity of plum trees grown under the stated environmental conditions (Rakićević et al., 2004; Pešaković, 2007).

CONCLUSION

The results of the study of the effects of different mineral fertilizer rates on the number of fungi in soil planted with plum trees (cv Stanley) infer the following:

— the number of the studied group of microorganisms was dependent on fertilizer rates, period of sampling and year of study;

— the applied fertilizers brought about an increase in the number of fungi, particularly in the variants that included highest nitrogen content;

— the effect of the applied fertilizers on the number of fungi in soil was most evident at the end of the growing season 2003.

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МИНЕРАЛНА ЂУБРИВА КАО ФАКТОР РЕГУЛАЦИЈЕ БРОЈНОСТИ ГЉИВА У ЗЕМЉИШТУ

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Резиме

У периоду 2003—2005. године у експерименталном засаду шљива Института за воћарство Чачак и у Одељењу за Микробиологију Агрономског факултета у Чачку, праћен је утицај различитих доза минералног ђубрива формулације 8:16:24 + 3% MgO (N_1 — 400 kg ha^{-1}; N_2 — 600 kg ha^{-1}; N_3 — 800 kg ha^{-1} и N_4 — 1000 kg ha^{-1}) на развој земљишних гљива. Као контрола коришћено је земљиште које није ђубрено. Свака од наведених варијаната ђубрења била је заступљена у три понављања. Величина основне огледне парцеле износила је 68 m^2.

Ефекат примењених ђубрива одређиван је три пута током вегетације, а праћен је путем утврђивања бројности гљива индиректном методом разређења на Чапековој хранљивој подлози. Резултати истраживања су показали да је примена минералних ђубрива изазвала повећање бројности гљива. Од свих испитиваних варијаната ђубрива најизраженији утицај показала је N_4 варијанта (варијанта са највишом дозом азота). Утицај примењених ђубрива је био најизраженији на крају вегетације и током 2003. године.

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GROWTH AND FRUIT BODY FORMATION OF *PLEUROTUS OSTREATUS* ON MEDIA SUPPLEMENTED WITH INORGANIC SELENIUM

ABSTRACT: Selenium is a trace mineral chemically related to sulfur and tellurium. In the body selenium combines with protein molecules to form selenoproteins and it is distributed in low concentrations and unequally in air, soil and water all over the world. Edible mushrooms are known to be selenium accumulators. Since mushrooms contain relatively high protein levels, and they can accumulate large amounts of selenium, it is reasonable to expect that selenium could be incorporated into proteins. The growth of mycelia and fruit body formation of different medicinal mushroom strains of *Pleurotus ostreatus* (Hk-35 and P_{70}) over the wide range of concentrations of inorganic form of selenium were examined. Mushrooms were cultivated on agar base media and on substrates based on sawdust. Vegetative growths of mycelium were measured as colony diameter in pure cultures supplemented with inorganic form of Se supplements, prepared as Na_2SeO_4 and Na_2SeO_3 in concentrations of: 1, 10, 25, 50, 75, 100 and 150 mg/l. Inorganic form of Se supplements, showed stimulation effects (in concentration of 1—50 mg/l) and toxic effects in higher concentration. On the standard industrial sawdust based substrate, supplemented with 100 mg/kg Na_2SeO_4 and Na_2SeO_3, accumulation of Se in fruit bodies was determined by the method of flameless atomic absorption spectrophotometer. The readings were performed on Varian SpectrAA-10 spectrophotometer equipped with VGA-76. Se as Na_2SeO_4 and Na_2SeO_3 was effectively taken up from substrates and accumulated in fruit bodies. Mushrooms accumulated selenium between 120 and 250 mg/kg dry weight. In mushrooms cultivated without Se supplement, Se contents were only about 1 mg/kg and in substrate about 0.1 mg/kg.

KEY WORDS: *Pleurotus ostreatus*, Selenium, Spectrophotometer, Substrate

INTRODUCTION

Selenium is a trace mineral chemically related to sulfur and tellurium. In the body selenium combines with protein molecules to form selenoproteins. Selenium deficiency is linked with numerous diseases: cancer, muscular dystrophy, malaria and cardiovascular disease including endemic cardiomyophaty

in selenium deficient regions (Nassir et. al., 1997). Selenium is distributed in low concentrations and unequally in air, soil and water all over the world (Barceloux, 1999). The soil in some parts of China and Russia has scant amounts of selenium. The amount of Se in soil, which varies by region, determines the amount of Se in the plant foods and meat. The daily value recommended for selenium by the FDA is 70 μg. When supplements are used, 100—200 μg daily of selenomethionine is recommended. Amounts greater than 200 μg should be avoided.

Fungi are known as accumulators some chemical elements, like Zn, Cd, As etc. Edible mushrooms are known to be selenium accumulators. Since mushrooms contain relatively high protein levels, and they can accumulate large amounts of selenium, it is reasonable to expect that selenium could be incorporated into the proteins (Gergely, 2006). The genus *Pleurotus* (higher *Basidiomycetes*) includes edible and medicinal species, some of them are cultivated and have important economical value and some have important medicinal properties: reduction of cholesterol levels, antitumor, antiviral, antibacterial and immunomodulating activity. *Pleurotus* species may present an excellent dietary source of some microelements because of their ability to absorb them from medium (Stajić, 2001).

The aim of the study was to determine the contents of mineral element (Se) in the cultivated mushrooms *Pleurotus ostreatus*.

MATERIAL AND METHODS

Growth and fruit body formation on media supplemented with selenium was investigated on mushroom strains (Faculty of Agriculture collection): *Pleurotus ostreatus* (Hk-35, P_{70}). Cultures were maintained at 4°C on malt agar base contained in screw slants with periodic transfers to fresh agar base (pH = 5.4 ± 0.2).

The response of fungi to inorganic selenium supplements (Faculty of Chemistry, University of Belgrade) was examined. Inorganic form of selenium supplements were prepared as Na_2SeO_4 and Na_2SeO_3 (1 mg/l, 10 mg/l, 25 mg/l, 50 mg/l, 75 mg/l, 100 mg/l, 150 mg/l) in Petri dishes with three replicates per treatment.

After agar sterilization at high preasure in an autoclave for 15 minutes at 121°C, the fragments of mycelia were inoculated and incubated at 25°C in the dark. The diameters of the formed colonies were determined 9 days after inoculation in order to assess the effects of Se on mycelial growth.

Seed for bags inoculation were prepared in glass jar, on wheat grain. Grains were cooked in water and dried with filter paper. To adapt pH at 6—7, CaCO3 was added into wheat. The prepared grains were put into jams and sterilized in autoclaves for 40 minutes at 121°C. After cooling, grains were inoculated with fragments from mycelia and incubated at 25°C in the dark for about 4 weeks, depending on the mushroom species and strains.

The substrates for *P. ostreatus* cultivation were prepared with sawdust (42%) as basic substratum (100%) and straw (29%), chopped oik (24%) and

gypsum (5%) were added to the basic substratum. The moisture content of substrate was adjusted to about 60%. Polipropylen bags contained substrate (1.5 kg/bag).

Selenium as Na_2SeO_3 and Na_2SeO_4 solution was supplemented at 100 mg/kg into 5 bags. The bags were sterilized, inoculated with seed and incubated at 26°C under 85% humidity in the dark for 20 days to allow full colonization of mycelia. All bags were then transferred to the green house for fruiting under daily irrigation and attenuated illumination. The substrate blocks were exposed to the temperature of 21—23°C and humidity 60—80%. The fruit bodies of both strains, developed completely and discharged spores from 50—60 days after the inoculation. The fresh mushrooms were stored frozen in boxes and kept at –20°C for a while. Melt fruitbodies of mushrooms were cut into pieces, dried at hot air (80°C, for few days) and then ground into a consistent powder.

The total selenium content in soil samples was determined by the method of flameless atomic absorption spectrophotometer, after its digestion with acids (HNO_3 and $HClO_4$) and reduction of Se^{6+} to Se^{4+} with 6MHCl. The readings were performed on Varian SpectrAA-10 spectrophotometer equipped with VGA-76.

RESULTS AND DISCUSSION

The effects of inorganic supplements on mycelial growth of mushroom species on agar base media and sawdust substrates were examined. The obtained results showed that the investigated *Pleurotus* strains have different abilities to absorb selenium by mycelia from medium where it is present in the form of Na_2SeO_3, Na_2SeO_4 and in different concentrations. They also differ in their ability to retain selenium in fruit body.

The investigated selenium sources and concentrations had different effect on the production of mycelial biomass in the investigated *Pleurotus* strains. In some cases, selenium did not show any effects on production and it was the same as in the control medium. However, in some cases, the presence of selenium in medium caused either an increase or a decrease of mycelial biomass production in comparison with the control.

— Mycelial growth on agar base media was measured as colony diameter in pure cultures supplemented with Na_2SeO_3 and Na_2SeO_4. Na_2SeO_4 (1 mg/l, 10 mg/l and 25 mg/l) stimulated the growth of *P. ostreatus*, strain Hk-35 (Fig. 1).

Mycelia at both strains showed slight inhibition at 150 mg/l. Growth of strain P_{70} was inhibited with all tested concentrations (Fig. 2).

Na_2SeO_3 stimulated mycelial growth of *P. ostreatus* Hk-35 at 1 mg/l and 10 mg/l. Not any of tested concentrations (1—150 mg/l) showed inhibition by 50? for *P. ostreatus* strains.

— Selenium as Na_2SeO_4 was effectively taken up from the substrates and accumulated in fruit bodies. The mycelia grew normally and young primordia of *P. ostreatus* formed well. There was no difference in morphology of fruit bodies grown on substrates with and without selenium supplements. Accumu-

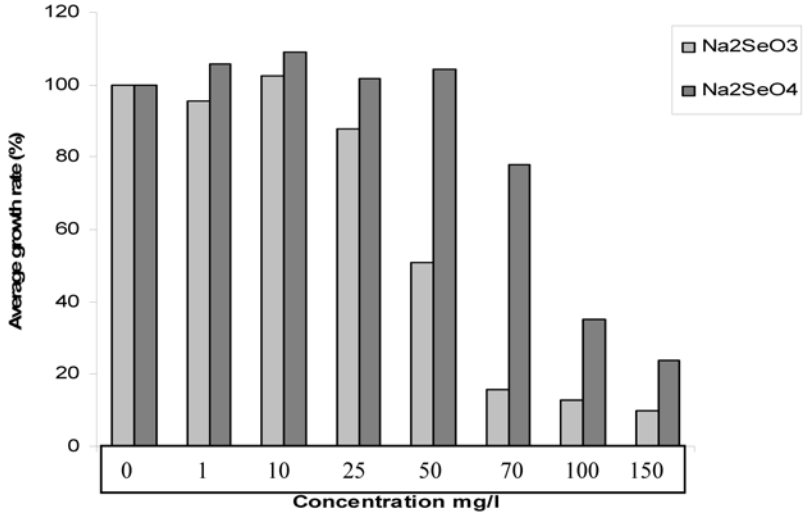

Fig. 1 — Mycelial growth of *Pleurotus ostreatus Hk-35* on agar base supplemented with Na_2SeO_3 and Na_2SeO_4

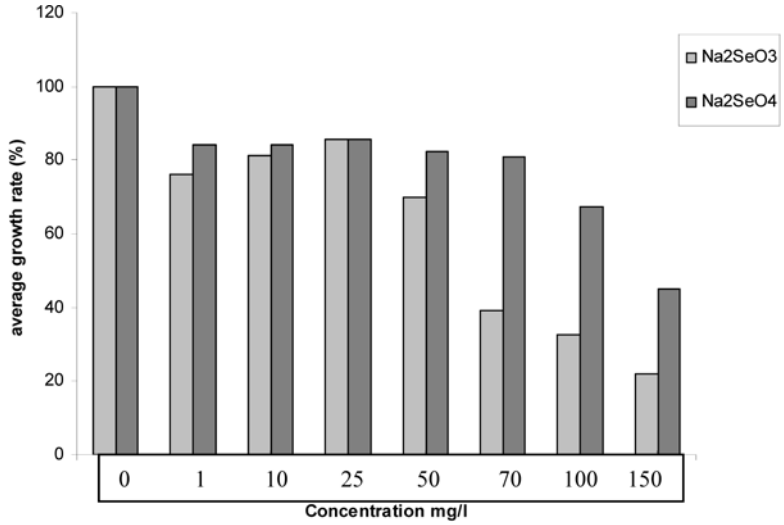

Fig. 2 — Mycelial growth of *Pleurotus ostreatus* P_{70} on agar base supplemented with Na_2SeO_3 and Na_2SeO_4

lation of selenium in fruit bodies was determined by electrochemical atomic absorbtion methods. The results showed that selenium as Na_2SeO_3 and Na_2SeO_4 was effectively taken up from the substrates and accumulated in fruit bodies. (Tab. 1)

Tab. 1 — Average content of selenium in mushroom fruit bodies (mg/g) grown on Se enriched substrate with 100 mg/kg d.w.

Mushroom strain	Natrium selenat	Natrium selenit	Control
Pleurotus ostreatus P70	169.3	213.0	0.85
Pleurotus ostreatus Hk-35	154.7	205.2	1.1

The total selenium content of the frozen and dried mushrooms depends on the mushroom strains and form of supplemented selenium. *P. ostreatus* accumulated selenium better from Na_2SeO_3 than from Na_2SeO_4. *P. ostreatus* accumulated selenium between 120 and 250 mg/kg. In mushrooms cultivated without Se supplement, Se content was 1 mg/kg, and in the control substrat it was 0.1 mg/kg.

CONCLUSION

Selenium enriched mushrooms are an excellent dietary source. Selenium as Na_2SeO_4 and Na_2SO_3 was effectively taken up from the substrates and accumulated in fruit bodies. Considering the foregoing results, further investigations can be made in the following areas:

— The study of selenium metabolic pathway and its forms, which are presented in *Pleurotus* species;

— Analyzing other cultivation media and finding the best one for maximum Se absorption by mycelia and the incorporation of organic selenium compounds in *P. ostreatus*;

— Considering both the medicinal properties of the *Pleurotus* species, and selenium antioxidative, antimutagenic and anticarcinogenic features, and the investigation of the effect of using Se-enriched fruit bodies in nutrition as well as their extracts.

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ПОРАСТ И ФОРМИРАЊЕ ПЛОДОНОСНИХ ТЕЛА ГЉИВЕ *PLEUROTUS OSTREATUS* НА СУПСТРАТУ ОБОГАЋЕНОМ НЕОРГАНСКИМ ЈЕДИЊЕЊИМА СЕЛЕНА

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Резиме

Селен је есенцијелни микроелемент, неопходан у малим количинама. Земљиште на територији Европе има низак садржај селена. Печурке садрже релативно висок ниво протеина, те стога могу да усвоје високе концентрације селена, који се инкорпорира у протеине формирајући селенопротеине. У раду је испитиван утицај већег броја различитих концентрација неорганских једињења селена на пораст и формирање плодоносних тела медицински значајне гљиве *Pleurotus ostreatus* (комерцијални сојеви Hk-35 и P_{70}). Пораст мицелијума на сладном агару праћен је мерењем пречника колоније чисте културе обогаћене неорганским једињењима селена. Коришћена неорганска једињења селена, у облику Na_2SeO_4 и Na_2SeO_3 (1—50 mg/l), стимулисала су пораст мицелијума, док су веће концентрације показале различит токсичан ефекат у зависности од концентрације додаваног једињења и соја гљиве. Печурке су затим гајене на хранљивом супстрату обогаћеном неорганским једињењима селена концентрације до 100 mg/kg. Садржај селена у плодоносним телима одређен је помоћу AAS (хидридни метод) након влажне дигестије и резултати су очитани на VarianSpectarAA-10 спектрофотометру са VGA-76 (помоћни апарат за испаравање) LSD тестом. Анализе су

показале да су гљиве упешно усвојиле селен у плодоносном телу. Тотални садржај селена у плодоносном телу печурака зависио је од испитиваног соја и врсте додаваног једињења. *Pleurotus ostreatus* је боље усвојила селен из Na_2SeO_3 него из Na_2SeO_4. Концентрација усвојеног селена се кретала између 120 и 250 mg/kg суве масе. У печуркама које су гајене на супстрату без додатка селена, садржај селена је износио свега око 1 μg/g, док се ова вредност за чист супстрат кретала око 0.1 mg/kg.

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INFLUENCE OF BIOACTIVE COMPOUNDS EXTRACTED FROM MUSHROOM *GANODERMA LUCIDUM* ON B AND T CELLS

ABSTRACT: *Ganoderma lucidum* (Leyss.: Fr.) Karst is one of the most often used mushrooms in traditional medicine of Far Eastern people. Because of its bitter taste and wooden build it is not suitable for nutrition, but the bioactive substances extracted from this mushroom possess very important medicinal characteristics. The aim of this experiment was to investigate the effects of different concentrations of isolated *Ganoderma lucidum* GL-I extract on the growth of JY (B) and Jurkat (T) cells. Obtained extracts were added to the cells in concentrations 1 mg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml and 100 pg/ml. JY and Jurkat cells were exposed to the action of bioactive compounds, b-glucans, during the incubation period of 72 h, at 37°C, in the atmosphere with 5% CO_2 and their number was counted. Among all tested concentrations of extract the most important influence showed concentration of 1 mg/ml, which reduced the number of B cells by 61.46%, while in the case of T cells their number was reduced by 57.14%.

KEY WORDS: b-glucans, bioactive polysaccharides, *Ganoderma lucidum*, Jurkat cells, JY cells

INTRODUCTION

Many mushrooms have long been valued as tasty, nutritious food by different societies worldwide. To the ancient Romans they were “the food of the Gods”; the Egyptians considered them as “the gift from the God Osiris”; the Chinese viewed them as “the elixir of life”. Mushrooms are popular and valuable functional food, low in calories and high in minerals, essential amino acids, vitamins and fibers. In the Orient, several thousand years ago, there was the recognition that many edible and certain non-edible mushrooms could have valuable health benefits (Bensky and Gamble, 1993, Hobbs, 1995).

Some of edible mushrooms demonstrate medicinal or functional properties, while others are known only for their medicinal properties, e.g. *Ganoderma lucidum*, commonly known as lacquered mushroom. It is a non-edible mushroom due to its coarse and hard texture and bitter taste. The historical evolution of usage of these essentially scarce, forest-obtained medicinal mushrooms certainly did not include whole mushrooms, but in the form of hot water extracts, concentrates, liquors or powders and used in health tonics, tinctures, teas, soups and herbal formulae (Smith et al., 2002). *Ganoderma lucidum* has been widely used in China (named Ling Zhi) and Japan (named Reishi, Mannentake) for thousands of years for the treatment of various diseases, including cancers. The fact that this mushroom earned itself names like "Sky Plant" and "Mushroom of the Universe", confirm its possibility of revitalization and curing of different illnesses. It acts antitumour, antiinflammatory, antiviral (e.g. anti-HIV), antibacterial, antiparasitic, immunomodulating and hepatoprotective, it has a role in blood pressure regulation, against cardiovascular disorders and chronic bronchitis, like kidney tonic and nerve tonic (Wasser and Weis, 1999). By current techniques a numerous bioactive compounds were isolated from different parts of the mushroom, among which polysaccharides, b-D-glucans, peptidoglycans and bitter triterpens were the most important. Pharmacologically, a number of the water-soluble polysaccharides have demonstrated antitumour and immunostimulating activities.

The effects of bioactive compounds isolated from mushrooms could be observed on different cell lines, such as T cell and B cell lines, which are specifically transformed. T cells belong to a group of white blood cells known as lymphocytes and play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, such as B cells and natural killer cells, by the presence of a special receptor on their cell surface called T cell receptors (TCR). B cells are lymphocytes that play a large role in the humoral immune response (as opposed to the cell-mediated immune response, which is governed by T cells). The principal functions of B cells are to make antibodies against antigens, perform the role of Antigen Presenting Cells (APCs) and eventually develop into memory B cells after activation by antigen interaction (Abbas et al., 2000).

MATERIALS AND METHODS

Dried carpophores of *Ganoderma lucidum* GL-I mushroom were used for this investigation. The samples were exposed to the hot water extraction of polysaccharides and alcohol precipitation, refined by dialyses and the obtained extracts were used for testing their influence on human B and T cells (Klaus, 2004).

Hot extraction of bioactive compounds

Powdered tissue was washed with 96% ethanol, filtered and dried in vacuum (at 40°C for 60 min.) until it turned into powder up to getting powder.

Dried filtercake was mixed with deionized water and glucans were extracted by autoclaving at 120°C for 20 min. Material was cooled down and centrifuged (10000rpm, at 4—9°C for 10min.). Supernatant was mixed with 2 vol. 96% ethanol and left at 4°C until precipitate was formed. After centrifuge, the collected pellets were dried in vacuum and the obtained powder was refined by dialysis.

Refining of bioactive compounds by dialysis

Bioactive compounds, polysaccharides, and b-glucans obtained by hot water extraction and alcohol precipitation from dry mushroom carpophore were purificated by dialysis against MQ water. Suspensions were dialysed for 24 h at room temperature. Dialysis is necessary for refining because low-molecular weight molecules will pass through membrane in solution, while high--molecular weight molecules, b-glucan will stay inside the membrane. After the dialysing content was centrifuged, 2 vol. 96% ethanol was added to supernatant and left at 4°C for a couple of hours. To remove supernatant, the centrifugation was applied again and the pellets were dried in vacuum. Dried pellets were dissolved in phosphate saline buffer (PBS) and used for further examination on JY and Jurkat cells.

Jurkat and JY cell lines

Jurkat cell line is an immortalized line of human lymphocyte cells that are used to study acute T cell leukemia, T cell signaling and expression of various chemokine receptors susceptible to viral entry, particularly HIV. Jurkat cells are also useful in science because of their ability to produce interleukin 2. Their primary use, however, is to determine the mechanism of differential susceptibility of cancers to drugs and radiation (A b b a s et al., 2000).

The JY cell line is a human B cell line transformed by Epstein-Barr virus (EBV); it does not produce EB virus but does produce Ig. They are a suspension cell line, though they are known to grow in clumps (A b b a s et al., 2000).

Jurkat and JY cells were incubated in plastic 28 cm^3 flasks filled by growing media RPMI 1640 with addition of 10% fetal calf serum (fcs), antibiotics (1% penicillin and 1% streptomycin) and 7.5% $NaHCO_3$ (buffer), at 37°C, in the 5% CO_2 atmosphere. After a seven day incubation, 10 ml of suspension were centrifuged for 10 min on 8000 rpm. 10 ml of fresh RPMI 1640 medium with 10% fcs, antibiotics and $NaHCO_3$ which were warmed up to 37°C were added to the obtained pellets. This suspension was used for making solution with 10^5 CFU/ml.

Loading the wells

For all experiment, plates with 24 wells were used. First row was control and did not contain any extract of mushroom. In the 2nd row 1 μg extract per

well was added; in the 3rd row 100 ng extract per well were added; in the 4th row 10 ng extract per well were added; in the 5th row 1 ng extract per well was added and in the 6th row 100 pg extract per well were added. The rows 3, 4, 5. and 6. were serially diluted by pipetting 100 µl from row 2. into row 3, from 3. into 4, etc. The starting suspension added to the 2nd row was obtained by adding 100 µl of mushroom extract into the 900 µl sterile PBS. JY and Jurkat cells were exposed to the action of bioactive compounds, b-glucans, during the incubation period of 72 h, at 37°C, in the atmosphere with 5% CO_2 and their number was counted with Fuchs-Rosenthal chamber.

RESULTS AND DISCUSSION

From 9g powdered dried *Ganoderma lucidum* Gl-I carpophore 0.0055 g polysaccharides, b-glucans were obtained through the treatment of hot water extraction, alcohol precipitation and dialysis refining.

After the incubation period, in the presence of certain amount of extract, number of cells were counted with Fuchs-Rosenthal chamber, to establish the changes in the number of cells.

Tab. 1 — Influence of different concentration of *Ganoderma lucidum* Gl-I extract on JY cells number and standard deviation

treatmant	extract concentration	number of JY cells (4 repeats)				average number	standard deviation
		1	2	3	4		
C	—	140	141	138	144	140.75	2.5
1	1 µg	47	57	59	54	54.25	5.25
2	100 ng	67	65	60	69	65.25	3.86
3	10 ng	76	78	75	76	76.25	1.25
4	1 ng	86	82	92	87	86.75	4.11
5	100 pg	88	86	93	92	89.75	3.30

It was established that average number of JY cells was changed in the presence of bioactive compounds extracted from karpophore *Ganoderma lucidum* Gl-I (Figure 1). In the presence of 1 mg/ml mushroom extract, the average number was reduced to 54.25 from 140.75, in comparison with the control, which didn't contain mushroom extract. The number of cells was reduced by 61.46% under the influence of 1 mg/ml mushroom extract. With the addition of 100 ng/ml mushroom extract, the average number of cells was reduced to 65.25, which represents a reduction of 53.64%. In the presence of 10 ng/ml of mushroom extract, the average number of cells was 76.25 and it was reduced by 45.82%. Addition of 1 ng/ml mushroom extract induced a reduction of cells number by 38.36%, and average number of cells was 86.75. The smallest change in the cells number appeared when 100 ng of mushroom extract were added; in that case average number of cells was reduced by 36.23% and it was 89.75 (Table 1).

On the basis of standard deviation and t-test, p-values were found and it was established that statistically significant difference existed between all treat-

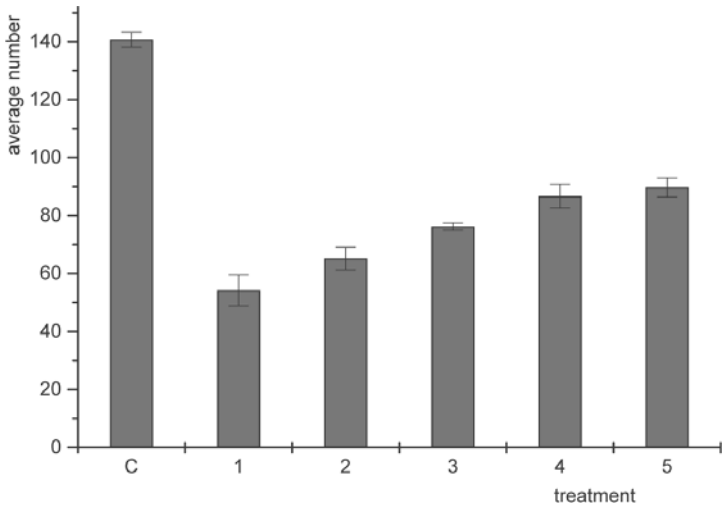

Fig. 1 — Changes of JY cells number in the presence of different concentration of *Ganoderma lucidum* Gl-I extract

ments, except the treatments 1—2. It means that differences in concentrations of added extract were important in all cases, except in the treatments 1—2. Compared to the control the biggest difference in the cells number was obtained in the treatment 1, when 1 mg/ml of mushroom extract was added to the suspension (Figure 1).

Tab. 2 — Influence of different concentration of *Ganoderma lucidum* Gl-I extract on Jurkat cells number and standard deviation

treatmant	extract concentration	number of JY cells (4 repeats)				average number	standard deviation
		1	2	3	4		
C	—	110	107	105	112	108.5	3.109
1	1 μg	44	47	49	46	46.5	2.08
2	100 ng	82	93	89	87	87.75	4.57
3	10 ng	88	87	88	90	88.25	1.25
4	1 ng	85	93	82	87	86.75	4.64
5	100 pg	94	98	85	93	92.5	5.44

In the presence of bioactive compounds extracted from karpophore *Ganoderma lucidum* Gl-I, average number of Jurkat cells was changed (Figure 2). The most important change appeared with addition of 1 mg/ml of mushroom extract when average number of cells was reduced to 46.5 from 108.5, in comparison with the control which didn't contain mushroom extract. The number of cells was reduced by 57.14% under the influence of 1 mg/ml mushroom extract. With addition of 100 ng/ml of mushroom extract average cells number was reduced by 19.12% and it was 87.75. Similar result was obtained with the addition of 10 ng/ml of mushroom extract when average cells number was reduced by 18.66% and it was 88.25. In the presence of 1 ng/ml of mushroom extract average number of cells was 86.75 and it was reduced by 20.05%. Ad-

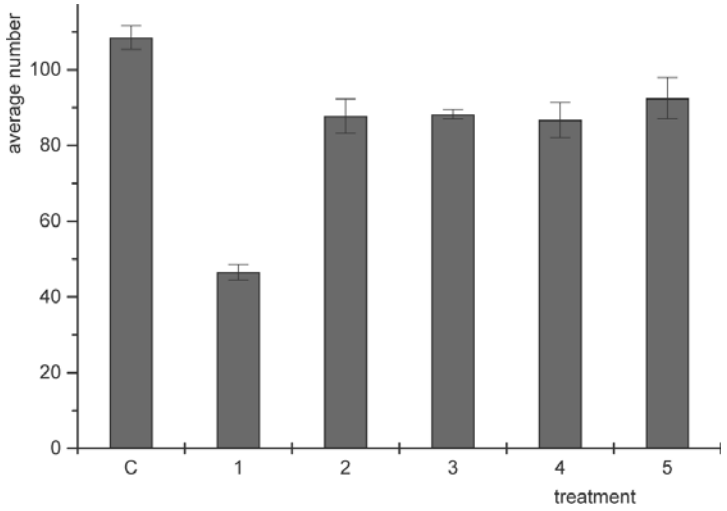

Fig. 2 — Changes of Jurkat cells number in the presence of different concentration of *Ganoderma lucidum* Gl-I extract

dition of 100 pg/ml of mushroom extract induced a reduction of cells number by 14.75% and average number of cells was 92.5 (Table 2).

On the basis of standard deviation and t-test, p-values were found and it was established that statistically significant difference existed between all treatments except treatments 2—3, 2—4, 2—5, 3—4 and 3—5. It means that differences in concentrations of added extract were important in all cases, except in treatments 2—3, 2—4, 2—5, 3—4 and 3—5. When compared to the control the biggest difference in the cells number obtained in the treatment 1 was when 1 mg/ml of mushroom extract was added to the suspension (Figure 2).

CONCLUSION

Ganoderma lucidum Gl-I is one strain of this species which could be found in the woods, but it is also suitable for artificial growing under controlled conditions. This kind of mushroom belongs to the very important medicinal mushroom thanks to its bioactive substances content, which has been confirmed for over thousands of years now; it is not edible because of its bitter taste and wooden build. This investigation showed that bioactive substances, polysaccharides, β-glucans, obtained through the processes of hot water extraction, alcohol precipitation and dialyses refining had influence on the reduction of B cells (a human B cell line transformed by Epstein-Barr virus) and T cells (an immortalized line of human T lymphocyte cells). Among all tested concentrations of mushroom extract, the most intensive influence showed concentration of 1 mg/ml, which reduced the number of B cells by 61.46%, while in the case of T cells their number were reduced by 57.14%.

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УТИЦАЈ БИОАКТИВНИХ КОМПОНЕНАТА ЕКСТРАКОВАНИХ ИЗ ГЉИВЕ *GANODERMA LUCIDUM* НА Б И Т ЋЕЛИЈЕ

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Резиме

Ganoderma lucidum (Leyss.: Fr.) Karst је једна од најчешће коришћених гљива у традиционалној медицини народа Далеког истока. Горког је укуса и дрвенасте грађе, па није погодна за исхрану, али биоактивне компоненте екстраковане из ове гљиве показују врло важне медицинске карактеристике. Циљ овог рада био је испитивање дејства различитих концентрација издвојеног екстракта гљиве *Ganoderma lucidum* GL-I на раст JY (Б) и Jurkat (Т) ћелија. Издвојени екстракт је додат ћелијама у концентрацијама 1 mg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml и 100 pg/ml. JY и Jurkat ћелије су инкубиране 72 h на 37°C у атмосфери са 5% CO_2 у присуству биоактивних компонената, полисахарида, б-глукана, а затим је утврђен њихов број. Од свих примењених концентрација екстракта највећи утицај је показала концентрација 1 mg/ml, која је у случају дејства на Б ћелије довела до смањења њиховог броја за 61.46%, а у случају дејства на Т ћелије број је смањен за 57.14%.

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INFLUENCE OF STRUCTURAL FEATURES ON IMMUNOSTIMULATING ACTIVITY OF GLUCANS EXTRACTED FROM *AGARICUS BLAZEI* MUSHROOM

ABSTRACT: High molecular weight β-D-glucans derived from *Basidiomycetes* cell walls are able to specifically activate cellular and humoral components of the host immune system. The aim of this paper was to examine immunomodulating activity of native, chemically and enzimatically modified glucans from *Agaricus blazei* mushroom and to determine which structural features are of primary importance for their stimulation referring to humane immune cells. The immunomodulating activities were tested *in vitro*, by stimulation of peripheral blood mononuclear cells (PBMCs) and measuring of interferon-gamma (IFN-γ) production by enzyme linked immunosorbent assay (ELISA). Measurements of immunomodulatory capacity of *Agaricus blazei* native glucans showed their expressive immunostimulating effect on activated PBMCs and synthesis of IFN-g. The results obtained after the stimulation of cells with 1M H_2SO_4 and 1M NaOH, the treated glucans showed that primary structure is of more importance than the tertiary structure of the triple helix for their immunostimulating activity and synthesis of IFN-γ. Glucans of lower molecular weight obtained after acid hydrolysis appeared as effective immunostimulators of PBMC's. The results obtained after the incubation of cells with 1,6 β-glucanase modified glucans suggest that β-(1,6) binding of glucose monomers probably has no importance for the production of imunostimulating effects, *in vitro*. This confirmed that β-(1,3) bonds are the primary determinants of immunomodulatory activities and stimulation of IFN-γ synthesis.

KEY WORDS: *Agaricus blazei*, β-D-glucans, IFN-γ, immunostimulating activity, PBMC's

INTRODUCTION

β-D-glucans are important secondary metabolites isolated from microorganisms, mushrooms and plants. They exhibit prophylactic and therapeutic properties and can function as biological response modifiers when administered to mammals. They have the ability to enhance or suppress both innate and

acquired immune response. The major immunopotentiation effects of these active substances include mitogenicity and stimulation of hematopoietic stem cells, such as T_H and T_C cells, B cells, macrophages, DC_s, and NK cells (Lull et al., 2005.).

The term "β-glucan" refers to the polymers which are generally composed of a linear backbone containing D-glucopyranosyl repeat units which are linked together by β-(1,3) and β-(1,4)-linkages. Some, but not all, exhibit β-(1,6)-side chains on the backbone. Glucans can assume a number of solution conformations depending upon the solvent system. For water soluble glucans, the two predominant conformations are single helix or triple stranded right--winding helix. In the fungal cell wall, most glucans comprise a three-dimensional network of β-(1,3-1,6)-glucans that are connected to the other carbohydrates, proteins and lipids (Young and Castranova, 2005).

The mechanism of the imunomodulating effect of glucans is not yet fully understood and probably depends on chemical characterisics, such as molecular weight, branching patterns, solubility in water and conformational features like the formation of helix (Freimund et al., 2003).

In this study we tried to determine which structural features of water soluble glucans are of primary importance for their *in vitro* immunostimulatory properties. Two types of glucan structure modification were applied, a chemical with 1M NaOH and 1M H_2SO_4 and enzymatic with 1,6 β-glucanase. NaOH changes the conformation of glucans from triple helix to single strand (Maeda et al., 1988). Glucans of lower molecular weight were obtained after acid hydrolysis with 1M H_2SO_4 (Dia et al., 2003) and modification with 1,6 β-glucanase.

MATERIAL AND METHODS

Glucans were extracted by hot water and alcohol precipitation from powder of fruit body of *Agaricus blazei* mushroom. Purification of extract was done by dialysis. The immunomodulating activity of native and modified glucans was tested *in vitro*, by PBMCs and measurement of IFN-g production was done by ELISA. Changes of molecular weight, after incubation with 1M NaOH, 1M H_2SO_4 and 1,6 β-glucanase, were observed by exclusion chromatography using Sephacryl S 200 (Kozarski, 2006.).

Extraction of water soluble glucan fraction

Up to 10% of dried powdered tissue was suspended in water. Glucans extraction was done by autoclaving 2 x at 121°C for 20 minutes. The extract was cooled down and centrifuged at 12325 x g for 20 minutes. Supernatants were collected and boiled to 10% of starting volume. Two volumes of 96% ethanol were added and left at 4°C overnight. Supernatant was decanted, washed 1 x with 70% ethanol and centrifuged at 12325 x g. Pellet was dried at 42°C. Purification was done by dialysis, against 2 l of destilled water, obtained by Millipore purification system (MilliQ) for 24 hours at room temperature.

Chemical modification

50 mg/ml of glucans were incubated in 1M NaOH and 1M H_2SO_4 at 37°C for 16 hours. Neutralization was done with 10M H_2SO_4 resp. 10M NaOH to pH 6.8—7.2. Glucans in 1M phosphat buffer saline (PBS) were used as control, under the same conditions.

Enzymatic modification

25 mg/ml of glucans in 5mM sodium acetate (NaAc) buffer at pH 5.4 were incubated with 6 mg/ml of 1,6 β-glucanase, Onuzuka R-10 (Yakult Honsha Co Ltd., Japan) for 1 hour at 55°C. Reaction was stopped by heating at 90°C for 30 minutes followed by precipitation of glucans by addition of 2 volumes of 96% ethanol.

Column chromatography

Size exclusion chromatography was done on a 1.5 x 90 cm column of Sephacryl S 200. 25 mg/ml of each glucan sample in MilliQ applied on column. Eluation was done using fast performance liquid chromatography (FPLC) system (Pharmacia) with degassed MilliQ water at flow rate of 0.5 ml/min. The eluents were collected by a fraction collector (Pharmacia), each 5 ml in a tube. The void volume was determined to be 60 ml in each fraction, and glucan content was semiquantified by the phenol-sulfuric acid method with glucose as a reference (Dubois et al., 1956). Protein content was determined using the Bradford method with bovine serum albumun (BSA) as a standard (Bradford et al., 1976) Glucan and protein contents were measured in 5 times concentrated fractions.

Cells isolation

Human PBMCs were prepared from buffy coats, obtained from various healthy donors. Buffy coats were diluted 1 x with PBS and centrifuged for 15 minutes at 2500 x g over a layer of Histopaque 1077 (Sigma). PBMCs were carefully collected at the interphase and washed with PBS. Cells were counted and resuspended at 5—10 x 10^6/ml in RPMI-1640 containing 10% fetal calf serum (FCS), 1% penicilin and 1% streptomycin.

Immunomodulatig activity

Glucan solutions were heated before application at 95°C for 20 minutes. Immunomodulating activity of the various glucans was tested by exposing stimulated PBMCs. As transcription activators, 1 ng/ml phorbol 12-myristate

13-acetate (PMA) and 0,5 µl/ml Ca-ionophore were added. After incubation for 48 hours at 37°C in 5% CO_2 atmosphere, medium was tested for IFN-g concentration by sandwich ELISA.

ELISA was performed in Nunc Maxisorp high affinity 96 well plates. Wells were coated overnight at 4°C wih 50 µl of mouse anti-human IFN-γ in PBS, pH 7. Blocking of non specific binding sites was carried out overnight at 4°C with 0,1% BSA and 1% skimmed milk protein in PBS. Plate was incubated for 2 hours at room temperature with samples diluted in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris buffered saline, pH 7.2—7.4, 0.2 µm filtered) and duplicates of standard, human IFN-g (BioSource) reconstituted with 50% glycerol, that was serially diluted in reagent diluent. The concentration of high standard was 1000 pg/ml. The 100 µl biotinylated goat anti-human IFN-g in reagent diluent was added to each well, followed by 1 hour incubation at room temperature. Streptavidin-HRP (BioSource) in 100 µl of reagent diluent was added to the wells and incubated for 20 minutes at room temperature. After each step, the plate was washed with wash buffer (0.05% Tween 20 in PBS, pH 7.2—7.4). Then 100 µl of substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was added to each well and incubated at room temperature. After 20 minutes, 50 µl of stop solution (1M H_2SO_4) was added. IFN-g production was measured at 415 nm using Benchmark microplate spectrophotometer (Bio-Rad).

RESULTS AND DISCUSSION

Size exclusion chromatography of *A. blazei* native glucans on Sephacryl S 200 showed the presence of one highest peak eluting together with the void volume of the column, indicating high molecular weight, over 80 kDa, and a few small peaks containing molecules of much lower size (Figure 1). In each fraction, the presence of protein was confirmed. This suggested that glucans can bound to protein or peptide residues and form proteoglucans. Running the glucanase digested glucans on the column showed a high fractionation of the glucan extract (Figure 1). This confirmed that glucans of *A. blazei* fruiting bodies predominantly had a β-(1,6)-backbone structure with β-(1,3)-side branches (K o z a r s k i, 2006).

Measurements of immunomodulatory capacity of *Agaricus blazei* native glucans showed that *A. blazei* glucans express immunostimulating effect on activated PBMCs and synthesis of IFN-g (Figure 2). Titers of IFN-g measured after stimulation of cells with acid-hydrolyzed fractions confirmed that glucans of lower molecular weight are as effective as non-hydrolyzed glucans. ELISA measurements of IFN-g titer obtained after the stimulation of PBMCs with 1M NaOH treated glucan showed that the immunostimulating activity was not changed (Table 1, Figure 2). This indicated that the primary structure of glucans is of more importance than the tertiary structure of the triple helix for their immunostimulating activity and synthesis of IFN-γ.

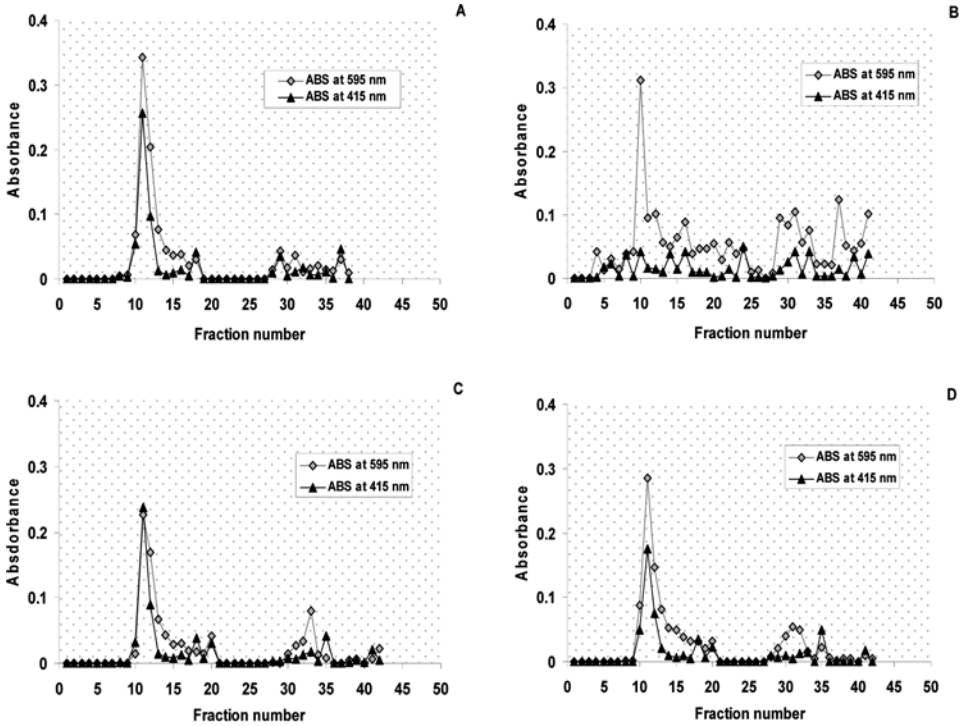

Fig. 1 — Separation of native and modified *A. blazei* (graphs **A**, **B**, **C, D**) fruiting body glucans on Sephacryl S 200. Glucans in PBS, **A**; with 1,6 b-glucanase modified glucans **B**; with 1M H_2SO_4 treated, **C**; and with 1M NaOH, **D**.

Tab. 1 — ELISA measurements of IFN-g titer obtained after the stimulation of PBMCs with *A. blazei* native, in 1 M PBS glucans (**Ab**) and 1M NaOH (**AbB**) and 1M H_2SO_4 treated glucans (**AbA**). Cell suspension in RPMI, with transcription activators, was used as control.

sample	*IFN-γ titer (pg/mL)*
Control	**56.09 ± 4.11**
Ab	**219.26 ± 29.45**
AbB	**215.95 ± 23.72**
AbA	**198.31 ± 25.09**

The resulting β-(1,3)-glucan fragments of high molecular weight (MW > 80 kDa) and small β-(1,3)-glucan fragments (MW < 80 kDa), left after glucanase degradation, showed a strong enhancement of immunostimulatory activity compared to the native glucans (Figure 3).

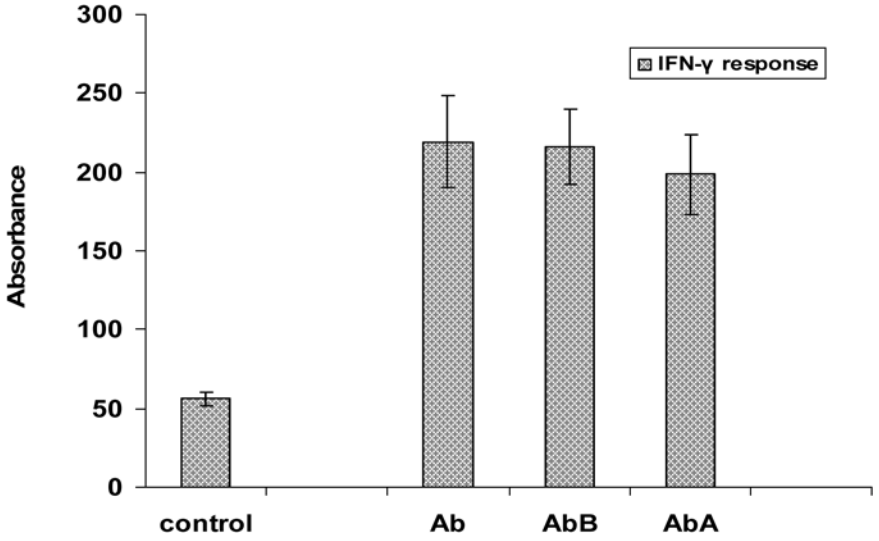

Fig. 2 — IFN-g response of stimulated PBMC's incubated with native, in 1M PBS, *A. blazei* glucans (**Ab**) and glucans which have been exposed to 1M NaOH (**AbB**) and 1M H_2SO_4 (**AbA**).

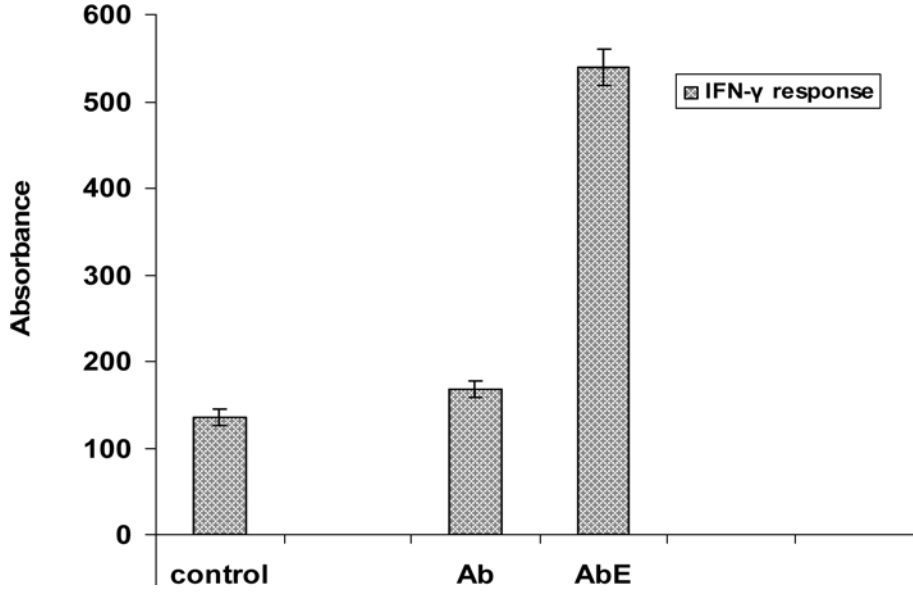

Fig. 3 — IFN-g response, after 48 hours, of stimulated PBMC's incubated with native, in 1M PBS, *A. blazei* glucans (**Ab**) and with 1,6 b-glucanase digested glucans (**AbE**).

Tab. 2 — ELISA measurements of IFN-g titer obtained after the stimulation of PBMCs with *A. blazei* native, in 1 M PBS glucans (**Ab**) and with 1,6 b-glucanase treated glucans (**AbE**). Cell suspension in RPMI, with transcription activators, was used as control.

sample	*IFN-γ titer (pg/mL)*
Control	**135.23 ± 9.30**
Ab	**219.26 ± 29.45**
AbE	**198.31 ± 25.09**

The obtained results suggest that β-(1,6) binding of glucose monomers probably has no importance for the production of imunostimulating effects, *in vitro*.

CONCLUSION

Measurements of immunomodulatory capacity of *Agaricus blazei* native glucans showed that *A. blazei* glucans express immunostimulating effect on the activated PBMCs and synthesis of IFN-g. The results obtained after the stimulation of cells with chemical and enzimatically modified glucans showed that primary structure is of more importance than the tertiary structure of the triple helix for their immunostimulating activity and synthesis of IFN-g. The obtained results confirmed that β-(1,6) binding of glucose monomers probably has no importance for the production of imunostimulating effects, *in vitro*. This suggests that β-(1,3) bonds are the primary determinants of immunomodulatory activities and stimulation of IFN-γ synthesis. The results confirmed that glucans of lower molecular weight are effective for stimulation of PBMCs and production of IFN-γ.

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УТИЦАЈ СТРУКТУРНИХ КАРАКТЕРИСТИКА НА ИМУНОСТИМУЛАТИВНУ АКТИВНОСТ ГЛУКАНА ЕКСТРАХОВАНИХ ИЗ ГЉИВЕ *AGARICUS BLAZEI*

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Резиме

β-D-глукани великих молекулских маса изоловани из ћелијског зида гљива из класе *Basidiomycetes* имају способност да специфично активирају целуларне и хуморалне компоненте имуног система домаћина. Имуномодулаторска активност мико-D-глукана у функцији је њихових хемијских карактеристика, као што су молекулска маса, степен гранања, растворљивост у води и терцијарна структура. Циљ овог рада је био испитивање имуномодулаторске активности нативних, хемијски и ензимски модификованих глукана гљиве *Agaricus blazei* и да се утврди која је структурна карактеристика од примарног значаја за стимулацију ћелија хуманог имуног система. Имуномодулаторска активност је тестирана *in vitro*, стимулацијом мононуклеарних ћелија крви из периферног крвотока (PBMC) молекулима глукана и мерењем количине синтетисаног интерферона-гама (IFN-γ) од стране стимулисаних ћелија ензимоимунотестом (ELISA). Мерењем имуномодулаторског капацитета *Agaricus blazei* нативних глукана показано је да ови молекули имају изражено имуностимулативно дејство на активиране PBMC ћелије и стимулацију синтезе IFN-γ. Стимулацијом ћелија глуканима који су претходно били парцијално хидролизовани 1M H_2SO_4 и 1M NaOH измерени титар IFN-γ се није значајно променио у односу на нативне молекуле. Глукани мањих молекулских маса, настали након киселе хидролизе, показали су се као ефикасни стимулатори PBMC ћелија. Мерењем титра IFN-γ насталог након инкубације активираних ћелија са 1,6 β-глуканазама модификованим глуканима потврђено је да су фрагменти β-(1,3)-глукана великих молекулских маса (ММ > 80 kDa) и мали фрагменти β-(1,3)-глукана (ММ <80 kDa), настали након ензимске модификације, испољили значајно повећање имуностимулативне активности у односу на нативне молекуле. Добијени резултати су указали да β-(1,6)-гликозидне везе немају значаја у испољавању имуностимулативног ефекта, *in vitro*.

Овим је потврђено да је за имуностимулативну активност и стимулацију синтезе IFN-γ од примарног значаја присуство β-(1,3)-гликозидних веза. Закључено је да је за имуномодулаторску активност ових молекула битна примарна структура, а не конформација троструког хеликса нативних молекула, као и да су молекули глукана мањих молекулских маса ефикасни стимулатори синтезе IFN-γ.

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MACROFUNGI OF THE ZASAVICA SPECIAL NATURE RESERVE

ABSTRACT: In the frame of biodiversity investigation of the Republic of Serbia, the investigation of the presence and the diversity of macrofungi of the Zasavica Special Nature Reserve (North Serbia) has been undertaken. Relatively poor generic diversity of macrofungi was recorded with domination of ecological group of wood-decaying species.

Even though being preliminary, our results point to the necessity of conservation and protection of recent fungal diversity but, in our opinion, not by making a so-called "Red list of endangered species", which, due to the lack of information and very poor evidence on this group of organisms in our country, are extremely unreliable and therefore disputable, but rather through the very short list of a few not endangered species, conditionally called "White list of not endangered fungal species", if such species recently exist et all.

KEY WORDS: Macrofungi, Zasavica Special Nature Reserve, Serbia.

INTRODUCTION

The investigations of the generic composition of mushrooms, macroscopic fungi, in Special Nature Reserve Zasavica, has been done sporadically. The obtained results have not been systematized so far, however, these are two completed publications representing mushrooms of Zasavica: a report published in the Monograph on Zasavica (2001) and a chapter in the book "Guide through the nature of SNR Zasavica", Chapter "Fungi". Only 30 dominant species of macrofungi have been described in the mentioned publications. During the course of the research camps in 2006 around 100 new species of mushrooms have been recorded in the Zasavica Special Nature Reserve Region. It should be emphasized that this paper represents only a part of permanent monitoring of mushrooms composition, taking place in SNR Zasavica. 150 species of macrofungi have been determined so far in the reserve.

Tab. 1 — Generic composition of the lignicolous macrofungi of the Zasavica River bank zone

Species	Familia	Substrate	Locality
***Fomes fomentarius* (L.: Fr.) Fr.**	*Polyporaceae*	on old trunk of *Populus nigra*; on living *Salix alba* tree; on dry fallen trunk of *Acer campestre*	Batar, Yovacha, Prekopac, Palyevine, Prekoyovacha, Treblyevine
***Polyporus squamosus* (Hudson): Fr.**	*Polyporaceae*	on *Salix alba* stumps and fallen trunks; on fallen branch of *Acer campestre*	Batar, Skelice, Vrbovac, Prekopac
***Meripilus giganteus* (Pers.: Fr.) Karst.**	*Polyporaceae*	on dead *Salix alba* trunk	Batar, Vrbovac
***Trametes versicolor* (L.: Fr.) Pilat**	*Polyporaceae*	on *Salix alba* stumps	Banovo Polye, Vrbovac
***Trametes hirsuta* (Wulfen: Fr.) Pilat**	*Polyporaceae*	on *Salix alba* stumps	Shumareva cyupriya
***Lenzites betulina* (L.: Fr.) Fr.**	*Polyporaceae*	on *Salix alba* stump	Ravanyska cyupriya
***Laetiporus sulphureus* (Bull.: Fr.) Murrill**	*Polyporaceae*	on living tree of *Salix alba*	Vrbovac, Treblyevine, Shiroka bara, Bara Ribnyacha
***Daedaleopsis confragosa* (Bol.: Fr.) J. Schroeter**	*Polyporaceae*	on strait standing dead *Salix alba* trunk	Vrbovac, Treblyevine, Banovo polye
***Pleurotus ostreatus* (Jack.: Fr.) Kummer**	*Tricholomataceae*	on living *Salix alba* trunk	Vrbovac, Batar
***Panus tigrinus* (Bull.: Fr.) Sing.**	*Tricholomataceae*	on fallen *Salix alba* trunks and in the basis of willow stumps	Kanal Bogar, Valyevac, Vrbovac
***Flammulina velutipes* (Curt: Fr.)**	*Tricholomataceae*	in the basis of *Salix alba* living tree	Batar, Polyansko, Treblyevine, Vrbovac
***Panellus stipticus* (Bull.: Fr.) Karsten**	*Tricholomataceae*	on old *Salix alba* stump	Valyevac, Vrbovac
***Ganoderma applanatum* (Pers.) Pat.**	*Ganodermataceae*	on fallen trunk of *Salix alba*	Vrbovac, Batar
***Ganoderma lucidum* (Curtis: Fr.) Karsten**	*Ganodermataceae*	in the basis of old stump and on fallen trunk of *Salix alba*	Batar, Treblyevine, Vrbovac, Prekopac, Yovacha, Prekoyovacha, Palyevine, Skelice

Stereum hirsutum **(Wild.: FR) S. F. Gray**	*Stereaceae*	on the stump of *Morus alba*	Ravnye, Prekopac
Stereum rugosum **(Pets.: Fr.) Fr.**	*Stereaceae*	on the stump of *Robinia pseudoacacia*	Banovo polye, Vrbovac
Coprinus micaceus **(Bull.: Fr.) Fr.**	*Coprinaceae*	on *Salix alba* stumps and fallen branches	Vrbovac, Batar
Coprinus disseminatus **(Pers.: Fr.) Fr.**	*Coprinaceae*	in the basis of stump of *Populus nigra*	Vrbovac, Skelice
Nectria cinnabarina **(Tode: Fr.) Fr.**	*Hypocreaceae*	on dry fallen branch of *Acer campestre*	Ravnye, Kanal Bogar, Vrbovac, Valyevac
Hirneola auricula-judae **(Bull.: St.-Am) Berk. (*Auricularia auricula-judae* (Bull.: St.-Am))**	*Auriculariaceae*	on fallen branch of *Robinia pseudoacacia*; on fallen branch of *Acer campestre*	Vrbovac, Batar, Prekopac, Yovacha
Schizophyllum commune **Fr.: Fr.**	*Schizophyllaceae*	on fallen *Salix alba* trunk; on dry fallen branch of *Acer campestre*	Batar, Vrbovac, Prekopac, Skelice, Preseka, Treblyevine, Yovacha, Pollyansko, Palyevine
Pholiota cerifera **(Karst.) Karst.**	*Strophariaceae*	on living *Populus nigra* tree	Banovo polye, Shumareva cyupriya
Phellinus igniarius **(L.: Fr.) Quel.**	*Hymenochaetaceae*	on living *Salix alba* trees	Vrbovac, Yovacha
Tremella mesenterica **Retz.: Hooker**	*Tremellaceae*	on fallen branch of *Robinia pseudoacacia*	Vrbovac, Batar
Peziza aurantia **(Pers.) (*Aleuria aurantia* (Pers.) Fuck.)**	*Aleuriaceae (Sarcoscyphaceae)*	on fallen *Salix alba* trunk; on fallen branch of *Robinia pseudoacacia*; on dry fallen branch of *Acer campestre*	Vrbovac, Preseka, Treblyevine, Batar, Prekoyovacha Shumareva cyupriya

Data considering the qualitative and quantitative composition of these important reducers of organic matter are lacking, as well as data about their significant role in the entire chain of the nutrition in natural environments. The fungi in general and especially lignicolous macrofungi, become recently a group of organisms of great biotechnological interest as potential producers of various biologically active agents (Matavuly, 1993, 1996a).

In our country the investigations of biodiversity (Stevanovicy et al., 1995) are in progress. The aim of this research was collecting necessary informations regarding this field in order to form a basis for the conservation and improvement of existing conditions in natural environments. Fungi are pointed as especially endangered group of organisms in Europe (Arnold and de Vries, 1993); Ing, 1993; Ivančević, 1995). Since data on macrofungi originating in the Zasavica speciae nature reserve are scarce (Matavuly et al., 2001), and the region has been classifical as protected area, it was found worthy and interesting to start with the evidence of fungal species as the beginning of more systematic and more detailed investigations of the presence, biology, ecology and conservation of these endangered organisms as a whole.

MATERIAL AND METHODS

Sporadic mycological investigations of the Zasavica region were done during the nine-year period, from 1997 to 2006. Seventeen localities within the investigated sector were chosen for the collection of samples of lignicolous macrofungi (Batar, Treblyevine, Vrbovac, Prekopac, Yovacha, Prekoyovacha, Palyevine, Skelice, Shiroka bara, Bara Ribnyacha, Sajak, Polyansko, Preseka), and other six sites were chosen for sampling macrofungi during 2006 (Valyevac Livade, Valyevac Shume, Lug, Pachya Bara; Turske Livade, Shiroka Bara).

The samples have been collected, prepared, and photo-documented by members of NGO "Pokret Gorana Sremska Mitrovica" preserved and stored in the Nature Reserve is natural collection. Fungi were identified on the basis of both morphological and anatomical properties of fruit bodies and according to specific chemical reactions using modern keys (Ainsworth et al., 1973; Bon, 1988; Božac, 1989; Cetto, 1979; Ellis and Ellis, 1991; Focht, 1979; Hermann, 1990; Moser, 1978; Phillips, 1983).

RESULTS AND DISCUSSION

Results of recording of fungal species in the Zasavica special nature reserve are displayed in Table 1 containing the list of noted macrofungi. Most of them can be found during the whole year, regardless of the season, except *Flammulina velutipes* which appears usually during the late winter and early spring seasons, *Coprinus micaceus* and *Coprinus disseminatus*, which do not grow only during the winter, and *Pholliota cerifera*, which belong to the group appearing during summer and autumn seasons.

Tab. 2 — Preliminary list of fungal species recorded on other six localities belonging to the wider zone of the Zasavica Special Nature Reserve

Sampling site	Valyevac Livade	Lug	Valyevac Shume	Pachiya Bara	Turske Livade	Shiroka Bara
Species	***Auricularia mesenterica***	***Ganoderma applanatum***	***Auricularia auricula judae***	***Corilopsis trogi***	***Agaricus silvaticus***	***Coprinus callinus***
	Coprinus auricomus	***Ganoderma australe***	***Colticia perennis***	***Daedaleopsis confragosa***	***Clitocyba vibecina***	***Coprinus domesticus***
	Coprinus flocculosus	***Marasmiellus ramealis***	***Datronia mollis***	***Exidia recisa***	***Coprinus sp.***	***Derocybe sp.***
	Lenzites betulinus	***Panaelous sphinctrinus***	***Ganoderma lucidum***	***Enteloma incanatum***	***Marasmius androsaceus***	***Entoloma incarnatum***
	Fomes fomentarius	***Phellinus trivialis***	***Hygrocibe unginosa***	***Hapalopilus rutilans***	***Marasmius bulliardi***	***Enteloma sp.***
	Macrolepiota procera	***Polyporus squamosus***	***Lentinus tigrinus***	***Heterobasidium anosum***	***Marasmiellus vaillanti***	***Hebeloma mesophaceum***
	Peroneutypa heteracantha	***Trametes versicolor***	***Mycena galopus***	***Hemimycena candida***	***Mycena pura var. lutea***	***Panaelopulus sp.***
	Agaricus silvicola	***Trametes hirsuta***	***Shizophyllum commune***	***Inotus hispidus***	***Oligoporus stipticus***	***Psathyrella condelleana***
	Ganoderma lucidum	***Xylaria longipes***	*Fomes fomentarius*	***Marasmius rotula***	***Pluteus cinereofuskus***	***Psathyrella conopileus***
	Marasmius androsaceus	*Auricularia mesenterica*	*Ganoderma australe*	***Monilina jansoni***	***Pluteus cervinus***	***Pseudocraterellus untulatus***
	Marasmius bulliardi	*Shizophyllum commune*	*Polyporus squamosus*	***Mycena pura v. pura***	***Scleroderma citrinum***	***Sarcoscypha austriaca***
	Marasmiellus vaillanti			***Mycena galopus***	***Polyporus adustus***	*Auricularia auricula judae*
	Shizophyllum commune			***Mycena polygrama***	*Auricularia mesenterica*	*Marasmiellus vaillanti*
				Peniophora rufomarginata	*Ganoderma lucidum*	*Panus tigrinus*
				Phellinus igniarius	*Macrolepiota procera*	*Shizophyllum commune*
				Psathyrella obtusata	*Xylaria longipes*	
				Poculum firmum	*Auricularia mesenterica*	
				Lentinus tigrinus	*Shizophyllum commune*	
				Peroneutypa heteracantha		

Out of 25 recorded lignicolous macrofungi, 8 species belonged to the *Polyporaceae* followed by 4 members of fam. *Tricholomatacea*; two of them belonged to families *Ganodermataceae*, *Stereaceae*, and *Coprinaceae*, and by one species to families: *Hypocreaceae*, *Auriculariaceae*, *Schizophyllaceae*, *Strophariaceae*, *Hymenochaetaceae, Aleuriaceae and Tremellaceae.*

Recording of fungal species in the wider zone of the Zasavica Special Nature Reserve during the 2006 (Table 2), another 60 species of macromycetes have been noted, most of them for the first time in the Zasavica region. Howewer, most of them belonged to the ecological group of wod-decaying fungi, which confirmed our earlier findings (M a t a v u l y et al., 2001). Lignicolous species were numerous in Lug and Valyevac Shume localities as expected since these two localities have been the most densely forested with the richest diversity of tree and shruby plant species.

Since fungi are one of the most important group of organisms playing irreplaceable role in the organic matter reduction and mineralization in the natural environments, it is necessary to undertake measures for conservation of existing fungal genofond. For the conservation and improvement of the existing fungal genofond it is necessary to conserve and if it is possible to reconstruct and improve the autochthonous, even rudimentary plant associations, in order to provide substrates and ecological niches for fungal appearance.

We also find the promotion of so-called "red lists of endangered fungal species" (I v a n c h e v i c y, 1995, 1996) in similar natural environments, drastically devastated by anthropogenic monoculture introduction, not justified, due to very limited knowledge regarding this group of organisms and due to the distinct lack of informations about both former and recent presence or absence of fungal species at this territory and for one longer period of time. Being a group of organisms most sensitive to the anthropogenic natural changes (pollution, decrease of ecological niches diversity, excessive exploitation, the eradication by fungicides, etc.), fungi are the most endangered organisms. From this reason, in our opinion, at this stage of our, more convenient and more justified would be to establish a (very) short "white list of not endangered" fungal species which would, we are convinced, much more adequately serve for the protection and conservation of this extremely important link in the matter cycle and energy flow, first of all in terrestrial ecosystems (M a t a v u l y et al., 1998).

CONCLUSION

During the 1997—2006 period, the presence and diversity of macrofungi species in the special nature reserve Zasavica have been surveyed. Mostly lignicolous species were recorded. Relatively poor generic composition can be explained by drastic anthropogenic devastation of autochthonous plant associations, and by reducing the vegetation along the Zasavica bank to the relatively small number of plant species, consequently causing the reduction of the diversity of ecological niches for growth of fungi, as a rule highly specified for dead or living plant substrate or for the plant symbiont. Higher number of

fungal species was recorded within the wider region of the Zasavica Special Nature Reserve.

Since in natural environments fungi have an important role as one of the most important group of mineralizators of organic matter, which is an important link in the matter cycle and energy flow through the ecosystem, it is necessary to prevent further devastation of fungal species diversity and to undertake measures for conservation of existing fungal genofond and for its diversity improvement by protection of autochthonous plant associations what would cause the protection of ecological niches diversity and consequently to that preservation of fungal species diversity.

From mycological point of view, the Special nature reserve Zasavica is not *terra incognita* any more, but for more reliable data, further systematic and more detailed investigations of the whole Zasavica region should be undertaken.

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МАКРОГЉИВЕ СПЕЦИЈАЛНОГ РЕЗЕРВАТА ПРИРОДЕ ЗАСАВИЦА

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Резиме

У оквиру истраживања биодиверзитета Републике Србије започета су истраживања присуства и разноврсности макрогљива у Специјалном резервату природе Засавица (Северна Србија, делом Административна територија АП Војводине). Установљен је релативно сиромашан генерички састав макрогљива са доминацијом лигниколних врста.

Иако прелиминарни, наши резултати указују на неопходност конзервације и заштите рецентног диверзитета макрогљива али, по нашем мишљењу, не установљавајући такозване "Црвене листе угрожених врста", које су, захваљујући недостатку информација и врло сиромашној евиденцији ових организама у нашој земљи, врло непоуздане, а тиме и дискутабилне, него пре установљавајући врло кратке листе од по неколико неугрожених врста, условно званих "Беле листе неугрожених врста гљива", уколико такве врсте данас уопште постоје.

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ROLE OF FUNGI IN BIODETERIORATION PROCESS OF STONE IN HISTORIC BUILDINGS

ABSTRACT: Fungal ability in production of pigments and organic acids have crucial role in discoloration and degradation of different types of stone in cultural heritage objects. Additionally, stone objects may support novel communities of microorganisms that are active in biodeterioration process. This investigation focuses on mycological analyses of microbial biofilm from two important buildings, made of granite and sandstone, and which were heavily colonized by fungi. The 23 fungal taxa including filamentous microfungi and yeasts with specific distribution on sandstone and granite substrate were isolated. Melanized fungi from *Dematiaceae* (*Deuteromycotina*) were dominant. The identified microfungi cause discoloration, as well as mechanical exfoliation of building stone material that was analyzed through mechanical hyphae penetration and production of dark pigments and organic acids.

KEY WORDS: biodegradation, cultural heritage, fungi

INTRODUCTION

Numerous factors affect the stone durability. Stone surfaces are continuously exposed to physical, chemical and biological degradation. Physical, chemical, and biological agents act in co-association, ranging from synergistic to antagonistic, to the deterioration. Among biological agents miroorganisms have critical importance, in stone deterioration. They can cause various damages on the stone surface, such as: formation of biofilm, chemical reactions with substrate, physical penetration into the substrate as well as pigments production. Numerous studies have been dealing with establishing the role of biological agents in the stone deterioration (Pochon and Jaton, 1968; May et al., 1993; Bock and Sand, 1993).

During the recent decades there has been a general concern about the deterioration of historic buildings. Along with chemical and physical factors, microbial growth plays an important role in this process (Suihko et al., 2007). Microbial colonization of stones depends on environmental factors such as water availability, pH, climatic exposure, nutrient sources, and petrologic

parameters such as mineral composition, type of cement, as well as porosity and permeability of rock material (Warscheid and Braams, 2000). The stone ecosystem is subject to harsh environmental changes, especially temperature and moisture, exerting extreme selective pressure on any developing microbial community (May, 2003). Bioreceptivity of stone depends on its structure and chemical composition, while the intensity of microbial contamination is determined by the climatic conditions and anthropogenic eutrophication of the atmosphere (Prito and Silva, 2005). Biofilm formation on clean surfaces usually starts with phototrophic organisms (algae, cyanobacteria) which use CO_2 from the atmosphere as their carbon source and sunlight as their energy source. Heterotrophic organisms (most bacteria and all fungi) need some organic source for their growth, which is provided by metabolites of phototrophic organisms or by air-borne deposition. It has been shown that very low nutrient requirements of some rock inhabiting heterotrophic microorganisms may be fulfilled by remains of polluted air and rain or animal remains and secretion (Suihko et al., 2007).

The aim of this work was to study the microfungi community on biodeteriorated stone samples originating from two historical buildings by using cultivation and microscope observations in order to evaluate the potential damage caused by fungal species.

MATERIAL AND METHODS

Sampling

Samples of stone were collected from two localities: "Monument of the Unknown Hero" (Avala Mountain near Belgrade) and "Brankov most" (Sava river, Belgrade), and two types of stone: granite and sandstone. After a careful observation visible alterations and degradation were mapped and after that the samples were taken. Granite and sandstone samples were taken for mycological analyses by swabbing surfaces with sterile cotton swabs. The samples were then stored at 4°C.

Cultural media and inoculations

Swab samples were diluted in 10 ml sterile distillated water and shaken mechanically for 10 minutes. Malt-streptomycin-agar (MSA) medium (MA according to Boot (1971), with 500 mg streptomycin per liter) was inoculated with 1 ml of the resulting suspensions. Each sample was repeated in triplicate. The plates were incubated at 24°C in thermostat. Isolation of the formed colonies was done successively, using standard mycological medium (Malt extract agar, Potato-dextrose agar and Czapek's solution agar). All the cultures were grown 7 days in thermostat at 24°C. Macroscopic and microscopic characteristics of the obtained isolates were examined. Identification of fungi was based on the macroscopic features of colonies grown on agar plates, and the micro-

morphology of the reproductive structures was identified identification keys (Arx, 1974; Ellis and Ellis, 1997; Pitt, 1979; Rapper and Fennel, 1965).

RESULTS AND DISCUSION

23 fungal taxa were identified analyzed samples from all (Table 1). The fungal species from both granite and sandstone substrata were *Alternaria* sp. 1, *Alternaria* sp. 2, *Cladosporium cladosporioides, C. sphaerospermum, Epicoccum purpurascens*, *Fusarium* sp., *Mycelia sterilia* (melanised), *Mycelia sterilia* (non-melanised) and one yeast taxa. Species occurring on granite substrata only were *Alternaria* sp. 3, *Aspergillus flavus, Aspergillus nidulans, Cunninghamela echinulata*, *Drechlera dematoidea*, some species from *Moniliales* order and *Mucor* sp. (Figs. 1 and 2). On the other hand, sandstone surface biofilm includes some species not present on granite: *Aureobasidium pullulans* var. *melanigerum*, *Fusarium oxysporum*, *Mucor racemosus*, *Paecilomyces variotii*, *Penicillium* sp., *Penicillium verrucosum* var. *cyclopium* and *Phoma* sp. The mentioned fungal species are typically soil fungi, which is in accordance with the results of Šimonovičova et al. (2004) who noted considerable number of the same genus and species. The identified microfungi cause discoloration, as well as mechanical exfoliation of building stone material that was analyzed through mechanical hyphae penetration and production of different pigments (*Aureobasidium, Cladosporium, Alternaria*) and organic acids (some species of genus *Aspergillus*, *Alternaria, Penicillium*). *Alternaria* species were among dominant microfungi on the mineral substrate (Šimonovičova et al., 2004). *Aspergillus versicolor*, *Aureobasidium pullulans*, *Alternaria alternata* and *Penicillium chrysogenum* were the most common species isolated from themineral substrates.

Tab. 1 — Micromycetes isolated from both deteriorated stone material

Micromycetes	***Stone material***	
	Granite	Sandstone
Alternaria sp. 1	*	*
Alternaria sp. 2	*	*
Alternaria sp. 3	*	
Aspergillus flavus	*	
Aspergillus nidulans	*	
Aureobasidium pullulans var. *melanigerum*		*
Cladosporium cladosporioides	*	*
Cladosporium sphaerospermum	*	*
Cunninghamela echinulata	*	
Drechlera dematoidea	*	
Epicoccum purpurascens	*	*
Fusarium oxysporum		*
Fusarium sp.	*	*
Moniliales	*	
Mucor racemosus		*
Mucor sp.	*	

Mycelia sterilia (non-melanized)	*	*
Mycelia sterilia (melanised)	*	*
Paecilomyces variotii		*
Penicillium sp.		*
Penicillium verrucosum var. *cyclopium*		*
Phoma sp.		*
yeast	*	*
total	16 taxa	16 taxa

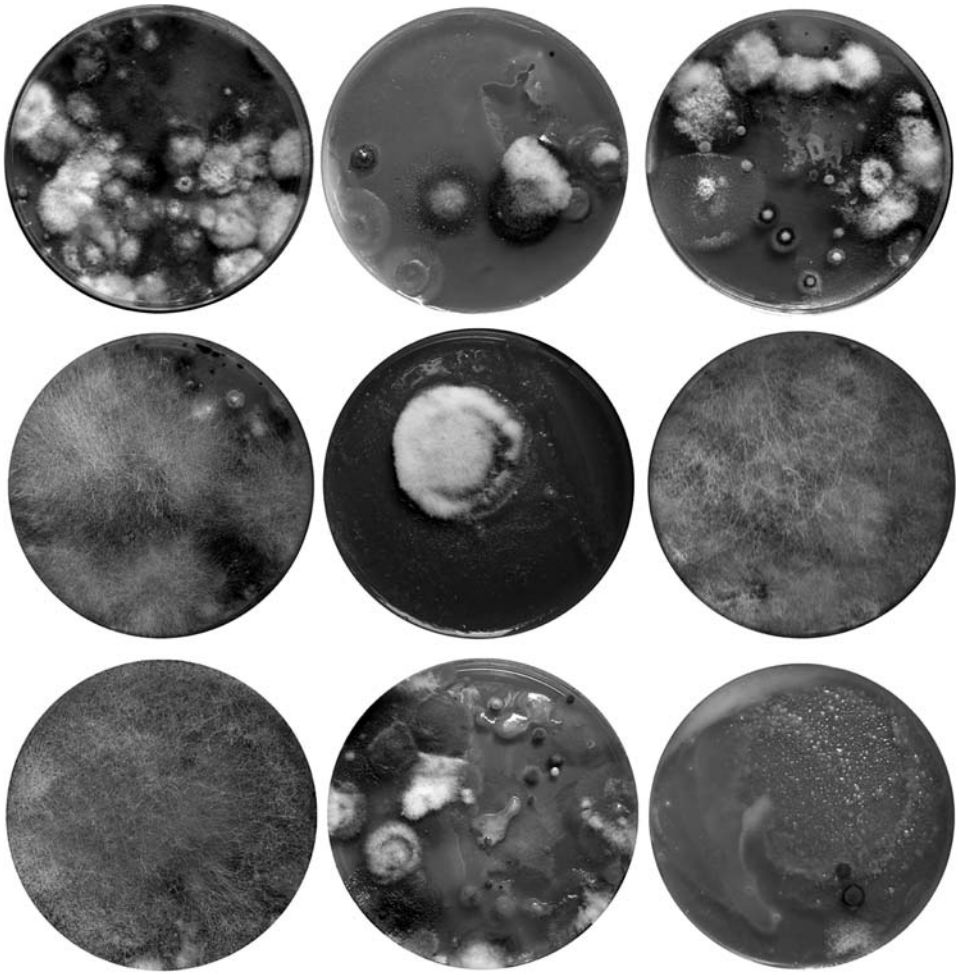

Fig. 1 — Mixed fungal cultures isolated from granite and sandstone swab samples

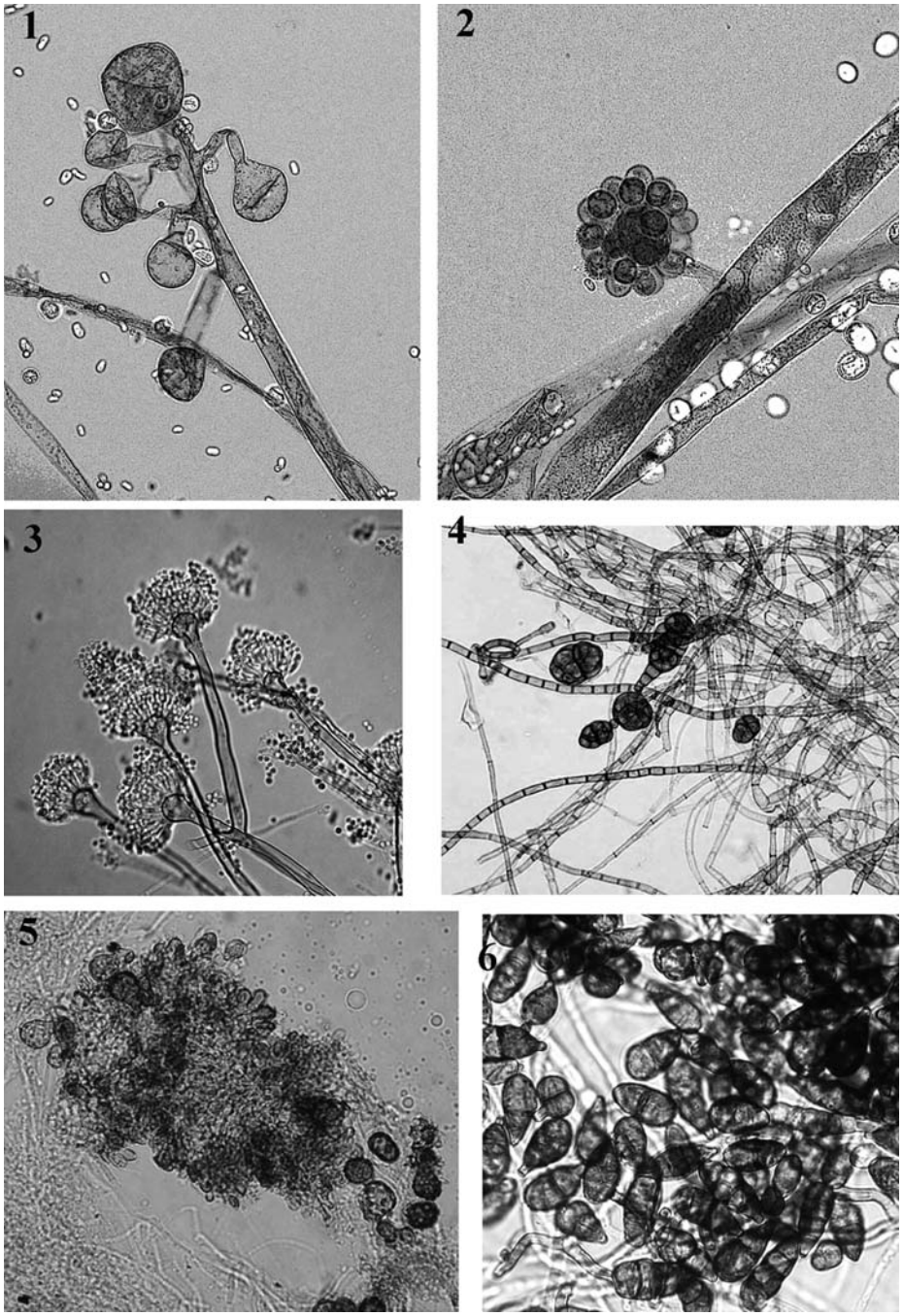

Fig. 2 — Micromycetes isolated: 1. and 2. *Cunninghamela echinulata* and 3. *Aspergillus nidulans* from granite stone; 4. *Alternaria* sp. 1, 5. *Epicoccum purpurascens* and 6. *Alternaria* sp. 2. from granite and sandstone

Biological infections and the intensity of biodeterioration processes are strongly influenced by water availability. This is determined by material-specific parameters, like porosity and permeability, environmental conditions of the site and exposure of the object (Warscheid and Braams, 2000). The establishment of fungi on rocks is possible even without the pioneering participation of phototrophic organisms. Fungi are especially concentrated in stone crusts. They are able to penetrate into the rock material by hyphal growth and biocorrosive activity, due to excretion of organic acids or by oxidation of mineral-forming cations, preferably iron and manganese. Their deterioration activities also include discoloration of stone surface, due to the excretion of melanin by dematiaceus fungi (Warscheid and Braams, 2000).

Recently, it has been apparent that fungi comprise a significant component of microbiota in a wide range of rocks including sandstone, granite, limestone, marble and gypsum (Burford et al., 2003). Šimonovičova et al. (2004) the reported presence of 36 different microfungi on stone in hypogean cemetery in Bratislava. In the Earth's lithosphere, fungi are of fundamental importance as decomposer organisms, being ubiquitous in subaerial and subsoil environments. The ability of fungi to interact with minerals, metals, metalloids and organic compounds through biomechanical and biochemical processes, makes them ideally suited as biological weathering agents of rock and building stone. Biological and mycological investigations are very important part of good conservation and cannot be ignored in modern conservation concept which includes close collaboration between art and science.

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УЛОГА ГЉИВА У ПРОЦЕСУ БИОДЕТЕРИОРАЦИЈЕ КАМЕНА ИСТОРИЈСКИХ ГРАЂЕВИНА

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Резиме

Способност гљива да интерреагују са минералима, металима и органским материјама чини их идеалним агенсима који путем механичких и биохемијских процеса делују деградирајуће на стене и грађевински камен. Пигменти и органске киселине, које продукују гљиве, имају есенцијалну улогу у дисколорацији и деградацији различитих типова камена културно-историјских објеката. Ова истраживања обухватају миколошку анализу биофилма пореклом са два историјска објекта у Београду: Споменика незнаном јунаку на Авали и Бранковог моста на Сави. Изоловано је укупно 23 таксона филаментозних гљива, са доминацијом меланизованих микромицета (*Alternaria* spp., *Aureobasidium pululans* var. *melanigerum*, *Cladosporium cladosporioides*, *C. sphaerospermum*, *Drechlera dematoidea*, *Epicoccum purpurascens*., *Phoma* sp.) и специфичним распоредом на гранитном супстрату и камену пешчару који су истраживани. Миколошка анализа биофилма са површине грађевинског камена је важна карика у спровођењу добре конзервације и не може бити изостављена у савременом концепту конзервације који укључује тесну сарадњу између конзерватора и микробиолога.

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MARINE FUNGI — DEGRADERS OF POLY-3-HYDROXYALKANOATE BASED PLASTIC MATERIALS

ABSTRACT: The search for new biosynthetic and biodegradable materials to save nonrenewable resources and reduce global pollution problems is an urgent task. Recently, materials like thermoplastic poly-3-hydroxyalkanoates (PHA), have been found synthesized by bacteria as storage materials. The major PHAs synthesized are poly-b-hydroxybutyrate (PHB), poly-b-hydroxyvalerate (PHV) and their copolymers. They are already commercially produced and used as BIOPOL™ (ICI, England). Their complete degradability by bacteria has already been shown. Today, oceans and estuaries serve as major landfills, and since fungi are an important part of the degrading microbiota, in order to prove their participation in the degradation process, a simple degradation test suitable for fungi and marine conditions had to be developed. Several solid media based on artificial sea water, differing in the content of non-alkanoate organics and supplemented with 0.1% PHA (or BIOPOL™) as a main source of carbon have been tested. The testing principle consists of clearing the turbid medium in test tube or plates caused by suspended granules of PHA.

All media tested supported the growth of fungi. For the discrete and transparent clearing of zones, a mineral medium with 0.01% peptone, 0.01% yeast extract, and 0.1% PHB or BIOPOL™ was finally chosen where the fine and evenly distributed turbidity is accomplished by a specific procedure. This method allows the investigation of degradability of PHA-based plastic materials as well as screening for fungal ability to depolymerise pure PHA homopolymers. Using this medium, 32 strains of marine yeasts and 102 strains of marine mycelial fungi belonging to different systematic and ecological groups were tested for their ability to degrade PHAs. Only about 4% of the strains were able to degrade BIOPOL™ and about 6% depolymerised pure PHB homopolymer. This is in sharp contrast to the results of our previous experiments with 143 strains of terrestrial fungi which showed that 55% are able to degrade BIOPOL™.

KEY WORDS: PHA, BHB, BIOPOL, marine fungi, degradation, screening method

INTRODUCTION

The decrease in natural fossil resources recently led to an increased search for renewable, that is biosynthetic raw materials. Furthermore, current

concern about environmental pollution caused by petrochemical-based plastics led to investigations for replacement of such nondegradable synthetic polymers by biodegradable ones (Hartley, 1987; Lafferty et al., 1988; Anderson and Dawes, 1990; Brandl et al., 1990).

In some bacteria poly-3-hydroxyalkanoates (PHAs) constitute a major bacterial carbon and energy storage material (Holmes, 1988; Fuller, 1990). Recently, poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyvalerate (PHV) often copolymerised as PHB-co-PHV, showing thermoplastic properties, which attracted great industrial interest. BIOPOL™, the new "biodegradable" plastic material is one of copolymers that has been already produced on a large scale, and is being used as packaging material (ICI, Imperial Chemical Industries, England; today Zenecca).

Environmentally, the most important property of PHAs is their full microbial degradability to CO_2, water and energy (ICI, 1990; Byrom, 1990). PHAs have been shown to be completely biodegradable by bacteria (Macrae and Wilkinson, 1958; Merrick and Doudoroff, 1964; Delafield et al., 1965; Lusti and Doudoroff, 1966; Hippe und Schlegel, 1967; Fedulova et al., 1980; Tanio et al., 1982; Nakayama et al., 1985; Fukui et al., 1988; Janssen and Harfoot, 1990; Molitoris et al., 1997; Matavulj and Molitoris, 2000; Matavuly et al., 2006). However, fungal participation in the degradation of PHAs and PHA-based plastics has been only indicated and mostly neglected with few exceptions (Delafield et al., 1965; Lepidi et al., 1972). Fungi have attracted more attention in this connection much later (Holmes, 1985; McLellan and Halling, 1988; Dave et al., 1990; Matavulj and Molitoris, 1990; 1991; 1992a; 1992b; Matavulj, 1991; Matavulj et al., 1994; Neumeier et al., 1994).

Fungi are an important part of degrading microbiota, playing a most active role in the mineralization of organic matter and in the element cycling. Equipped with extracellular multienzyme complexes, fungi are most efficient, especially in breaking down the natural polymeric compounds. By means of their hyphal systems they are also able to colonize and penetrate substrates rapidly and to transport and redistribute nutrients within their mycelium.

The ultimate fate of many organic materials is mineralization by microorganisms. The ever-increasing human demand for materials leads to a concomitant increase in waste production. The fact that marine environments (sea- and estuarine waters, sea-bed sediments, coastal sand/rock/soil areas) serve today as major landfills, it also means that the new generation of biosynthetic plastics will probably have to be degraded there as well. This led us to investigate the potential of marine fungi to degrade biosynthetic plastics, in our case BIOPOL™ and its available individual components.

Since fungi participate significantly in microbial degradation processes and since their role in the transformation of organic matter, particularly in marine habitats, is mainly unknown, a simple screening method for fungal degradation of BIOPOL™ and its components (PHB homopolymer and triacetin) was developed. The method was to provide at least semiquantitative data from screening of high numbers of marine fungi under simulated marine conditions

(mineral composition of water, level of salinity, pH). Similarly, a screening method for terrestrial fungi under terrestrial conditions has already been developed and reported recently (Matavulj and Molitoris, 1992a,1992b).

MATERIAL AND METHODS

Media

During the development of media for fungal growth and detection of PHA degradation in simulated marine conditions, three media with low content of non-PHA organic nutrients were tested comparatively:

1) Glucose-peptone-yeast extract agar (GPY), containing 0.1% glucose, 0.05% peptone, and 0.01% yeast extract (Molitoris and Schaumann, 1986).

2) Reduced glucose-peptone-yeast extract agar (rGPY): 0.01% glucose, 0.01% peptone, and 0.01% yeast extract.

3) Basal mineral medium with peptone and yeast extract (MPY):

Mineral salts: 0.14% $NaN0_3$, 0.10% $NH_4H_2P0_4$, 0.10% KH_2P0_4, 0.06% K_2HP0_4 x $3H_2O$, 0.04% $MgS0_4$ x 7H2O, 0.02% $CaCl_2$ x $2H_2O$.

Organic substances: 0.01% peptone, 0.01% yeast extract.

Microelement solution A: (1 ml/l medium): 0.40% $CUS0_4$ x $5H_2O$, 0.40% $ZnS0_4$ x $7H_2O$, 0.40% Na_2Mo0_4, 0.20% $MnS0_4$ x H_2O, 0.20% $CoCl_2$ x $6H_2O$, 0.20% H_3B0_3, 0.10% KI, 0.10% Na_2Se0_3 x $5H_2O$, 0.10% Na_2Wo_4 x $2H_2O$, 0.10% $KAI(S0_4)_2$ x $12H_2O$.

Microelement solution B: (1 ml/l medium): 0.05% $FeS0_4$ x $7H_2O$.

In all cases 1.60% agar and artificial seawater were used for the preparation of the media. The pH was adjusted to 7.0 before autoclaving at 121°C for 20 min.

The composition of artificial seawater was, as it was described earlier (Lorenz and Molitoris, 1992) resulting in the following concentrations (weight/volume of distilled water): NaCl 2.270%, KH_2P0_4 0.788%, $MgS0_4$ x $7H_2O$ 0.700%, $MgCl_2$ x $6H_2O$ 0.550%, KCl 0.065%, NaBr 0.010%, H_3B0_3 0.003%, $SrCl_2$ x $6H_2O$ 0.0015%, $C_6H_80_7$ x H_2O 0.001%. $CaCl_2$ was added as a separate solution to make the final concentration of 0.150%.

A modified solution of microelements as proposed by Balch and Wolfe, 1976, and Balch, 1979, was added to give final concentrations of: $C_6H_9N0_6$ (titriplex I) 0.0015%, $MnS0_4$ x H_2O 0.0005%, $Ni(NH_4)_2(S0_4)_2$ 0.0002%, $FeS0_4$ x $7H_2O$ 0.0001%, $CoCl_2$ x $6H_2O$ 0.0001%, $ZnS0_4$ x $7H_2O$ 0.0001%, $CUS0_4$ x $5H_2O$ 0.0001%, $KAl(S0_4)_2$ x $12H_2O$ 0.00001%, Na_2Mo0_4 x $2H_2O$ 0.00001%, Na_2W0_4 x $2H_2O$ 0.00001%, Na_2Se0_3 x $5H_2O$ 0.00001%, KI 0.000005%.

Preparation of assay medium

Powdered BIOPOL™ ("Bottle formulation") (1.00 g) or powdered PHB homopolymer (1.00 g) was suspended in 100 ml of seawater, sonicated for 10 min (Bandelin Sonorex RK 106 S) and autoclaved separately. After sonication

for another 10 min, the opaque suspension was added aseptically to 900 ml of hot sterile medium, which was then sonicated for another 10 min, agitated, cooled down to 50°C and poured into sterile Petri plates or test tubes, which resulted in a fine and evenly distributed turbidity caused by the PHA.

During autoclaving of the media, precipitates of mineral salts occurred, which somehow interfered with the readability of the clearing reaction in plates, whereas no interference occurred in test tubes where the precipitate was settled at the bottom. To prevent the sedimentation of the fine PHA particles during solidification of the media, test tubes were cooled quickly in a cold water bath.

For preliminary experiments BIOPOL™ in granulated form or strips of BIOPOL™ shampoo bottles (Wella, Germany), were also used for media with the following modifications: 1% solution of BIOPOL™ in hot chloroform was converted to a 1% ethanol solution by adding 150 ml of 96% ethanol and boiled until the volume was reduced to 100 ml. Similarly, carefully adding hot distilled water and reducing the volume by boiling, 100 ml of a non-homogenous aqueous solution of BIOPOL™ was obtained. This was then homogenized by sonication as described above.

Granulated BIOPOL™ (Imperial Chemical Industries = ICI, England, lot MBL 100/1320) and powdered BIOPOL™ (PHB-co-HV) copolymer with approximately 8% PHV, 9% triacetin, 3% titanium dioxide, and 1% boron nitride) were kindly supplied by ICI, England.

Biological material

134 pure strains of marine fungi or marine fungal isolates (Culture collection Botanical Institute, University of Regensburg), belonging to different systematical and ecological groups (Tab. 1) were used for testing the three basic media. The halotolerant strain *Penicillium simplicissimum* (IMI 300465) was used as reference strain for its known high PHB-degrading activity (McLellan and Halling, 1988; Matavulj and Molitoris, 1992a, b).

Cultivation

The fungal strains were cultured on GPY agar at 22°C. Approximately 3 x 3 mm squares from the actively growing area of the agar plates were used as inoculum. Yeasts were transferred by an inoculation loop. All cultures (Petri plates, 9 cm diameter and screw-capped test tubes, 20 x 100 mm) were incubated at 22°C and approximately 65% relative humidity with a diurnal periodicity of 12 hours light and 12 hours darkness.

Evaluation of degradative activity

The fungal ability to degrade BIOPOL™ and to depolymerize pure PHB homopolymer was determined by recording the diameter in mm of clear

formed in the turbid medium in agar plates or the depth of clearing in mm of the opaque media in test tubes at daily (first week), or weekly intervals up to 9 weeks.

Tab. 1 — Systematic and ecophysiological affiliation of the marine fungi investigated, and their capability to degrade BIOPOL™, pure PHB, and triacetin.

Systematic affiliation				Ecophysiological affiliation				Number (percentage) of Degrading strains					
Tax.	Str.	Spec.	Gen.	OM	FM	MI	NI	BP	BP%	PHB	PHB%	TA	TA%
D	65	43	29	0	15	17	33	4	6.2	5	7.7	60	92.3
Dy	16	10	4	0	15	1	0	0	0.0	1	6.2	16	100
A	46	33	23	0	6	5	35	2	4.4	2	4.4	43	93.4
Ay	6	1	1	0	6	0	0	2	33.3	2	33.3	6	100
B	23	9	3	5	3	0	15	0	0.0	1	4.3	21	91.3
By	10	8	2	5	3	2	0	0	0.0	0	0.0	10	100
T	134	85	55	5	24	22	83	6	4.5	8	6.0	124	92.5
Ty	32	19	7	5	24	3	0	2	6.3	2	6.3	32	100

Legend:
D = Deuteromycotina, Dy = asporogenous yeast; A = Ascomycotina, Ay = ascomycetous yeast, B = Basidiomycotina, By = basidiomycetous yeast, T total fungi investigated, Ty = total yeeasts investigated; OM = obligate marine, FM = facultative marine, MI = marine isolates, NI = no information available; BP = BIOPOL™, PHB = poly-b-hydroxybutyrate, TA = triacetin. Numbers of strains (Str.), species (Spec.), and genera (Gen.) given for the yeasts investigated (Dy, Ay, By) are included in the respective total numbers given for the Deuteromycotina (D), Ascomycotina (A) and Basidiomycotina (B).

RESULTS AND DISCUSSION

All fungal strains grew on all three tested media. Fungal PHA degradation rate, however, differed depending on the medium and the PHA carbon source used (Fig. 1). All marine isolates showing degradative activity under the experimental conditions applied, exhibited the highest rates of BIOPOL™--degrading activity if grown on MPY medium (Fig. 1A, B, D, E, F, G) except for *Gliomastix sp.* where this medium resulted in lower activity (Fig. 1C).

None of the active strains of the yeast *Debaryomyces hansenii*, showed PHA degrading activity when grown on GPY medium with 1% glucose (Fig. 1E, 1F), even after 65 days of incubation. *Debaryomyces hansenii* M-111 and *Fusarium merismoides* M-46 degraded BIOPOL™ only when it was offered as the sole source of carbon in MPY medium. Clearing activity was not apparent for 10 days in the former instance (Fig. 1G) or 40 days in the latter (Fig. 1E).

It may be noted that generally PHA breakdown decreased with increasing concentrations of glucose in the medium (e.g. Fig. 2D). It appears that glucose, which is metabolized earlier or more easily, inhibits breakdown of the PHAs investigated. This is in agreement with the work of Jendrossek et al., 1992, on bacteria, who reported an inhibition of PHB depolymerase formation when other organic substrates in addition to PHB were added to the

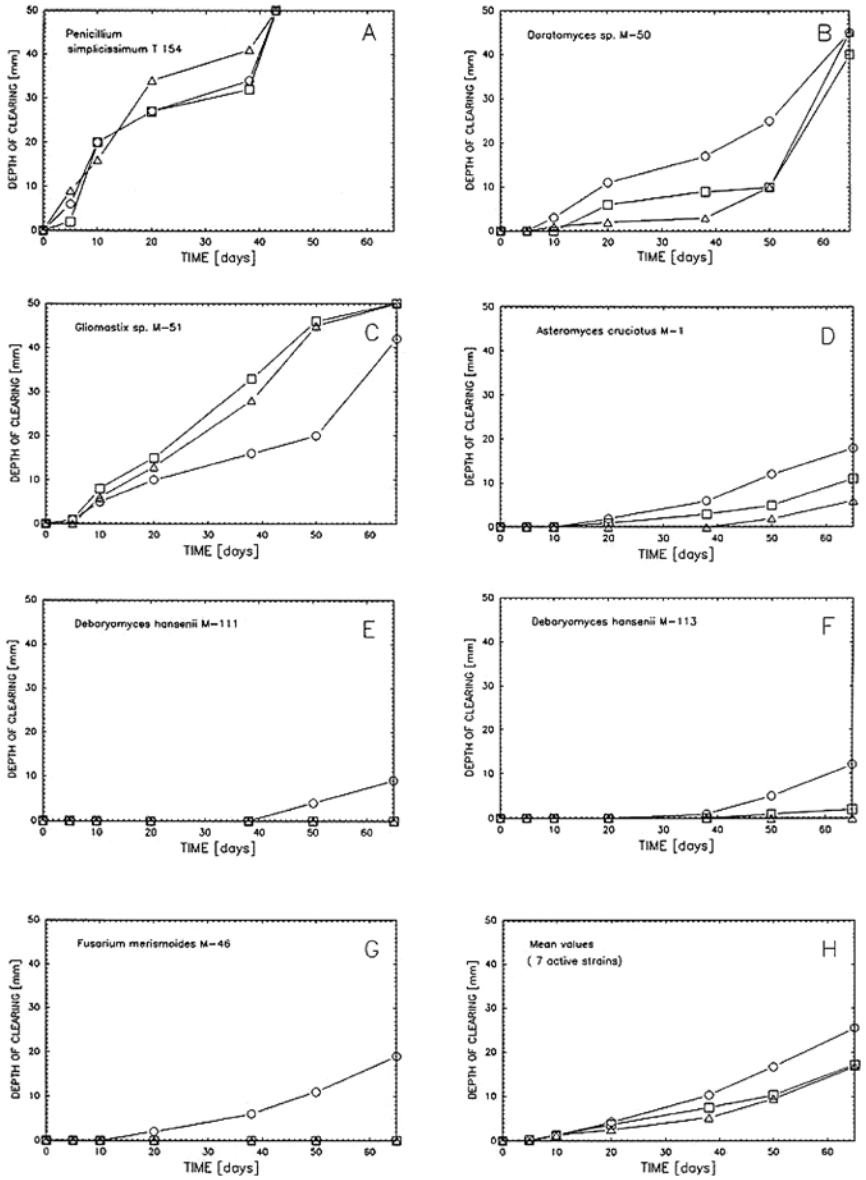

Fig. 1 — Fungal degradation of BIOPOL™ (mm clearing of turbid medium in *test tubes) in different media (GPY = triangles, rGPY = squares, mPY = circles). Fig. 1A: Penicillium simplicissimuum* as terrestrial reference strain. Fig. 1B to 1G: six marine isolates. Fig. 1H: Mean values of all seven active strains tested. Incubation period 65 days.

medium. In both cases this may be explained as a repression of depolymerase synthesis or secretion.

For all further investigations, MPY medium with 0.1% of BIOPOL™ was chosen for the following reasons: a) it supported the growth of all fungi tested,

b) most of the active fungi showed the highest rate of BIOPOL™ degradation on MPY medium, c) some of the fungal strains tested expressed BIOPOL™ — degrading activity only on MPY medium.

In order to test all strains for their ability to degrade the individual organic components of BIOPOL™ ("bottle formulation"), two of its components, the pure PHB homopolymer or triacetin (9% triacetin in commercial BIOPOL™), (P ü c h n e r, 1988) were offered as the main source of carbon in another series of experiments.

Figure 2 shows the typical BIOPOL™ degradation pattern as a clearing zone around the fungal colony in agar plates (Fig. 2a, 2b) and in test tubes (Fig. 2c). When BIOPOL™ was used as a PHA substrate, agar medium clearing was incomplete and the borderline of the clearing area was less defined.

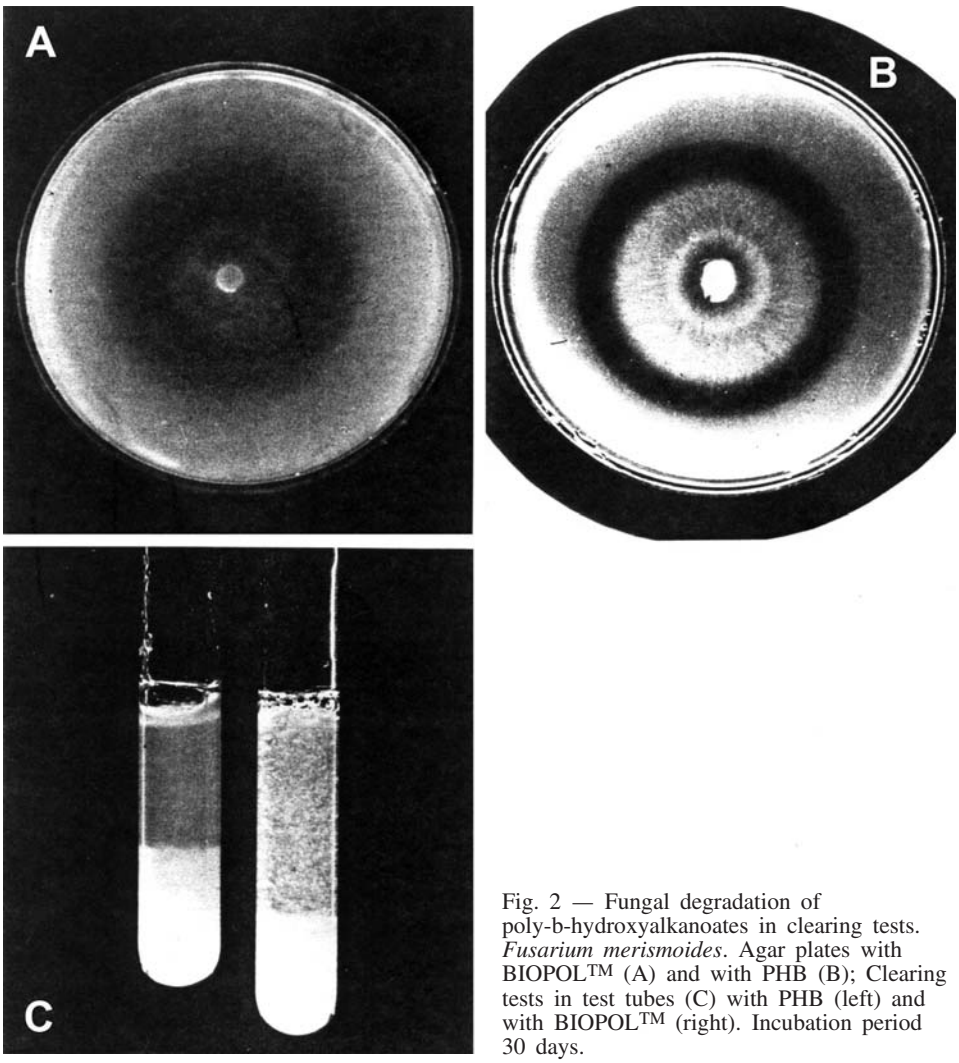

Fig. 2 — Fungal degradation of poly-b-hydroxyalkanoates in clearing tests. *Fusarium merismoides*. Agar plates with BIOPOL™ (A) and with PHB (B); Clearing tests in test tubes (C) with PHB (left) and with BIOPOL™ (right). Incubation period 30 days.

However, pure PHB homopolymer was offered, however, a characteristic and sharp border of the clearing zone could be observed.

In our work on PHA degradation by terrestrial fungi (Matavulj and Molitoris, 1992a) we recommended usage of test tubes instead of Petri plates for the PHA clearing test because the clearing reaction on Petri plates may be concealed by the growing mycelium, particularly in the case of working with fast-growing strains. The same applies to marine fungi. Since BIOPOL™ contains several components including PHB and PHV as a heteropolymer (79% PHB, 8% PHV, 9% triacetin, 3% titanium dioxide, 1% boron nitride), it is important to know whether there are differences between a medium containing the PHA and the pure PHB homopolymer for the purpose of specifying depolymerase activity.

When pure PHB was included as substrate in MPY medium, the medium was turbid and after incubation with active strains two types of reaction occurred: a) no clearing at all (no PHB degradation), b) complete clearing (PHB degradation). When triacetin was included in the MPY medium, the medium appeared to be only slightly turbid. After incubation with fungal strains the growth occurred in all cases, and in almost all cases the slight turbidity disappeared as well, which was considered as an indication of breakdown of this substance.

When BIOPOL™ was included in the MPY medium, which resulted in a turbid medium, 3 types of reaction occurred after growth of fungal strains: a) no clearing at all, b) a slight turbidity left, c) total clearing. Based on the above mentioned results, our interpretation is that in case a) no depolymerization activity towards PHB and PHV is present, in case b) only the PHB moiety and triacetin are depolymerized, in case c) PHB, PHV and triacetin are depolymerized. Final proof, however, must result from the experiments using pure PHV, which was not available to us.

The results of testing 134 marine fungal isolates for the degradation of BIOPOL™, PHB homopolymer and triacetin are summarized in Table 1. Out of total 134 strains tested, belonging to 85 species and 55 genera of different systematical and ecological groups, only 6 strains (4.5%) were able to degrade BIOPOL™, 8 strains (6.0%) depolymerize PHB homopolymer, and 124 strains (92.5%) were able to use triacetin as the sole carbon source. This is in sharp contrast to our results with terrestrial fungi where about 55% are able to degrade BIOPOL™, and about 68% depolymerize PHB homopolymer. Triacetin was also degraded by more than 90% of the terrestrial fungi tested. The easy degradability of triacetin could be an important initial step in the destruction of this plastic material in nature, especially under oligotrophic marine conditions (Matavulj and Molitoris, 1992a).

Recent work on bacterial degradation of PHAs by Jendrossek et al. (1992) shows that only about 10% of the aerobic PHB-utilizing bacteria were also able to degrade pure PHV homopolymer. Furthermore, Doi et al. (1990) report that the rate of polyester degradation (followed as film erosion) by extracellular PHA-depolymerase, purified from the bacterium *Alcaligenes eutrophus*, was strongly dependent on the composition of the polyester. They observed that the presence of 3-hydroxyvalerate units significantly retarded the

degradation of the polyester, in comparison with the rate of pure PHB degradation.

Another important question was whether the PHA-degrading activity shows any correlation with the systematic or ecologic groups of fungi investigated. Owing to the low number of active strains in marine fungi, it is difficult to obtain statistically significant correlations. However, from Tables I and II, it can be seen that the degrading activities are not evenly distributed among the different groups of marine fungi investigated. Table II seems to indicate that the PHA-degrading activity in marine fungi is higher among the *Deuteromycotina* (BIOPOL™ — 6,15%, PHB — 7.69%) than in other groups.

Observing the distribution of the PHA-degrading activity among several members of a given genus, or a given species, one can see from table II that out of five strains of *Asteromyces cruciatus* investigated, only strain M-1 showed the ability to degrade both BIOPOL™ and PHB. Out of 9 strains of the genus *Candida*, only one strain of *Candida guillermondii* (M-122)-depolymerized PHB. The yeast *Debaryomyces hansenii* was represented in our screening by 6 strains, of which only two showed the ability to degrade both PHAs. The investigation of 23 *Basidiomycotina* representing 3 genera and 9 species, showed that only one strain of *Nia vibrissa* (M-167) was able to depolymerize PHB.

CONCLUSION

The data shown indicate that the ability to degrade PHAs is not wide represented in marine fungi. This is in contrast to the results obtained from the investigation of terrestrial fungi. Moreover, our data give no indication that this property is a taxonomic characteristics of certain species or genera of marine fungi, nor that it is a property of certain ecological groups. It rather seems to be a physiological property of individual “physiological strains” of marine fungi.

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МОРСКЕ ГЉИВЕ — РАЗЛАГАЧИ ПЛАСТИЧНИХ МАТЕРИЈАЛА ПРОИЗВЕДЕНИХ НА БАЗИ ПОЛИ-3-ХИДРОКСИАЛКАНОАТА

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Резиме

Трагање за биосинтетичким и биодеградабилним пластичним материјалима је актуелан задатак научника из разлога очувања необновљивих ресурса и смањења глобалног загађивања. Недавно су нађени такви материјали, у виду термопластичних поли-3-хидроксиалканоата (ПХА), које синтетишу бактерије и нагомилавају као резерве енергије и угљеника. Најчешће синтетизовани ПХА су поли-б-хидроксибутират (ПХБ), поли-б-хидроксивалерат (ПХВ) и њихови кополимери. Они се данас комерцијално производе и користе у облику биопола (BIOPOL™, ICI, England) и већ је експериментално показана њихова потпуна раз-

градљивост од стране бактерија. Пошто морске и бракичне воде данас служе као главне депоније отпада, и пошто су гљиве важан део популације разлагача, да би се доказало њихово учешће у процесима разградње ПХА требало је разрадити једноставан тест за ту активност гљива у маринским условима. Компоновано је неколико агаризованих вештачких подлога на бази вештачке морске воде, које су се међусобно разликовале у садржају органског супстрата неалканоатног порекла. Све су садржале 0,1% ПХА (или BIOPOL™) као главни извор угљеника. Тестирање активности гљива заснивало се на њиховој способности да помоћу ензима деполимераза просветљавају чврсту подлогу која је била замућена фино диспергованим гранулама ПХА у петри плочама или тест епруветама.

Тестиране гљиве су успешно расле на свим коришћеним подлогама. За коначни тест просветљавања подлоге изабрана је минерална подлога са 0,01% пептона, 0,01 квашчевог екстракта и 0,1% ПХБ или BIOPOL-а. Овај метод омогућава испитивање разградљивости пластичних материјала произведених на бази ПХА, као и скрининг својства изолата гљива да деполимеризују чисте ПХА хомополимере. Коришћењем ове подлоге тестирана је способност разградње ПХА код 32 соја маринских квасаца и 102 соја маринских мицелијалних гљива које су припадале различитим систематским и еколошким групама. Резултати показују да су само 4% од сва 134 соја испитиваних гљива биле способне да разграђују BIOPOL, а око 6% да деполимеризују чист ПХБ. Добијени резултати су у супротности са нашим претходним резултатима испитивања 143 соја терестричних гљива од којих је преко 55% показало способност разградње BIOPOL-а.

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CHRONIC CANDIDIASIS — PATHOGENESIS, SYMPTOMS, DIAGNOSIS AND TREATMENT

ABSTRACT: The yeast named *Candida* normally colonises the gut and vagina without causing any sign of its presens. It is a commensal and opportunic fungus but in certain conditions it turns to be pathogenic, causing chronic disturbances in any part of the body. The pathogenesis is complex, signs and symptoms are non-specific. The colonisation is difficult to distinguish from invasive disease. The current diagnostic methods do not always allow a definitive diagnosis to be made. Treatment is complex, individual and no protocol can be created. The author tries to give an overview of the *Candida* related problem.

KEY WORDS: *Candida*, chronic Candidiasis, yeast infection,

INTRODUCTION

This presentation deals with the pathogenesis, symptoms, diagnosis and treatment of chronic candidiasis. It is based on the author's over thirty years long experience and it is supplemented with data from the bibliography. In order to put the problem into historical perspective, a short chronological survey about the perception of pathogens and the body's milieu comes next.

Louis Pasteur (1822—1895), chemist and microbiologist, linked different infectious diseases to different pathogens. He believed that these infectious organisms were the most important etiologic factors and nothing else was needed for a disease to develop. He discovered that penicillin had antibiotic effect against many bacteria. At the same time Claude Bernard (1813—1878), physiologist, discovered the importance of the body's internal environment, and taught that all the components of the blood must be in balance, if not, the pathogens could cause infection. To maintain the homeostasis means to stay healthy and to be protected from the pathogens. These two different approaches to the infectious diseases were the root cause of their disagreement. At the end of his life, Pasteur recognized that Bernard had been right, saying: "The pathogen is nothing, the terrain is everything." Elie Metchnikoff (1845—1916) biologist, microbiologist and pathologist, studied the body's bowel flora

and found out that there was an antagonism between *Lactobacillus acidophilus* and bacteria causing putrefaction. He believed that the products of putrefaction were absorbed and intoxicated the body causing early aging. To prevent this process, he recommended increased intake of yogurt, containing a lot of living friendly bacteria. He discovered the white blood cells and their role in protecting the body from infection. He believed, as Bernard, that the correct way to deal with infectious diseases was the enhancement of the body's own defenses.

These were the data from the 19th century. What happened in the 20th?

The influence of Louis Pasteur was dominant. Discovering and using antibiotics of broad spectrum and thanks to the vaccinations, a lot of infectious diseases were eradicated and many lives were saved. But what is the side effect of this success? The overgrowth of fungus and bacteria resistant to antibiotics. As the medical treatments are getting more and more aggressive (not only antibiotics, but hormones, immunosuppressive- and chemotherapy), destroying the body's own already weakened defenses by the western life style, we must face up to the serious upcoming problems. As quoted often in the bibliography, the presence of fungal infection among the patients in the intensive care units often causes death of immunocompromised patients. These fungal infections are iatrogenic, so should be prevented in a proper way. The conventional western medicine most often uses symptomatic therapy — for infection antibiotics, for pain painkillers, for inflammation anti-inflammation drugs, etc. Researchers in conventional medicine try to develop more efficient and aggressive drugs against the resistant pathogens. On the contrary, doctors who are familiar with complementary medicine try to find out the cause of the imbalance and help the body to build up the weaken defenses. Restoring the homeostasis, the body will cure itself. The WHO in 1996 emphasized the significance of integrative medicine for the 21st century. Now it is time to change the paradigm of Pasteurian approach to the infection, and investigate the patient's status in a holistic way.

The present western way of life (unhealthy diet, harmful influence of the environment, lot of stress, etc.) leads to the long term imbalance of the homeostasis. In such a case *Candida* may overgrow, causing with its metabolic products — mycotoxins — disturbances in different organs all over the body, and the patient "feels sick all over". The symptoms come from the local *Candida* overgrowth in the gastrointestinal tract, vagina or urinary bladder and from the damages of different organs. As the symptoms do not relate to any well described disease, the patients are considered to be psychosomatic — their health problems are related to their brain. Moreover, many doctors consider the *Candida* related problems as a fad.

Dr. Orion Truss, a specialist in internal medicine and allergy, published the "The Missing Diagnosis" in 1983. He was the first to describe the concept of chronic Candidiasis based on his twenty years clinical experience.

At the same time Dr. William G. Crook published his own experience in the book "The Yeast Connection". Their works were considered as pseudo--scientific for lacking double-blind and placebo-controlled clinical trials. The author of this article had more then thirty years clinical experience which confirms that Truss and Crook were right.

"The Mycotoxin Blue Book" lists disorders that mycotoxins cause in animals when feed is infected with fungi. It is interesting that the symptoms in these animals are the same or very similar to those experienced in people suffering from *Candida* overgrowth. Adding 'Mycosorb' to the infected feed prevents the intoxication of the animals by adsorbing the mycotoxins. These are objective data and not a whim. The existence of *Candida* related problems are denied by those not up-to-date on recent medical, microbiological, biochemical literature.

PATHOGENESIS

For most of the patients the chronic candidiasis is iatrogenic in origin, and the western lifestyle also leads to the imbalance of friendly bacteria, *Candida* and host immune system interactions. The compromised immune system or lack of friendly bacteria may lead to *Candida* overgrowth in the gut or vagina causing local symptoms and functional disorders in other organs — the benign round yeast form of *Candida* converts into invasive budding mycelial form. However, *Candida* can cause functional disorders without causing a classical local infection, but so called dysbiosis or "silent infection" of the gut or vagina. In this case the symptoms are due to the absorbed metabolic products called mycotoxins produced by *Candida* and chronically released into the bloodstream.

Candida produces certain molecules which interfere with normal human cellular metabolism, such as alcohol, acetaldehyde, ammonia and uric acid. *Candida* also externalizes a protein-digesting enzyme (proteinase) which lyses secretory IgA of the mucosa and humoral immunoglobulins, keratin and collagen as well. Proteinase also destructs the microvilli of intestine and leads to lack of mucosal digestive enzymes causing maldigestion and malabsorption. The damage of the intestinal mucosa results in leaky gut (increased permeability of the intestine) with increased absorption of debris (large undigested food particles, bacterial and yeast components and various toxic chemicals) from the gut and in overstimulation of the immune system (polyclonal T- and B-lymphocyte activation and production of excessive levels of cytokines and other inflammatory effectors) that may lead to several systemic inflammatory disorders, allergy and autoimmune diseases. Gliotoxin produced by *Candida* suppresses human immune function. The combination of mucosal damage, destruction of IgA and humoral immunoglobulins and immunosuppression, allows the ongoing chronic infection caused by other microbes.

CLINICAL PICTURE

As the pathogenesis is very complex, the patients may have very different symptoms depending on which organs are involved. Any organ seems susceptible to the effect of yeast products released to the blood stream. Symptoms change in intensity and complexity at different times in an illness, just as they vary in different individuals with the same illness. The clinical picture

will not be identical in every patient, or in the same one at different stage of the illness. The same variety of symptoms allows this condition to masquerade under a number of different diagnoses. This is the reason why the patients are usually misunderstood, sent to psychiatric consultation but the tried psychiatric treatments are futile.

The symptoms can be limited at the site of infection of the intestinal tract, vagina, or urinary bladder. Other symptoms are caused by the candida products released to the blood stream, or have allergic origin. Almost every organ is capable of allergic or toxic response, resulting in clinical manifestations as varied as the combinations of affected tissues. But, it is difficult to prove which symptoms come from allergic or toxic reaction. To make the problem even more complex, most of the patients have at the same time symptoms of maldigestion and nutrition deficiency, or some other illness not related to *Candida*. All these circumstances render the *Candida* syndrome confused. The following symptoms can be experienced:

Local symptoms of the intestinal tract: coated tongue, oedema of the tongue, oral thrush, heartburn, abdominal discomfort or pain, distension, bloating (with or without odor), constipation, diarrhea, mucus in the stool, rectal itching.

Local symptoms of the vagina: yoghurt like discharge with itching.

Local symptoms of urinary bladder: distension and bloating of empty bladder, urgency, burning and frequency of urination.

Other symptoms of allergic or toxic origin to mention but a few:

Nervous system: headache, depression, uncontrollable crying, anxiety, loss of memory and concentrating ability, sleeping disorder, sleepiness during the day, lethargy, fatigue, lack of energy, drinkless drunkenness.

Skin: hair loss, dry skin, itching, urticaria, dermographism, eczema.

Respiratory system: cough asthma.

Cardiovascular system: palpitation, instable blood tension, cold arms and legs.

Immunsystem: recurrent or chronic bacterial or virus infections.

Locomotor system: joint swelling and stiffness, muscle pain.

Endocrine system: hypoglicaemia, need to eat often, abnormal craving for sweets, overweight, feeling better after eating sweets but immediately bloating, reduced or lack of sexual desire, menstrual problems.

Other symptoms: food and chemical intolerance.

DIAGNOSTIC PROCEDURE

To prove the connection between the symptoms and candida is not easy, because presently available diagnostic methods are imperfect. The results should be always considered individually in relation to the clinical picture.

Many published studies have shown in everyone's blood the presence of candida antibodies (IgM, IgG) which can be measured, as *Candida* is normally present in the body. That is why the early skin prick test is normally positive to *Candida*. By the early age of six months almost 90% of the babies have po-

sitive early skin test to *Candida*. This is the result of a good humoral immune function. When the immune system is weakened (it can be also caused by *Candida* products), and the level of antibodies against candida is very low or absent in the blood stream, the positive early skin test turns to negative. But, antibodies are not actively involved in protecting the body against the yeast. The cellular immunity is responsible normally to eliminate *Candida* from the tissues, so the late skin test to *Candida* is positive normally. But, in the case of candidiasis, the late skin prick test to *Candida* is negative showing the impaired cellular immune function. Successful anti-*Candida* treatment and stimulation of the cellular immunity will turn the negative late skin test to positive.

Cultures for *Candida* obtained from mouth, stool or vagina can be normally positive, but negative ones do not rule out the diagnosis of chronic candidiasis, just show the absence of *Candida* in those samples.

The level of blood sugar can be low — hypoglycemia. The level of alcohol, transaminases of the liver, uric acid, and cholesterol can be high.

Some diagnostic methods used by complementary medicine can be added as bio-resonance, kinesiology, dark field microscopic blood analysis, etc.

Sometimes, the successful anticandida "ex juvantibus" treatment of the disease of "unknown etiology", will confirm the fungal connection.

TREATMENT

Treatment will be discussed in a general way as it is very complex and should be individually formed. The first aim is to discover the causes of the homeostasis imbalance and eliminate them. Then, the following should be considered parallely: to stimulate the immune system, to support the detoxification of the body, to kill and eliminate the dead *Candida*, and to substitute the nutrition deficiency. Often, the treatment takes time, sometimes several months or years. In most of the cases the combination of antifungal drugs with the treatment methods of the complementary medicine is recommended or required. Long term changes in lifestyle are required.

PREVENTION

For everyone, who has suffered from *Candida* infection or not yet, it is recommended to take more attention to the conditions leading to dysbiosis or *Candida* overgrowth and avoid them as much as possible. Try to maintain good health condition, the homeostasis of the body. For medical doctors it is advisable to think about the possibility of *Candida* infection as a side effect of some therapy (antibiotic, corticosteroids, birth control pills, immunosuppressive therapy, chemotherapy, etc) and as a cause of health problem, before sending the patient to psychiatrist. Holistic approach to every patient should be followed.

The author's case studies:

1. Case of seronegative polyarthritis: 60 years male was diagnosed as seronegative polyartritis, but he has never believed in his diagnosis. He kept trying to find out the cause of his disease. One day the vet prescribed for his very old dog an antibiotic for pneumonia, recommending to supplement it with antifungal drug in order to prevent fungal infection for the case of survival. The dog died, leaving behind the drugs. The master decided to take the antifungal ketokonazole. After a week of treatment he felt better, the bloating in the bowel and the diarrhea disappeared, the movements in his joints improved, the painful swelling of them almost disappeared. He asked his doctor to prescribe him more ketokonazole but the latter resisted saying: It isn't a protocol to treat the polyarthritis with ketokonazole.

2. Case of chronic urticaria: thirty years female was suffering of chronic urticaria for several years. All results of the investigations were negative except the bile — *Candida albicans* was cultured, but no doctor took it seriously. Several days after the beginning of anticandida regime urticaria disappeared. She was free of it for five years. Then she got antibiotics for tooth inflammation for a period of five days. She took no attention to the diet, did not take antifungal drugs and friendly bacteria. After three weeks urticaria returned.

3. Case of vitiligo, chronic urticaria and acute asthmatic attack: twenty six years old male had vitiligo since he was 15. It began after a stress. Later every stress expanded the vitiligo. At the age of 25 urticaria appeared and remained chronic. One year later an asthmatic attack led him to the doctor who gave him steroids but without effects. His tongue was coated with *Candida*. Bioresonance testing showed a severe allergy to *Candida*. After the beginning of anticandida regime urticaria disappeared, together with the asthmatic attacks and the vitiligo began slowly to decrease.

4. Case of chronic alcoholism without alcohol consumption: fifty years old female was suffering for years of the symptoms of chronic alcoholism but never drank. Several times she needed hospitalization. Every time the level of blood alcohol was high, as the liver enzymes. The doctors did not clear up. Only the bioresonance testing showed allergy to *Candida*. The anticandida regime was successful. After six months no alcohol was detected in the blood. She feels healthy for more than two years.

5. Case of cirrhosis hepatis: fifty-eight years old female was hospitalized for haematemesis — vomiting fresh blood. The diagnosis was decompensation of cirrhosis hepatis and bleeding from the oesophageal varices. *Candida albicans* was cultured from her tongue. Two months of antifungal treatment compensated her — ascites and swelling of legs disappeared, liver enzymes turned to normal, but the varices of the oesophagus remained. She felt healthy. After three months she died after a severe bleeding from the varices but with normal liver function.

In all of these five cases the role of *Candida* was unrecognized by the doctors of conventional medicine. But these cases do not mean that every polyarthritis, urticaria or chirrosis hepatis are related to *Candida*, but this possibility should be considered.

CONCLUSION

Candida is a commensal saprophyte fungus, but turns to pathogenic by changes in the internal balance of the body. So, fungal problems are secondary to other primary imbalances related to the general health of the immune system. It is difficult to diagnose the *Candida* overgrowth, in lack of unequivocal objective tests to verify its existence and the relation between the *Candida* and the symptoms. Laboratory results may be *Candida* positive, but it does not mean that the health problem is related to *Candida* overgrowth; neither can negative results exclude it. Therefore, one should start out with a high index of suspicion in making diagnosis of fungal infection. No treatment protocol can be established; healing must be individual, sometimes lasts quite long and may require lifestyle changes. Treating only the fungal infection may help relieve some of the symptoms, but the persistence of underlying imbalance will allow the condition to be chronic. The recovery of the immune defense system's function is the first aim. To kill *Candida* is not sufficient.

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ХРОНИЧНА КАНДИДИЈАЗА — ПАТОГЕНЕЗА, СИМПТОМАТОЛОГИЈА, ДИЈАГНОСТИКА И ТЕРАПИЈА

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Резиме

Квасна гљивица звана кандида нормално колонизира пробавни тракт и вагину, не дајући никакве знаке свог присуства. Она је коменсална и опортунистичка гљивица, која се под одређеним условима може преобразити у патогену,

узрокујући хроничне поремећаје у функцији различитих органа. Патогенеза је комплексна, а знаци поремећаја су неспецифични. Тешко је одредити границу између нормалног стања колонизације и патолошког стања инвазије. Данас постојеће дијагностичке методе нису увек у стању да потврде дијагнозу хроничне кандидијазе. Не постоји јединствени протокол лечења јер је оно комплексно и индивидуално за сваког пацијента. Аутор рада приказује проблематику везану за хроничну кандидијазу.

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DERMATOMYCOSIS — A POTENTIAL SOURCE OF ZOONOTIC INFECTION IN CITIES

ABSTRACT: Skin infections in animals and humans represent a complex syndrome that is, from etiological point of view, often hard to solve. Skin is one of the largest organs and plays an important role in the organism of mammals. Depending on the age, it presents 5—8% of total body mass in humans, 12% of adult animals, and even up to 24% in newly born animals.

The population of pets and stray dogs in cities provides a possibility for contacts between animals and/or humans, which is a mode of transmission for some diseases. Fungal diseases in dogs caused by dermatophyte are zoonosis, contagious infections that affect dogs and cats, but also other animals, as well as humans. There has been an overall increase in the number of the cases caused by anthropophilic fungi from the strain of *Microsporum* spp. and *Trichophyton* spp.

Our research was related to animals with different changes on skin manifested by hairless surfaces, dandruff, red skin and extensive pruritus. The objective of our examination was the analyses of scarified samples and swob from canine skin with the aim to determine whether the changes were caused by dermatophytosis. Our aim was to point out the possibility of transmission of mycotic infections from animals to humans in households.

For this mycological examination swobs and skin scarifications from dogs, were used. The samples were streaked on Sabo and dextrose agar and incubated at 25°C for at least 21 days. In one year period 81 samples were collected out of which 11 (13.58%) were positive for dermatophyte. In all samples *Microsporum canis* was isolated.

The findings of *Microsporum canis* in 13.58% of the examined samples indicate the importance of mycological skin disorder in pets. Having in mind that this is a zoonosis, the findings point to a possibility of exposing the humans to fungal infection, affecting almost all pet owners, especially in urban environment.

KEY WORDS: dermatophytes, dogs, zoonosis, city

INTRODUCTION

Humans have moved to cities from smaller settlements, where life style was closely connected to nature. However, in the cities nature is preserved only in a form of small green areas, parks or gardens. Human need for nature is obvious, so there is a constant striving to maintain some parts of nature in

the surrounding. Part of this is the practice of co-habiting with different pets. It is questionable whether the owners are informed about the unwanted consequences of keeping the animals in their households. Urban environment has specific characteristics when speaking about raising the pets: households are not large, what gives a possibility for closer interaction between humans and animals; they spend more time together than when living in natural environment. The potential for human exposure to transmitting of different microorganisms and parasites (L e f e b v r e a et al., 2006) from animals is very high and vice versa.

Over 300 species of fungi have been reported to be animal pathogens (O u t e r b r i d g e, Catherine A., 2006). Actual mycological pathogens of skin can be divided based on what layers of the skin they infect: surface layer — cutis, hair coat or claws. Superficial mycosis include dermatophytosis (predominantly *Microsporum* and *Trichophiton* species, but also *Malassezia* spp. and *Candida* spp. and *Trichosporon*) (O u t e r b r i d g e, Catherine A., 2006, P o p o v i ć, N., L a z a r e v i ć, M., 1999). Subcutaneous and deep systemic mycosis will not be reviewed in this paper. Cutaneous tissue may be infected when fungal organisms contaminate or colonise epidermal surface or hair follicles. When the integrity of skin barrier is disrupted, than the host is disposed to the infection. Canine and feline skin and hair coats can be transiently contaminated with a large variety of saprophytic fungi from the environment and some of them can cause opportunistic infections (S t o j a n o v et al., 2007, O u t e r b r i d g e, Catherine A., 2006).

Based on the experiences and data from literature, the objective of our research was to examine scarification and swabs from changed skin, with the aim to point out a possibility of transferring fungi infection from animals on humans in specific urban conditions of pets co-habitation.

MATERIAL AND METHODS

Our research was done on dogs with different skin infections. Clinical findings were not specific, and the etiology of a disease could not be determined. Effective therapy could not be applied. The findings differed in the intensity and type of changes that were manifested as more or less pronounced hair loss (sometimes even more than 50% of body). The signs of dandruff or seborrhea were visible and separated from deeper layers of cutis. The skin was reddish, with purulent pustules and expressed pruritus. In one year period there were 81 samples collected. Swob and scarifications of dog skin (C a b a n e s, F. J. et al., 1996) brought to the laboratory were used for the determination of the etiology of the disease. The samples were streaked on Saboraud dextrose agar and incubated at 25°C for 10 to 21 days. The colonies were identified according to their shape and colour, and the classification was done according to their conidia, macroconidia and conidiophores (Q u i n n, J. P. et al., 2002).

RESULTS AND DISCUSSIONS

From the total 81 of samples, there were 11 (13.58%) samples positive on the presence of dermatophyte. Beside the fungal organisms that are the causal agent of superficial mycosis, the presence of a large number of different saprophyte fungi was determined originating from the environment, but not dermatophyte. They were present in more than 90%, i.e. in 74 samples (91.35%) isolated in single or mixed microflora nondermatophyte fungi. The results are displayed in Table 1.

Tab. 1 — The results of the examination of swabs and skin scarifications

Isolate	Number of the samples	Number of positive findings (%)
Mycrosporum spp.	81	11 (13.58%)
Trichophyton spp.	81	Not isolated
Saprophytic fungi	81	74 (91.35%)

The most important dermatophyte that cause the infections of skin are those that belong to keratophyl *Microsporum*, *Trichophyton* and *Epidermophyton* spp. The mentioned dermatophyte were divided in 3 or 4 groups depending on the natural environment where they can be found. In Table 2 the most important kinds of dermatophyte depending on their natural environment are displayed (Outerbridge, Catherine A., 2006, Chabassea, D., Piheta, M., 2008).

Tab. 2 — Most important dermatophyte kinds, depending on their habitat and host

Habitat of dermatophyte	Dermatophyte	Host
Zoophilic dermatophyte	*Microsporum canis*	Dogs and cats
Geophilic dermatophyte	*Microsporum gypseum* *Trichophyton mentagrophytes*	Soil
Sylvatic (forest) dermatophyte (specially adapted zoophil dermatophyte)	*Trichophyton mentagrophytes* *Trichophyton erinacei* *Trichophyton persicolor*	Rodents and hedgehogs
Anthrophilic dermatophyte	*Trichophyton tonsurans* *Epidermofyton floccosum*	Humans (dogs and cats)

In this study, the presence of only one dermatophyte of *Microsporum* (*Microsporum canis*) was detected. No fungi from *Trichopyton* were cultured. According to the data in literature *Microsporum canis* is responsible for 97—100% of cutaneous mycosis in Italy (Mancianti et al., 2003).

Saprophyte fungi, i.e. fungi from the soil and the environment were isolated in a high percentage (91.35%) and the most frequent were *Aspergilus* sp., *Penicillium* sp., *Alternaria* sp., *Mucor* sp. and *Fusarium* sp. Their presence was determined in mixed and single flora in the material where dermatophyte was not isolated, however, they were present, though in a smaller quantity, in the materials that were positive on the presence of causal agents of dermatomycosis. The growth of saprophyte fungi was so abundant, that the precise identification of dermatophyte colonies was often difficult.

In our study only one kind of dermatophyte *M. canis* was isolated and this finding corresponds to data reported by a group of authors (Manciantiet al., 2008) who examined different kinds of therapy. It is important to stress that these authors isolated *M. canis* from the surface of fur, i.e. from the skin of many asymptomatic animals. The findings of dermatophyte on the skins of dogs with no clinical symptoms were also reported by other authors (Ates et al., 2008) in 2.6% cases. However, in their examination the presence of zoophilic dermatophyte *M. canis* was not detected, but geophylic (*M. gypseum*) and forest dermatophyte (*Trichophyton mentagrophytes*) were isolated. The authors (Ilkit et al., 2007) in an area where dogs were analysed on the presence of dermatophyte, also analysed the etiology of dermatological infections in children. It was reported that the changes on the skin, first of all on the head, were caused by the infection with *Microsporum canis*. The results of two aforementioned research point out that dogs can be the carriers of different dermatophyte, but are not a direct cause of dermatomycosis in the humans. Fungi superficial in the humans may be caused by geophylic, zoophilic and antrophilic dermatophyte (*Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton tonsurans* etc.) (Tomson, Nevianna, Sterling, Jane C., 2007).

An overwhelming survey on different dermatophytes in pets is displayed in the work of Hoppmann and Barron (2007), where the presence of dermatomycosis was studied in rodents. In the urban environment there are many different exotic animals and one of them are the rodents. Mice, rats, hamsters, gerbils, chinchilla and other animals are often kept as pets. They may be symptomatic or asymptomatic carriers of different dermatophyte (*M. gypseum, T. mentagrophytes, M. canis*). The owners of these pets are not often aware of their exposure to these zoonosis, because clinical signs of dermatomycosis in the exotic animals is a consequence of stress, bad feed and immunodeficiency.

There are authors (Chen Cheng-Hsu et al., 2008) who explained the risks of keeping pets in the urban environment and found a connection between the skin infection of a patient and her dog pet. Before the clinical symptoms were obvious, the patient with renal transplantation received immuno-suppressive agents. The causal agent of changes on skin was fungi *M. canis* that was also found on her pet dog, which was however asymptomatic. The author's opinion is that dermatomycosis would not have occurred if there was not a disbalance of immune system caused by immunosuppressive agents. This finding points to a continuous exposure to dermatophyte and risks in the cases of co-habiting pets.

CONCLUSION

In our research dermatophyte was determined in 13.58% of samples. The etiology of the disease pointed to these microorganisms, so the therapy could be prescribed. One kind of fungi, from the genus of *Microsporum* (*Microsporum canis*), was detected. Saprophyte fungi were isolated in a great number (91.35%) of samples which revealed contamination of fur and skin in

dogs. However, the presence of dermatophyte may also be "hidden" under this finding.

The presence of dermatophyte without symptoms points out the risk of keeping pets in the urban conditions, where the humans are exposed to closer contact with the animals than it is usual in nature. Therefore, it is important that every pet owner should be acquainted with the risk of keeping pets.

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ДЕРМАТОМИКОЗЕ ПАСА КАО ИЗВОР ЗООНОЗА У ГРАДСКОЈ СРЕДИНИ

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Резиме

Болести коже животиња и људи чине комплексан синдром који етиолошки често није јасан и који из тих разлога није лако решити. Кожа представља један од највећих органа чије су многобројне физиолошке функције важне за организам сисара. У зависности од старосне доби њена маса се може кретати од 5—8% укупне телесне масе код људи, до 12% код одраслих животиња и чак до 24% код новорођене штенади.

Присуство кућних љубимаца у градској средини као и паса луталица, може допринети већем међусобном контакту животиња и/или људи, чиме се повећава вероватноћа преношења неких од биолошких фактора болести коже. Гљивична обољења коже паса узрокована дерматофитима је зоонозна, контагиозна инфекција која угрожава, поред паса и мачке, друге животиње укључујући и људе. Највећи број микоза паса изазван је гљивицама које проузрокују *Microsporum* spp. и *Trichophyton* spp.

Наше истраживање је било везано за псе који су имали различите форме промена на кожи које су се манифестовале појавом обездлачених површина, перутањем коже, црвенилом и израженим пруритусом. Предмет испитивања је била миколошка анализа узетих скарификата и брисева са промењених места на кожи паса са циљем да се утврди да ли су настале промене изазване дерматофитима и, ако јесу, о којим се врстама ради. Исто тако циљ нам је да укажемо и на могућност преношења гљивичних инфекција са животиња на људе у специфичним градским условима одгајања кућних љубимаца.

За миколошко испитивање користили смо брисеве и скарификате коже паса који су долазили на лабораторијску анализу како би се утврдила етиологија обољења. Узети узорци засејани су на Сабуро декстрозни аграр и инкубирани на 25 степени најмање 21 дан. У току једне године испитан је 81 узорак од којих је 11 (13,58%) било позитивно на дерматофите. Код свих позитивних налаза изолован је *Microsporum canis*.

Налаз *Microsporum canis* код 13,58% испитаних узорака показује да су дерматофите значајан узрочник кожних обољења кућних љубимаца. Исто тако, с обзиром да се ради о зоонози, њихов налаз указује на могућност експонирања људи гљивичним инфекцијама, а пре свега власника, што је посебно потенцирано у условима гајења животиња у урбаној средини.

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THE CLINICAL AND PATHOMORPHOLOGICAL DIAGNOSIS OF MYCOTOXICOSIS IN DIFFERENT SWINE CATEGORIES

ABSTRACT: The issue of mycotoxins and mycotoxicosis in veterinary medicine is directly connected to the usage of mouldy and/or adversely stored grains (corn, wheat, barley) used in animal feed. In swine production, in our geographical region, the most common are mycotoxicosis caused by zearalenon (F-2 toxin), but aflatoxins, ochratoxin and trichothecenes can also be found. For the known mycotoxins of clinical importance, the response is usually subacute or chronic and the presenting clinical signs are often vague. Mostly the problems are expressed only as alterations of the reproductive cycle, reduced feed intake and slow growth. However, if we consider the clinical signs and pathomorphological picture of mycotoxicosis in different swine categories (breeding animals, suckling and weaned piglets, fatteners), the age dependent changes can be found. Some mycotoxins have hepatotoxic, nephrotoxic and immunosuppressive effects, which further complicate the clinical and pathomorphological picture and diagnosis of mycotoxicosis in swine.

The material for this research included the samples provided from ten swine farms. In different swine categories health disorders, resembling to the problem with mycotoxins were detected. The applied research methods included clinical evaluation and pathomorphological examination and laboratory microbiological feed testing, in order to examine the presence of fungi and some mycotoxins (aflatoxins, zearalenon, ochratoxin A and trichothecenes).

On the basis of the obtained results, it may be concluded that the most frequently detected mycotoxin in the examined feed samples was zearalenon. The presence of mycotoxin in feed was directly connected to the reproductive failures and diagnosed health disorders in the examined swine categories (vulvovaginitis, skin necrosis, pneumonia, gastroenteritis).

KEY WORDS: diagnosis of mycotoxicosis, swine feed, zearalenon

INTRODUCTION

Mycotoxins are secondary metabolites of moulds, and so far, approximately 400 secondary metabolites with toxigenic potential produced by more than 100 moulds have been reported (Kabak and Dobson, 2006). Several fungi, mainly belonging to the genera: *Aspergillus, Penicillium, Fusarium* and

Alternaria, are detected most frequently. The issue of mycotoxins and mycotoxicoses in veterinary medicine is directly connected to the usage of mouldy and adversely stored grains (corn, wheat, barley) in animal feed (O s w e i l e r, 2006).

In the swine production, in our geographical region, the most common are mycotoxicosis caused by zearalenon (F-2 toxin, ZEA), but also aflatoxins (AF), ochratoxin (OCT) and trichothecenes occur. The problems are expressed mostly only as alterations of the reproductive cycle, reduced feed intake and slow growth (G o n z a l e s and R o d r i g u e z, 2008).

The clinical and patomorphological picture of mycotoxicoses in swine depends on the age and category (breeding animals, suckling and weaned piglets, fatteners). Beside this, some mycotoxins have hepatotoxic, nephrotoxic and immunosuppressive effects (K a b a k and D o b s o n, 2006), which can further complicate the clinical and pathomorphological picture and diagnosis of mycotoxicosis in swine. Immunosuppressive effects of mycotoxins are of special interest and may have significant influence on the occurence of infective diseases of pigs (O b r e m s k i et al., 2008). The connection between clinical cases of common diseases (swine erysipelas, swine disentery and salmonellosis) influenced by AF under experimental conditions are detected (O s w e i l e r, 2006).

MATERIAL AND METHODS

The material for this research included the samples collected from ten swine farms, with different swine categories where health disorders resembling to the problem with mycotoxins were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: epidemiological and clinical evaluation, pathomorphological examination and laboratory microbiological feed testing, in order to examine the presence of fungi and evaluation to detect the presence of mycotoxins by the method of thin layer chromatography.

RESULTS

In seven examined cases, the presence of ZEA in different swine feed was detected. Depending on the swine category (suckling piglets, weaned piglets, fatteners and breeding animals) the clinical symptoms in diseased animals and patomorphological changes in dead pigs were evaluated.

In the clinical cases of ZEA mycotoxicosis in the suckling piglets, the occurence of neonatal diarrhoea already in the first 3 days of life after farrowing were detected. These health problems did not improve after the medical treatment with antibiotics. In great number of just farrowed piglets the most prominent clinical sign was vulvovaginitis (swelling and reddening of the vulva). Beside this, in two cases, a large number of small, weak and splayleg piglets were noticed. Applying patomorphological examination on the dead suckling piglets the prominent changes on mucosal surfaces of the digestive tract (*Gas-*

troenteritis haemorrhagica), fewer number of pale kidneys, necrotic and distrophic processes on liver tissue were detected. By laboratory testing of the available swine feed grains from the stables intended for different swine categories, the presence of ZEA in different concentrations was detected (from 0.72 to 6.4 mg/kg).

The ZEA mycotoxicosis in the weaned piglets was clinically characterised with signs of pneumonia, slow growth, vulvovaginitis and necrosis of the tails, sporadically with diarrhoea and rectal prolapses. The patomorphological examination of the dead weaners revealed the following lesions: bleeding on the mucosal surface of the digestive tract (*Gastroenterotyphlocolitis haemorrhagica*), pleuropneumonia and pneumonia with the sings of purple to gray areas of consolidation, hepatomegalia, focal nephritis and rectal prolapses. Etiologically the pneumonia was caused by *Actinobacillus pleuropneumoniae, Haemophilus suis*, and *Mycoplasma hyopneumoniae.* Another problem that was frequently observed was digestive infection caused by enteropathogenic *Escherichia coli*, which might have been promoted by the presence of mycotoxin (ZEA 0.8 mg/kg) and a high number of different fungi species in the weaners feed (*Fusarium, Penicillium, Aspergillus, Rhisopus*). During evaluation of the farm storage facility, approximatelly 20% of mouldy wheat was found. Having in mind this fact and the clinical symptoms observed in the piglets (necrosis of tails and ears, vomiting and diarrhoea) a justifed suspicion on the presence of ergot alkaloids was made.

In the fatteners, clinical symptoms included vulvovaginitis, bronchopneumonia, bloody diarrhoea, and sporadically rectal prolapses. One of the characterictics of this swine category was transient feed refusal. The patomorphological examination revealed the lesions on the mucosa of digestive organs (*Gastroenteritis haemorrhagica, Dysenteria suum*), pleuropneumonia and pericarditis. After laboratory testing of thecorn samples the presence of ZEA from 0.5 to 0.8 mg/kg was detected.

Only in one case the presence of ZEA was detected and the following clinical symptoms were noticed in the suckling piglets and sows: reduced feed intake, agalactia and endometritis. On the another farm, the clinical symptoms in sows consisted of reproductive disorders with the increased number of rebreeding (= 20%), lower rate of conception (from 93% to 77%), increased number of deadborn (0.85 per litter) and mummified piglets, and decreased litter size. However, it should be noted that apart from the presence of ZEA in feed (6.4 mg/kg), the infection with parvovirus was also diagnosed on the farm, which further complicated the diagnosis.

On two swine farms, in the feed samples the presence of two mycotoxins, ZEA (0.1—4 mg/kg) and OCT-A (0.080—0.12 and 0.5 mg/kg), were simultaneously detected. The following feed samples were tested: corn, piglets first and second feed, feed for pregnant sows and boars. The piglets showed the following clinical signs: feed refusal, reduced growth, diarrhoea, pneumonia. The pathomorphological changes were not different from the aforementioned signs observe on the other farms that consumed feed contaminated with ZEA. However, in sows the reproductive disorders were clinically evident: rebreeding (27%), infertility (20%), anestrous (10.6%) and freqent endometritis.

The presence of AFB1 in the first feed for piglets (0.018 mg/kg) was detected only in one case. Microbiological feed testing detected 3-fold increase in the number of fungi from genera *Penicillium, Aspergillus, Rhisopus* as compared to the level set by the regulation. In the weaned piglets clinical and patomorphological lesions characteristic for the enteropathogenic *Escherichia coli* infection were diagnosed. It can be assumed that this was provoked by the presence of mycotoxin in feed.

Simultaneous presence of several mycotoxins (ZEA, AFB1, AFG1, OCT) was established only in one examined case. The above mentioned mycotoxins were detected in the feed for pregnant and lactating sows (ZEA 0.8 mg/kg; AFB1 0.008 mg/kg; AFG1 0.02 mg/kg; OCT 0.2 mg/kg), in the supplemental feed for fatteners (ZEA 4 mg/kg; AFB1 0.008 mg/kg; AFG1 0.016 mg/kg; OCT 0.5 mg/kg), corn (ZEA 4 mg/kg; AFB1 0.008 mg/kg; AFG1 0.002 mg/kg; OCT 0.2 mg/kg), sunflower pellets (ZEA 4 mg/kg; AFB1 0.016 mg/kg; AFG1 0.008 mg/kg; OCT 1.0 mg/kg), soyabean pellets (ZEA 2.0 mg/kg; AFB1 0.016 mg/kg; AFG1 0.008 mg/kg; OCT 1.0 mg/kg) and animal yeast (ZEA 0.8 mg/kg; AFB1 0.016 mg/kg; AFG1 0.02 mg/kg; OCT 3 mg/kg). Clinical signs were noticed in the piglets and sows. In the sows, gravidity period and farrowing time were prolonged, cases of agalactia (sudden loss of milk and lying on the udder), a small number of stillbirths and mummified piglets were noticed. The newborn piglets were described as weak, nonviable, with diarrhoea. The diseased piglets lived only 4 days after birth. They probably died due to hypoglicemia, because sows did not have enough milk or the piglets were too weak and did not have enough strenght for milk suckling. Sporadically, the occurence of splayleg was observed. By patomorphological examination of the dead suckling piglets the following lesions were evident: pale kidneys, hepatic damage (yellowish color), gastric ulcers, diffuse haemorrhage on internal organs and the brain.

DISCUSSION

On the basis of the obtained results, it may be concluded that the most frequently detected mycotoxin in the examined feed samples was ZEA. The presence of mycotoxin in feed was directly connected to the reproductive failures and health disorders (vulvovaginitis, skin necrosis, pneumonia, gastroenteritis) diagnosed in the examined swine categories. Zearalenone is a mycotoxin which acts as estrogen, binding competitively to estrogen receptors of the uterus, mammary gland, liver and hypothalamus (G a j e c k i, 2002). Pigs are the most susceptible to the presence and negative effects of ZEA (D i e k m a n and G r e e n, 1992; O b r e m s k i et al., 2003). In our research, the perinatal hyperestrogenic syndrome was a constant clinical sign in the suckling piglets. This is certainly the consequence of mycotoxins presence in the feed for sows, i.e. during the pregnancy and the presence of its excreted metabolite in milk of the exposed sows.

The toxic effect of mycotoxins depends on a number of factors: the intake levels, duration of exposure, toxin species, mechanisms of action, meta-

bolism and defense mechanism. The consumption of mycotoxin-contaminated feed leads to the induction of teratogenic, cancerogenic, oestrogenic, neurotoxic and immunosuppresive effects in the organism (K a b a k et al., 2006). The influence on immune system is of special interest in the swine industry. The technology on swine farms demands frequent vaccinations, especially in piglets and sows which may be a problem in the case of immunocompromised animal. From the obtained results, several examples of immunosuppresive effect can be presented. For instance, the occurence of enteroxemia in piglets, despite the fact that dams were vaccinated twice during gestation.

Aflatoxins (B1, B2, G1, G2) are recognised as immunomodulating agents, and when AFB1 is metabolised by mammals it occurs in milk as M1. It is assumed that AFB1 is the most toxic fraction (O s w e i l e r, 2006; Ž i v k o v - - B a l o š et al., 2008). Gross lesions associated with porcine aflatoxicosis include liver fatty degeneration and necrosis: clay-colored liver with hemorrhages; the fibrosis develops, characterised by a firm hard liver with accentuated lobular patern (O s w e i l e r, 2006). In our research, it was discovered the persistant presence of various infections, which reacted poorly or failed to react on the applied antimicrobial therapy (endometritis, pneumonia). Although the examined feed samples did not contain trichothecenes, their strong tendency to induce feed refusal and vomiting in swine should be stressed, which makes them somehow self-limiting as toxins (O b r e m s k i et al., 2008).

The ochratoxin is a nephrotoxic and immunosuppresive mycotoxin, and principal effects are manifested on proximal renal tubules. However, the clinical signs which include polydipsia, polyuria, reduced growth and lower feed efficiency can not be associated with sole ochratoxicosis. The true control should be carried out in the abattoir. It is assumed that pale and firm kidneys (nephrosis and interstitial fibrosis) and gastric ulcers are commonly associated with ochratoxin in endemic areas (O s w e i l e r, 2006). Our results indicate the occurence of slow growth, the increased feed consumption and significant difference in the weight of fatteners as a consequence of mycotoxin feed contamination.

For the known mycotoxins of clinical importance in the swine production, the response is usually subacute or chronic and the presenting signs are often subtle and vague (G o n z a l e s and R o d r i g u e z, 2008). As a consequence of immunosuppresive action of mycotoxins, clinical and pathological lesions correspond to the infective diseases of different etiology (O b r e m - s k i et al., 2008). Dietary deficiencies of protein, selenuim and vitamins have been sugested as predisposing factors in the mycotoxicoses. Combinations of several mycotoxins may potentiate the action of one other, or at least exert an additional effect (O s w e i l e r, 2006). The obtained results strongly support the interaction between the mycotoxins and infective agents.

In the last five years, the swine industry in our country was exposed to the most unfavourable conditions. As a consequence, most of the pig producers blend mycotoxin-contaminated feed with the sound one in such a proportion that animals consume it without any obvious adverse effects on the growth and reproduction. However, economic losses that occur due to usage of blended feed probably stay unknown because low concentrations of several

mycotoxins may interact in ways that are difficult to detect reducing the performance. The continuous intake of small amounts leads to chronic intoxication which is characterised by the loss of weight and insufficient weight gain, fertility disorder or increased susceptibility to infectious diseases. As feedstuffs are mostly contaminated with several different mycotoxins simultaneously, it may be assumed that mycotoxicoses are multicausal (multitoxic) diseases (Diekman and Green, 1992; Osweiler, 2006).

Because of detrimental effects of mycotoxins, a number of strategies have been developed to decontaminate and detoxify mycotoxin-contaminated feed. They may include inhibition of mycotoxin adsorption in the gastrointestinal tract. One of the most recent approaches to the prevention of mycotoxicoses is the addition of non-nutritional adsorbents in the feed that bind mycotoxins in the gastrointestinal tract and reduce their bioavailability. The activated carbons, aluminosilicate, zeolites, bentonites and certain clays are well known. A novel strategy to control the problem of mycotoxicoses in animals is the application of microorganisms, yeasts (Eubacterium-BBSH 797; Trichosporon MTV, 115) capable of biotransforming mycotoxins into nontoxic metabolites (Kabak et al., 2006). The basic preventive measures in order to protect the animals are usage of healthy feed and proper storage and condition management of animal feed. Certainly, if mycotoxicosis occurs or is suspected, the first action should be the change of the source of feed. Mycotoxicoses are generally a herd problem and not amenable to individual treatment. Practical preventive program should be the part of every swine management program.

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КЛИНИЧКА И ПАТОМОРФОЛОШКА ДИЈАГНОСТИКА МИКОТОКСИКОЗА КОД РАЗЛИЧИТИХ КАТЕГОРИЈА СВИЊА

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Резиме

Примена плеснивих и/или неадекватно ускладиштених житарица у исхрани животиња (кукуруз, пшеница, јечам) директно се повезује са проблемом микотоксина и микотоксикоза у ветеринарској медицини. На нашем географском подручју у свињарској производњи су најчешће микотоксикозе узроковане зеараленоном (Ф-2 токсин), али су значајни и афлатоксин, охратоксин и трихотецени. За микотоксикозе које су од клиничког значаја ток обољења је најчешће субакутан или хроничан и клинички знаци су нејасни. У највећем броју случајева здравствени проблеми се огледају у поремећају репродуктивног циклуса, успореном расту и смањеној конзумацији хране. Међутим, када је у питању клиничка и патоморфолошка манифестација микотоксикоза у свињарској производњи постоји изражена специфичност узраста (приплодне јединке, прасад на сиси и одгоју и товљеници). Поред тога, поједини микотоксини имају изражен хепатотоксични, нефротоксични и имуносупресивни ефекат, што даље компликује клиничку и патоморфолошку манифестацију обољења и дијагностику микотоксикоза код свиња.

Материјал за испитивање је обухватао десет фарми свиња, на којима су регистровани здравствени проблеми код различитих категорија свиња а који су указивали на потенцијални проблем микотоксикозе. У оквиру примењених метода обављени су клиничка испитивања, патоморфолошки преглед угинулих јединки и микробиолошко испитивање хране у циљу установљавања присуства плесни и микотоксина (афлатоксин, зеараленон, охратоксин А и трихотецени). Постигнути резултати испитивања указују да је у храни за свиње најчешће установљено присуство микотоксина зеареленона. Присуство микотоксина у храни доводи се директно у везу са поремећајем репродуктивне ефикасности и различитим поремећајима здравственог статуса код испитиваних категорија свиња (вулвовагинити, некрозе коже, пнеумоније и гастроентерити).

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EFFECT OF CULTIVATION CONDITIONS ON LIGNINOLYTIC ENZYME PRODUCTION BY *GANODERMA CARNOSUM*

ABSTRACT: *Ganoderma carnosum* has been found in Europe only, at coniferous trees and it is difficult to distinguish it morphologically from *G. lucidum*. Since *G. carnosum* has not been studied biochemically yet, the aim of this study was to analyse its ability to produce Mn-dependent peroxidase (MnP), versatile peroxidase (VP) and laccase (Lac) under conditions of solid-state fermentation of selected plant raw materials as carbon sources, in the presence of different nitrogen concentrations in the medium. Wheat straw, corn stem, oak and grapevine sawdust were the analysed plant raw materials. Nitrogen source in synthetic medium was NH_4NO_3 and its concentrations were: 10mM N and 20 mM N. Enzyme activity was determined spectrophotometrically, using ABTS and phenol red, as the substrates for Lac and Mn-oxidizing peroxidases, respectively. Maximum level of MnP activity (56.82 U/l) was obtained in the medium with wheat straw and nitrogen concentration of 10 mM. Best carbon source for VP production was grapevine sawdust at nitrogen concentration of 10 mM (80.80 U/l). The obtained Lac activity was very low in the medium with wheat straw (1.80 U/l), while it was not detected in the presence of other three analyzed plant raw materials. Maximum of total protein content (0.06 $mgml^{-1}$) was noted in the medium where oak sawdust was carbon source and nitrogen concentration was 20 mM.

KEY WORDS: *Ganoderma carnosum*, laccase, Mn-dependent peroxidase, nitrogen concentration, plant raw materials, versatile peroxidase

INTRODUCTION

White-rot fungi are capable of degrading all basic wood polymers, due to their ability to synthetise relevant hydrolytic and oxidative extracellular enzymes. These enzymes are responsible for the degradation of cellulose, hemicellulose and lignin into low-molecular-weight compounds that can be assimilated for fungi nutrition (Songulashvili et al., 2007). Due to their low substrate specificity, ligninolytic enzymes can oxidize a wide range of compounds

with structural similarities to lignin, so they have important role in bioremediation of various toxic compounds in soil and waste waters (Winquist et al., 2008). The enzymes produced by *Ganoderma* species as white-rot ones are: lignin peroxidases (LiP), Mn-oxidizing peroxidases (Mn-dependent peroxidases (MnP) and versatile peroxidases (VP)), and laccases (Lac). Contrary to *G. lucidum*, which ligninolytic system is extensively studied during the last few decades, the other species of this genus are not adequately investigated. *Ganoderma carnosum* Pat. (syn. *G. atkinsonii* Jahn, Kotl. and Pouz.) has only been found in Europe only and it is difficult to distinguish it morphologically from *G. lucidum*. This species belongs to the *G. valesiacum* complex together with *G. oregonense*, *G. tsugae* and *G. valesiacum*. This group is apparently restricted to coniferous forests in the Northern Hemisphere (Moncalvo et al., 1995). *G. lucidum*, a white-rot Basidiomycetes is widely distributed worldwide and grows predominantly on deciduous trees (*Quercus*, *Acer*, *Alnus*, *Betula* etc.) and rarely on coniferous trees (*Larix*, *Picea*, *Pinus*) (Wasser and Weis, 1997). In contrast to *G. lucidum*, this species has not been studied biochemically yet (Keller et al., 1997). Therefore, the aim of this study was to research the effect of different plant raw materials used as carbon sources, and different nitrogen concentrations on the enzyme production by *G. carnosum* under the conditions of solid-state cultivation.

MATERIAL AND METHODS

Organism and growth conditions

Ganoderma carnosum, collected from conifer tree in Rožaje (Montenegro), was used in this study. The culture was preserved on malt agar medium, in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (Serbia).

The inoculum was prepared by inoculation of 100 ml of synthetic medium (glucose, 10.0 gl^{-1}; NH_4NO_3, 2.0 gl^{-1}; K_2HPO_4, 1.0 gl^{-1}; NaH_2PO_4 x H_2O, 0.4 gl^{-1}; $MgSO_4$ x $7H_2O$, 0.5 gl^{-1}; yeast extract, 2.0 gl^{-1}; pH 6.5) with 25 agar discs (Ø 0.5 cm) taken from 7 day-old *G. carnosum* culture. Incubation was performed at room temperature (22±2°C), on a rotary shaker (160 rpm), during 7 days. The obtained biomass was washed 3 times by sterile distilled water (dH_2O) and homogenized with 100 ml of sterile dH_2O in a laboratory blender.

Effect of different plant raw materials as carbon sources and nitrogen concentrations on the production of laccase and Mn-oxidizing peroxidases

Analyzed plant raw materials were: wheat straw, corn stem, oak and grapevine sawdust. Solid-state fermentation was carried out at 25°C in 100 ml flasks containing 2g of analysed plant residue soaked with 10 ml of the modi-

fied synthetic medium, without glucose, and with one of the two tested concentrations of nitrogen (10 mM and 20 mM) in the form of NH_4NO_3 and pH 5.0. Thus, the prepared flasks were inoculated with 3 ml of homogenized inoculum. Samples from flasks were harvested after 7 days of cultivation, and extracellular enzymes were extracted by stirring of samples with 50 ml of dH_2O on a magnetic stirrer for 10 min at temperature of 4°C. The obtained extracts were separated by centrifugation (4°C, 3000 rpm, 15 min), and the obtained supernatants were further used for measurements of the Lac, MnP and VP activity, as well as total protein content. Three replications for each analysed plant residue and nitrogen concentration were prepared, in order to decrease statistical error.

Enzyme activity assays

Laccase activity was assayed spectrophotometrically, using 50 mM ABTS (e_{436} = 29300 $M^{-1}cm^{-1}$) as a substrate, in a phosphate buffer (pH 6.0). The reaction mixture contained: buffer, ABTS, and sample (V_{tot} = 1 ml).

Mn-oxidizing peroxidases activities were determined with 3 mM phenol red (e_{610} = 22000 $M^{-1}cm^{-1}$) as a 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. The reaction mixture contained: buffer, sample, 2 mM H_2O_2, and phenol red, with or without 2 mM $MnSO_4$, for MnP and VP, respectively (V_{tot} = 1 ml). Reaction was stopped by adding 2 M NaOH. Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 mmol of substrate per minute. An UV-160 A Spectrophotometer (Shimaden) was used for these assays.

Determination of total proteins

The amount of total proteins was determined by means of a standard curve obtained from solutions containing bovine serum albumin at known concentrations (0.00; 0.01; 0.02; 0.03; 0.04; 0.05; 0.06; 0.07; $mgml^{-1}$), Bradford's reagent (0.2 ml), and sufficient water to complete a final volume of 1 ml. The mixture contained 0.80 ml of the sample and 0.20 ml of Bradford's reagent, and absorbance was measured at 595 nm after reaction for 5 minutes, at room temperature. Total protein content is given in $mgml^{-1}$.

RESULTS AND DISCUSSION

After 7 days of solid-state cultivation of *G. carnosum*, the activity of MnP and VP was detected in the media with all tested plant raw materials and both analysed nitrogen concentrations. In contrast to them, Lac activity was detected only in the medium with wheat straw as a carbon source (Fig. 1).

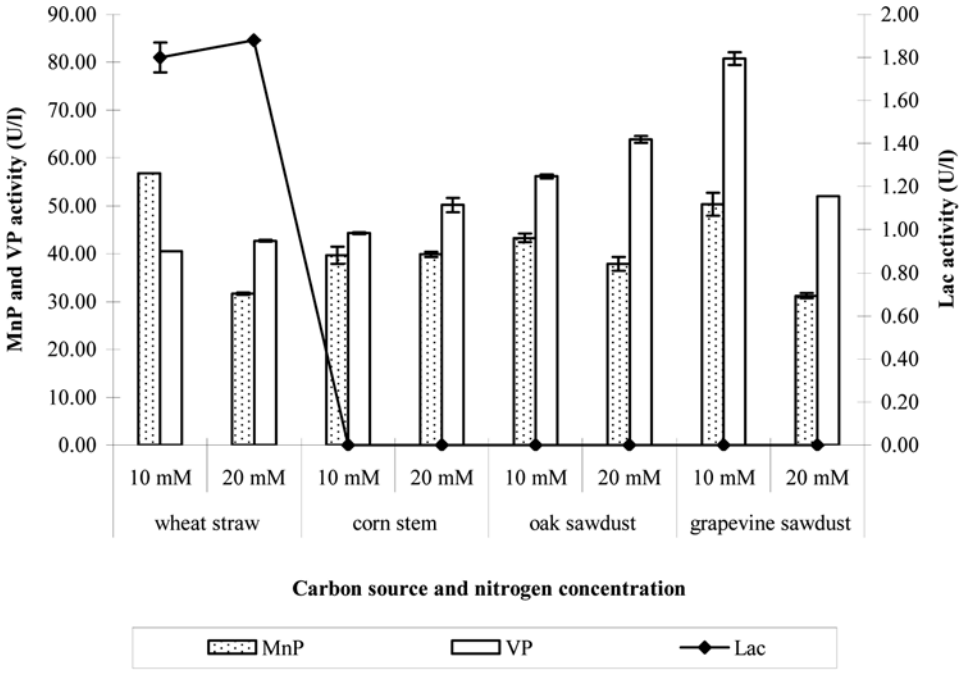

Fig. 1 — MnP, VP and Lac production depending on carbon source and nitrogen concentration

The maximum of MnP activity (56.82 U/l) was obtained in the medium with wheat straw and nitrogen concentration of 10 mM, which is in accordance with the results of Camarero et al. (1996). An increase in the nitrogen concentration to 20 mM has led to a decrease of MnP production (31.64 U/l) (Fig. 1). This can be explained by the fact that nitrogen limitation in the medium is trigger for ligninolytic enzyme production (Hammel, 1997). Numerous studies have also shown that high nitrogen levels repressed ligninolytic enzyme production in *Phanerochaete chrysosporium*, *Trametes versicolor* and *Pycnosporus cinnabarinus* (Buswell et al., 1984; Eriksson et al., 1990; Tekere et al., 2001). However, in *Pleurotus ostreatus* high concentration of nitrogen in the medium did not repress but slightly stimulated mineralization of lignin, as compared to the nitrogen — limited medium (Kaal et al., 1995). Similar, but slightly lower value for MnP activity was detected in the medium with grapevine sawdust, at nitrogen concentration of 10 mM (50.34 U/l), while at nitrogen concentration of 20 mM MnP activity was significantly lower (31.18 U/l). Grapevine sawdust was also good substrate for Mn-oxidizing peroxidases production by *P. ostreatus* and *P. pulmonarius* (Stajić et al., 2006). The level of MnP production was lower in the media with corn stem and oak sawdust as carbon sources than in the two afore mentioned carbon sources. The measured values were similar in these media and ranged between 37.90 and 43.30 U/l (Fig. 1).

The VP activity profile showed that optimal carbon source was grapevine sawdust with nitrogen concentration of 10 mM (80.80 U/l). This is in accordance with some earlier results (S t a j i ć et al., 2006) which showed that grapevine sawdust is good substrate for VP production by *P. ostreatus* and *P. pulmonarius*. Production of VP has decreased with increasing the nitrogen concentration to 20 mM (52.04 U/l). Relatively high values for VP production were detected in the medium with oak sawdust as carbon source and both nitrogen concentrations (56.19 U/l and 63.86 U/l at the nitrogen concentration of 10 mM and 20 mM, respectively). Slightly lower VP activity was noted in the medium with corn stem as carbon source, but the minimum of VP production (40.45 U/l) was obtained in the medium with wheat straw, at the nitrogen concentration of 10 mM, contrary to MnP production which had the peak of activity in this medium. One more difference observed in relation to MnP production is that higher nitrogen concentration has led to an increased VP production, in all analysed media, exept the medium with grapevine sawdust.

Laccase production was detected only in the medium with wheat straw as carbon source, and obtained activities were similar at both nitrogen concentrations (1.80 U/l at 10 mM N and 1.88 U/l at 20 mM N) (Fig. 1). These results show that either the cultivation conditions or the selected species were reason for low level of Lac production (S t a j i ć et al., 2009).

Maximum of total protein content of 0.060 mgml^{-1} was detected in the medium with oak sawdust and nitrogen concentration of 20 mM, and the mini-

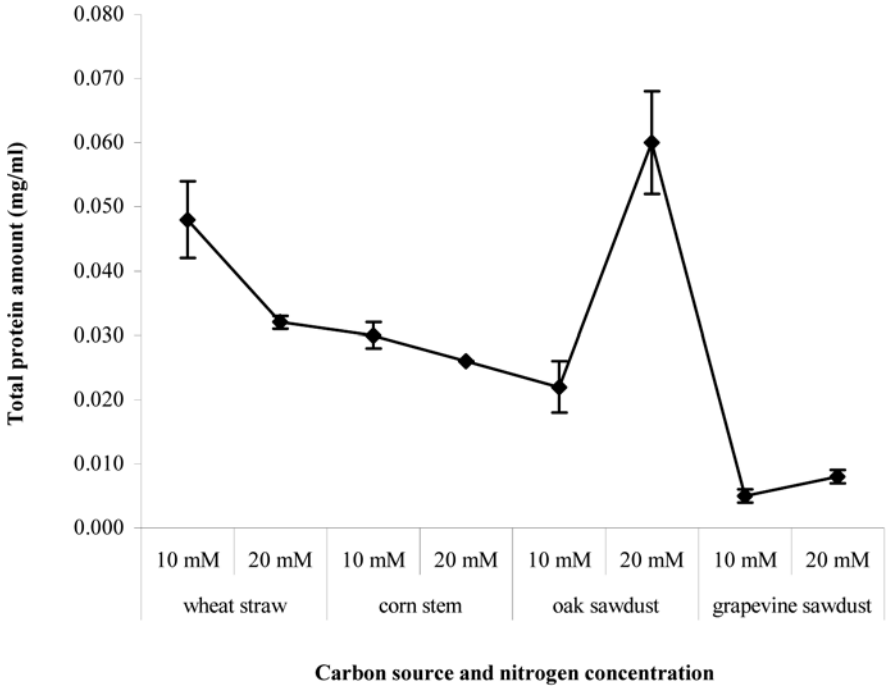

Fig. 2 — Effect of selected plant raw materials and nitrogen concentrations on total protein content

mum one of 0.005 $mgml^{-1}$ is measured in medium with grapevine sawdust and nitrogen concentration of 10 mM (Fig. 2).

CONCLUSION

According to the presented results, it can be concluded that different plant residues used as carbon sources, as well as nitrogen concentrations, considerably affect the production of MnP, VP and Lac and total protein content by *G. carnosum*, during solid-state cultivation. Moreover, *G. carnosum* is a far weaker producer of ligninolytic enzymes than *G. lucidum*.

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УТИЦАЈ УСЛОВА КУЛТИВАЦИЈЕ НА ПРОДУКЦИЈУ ЛИГНИНОЛИТИЧКИХ ЕНЗИМА КОД *GANODERMA CARNOSUM*

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Резиме

Врсте рода *Ganoderma* су продуценти многих биолошки активних супстанци па су објекти проучавања многих медицинских и фармацеутских студија. У новије време се све више пажње поклања упознавању њиховог лигнинолитичког ензимског система, у циљу успешне примене у различитим биотехнолошким процесима. За разлику од *G. lucidum*, чији је лигнинолитички систем интензивно проучаван, остале врсте овог рода су ретко биле објекти истраживања. *G. carnosum* се тешко морфолошки разликује од *G. lucidum*. и још увек није биохемијски проучена, па је циљ нашег истраживања био анализа продукције Mn-оксидујућих пероксидаза и лаказа у условима чврсте културације, на различитим биљним остацима (пшенична слама, стабљике кукуруза, пиљевина храста и винове лозе) у присуству азота у облику NH_4NO_3 и у концентрацијама од 10 mM, односно 20 mM. Максимум продукције Mn-зависне пероксидазе добијен је на пшеничној слами при концентрацији азота од 10 mM (56.82 U/l). Пиљевина винове лозе и концентрација азота од 10 mM били су оптимални за синтезу верзатил пероксидазе (80.80 U/l). Добијена активност лаказа је била изузетно ниска у медијуму са пшеничном сламом (1.80 U/l), док на осталим биљним остацима није забележена. Максимални садржај укупних протеина је био највећи у медијуму са пиљевином храста као извором угљеника и концентрацијом азота од 10 mM.

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THE INFLUENCE OF TECHNOLOGICAL PARAMETERS ON MALT QUALITY PRODUCED FROM DIFFERENT TRITICALE VARIETIES

ABSTRACT: The analysis of eight different triticale varieties, from selective experiments, crop 2007, from Rimski Šančevi (Serbia) location was performed. On the basis of results obtained from triticale and produced triticale malt analysis as well as during micromalting, technological quality of investigated triticale varieties was evaluated. The aim of the work was to determine if the gibberellic acid added during the germination phase had a positive influence on the degradation of triticale grain during micromalting. Based on the obtained results it was concluded that triticale variety has the most important influence on the quality of produced triticale malt.

INTRODUCTION

Triticale (*Triticosecale* spp. Wittmack) is the first manufactured cereal derived from an amphiploid between wheat (*Triticum* spp.) and rye (*Secale* spp.). Research efforts have resulted in triticale becoming environmentally more flexible than other cereals, showing better tolerance to many diseases and pests than its parental species and being capable of producing much higher yields and biomass than other cereals. Consequently, the significant increase in the area of triticale production over recent years is a testimony to its future potential (Glatthar et al., 2003).

Triticale shows a number of advantages for the grower. The main distinguishing features are as follows: higher grain yield even in unfavorable conditions, higher test weight, resistance to soil-climatic conditions, tolerance to dryness, tolerance to more acid soils and lower requirement of nutrient substances. Also, it does not need as much fertilizer when compared to types and

varieties providing the same yields (K u č e r o v á, 2007). Triticale has a lower susceptibility to diseases and pests which attack rye and wheat and this reduces the necessity of chemical protection against harmful agents (O e t t l e r, 2005).

Adjuncts are used in brewhouse operations to increase extract yield and beer stability, and possibly to reduce brewing costs (A g u, 2002). The use of cereal-based brewing adjuncts to partially substitute malt in the grist is becoming a standard procedure in brewing today (G l a t t h a r et al., 2003). It is estimated that the current share of mixed grists of malt and adjuncts reaches 90% (G l a t t h a r et al., 2002). Despite the undisputed economic role of adjunct utilization, beer quality is based on wort composition rather than wort price. Thus, the brewer needs to ensure that wort prepared from mixed grists of malt and adjuncts does not diminish the traditionally high quality standards (G l a t t h a r et al., 2003).

In addition to such agricultural considerations triticale shows promising brewing properties (G l a t t h a r et al., 2003). Some triticale lines contain high levels of amylolytic activity in their unmalted natural form, in conjunction with lower levels of proteolytic activity. Because of this and the low gelatinization range (59—65°C), triticale is capable of degrading its own starch content with efficiencies roughly equal to those of barley malt. Based on these facts, it can be assumed that triticale could be used as a brewing adjunct that would provide high malt ratios (> 30—50%) without the need for the addition of microbial enzymes during mashing, as currently practiced (G l a t t h a r et al., 2002).

Triticale's high α-amylase activity has a positive side for malting and brewing. Using the same malting conditions, P o m e r a n z et al. (1970) compared the malting quality of several triticales from the United States and Canada with U.S. barleys. Triticale had higher malt losses, but higher malt extracts, higher diastatic power, and higher α- and β-amylase activity than barley. G u p t a et al. (1985) confirmed the high malting value of triticale. Additionally, both duration of germination and steeping moisture significantly influenced malt losses; the highest malt losses and the highest enzymatic (amylase and protease) activity were achieved when 42% steeping moisture, instead of 38%, and 4 to 6 days of germination, in the presence of gibberelic acid, were used.

MATERIALS AND METHODS

Eight different triticale varieties, crop 2007, from Rimski Šančevi location (Serbia), were investigated. The investigations included: (a) triticale analyses; (b) micromalting of triticale; (c) following the changes during micromalting; and (d) analyses of the obtained triticale malts. Triticale and triticale malt analyses were performed using the standard European Brewery Convention, Analytica — EBC (1998) and/or MEBAK (1997) methods. Micromalting was carried out by the standard procedure (S c h u s t e r et al., 1999) using the micromalting plant "Seeger" (Germany). Tap water was used during micromalting. Micromalting scheme of barley samples is presented in Table 1.

Tab. 1 — Micromalting scheme of triticale samples

1st day	Immersion steeping for 6 hours, t = 15°C; Dry steeping for 18 hours, t = 15°C
2nd day	Immersion steeping for 4 hours, t = 15°C Dry steeping for 20 hours, t = 15°C
3rd day	Immersion steeping for 2 hours, t = 15°C Dry steeping for 22 hours, t = 15°C Moisture correction to 44.5% by spraying with water
4th day	Germination at 15°C, turning over
5th day	Germination at 15°C, turning over
6th day	Germination at 15°C, turning over
7th day	Germination at 15°C, turning over Drying according to the given programme
8th day	Drying finished, malt degermination, measuring and packing in polyethylene bags

RESULTS AND DISSCUSION

Results of triticale analyses, crop 2007, from Rimski Šančevi location are given in Table 2.

Tab. 2 — Triticale analyses, crop 2007, Rimski Šančevi location

Triticale sample	1	2	3	4	5	6	7	8
	NST3/07	NST4/03	NST5/06	NST6/06	NST7/06	NST8/05	NST13/06	NST14/ 06
Mechanical analysis								
Sorting:								
Sieving test, > 2.8 mm, %	54.3	40.8	55.6	53.1	35.3	12.3	65.6	56.3
Sieving test, > 2.5 mm. %	36.3	43.7	31.9	35.3	47.5	55.2	26.1	32.2
1st class grain, %	90.6	84.5	87.5	88.4	82.8	67.5	91.7	88.5
2nd class grain, %	6.7	12.3	7.4	7.6	12.4	26.0	5.2	7.8
3rd class grain, %	2.4	3.2	5.1	4.0	4.8	6.5	3.1	3.7
Thousand-kernel weight, g DM*	40.30	30.32	40.79	40.31	36.26	31.97	44.03	41.30
Test weight. g/hl	80.0	77.5	75.3	76.4	79.1	77.9	79.4	78.8
Physiological analysis								
Germination energy, 3 days. %	96	94	97	95.5	95	99	92	95.5
Water sensitivity, 3 days. %	0	6.5	0	0	0	1	0	0
Germination energy, 5 days. %	97.5	96	97.5	97.5	97.5	99.5	93	96
Water sensitivity, 5 days. %	0	2	0	0	0	0.5	0	0
Chemical analysis								
Moisture content of grain, %	10.48	9.85	10.30	10.36	10.05	9.98	10.19	10.07
Protein content, % DM	12.71	11.17	9.73	9.22	11.85	9.56	10.80	13.04

* DM — dry matter

The results of triticale analyses, presented in Table 2, show that the investigated varieties have high thousand-kernel weight (40.30 to 44.03 g dry matter (DM) except for the samples 2, 5 and 6 and high share of 1st class grains in the samples 1 and 7 (above 90%).

According to the results of physiological analysis, all samples were characterized with satisfactory and good germination and low hydrosensibility except for the sample 7.

The moisture content in all triticale samples was normal, which is important for safe storage. The protein content was very good (bellow 12% dry matter) except in the samples 1 and 8.

The results of triticale micromalting without and with the addition of gibberellic acid are given in Table 3 and 4. The steeping conditions of all barley samples were the same, and regarding the moisture change of grains, the steeping was performed uniformly in all samples. Appropriate steeping level (44.5%) was achieved in all samples. In the samples treated with gibberellic acid much higher steeping level was achieved than in the samples without the addition of gibberellic acid (approximately 1%, depending on triticale vairety). In all triticale varieties treated with gibberellic acid, the malt moisture content was lower than in the non-treated samples. Lower total malting losses were obtained in the samples with the addition of gibberellic acid (approximately 1—2% dry matter).

Tab. 3 — Results of micromalting triticale varieties, crop 2007, Rimski Šančevi location

Triticale sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	NST3/07	**NST4/03**	**NST5/06**	**NST6/06**	**NST7/06**	**NST8/05**	**NST13/06**	**NST14/ 06**
Moisture after 24 h of steeping, %	34.72	34.67	33.45	34.19	33.57	34.48	33.97	33.48
Moisture after 48 h of steeping, %	42.47	42.65	41.29	41.95	41.21	42.37	41.61	41.22
Moisture after 24 h of steeping, %	47.47	47.09	45.90	46.96	46.14	46.54	46.48	46.02
Moisture content of green malt, %	46.99	47.40	45.24	47.25	45.85	47.12	46.33	46.16
Malt moisture content, %	4.28	4.13	4.20	4.20	4.35	4.10	4.35	4.40
Malting losses, % DM*	11.46	12.58	10.72	11.72	9.40	10.51	11.60	10.49
— respiration losses, % DM	7.61	7.69	7.09	7.02	6.21	7.53	7.13	7.09
— rootlets, % DM	3.85	4.89	3.63	4.70	3.19	2.98	4.47	3.40

* DM — dry matter

Tab. 4 — Results of micromalting triticale varieties with the addition of gibberellic acid, crop 2007, Rimski Šančevi location

Triticale sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	NST3/07	**NST4/03**	**NST5/06**	**NST6/06**	**NST7/06**	**NST8/05**	**NST13/06**	**NST14/ 06**
Moisture after 24 h of steeping, %	34.75	34.60	33.22	33.39	33.25	34.27	33.84	33.65
Moisture after 48 h of steeping, %	42.92	42.99	41.95	41.84	41.67	42.89	41.98	41.90
Moisture after 24 h of steeping, %	48.52	48.15	47.30	47.50	47.12	48.25	47.39	47.26
Moisture content of green malt, %	48.04	47.60	46.89	47.56	47.01	47.56	46.68	47.31
Malt moisture content, %	3.94	3.91	3.70	3.88	3.96	3.74	3.98	3.95
Malting losses, % DM*	10.49	10.37	9.48	11.49	8.97	10.16	9.07	9.36
— respiration losses, % DM	6.70	6.60	6.53	7.08	6.04	6.58	6.13	6.43
— rootlets, % DM	3.79	3.78	2.95	4.40	2.94	3.58	2.94	2.94

* DM — dry matter

The analytical quality parameters of malts produced from the triticale varieties without and with the addition of gibberellic acid are displayed in Tables

5 and 6. The test weight of malt was lower in all triticale malt samples treated with gibberellic acid that points to better degradation. The thousand-kernel weights in the samples without and with the addition of gibberellic acid were similar.

The extract content in the fine grist, was very high (above 81% dry matter) in all samples, which is very important for the application of triticale in brewing. Saccharification was very good (bellow 10 minutes) in all samples.

The viscosity of wort and extract difference of finely and coarsely ground malt are the most important parameters of cytolytic degradation, i.e. the most important component is b-glucan; and the lower the parameter, the better the cytolytic degradation. The viscosity of worts produced with the addition of gibberellic acid was lower than in the non-treated samples.

The degradation degree of proteins, expressed as Kolbach indice, presents the percentage share of soluble nitrogen in total nitrogen (S c h u s t e r et al., 1999). In the three samples (1, 5 and 8) not treated with gibberellic acid protein degradation was adequate for malt used in brewing, whereas in the other samples protein degradation was excellent. In all samples treated with gibberellic acid excellent protein degradation was achieved (much higher soluble nitrogen levels). In separate studies, G u p t a et al. (1985) and P o m e r a n z et al. (1970) found that worts obtained from triticale malts were high in nitrogenous compounds and dark in colour, indicating high malt proteolytic activity. B l a n c h f l o w e r and B r i g g s (2006) confirmed that addition of gibberellic acid during the germination of triticale increases protein degradation.

The relative extract VZ 45°C points to activity of proteolytic and other enzymes, with the exception of a-amylase, and is the measure of malt degradation. The standard value for barley malt is 36%. Regarding the presented results of VZ 45°C for the investigated malts, all samples (with and without the addition of gibberellic acid) had very good degradation according to this parameter.

Tab. 5 — Results of malt analyses obtained from triticale varieties, crop 2007, Rimski Šančevi location

Triticale sample	1	2	3	4	5	6	7	8
	NST3/07	NST4/03	NST5/06	NST6/06	NST7/06	NST8/05	NST13/06	NST14/ 06
Malt analyses								
Extract content, fine grist, % DM*	83.86	85.25	86.63	85.02	84.11	85.02	83.73	82.98
— Saccharification. min	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
— Wort clarity	opal	opal	clear	slightly opal	slightly opal	slightly opal	opal	opal
— Filtration. min	> 60	> 60	29	> 60	> 60	> 60	> 60	> 60
— Wort color, EBC units	13.0	10.0	7.0	8.0	7.0	9.5	8.5	8.5
— pH of wort	5.84	5.85	5.84	5.83	5.85	5.80	5.86	5.83
— Soluble nitrogen, mg/100 ml	79.80	87.29	99.40	93.80	76.65	68.25	87.15	92.05
— Soluble nitrogen, % DM	0.71	0.78	0.89	0.84	0.68	0.61	0.78	0.82
— Viscosity, mPa·s, 8.6%e	2.132	2.070	2.361	2.347	1.977	2.087	2.088	2.081
Extract difference, % DM	2.24	1.88	3.24	3.04	1.66	2.07	2.07	1.99
Kolbach indice, %	35.01	43.53	56.99	56.72	36.08	39.73	45.00	39.37

Hartong VZ 45°C, %	48.19	52.93	47.85	53.27	42.56	48.11	56.64	57.56
Test weight, kg/hl	79.3	77.0	74.8	75.9	78.5	77.3	78.7	78.1
Thousand-kernel weight, g DM	35.94	31.60	38.40	36.57	32.58	28.37	38.10	37.14

* DM — dry matter

Tab. 6 — Results of malt analyses obtained from triticale varieties with the addition of gibberellic acid, crop 2007, Rimski Šančevi location

Triticale sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	NST3/07	**NST4/03**	**NST5/06**	**NST6/06**	**NST7/06**	**NST8/05**	**NST13/06**	**NST14/0 6**
Malt analyses								
Extract content, fine grist, % DM*	83.73	85.68	86.98	85.28	83.51	86.48	83.50	81.57
— Saccharification. min	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
— Wort clarity	opal	opal	slightly opal	opal	opal	opal	opal	opal
— Filtration. min	> 60	> 60	> 60	> 60	> 60	> 60	> 60	> 60
— Wort color, EBC units	13.0	11.0	10.0	10.0	6.5	8.5	7.5	8.5
— pH of wort	5.84	5.88	5.85	5.87	5.84	5.81	5.87	5.88
— Soluble nitrogen, mg/100 ml	102.20	121.80	124.60	107.10	113.75	144.90	108.15	129.50
— Soluble nitrogen, % DM	0.91	1.08	1.11	0.95	1.01	1.29	0.96	1.15
— Viscosity, mPa·s, 8.6%e	1.870	1.895	2.154	2.181	1.781	1.809	1.828	1.916
Extract difference, % DM	2.41	2.45	3.09	3.29	1.80	1.95	2.01	2.64
Kolbach indice, %	44.66	60.61	71.07	64.55	53.29	84.11	55.62	55.07
Hartong VZ 45°C, %	49.65	52.64	49.91	54.46	46.48	49.96	62.17	63.54
Test weight, kg/hl	51.3	51.3	51.2	52.5	48.5	51.0	51.4	51.7
Thousand-kernel weight, g DM	36.22	31.92	37.08	36.47	32.98	28.33	39.66	37.81

* DM — dry matter

CONCLUSIONS

All malt samples produced from different triticale varieties had high extract content. Saccharification was under 10 minutes for all samples, which indicates good activity of amylolytic enzymes. All produced triticale malt samples had high proteolytic degradation. Total degradation expressed as Hartong index VZ 45°C was very good in all triticale malt samples.

The obtained results from triticale and triticale malt analysis, as well as from micromalting confirmed that all triticale varieties could be considered as barley malt replacement in beer production. Gibberellic acid had possitive influence on triticale grain degradation during micromalting.

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УТИЦАЈ ТЕХНОЛОШКИХ ПАРАМЕТАРА НА КВАЛИТЕТ СЛАДА ПРОИЗВЕДЕНОГ ОД РАЗЛИЧИТИХ СОРТИ ТРИТИКАЛЕА

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Резиме

Извршена је анализа осам различитих сорти тритикалеа из селекционих огледа, жетве 2007. године, са локалитета Римски Шанчеви. На основу анализа узорака тритикалеа, добијених сладова као и током микросладовања изведени су закључци о технолошком квалитету испитиваних сорти тритикалеа. Циљ рада је био да се утврди да ли гиберелинска киселина позитивно утиче на разградњу зрна тритикалеа током микросладовања. Извршено је микросладовање под стандардним условима и анализирани су добијени сладови. У току сладовања додата је гиберелинска киселина у циљу поспешења разградње зрна тритикалеа. На основу добијених резултата може се закључити да сорта тритикалеа има највећи утицај на квалитет произведеног слада.

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EFFECT OF YEAST STORAGE TEMPERATURE AND FLOUR COMPOSITION ON FERMENTATIVE ACTIVITIES OF BAKER'S YEAST

ABSTRACT: Baker's yeast is a set of living cells of *Saccharomyces cerevisiae.* It contains around 70—72% of water, 42—45% of proteins, around 40% of carbohydrates, around 7.5% of lipids (based on dry matter), and vitamin B-complex. On the basis of yeast cell analysis it can be concluded that yeast is a complex biological system which changes in time. The intensity of the changes depends on temperature. Yeast sample was stored at 4°C i 24°C for 12 days. During storage at 4°C, the content of total carbohydrates decreased from 48.81% to 37.50% (dry matter), whereas carbohydrate loss ranged from 40.81% to 29.28% at 24°C. The content of trehalose was 12.33% in the yeast sample stored at 4°C and 0.24% at 24°C. Loss of fermentative activity was 81.76% in the sample stored at 24°C for 12 days. The composition of five samples of 1st category flour was investigated. It was found that flours containing more reducing sugars and maltose enable higher fermentation activities. The flours with higher ash content (in the range 0.5—0.94%) had higher contents of phytic acid. Higher ash and phytic contents in flour increased the yeast fermentative efficiency. In bakery industry, a range of ingredients has been applied to improve the product's quality such as surface active substances (emulsifiers), enzymes, sugars and fats. In the paper, the effect of some ingredients added to dough (margarine, saccharose, sodium chloride and malted barley) on the yeast fermentative activity was studied. The mentioned ingredients were added to dough at different doses: 0.5, 1.0, 1.5 and 2.0%, flour basis. It was found that the investigated ingredients affected the fermentative activity of yeast and improved the bread quality.

KEY WORDS: baker's yeast, fermentative activity, malted barley, margarine, sodium chloride, saccharose

INTRODUCTION

The quality monitoring of wheat grains and wheat flour has been intensively performed in order to assure good quality of bread and other bakery products (Pejin et al., 2007). The quality of grains and flour depends on spontaneous (active and passive) chemical, physical, biochemical and microbiological processes which develop in the grains (Gestenkorm et al.,

1990). The grain composition is determined by variety, applied agro technical measures and climatic conditions during vegetation. In the process of grain milling, different types of flour are produced.

Wheat flour is the major ingredient in many products and consequently it exerts a major effect on their quality. It is also a complex biological entity and, as such, varies significantly with the source of the wheat. As a complex system, wheat flour contains a multitude of compounds. These include: moisture 14%, proteins 7—15% (albumins, globulins, prolamin, glutelin), starch 63—72% (amylopectin, amilose), non-starchy polysaccharides 4.5—5.0% (pentosans and beta glucans), lipids 1%, as well as vitamins (thiamin, riboflavin, niacin), minerals (iron, sodium, potassium, calcium, magnesium, copper, zinc) (Mirić and Pejin, 2008). Apart from the flour basic ingredients (wheat flour, yeast, salt and water), a large number of optional ingredients such as sugar, hydrocolloids, skimmed milk, fat, acids, bran, emulsifiers and gluten are included in bread formulation to improve its nutritional, shelf life and organoleptic properties (Gujral and Singh, 1999). All the ingredients have specific functions. Salt gives flavour to bread and also acts as a dough strengthener, but in concentrations over 1.5% exerts an inhibitory effect on yeast activity, either by its osmotic pressure or by a specific chemical effect. Fat act as a plasticizer, gives softness, improves volume and has anti-staling properties when added to bread formula. Sugar (saccharose) positively affects the carbon-dioxide production in dough when added at 5% flour basis, however, higher concentrations were found to decrease CO_2 production by increasing the osmotic pressure. Yeast is an indispensable ingredient in the production of many bakery products. In bread production, yeast has three important functions: it ferments the dough, modifies the dough structure and contributes to the formation of flavour. During fermentation, carbon-dioxide modifies the dough structure causing physico-chemical changes in gluten network and other proteins giving the characteristic porosity of bread crumb. Glutation excreted from yeast cells reduces the disulphide bonds in gluten thus improving dough rheology properties. The formation of bread aroma depends on fermentation processes in dough and conditions during baking. The fermentation by-products are numerous: esters, organic acids, alcohols and other carbonile compounds and they have important role in the formation of bread aroma (Pejin et al., 2008).

The aim of the study was firstly to investigate the changes in the composition of yeast during storage under different conditions (12 days, at 4°C and 24°C). Another goal was to investigate how the changes in the composition affected the fermentative activity of *Saccharomyces cerevisiae*. Four samples of flour (A, B, C and D) were used in the experiment. The content of ash, wet gluten, maltose and amylograph peak viscosity were determined to estimate the α-amylase activity of the flour samples. The effect of flour quality category (I, II and III category) on the yeast fermentative activity was also considered. The effects of some optional ingredients used in the bakery industry (margarine, saccharose, salt, malted barley) on the yeast fermentative activity and bread quality were also studied.

MATERIALS AND METHODS

Materials

The examined wheat samples represented an average quality of wheat harvested in 2008 and intended for production of bread flour (1st, 2nd and 3rd category). Commercial baker's yeast *Saccharomyces cerevisiae* available on domestic market was used for the determination of fermentative activity. Commercially available, food-grade margarine, saccharose, malted barley and salt, from domestic production were used.

Methods

Standard methods, according to the Regulations on methods of physical and chemical analyses for quality control of wheat, were used to determine the quality of milling and bakery products, pasta and frozen dough (*Pravilnik o metodama fizičkih*…, 1988). Phytic acid content in flour was determined by the method for rapid determination according to Haug and Lantzsch (1983). Fermentative activity of yeast in dough was determined as a volume of carbon dioxide produced during 2 h per one gram of dry matter (Kaluđerski and Filipović, 1998). The content of total carbohydrates and trehalose was determined according to the modified method described by Trevelyan and Harrison (1979).

RESULTS AND DISCUSSION

The dependence of baker's yeast composition on storage time and temperature is presented in Table 1.

Tab. 1 — Influence of temperature and duration of storage on yeast composition

Days	Total carbohydrates (% dry matter)		Trehalose (% dry matter)		Proteins (% dry matter)	
	Yeast stored at 4°C	Yeast stored at 24°C	Yeast stored at 4°C	Yeast stored at 24°C	Yeast stored at 4°C	Yeast stored at 24°C
0	40.81	40.81	12.33	12.33	43.20	43.20
2	40.69	40.47	12.26	9.85	43.15	41.07
6	40.36	40.33	12.15	9.60	43.14	39.28
8	38.14	31.24	10.80	0.49	43.12	37.92
12	37.50	29.28	9.68	0.24	43.10	36.98

According to the results, over the given period of time, the protein content decreased by 0.1% and content of total carbohydrates decreased by 3.31% in the yeast stored at 4°C. The loss of trehalose content was 2.65%. All these indicated that the storage at 4°C did not affect the yeast protein content but decreased the total carbohydrate content, presumably due to cell respiration

and consequent decomposition of these compounds (P e j i n et al., 2005). When yeast was stored at 24°C the content of total carbohydrates was lowered by 11.56%, trehalose by 11.09% and proteins by 7.22%. Such massive decreases were caused by the temperature increase. Higher temperature intensified the cell respiration and enzyme activities enabling rapid decomposition of reserve carbohydrates and proteins in the cells (V a n D i j c k et al., 1995).

Figure 1 represents the effect of temperature and storage period on the fermentative activity of baker's yeast.

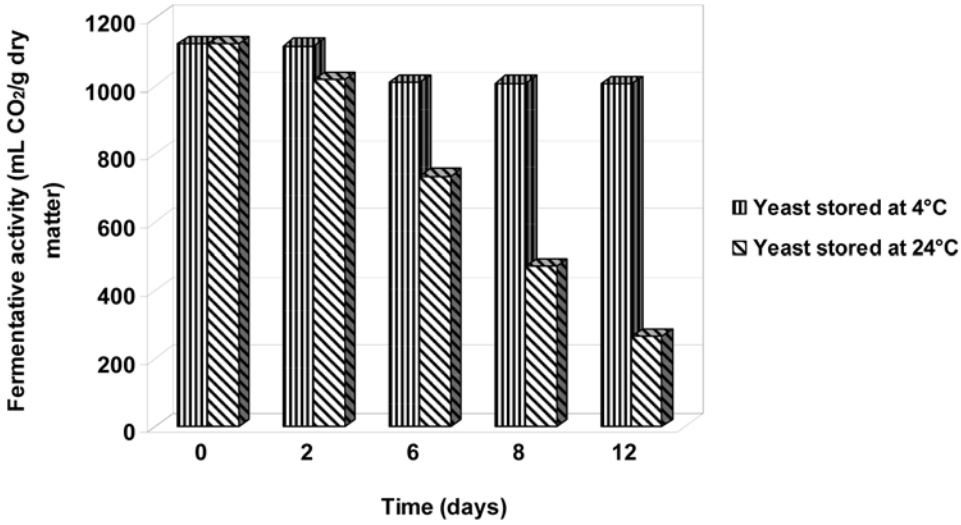

Fig. 1 — Influence of storage temperature and duration on yeast fermentative activity

Fermentative activity of baker's yeast was strongly influenced by storage temperature and period especially at higher temperatures (Fig. 1). The prolongation of storage period at 24°C caused a constant decrease in the fermentation activity. After 12 days, the fermentation activity was 22 ml/g dry matter. CO_2 whereas yeast stored at 4°C had activity of 1150 ml/g dry matter. This means that higher storage temperature caused 5.45-fold decrease in the fermentative activity. This can be explained by the fact that storage at this temperature decreased the protein content from 43.20% to 36.98% (Tab. 1). The observed influence of protein content in yeast cells and their fermentative activity is in agreement with the findings of Š v e c and H r u š k o v a (2004). It was also observed that trehalose content influenced the yeast fermentative activity. The yeast with lower trehalose content (0.24%) had very low activity (220 ml CO2/g dry matter). According to the results of V a n D i j c k (1995), trehalose protects yeast cells from stress. They concluded that trehalose content over 10% provides efficient beginning of fermentation.

Further investigations included testing five samples of wheat flour type 500. The quality parameters of the samples are given in Table 2. The flour samples had low ash contents and were all classified into B1 quality group. The sample A had the lowest wet gluten content (24%), the lowest reducing

sugar content (1.94%), maltose content (0.51%) and peak viscosity (675 B.U.). The highest in reducing sugar content (2.96%), maltose content and peak viscosity was the flour sample D. Although classified in the same quality group, there were differences in the other quality parameters within the flour samples. High values for maltose content and peak viscosity imply to increased α-amylase activity. α-Amylase degrades starch to maltose which is necessary for the activity of yeast cells (P a n d e r o et al., 2005).

The fermentative activities of the two yeast samples attained for the flour samples of different quality are presented in Fig. 2. It was observed that the yeast 1 produced the lowest activity in all flour samples as compared to the yeast 2. It was also found that higher contents of maltose and reducing sugar in flour (samples D and E) enhanced the fermentative activities of both yeast samples. This is due to the fact that higher sugar contents enabled yeasts to produce more CO_2.

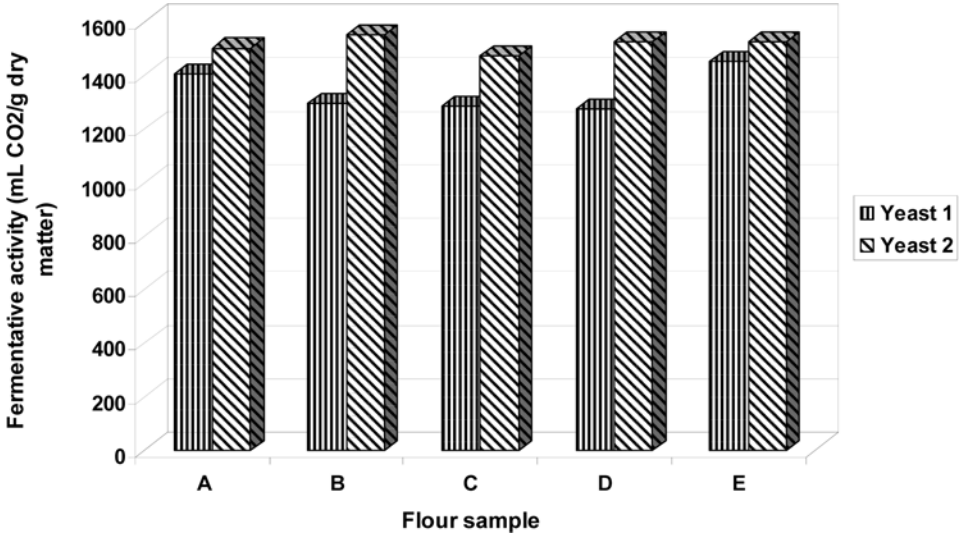

Fig. 2 — Influence of Ist category bread variety on yeast fermentative activity

Tab. 2 — Technological parameters of flour quality

Flour sample	Ash (% dry matter)	Wet gluten (%)	Reducing sugars (% dry matter)	Maltose number	Maximum viscosity (AJ)
A	0.55	24.0	1.94	0.51	675
B	0.40	30.0	2.70	1.03	965
C	0.48	29.0	2.83	1.19	1935
D	0.42	27.0	2.96	1.16	2100
E	0.39	29.0	2.83	1.13	1800

It was also examined how flours of different quality classes (1st, 2nd and 3rd) affected the fermentative features of baker's yeast.

Table 3 contains the quality parameters of flours belonging to the three quality classes. For each quality class, two samples of flour with varying content of phytic acid were analyzed. The first flour sample from the 1st class had ash content of 0.50% and phytic acid content 6.64 mg/g dry matter whereas the second sample had ash content of 0.47% and phytic acid 4.25 mg/g dry matter. This indicated that flours containing more ash also contain more phytic acid. The enhancement of fermentative activity in flours with increased phytic content is the result of enzymatic degradation of phytic acid in dough during fermentation. Phytase degrades phytic acid to m-inositole and phosphate (Fretzdoff, Brummer, 1992; Haraldsson et al., 2005; Febles et al., 2002). The flours from 2nd and 3rd quality class also had two samples with varying content of phytic acid. The results proved the positive correlation between the higher phytic acid content in flour and fermentative activity of yeast.

Tab. 3 — Influence of different flour categories on baker's yeast fermentative activity

Parameter	1st category flour		2nd category flour		3rd category flour	
Ash (% dry matter)	0.50	0.47	0.59	0.59	0.91	0.94
Wet gluten (%)	25.70	28.50	29.40	28.10	31.20	28.70
Maximum viscosity (AJ)	530	645	540	730	730	410
Phytic acid (mg/g dry matter)	6.69	4.25	2.31	8.00	7.42	6.65
Fermentative activity (mL CO_2/g dry matter)	885.73	765.89	820.13	892.84	899.82	931.22

In order to improve the quality of bread and bakery products, manufacturers include various optional ingredients that modify the baking performance of wheat flour. The choice of improver depends on the chemical, biochemical and technological quality of flours and the desired attributes of end-products (Beleslin, 1980). These ingredients are supposed to influence the fermentative activity of yeast. The effect of most commonly used bakery ingredients (margarine, saccharose, salt and malted barley) on yeast fermentative activity was examined. The following doses were used: 0.5, 1.0, 1.5 and 2.0% flour basis. The obtained results are displayed in Table 4.

Tab. 4 — Influence of additives in dough on baker's yeast fermentative activities

Additive	% of additive in flour	Fermentative activity (mL CO_2/g dry matter)	Bread volume (mL)
0	—	1053	650
Margarine	0.5	1194	670
	1.0	1149	780
	1.5	1119	700
	2.0	1118	700
Maltex	0.5	1164	670
	1.0	1198	720
	1.5	1264	780
	2.0	1273	830

Saccharose	0.5	1180	670
	1.0	1192	700
	1.5	1257	780
	2.0	1278	850
NaCl	0.5	1110	630
	1.0	970	640
	1.5	820	590
	2.0	760	500

Analysing the given data, it could be concluded that margarine at 0.5 and 1.0% positively influenced the yeast fermentative activity and bread quality (specific volume and penetrometer number) whereas higher doses impaired these parameters. Malted barley was found to positively influence the fermentative activity and bread quality at all investigated doses. The composition and enzyme content of malted barley enables more efficient starch degradation in dough providing yeast with products easy to metabolise which favour CO_2 production and fermentation. All tested doses of saccharose positively affected the fermentation activity and bread quality. Close contact of yeast cells with sodium chloride may cause cells to lose free water which results in the death of cells (Sekulić, Popović, 1996). From the data presented in Table 4, it is evident that sodium chloride exerted an inhibitory effect on the fermentative activity. Similar findings were reported by Baker et al. (1986).

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УТИЦАЈ ТЕМПЕРАТУРЕ СКЛАДИШТА И САСТАВА БРАШНА НА ФЕРМЕНТАЦИЈУ ПЕКАРСКОГ КВАСЦА

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Резиме

Пекарски квасац је скуп живих ћелија *Saccharomyces cerevisiae*. Он садржи око 70—72% воде, 42—45% протеина, око 40% угљених хидрата, око 7,5% липида (рачунато на суву материју) и витамина В-комплекса. На основу састава ћелија може се закључити да су ћелије квасца сложен биолошки систем који се мења са временом. Интензитет промена зависи од температуре на којој се квасац чува. Узорак квасца чуван је 12 дана на 4°С и 24°С. Садржај укупних угљених хидрата у квасцу чуваном на 4°С током 12 дана смањио се од 48,81% на 37,50% у сувој

материји, а код квасца чуваног на 24°C садржај укупних редукујућих угљених хидрата смањио се од 40,81% до 29,28 %. Садржај трехалозе код квасца чувана на 4°C је био 12,33%, а на 24°C је био 0,24%. Квасац чуван на 24°C током 12 дана изгубио је 81,76% ферментативне активности. Испитиван је састав пет узорака брашна I категорије и резултати ферментативних снага са два узорка квасца су показали да брашна са вишим садржајем редукујућих шећера и малтозе дају више вредности ферментативних снага. Брашна са вишим садржајем пепела (од 0,5 до 0,94%) садржала су више фитинске киселине. Са повишењем садржаја пепела и фитинске киселине расла је и ферментативна снага квасца. У пекарској производњи, у циљу побољшања квалитета уносе се у тесто површински активне материје (емулгатори), ензими, шећери, масноће. У раду је испитиван утицај маргарина, сахарозе, натријум хлорида и малтекса на ферментативне активности пекарског квасца. Наведени додаци додавани су у следећим концентрацијама: 0,5; 1,0; 1,5 и 2,0% на брашно. Такође је испитано како промене брзине мешења теста утичу на ферментативне активности пекарског квасца. Сви испитивани додаци у свим испитиваним садржајима неповољно утичу на ферментативну активност квасца, а повољно утичу на квалитет произведеног хлеба.

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ETHANOL PRODUCTION USING *SACCHAROMYCES CEREVISIAE* CELLS IMMOBILISED ON CORN STEM GROUND TISSUE

ABSTRACT: Cell immobilisation in alcoholic fermentation has been extensively studied during the past few decades because of its technical and economical advantages over those of free cell systems. A biocatalyst was prepared by immobilising a commercial *Saccharomyces cerevisiae* strain (baker yeast) on corn stem ground tissue for use in alcoholic fermentation. For this purpose, the yeast cells were submitted to the batch tests "in situ" adsorption onto pieces of the corn stem ground tissue. Cells immobilisation was analysed by optical microscopy. It was determined that the addition of the corn stem ground tissue led to an increase of the pH value, total dissolved salts content, and sugar content in fermentation medium. The addition of 5 and 10g of the corn stem ground tissue per liter of medium, increased ethanol yield, decreased amount of residual sugar and the cells immobilisation was effective. Corn stem is one of the abundant, available, inexpensive, stable, reusable, nontoxic celulosic biomaterial with high porosity, which facilitates the transmission of substrates and products between carrier and medium. The prepared immobilised biocatalyst showed higher fermentation activity than free cells. The results indicate that corn stem might be an interesting support for yeast cell immobilisation, and also a cheap alternative recourse of mineral components with possibility of application for improving ethanol productivities.

KEY WORDS: corn stems ground tissue, ethanol, fermentation, immobilization, yeast

INTRODUCTION

In recent years, cell immobilisation techniques have become increasingly important and are being successfully applied in production of alcohol (ethanol, butanol and isopropanol), organic acids (malic, citric, lactic and gluconic acids), enzymes (cellulase, amylase, lipase and others), and biotransformation of steroids for wastewater treatment, and food applications (beer and wine) (Reddy et al., 2008). Yeast cell immobilisation method by surface adsorption, seem to be more reasonable than other methods ("entrapment within a

porous matrics", "containment behind a barrier" and "self aggregation"), because of the fact that the yeast cell growth is not significantly affected, and some yeast cells can be washed out of the fermentation system and be continuously renewed. In addition, such supporting materials are readily cleaned and microbial contamination can be effectively prevented (Bai et al., 2008, Verbelen et al., 2006). Cells have been immobilised by the surface adsorption on a variety of natural and synthetic supports (Yu et al., 2007). The main factor that influences the immobilisation behaviour of the yeast cells and their productivity are thought to be the surface characteristics of the carrier including pore size, water content, hydrophilicity and magnetism (Fuji et al., 1999). Much of the nutrient material is stored in parenchyma cells of the corn stems. Corn stems remaining in the field after harvest contain 43% polysaccharide consisting mainly of cellulose and hemi cellulose, 29% lignin, 7% proteins, 5% ash, and 16% others (Beltron-Garcia et al., 2001). The corn stem ground tissue consists of parenchymatous cells, and has honeycomb microstructure (Battacharya and Henrich, 2006). Corn stem is a low cost, environmentally friendly, sustainable and abundantly available lignocellulosic raw material in many world regions (Fuji et al., 1999; Reddy and Yang, 2005; Thamae et al., 2008). Corn stem is one of the most promising renewable feedstock, not only for the biological conversion to fuels and chemicals, but also as a forage for ruminants (Anderson and Akin, 2008), and a source of fibers for manufacturing pulp for paper (Reddy and Yang, 2005). The aim of this study was to immobilise *Saccharomyces cerevisiae* cells on corn stem ground tissue and evaluate the biocatalyst produced for efficiency to perform alcoholic fermentation.

MATERIAL AND METHODS

Corn stalks of NS 640 maize hybrid were collected from ready-to-harvest corn fields from Budisava site, Republic of Serbia. In order to increase specific surface area of the carrier, the stalks were manually cleaned to separate the fibrous tissue and nodes from the pith tissue (Reddy and Yang, 2005). The outer ring was easily peeled by knives from the pith (Thamae et al., 2008). The corn stem ground tissue of the above ground internodes (7^{th}—10^{th}), cut into slices with a diameter 1.5—2 cm (width) and 0.5 cm long, with density 0.05 g/cm^3, and 8.81% measure content, was used as a support material (Brányik et al., 2005; Jung and Calser, 2006, Vasconcelos et al., 2004). The synthetic culture medium used for the fermentation consisted of (g/l) 76.5 g/l glucose, 1$(NH_4)_2SO_4$, 1KH_2PO_4, 5$MgSO_4$ and 4 yeast extract, and the pH was adjusted at 4.5 by the addition of H_2SO_4 prior to sterilisation. The fermentation medium in the absence and presence of 10 g/l of support was sterillised by autoclaving at 120°C for 30 min. Active microorganism was a commercial *Saccharomyces cerevisiae* strain (Alltech-Fermin, Serbia), commonly used in Serbian baking industry, in the form of pressed blocks (70% w/w moisture) (Plessas et al., 2007). An amount of 40 g of wet pressed yeasts was suspended in 200 ml sterilised 0.9% NaCl solution. In order to

obtain continually the same inoculums, the yeast cell concentration in this suspension was determined by Neubauer camera counting and then, the appropriate aliquots were added to the fermentation medium (Vasconcelos et al., 2004). All the fermentations were performed in duplicate, under anaerobic conditions at 30°C, in 500 ml Erlenmeyer flasks containing 200 ml of the same medium, inoculated with $1 \pm 0.1 \times 10^8$ yeast cells/ml. The flasks were maintained in rotary shaker at 120 rpm for 72 h (Santos et al., 2008).

Fermentation kinetics were monitored by measuring the weight of produced CO_2, residual sugars and ethanol concentration of the fermenting liquids at various time intervals (3, 6, 9, 24, 48 and 72 h from the beginning of fermentation). The concentrations of ethanol and residual sugar were measured spectrophotometrically (Parmanik, 2004). Ethanol was determined by measuring optical density at 600 nm after standard distillation using dichromate solution (Captui et al., 1968). Residual sugar was determined by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1958).

In order to examine the influence of carrier addition on chemical composition of the fermentation medium, a set of extraction experiments was performed following the fermentation procedure, only without the addition of yeast cells. For this purpose the corn stem pith was ground on a laboratory conical mill Miag-Braunschweig, type Doxy 71b/4 at 1375 r/min, until the particle size was less than 1000 μm. pH value, conductivity, total dissolved salts content from the synthetic medium in the absence and presence of and 10 g/l of support were monitored by a laboratory multiparametar analyzer Consort C863 (Consort, Belgium) and sugar content was determined by DNS method at the certain time intervals following the fermentation media sampling.

Carl Zeiss optical microscope connected to a Cannon S50 camera was used to capture yeast cells immobilised onto corn stem ground tissue. The mass of cells adsorbed onto the support particles was quantified by gravimetric method (Santos et al., 2008) using analytical balance; model Tehtnica Sauter, Type 414, Slovenia. Cell retention onto the corn stem ground tissue (R, g/g) was calculated as the ratio of dry mass of cells immobilised in the carrier (g) to the carrier mass. The immobilisation efficiency (Y_i, g/g) was calculated as the ratio of dry mass of cells immobilised in the carrier (g) to the dry mass of total cells (g).

RESULTS AND DISCUSSION

After autoclaving and 24 h of extraction, considering the carrier addition of 5 and 10 g per liter of the synthetic medium, the values of the following parameters increased: the sugar content by 2.1 and 10.2 g/l, pH value by 0.4 and 0.8 units, conductivity by 90 and 190 mS/cm and total dissolved salts content by 40 and 90 g/l respectively, and with no further change in time.

The addition of 5 and 10 g of the corn stem ground tissue per liter of medium decreased the amount of residual sugar (Fig. 1a). The results presented at Fig. 1a suggest that immobilised cells consume almost all available sugar during the first 24 h of fermentation. The ethanol concentrations by the end of

the fermentation were 4.91% (v/v) for free cells and 5.03% (v/v), 5.1% (v/v) for immobilised cells by the addition of 5, 10 g/l carrier, respectively (Fig. 1a). An increase in the ethanol concentration, especially in the sample with 10 g/l of the corn stem pith per liter of medium (Fig. 1a), is probably caused by the fermentation of small amounts of sugar extracted from the carrier. The dynamics of CO_2 production was in correlation with the ethanol production. Low fermentation times indicated that no period was needed for adaptation of biocatalyst in the fermentation environment. The immobilised yeast showed an important operational and stability without any decrease of its activity. The observed pH behavior during the fermentation (Fig. 1b) was, indeed, expected considering the established alkaline nature of the examined carrier.

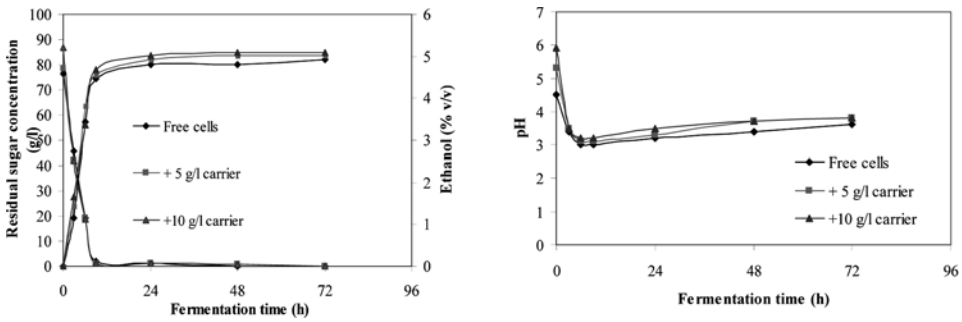

Fig. 1 — a) Residual sugar and ethanol concentration versus fermentation time, b) Variation of pH during the fermentation time

The immobilisation efficiency (Yi) and retention (R) of *S. cerevisiae* cells by prepared corn ground tissue increased along the fermentation, reached maximum values (Yi = 0.13 g/g, R = 0.23 g/g for the addition of 5 g/l carrier; Yi = 0.21 g/g and R = 0.24 g/g for the addition of 10 g/l carrier) after 9h and then decreased (Fig. 2). Cell immobilisation was presented by optical micrographs (Fig. 3) showing that yeast cells are densely and homogenously adhered onto the surface of the carrier, as a result of natural entrapment into the honeycomb cellulosic material of parenchymatous tissue and physical adsorption by electrostatic forces or covalent binding between yeast cell membrane and the carrier. As it is demonstrated by the uniform cell growth onto the surface of corn stem parenchymatous cells walls (Fig. 3), the cells immobilisation was effective, suggesting possible recycling of cells in repeated batch runs, also taking into account that cells grow even after 72 h of fermentation (Fig. 3b).

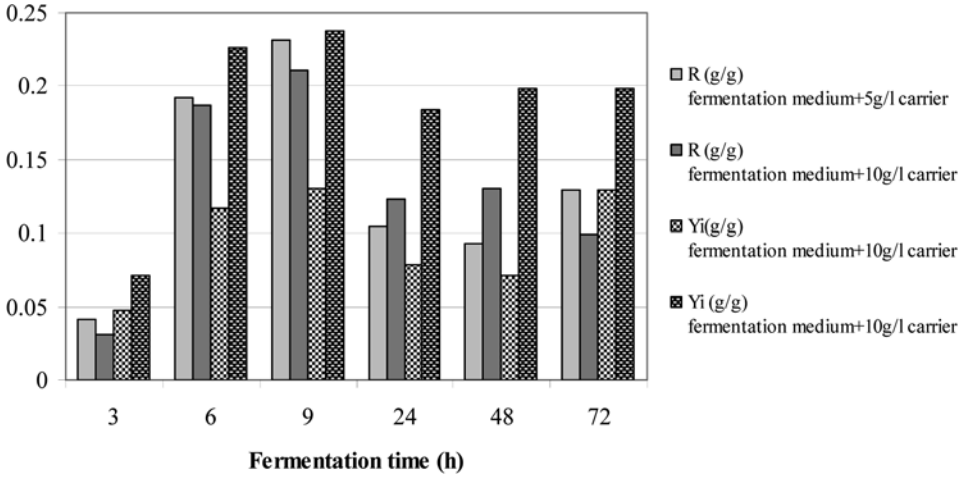

Fig. 2 — Cell retention (R, g/g) and immobilisation efficiency (Yi, g/g) versus fermentation time

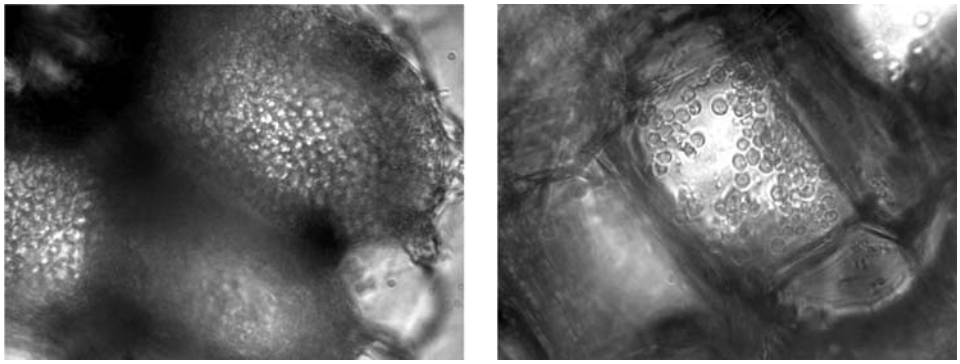

Fig. 3 — Optical microphotograph of *Saccharomyces cerevisiae* cells (400 x) immobilised onto corn stem ground tissue done after: a) 9 h and b) 72h of fermentation.

Tab. 1 — Fermentation parameters (average values) obtained in batch fermentations with *Saccharomyces cerevisiae cells* immobilised on various carriers, at 30°C

Carrier	Medium	Initial sugar (g/l)	Ferm. Time (h)	Residual sugar (g/l)	Ethanol (g/l)	Ethanol productivity (g/ld)	Conversion (%)
Mineral kissiris (Kana et al., 1989)	Glucose	113	16	7.5	48	74.3	93.4
Delignified cellulosic materials (Iconomou et al., 1996)	Molasses/ sucrose	172	36	10.2	104	69.3	94
	Glucose	350	67	68	144	51.5	80.5
Gluten pellets (Bardi et al., 1996)	Glucose	119	15	12.5	39.5	63.2	89.5
	Grape must	206	17	18.9	83.7	118	90.1
Dried figs (Brkatrou et al., 2002)	Wort	129	18	0	47.4	64	100
	Glucose	120	45	1.4	45	24	98
Quince pieces (Kountiras et al., 2003)	Grape must	185	28	0.1	84	72	99.9
Apple pieces (Kountiras et al., 2001)	Grape must	206	80	30.8	85	26	85
Orange peel (Plessas et al., 2007)	Glucose	125	9	4	51.4	128.3	96.8
	Molasses/ sucrose	128	14	2	58.9	100.1	98.4
	Raisin extract	124	12	2.3	55.3	110.4	98.1
Watermelon rind pieces (Reddy et al., 2008)	Grape must	202	64	tr	87.0	45.8	100
Corn ground tissue (5 g/l) present study	Glucose	78.6	24	1.4	38.6	38.6	98.2
Corn ground tissue (10 g/l) present study	Glucose	86.7	24	1.4	39.6	39.6	98.4

The biocatalyst was equally efficient for alcoholic fermentation, with other biocatalysts prepared by yeast immobilisation on natural, food grade materials that have been extensively studied, such as dignified cellulosic materials, gluten pellets, pieces of fruit etc. (Table 1) (Plessas et al., 2007; Reddy et al., 2008). The results demonstrated that the corn stem pith could be alternative resourse of mineral components and also an interesting support for cell immobilisation, with possibility of application for improving ethanol productivities. The prepared immobilised biocatalyst showed higher fermentation activity compared to free cells. The advantage of the corn ground tissue as yeast cells carrier is high porosity, which facilitates the transmission of substrates and products between carrier and medium. Still it is necessary to make more detailed studies to clarify the mechanism of *S. cerevisiae* cells attachment and deattachment from support particle. Further investigation on specific food applications using this biocatalyst would be interesting.

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ПРОИЗВОДЊА ЕТАНОЛА ПОМОЋУ ЋЕЛИЈА *SACCHAROMYCES CEREVISIAE* ИМОБИЛИСАНИХ НА ПАРЕНХИМСКОМ ТКИВУ СТАБЉИКЕ КУКУРУЗА

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Резиме

Примена имобилисаних ћелија у алкохолној ферментацији је веома актуелна истраживачка тематика, услед многобројних техничких и економских предности у односу на ферментацију помоћу слободних ћелија. У овом раду је испитана могућност примене имобилисаних ћелија квасца *Saccharomyces cerevisiae* на паренхимском ткиву кукурузовине, у алкохолној ферментацији. Процес имобилизације ћелија праћен је оптичким микроскопирањем и мерењем суве материје имобилисаног квасца. Установљено је да додатком носача у ферментациону подлогу расте pH вредност, као и садржај соли и шећера услед екстракције ових компонената из кукурузовине. Додатком стабљике кукуруза у количини 5 g и 10 g на литар подлоге за ферментацију остварују се виши приноси етанола, висок степен конверзије шећера, већа брзина ферментације, постиже се висок степен имобилизације ћелија квасца. Стабљика кукуруза је расположив, јефтин, стабилан, поновно употребљив, нетоксичан и механички стабилан целулозни биоматеријал високог степена порозитета који олакшава пренос масе супстрата и продуката између медијума и носача. Резултати овог рада указују на чињеницу да је стабљика кукуруза ефикасан носач за имобилизацију ћелија квасца, али такође и додатни извор хранљивих материја неопходних квасцу током ферментације, чија је примена у производњи етанола економски и еколошки оправдана.