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XEROPHILIC FUNGAL GENUS WALLEMIA – BIOACTIVE INHABITANTS OF MARINE SOLAR SALTERNS AND SALTY FOOD

ABSTRACT: *Wallemia* is a genus of cosmopolitan xerophilic fungi, frequently involved in food spoilage of particularly sweet, salty, and dried food. Until recently, only a single species, *Wallemia sebi,* was recognized in the genus. When a large group of strains globally collected in salterns and other different ecological niches was analyzed on the level of physiological, morphological and molecular characteristics, a new basidiomycetous class, Wallemiomycetes, covering an order of Wallemiales was proposed and three *Wallemia* species were recognized: *W. ichthyophaga, W. sebi* and *W. muriae*. *Wallemia ichthyophaga* was recognized as the most halophilic eukaryote known, thus representing an appropriate eukaryotic model for in depth studies of adaptation to hypersaline conditions. Our preliminary studies indicated that all three *Wallemia* species synthesized a yet undescribed haemolytic compound under, surprisingly, low water activity conditions. Due to the taxonomic status w hich was unrevealed only recently, there were so far no reports on the production of any bioactive compounds by the three newly described species. The article aims to present the taxonomy, ecology, physiology and so far described molecular mechanisms of adaptations to life at low water activity, as well as bioactive potential of the genus *Wallemia*, a phylogenetically ancient taxon and a taxonomic maverick within Basidiomycota.

KEY WORDS: fungi, *Wallemia* spp., taxonomy, low water activity, xerophiles, halophiles, osmoadaptation, secondary metabolites

ABBREVIATIONS

AAS, atomic absorption spectroscopy; a_w, water activity; EPS, extracellular polysaccharides; GPD1, glycerol-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; ITS rDNA, internal transcribed spacer regions ribosomal deoxyribonucleic acid; NMR, nuclear magnetic res-

onance; PTS2, peroxisomal targeting sequence; SSU rDNA, small subunit ribosomal deoxyribonucleic acid; UV, ultraviolet.

INTRODUCTION

Water, with its central role in biological processes, is the key life-limiting parameter. Therefore, low amount of biologically available water (low water activity [a_w]) represents one of the most pervasive stresses for biological systems, as only specially adapted organisms can thrive at such conditions. Tolerance of low water-activity is apparent in only ten out of 140 known orders of fungi, most of them belonging to the Ascomycota (de Hoog, Zalar et al., 2005), while xerotolerance is rare in the phylum Basidiomycota. Xerophilic fungi are capable of growth at water activity below 0.85, corresponding to 17% NaCl, or 50% glucose added to the growth medium (Gunde-Cimerman, Oren et al., 2005). Fungi are not only being capable of growing at low a_w, but they also show preferences for certain chemical nature of the solute lowering the a_w (de Hoog, Zalar et al., 2005; Gunde-Cimerman and Plemenitaš, 2006), as xerophilic fungi are able to live in the environments rich in sugar, while halophilic live in the environments rich in salt. Halotolerance and extreme halotolerance describe the salt-adaptable fungi that do not necessarily require salt (NaCl) for viability, but are able to sustain a range of different salt concentrations, even across the whole salinity range – from fresh water to NaCl saturation (Gunde-Cimerman and Plemenitaš 2006). An obligate halophilic character is possessed by only few fungal species that exhibit superior growth on media with NaCl as controlling solute (Wheeler, Hocking et al., 1988; Zalar, de Hoog et al., 2005; Kralj Kunčič, Kogej et al., 2010).

Xerophilic fungi were first only known as domestic extremophiles that contaminate food preserved by the reduction of biologically available water by means of drying, freezing or adding solutes (Pitt and Hocking 1977; Pitt and Hockering, 2009). Natural saline and hypersaline environments, where high concentrations of NaCl are prevailing, were believed to be populated almost exclusively by bacteria, archaea and eukaryotic alga *Dunaliella salina* (Rodriguez-Valera, Ruiz-Berraquero et al., 1981; Schiewer, 1991; Oren, 2005), until fungi as active inhabitants of solar salterns were first reported (Gunde-Cimerman, Zalar et al., 2000).

Later numerous halotolerant and extremely halotolerant fungi (Zalar, de Hoog et al., 1999; Gunde-Cimerman, Zalar et al., 2000; Butinar, Santos et al., 2005; Zalar, de Hoog et al., 2007; Zalar, Frisvad et al., 2008; Zalar, Gostincar et al., 2008), and only two halophilic representatives, both from genus *Wallemia* (Zalar, de Hoog et al., 2005) were isolated from hypersaline water of the Sečovlje solar salterns (Adriatic coast, Slovenia). Since the first discovery of fungi in salterns, numerous fungal species thriving in extremely saline environments around the

globe have been described, amongst them xerophilic and halophilic species of the genus *Wallemia*, and are in the focus of the above review.

Ecology of the *Wallemia* spp.

Fungi from genus *Wallemia* are frequently involved in food spoilage of particularly sweet, salty, and dried food (Samson, Hoekstra et al., 2002), and are also often isolated from indoor or outdoor air (Takahashi, 1997), soil (Domsch, Gams et al., 1990) and sea salt (Das Sarma, Klebahn et al., 2010). Until recently (Zalar, de Hoog et al., 2005), a single cosmopolitan species, *W. sebi,* that was isolated from jams, dates, bread, cakes, salted beans, maize flour, crystalline sugar, fish, bacon, fruits, soil, hay and textiles around the globe (Eduard, Lacey et al., 1990; Hanhela, Louhelainen et al., 1995; Zeng, Westermark et al., 2004; Zalar, de Hoog et al., 2005), have been recognized in the genus. *Wallemia sebi* commonly causes allergological problems resulting in farmer's lung disease (Lappalainen, Pasanen et al., 1998; Roussel, Reboux et al., 2004) and has been proved to be, although rarely, the causative agent of cutaneous or subcutaneous infections in humans (de Hoog and Guarro, 1996).

In order to clarify unresolved phylogenetical position of the genus *Wallemia* within the fungal kingdom (Wu, Tsumura et al., 2003), as well as its taxonomy and ecology, a large group of strains collected globally from food preserved with low a_w (e.g. peanuts, cakes, dried fish), different extreme ecological niches (hypersaline waters of the Dead Sea and salterns of the Red Sea, Dominican Republic and Slovenia), and from some medically relevant samples (e.g. chronic ulcerative skin lesion of human and hay sample associated with livestock toxicosis) were studied. Morphological and physiological characteristics were analyzed, as well as the sequence data of ribosomal DNA internal transcribed spacer regions 1 and 2 (ITS1 and ITS2 rDNA) including the 5.8S ribosomal DNA (5.8S rDNA). Based on the unique morphology, evolution and xerotolerance, a new basidiomycetous class, Wallemiomycetes, covering an order Wallemiales was proposed (Zalar et al., 2005). In addition, molecular data from six nuclear genes (18S, 25S, and 5.8S rDNA and genes coding *rpb1, rpb2* and *tef1* nuclear proteins) reinforced the isolated position of *Wallemia* in the Basidiomycota and suggested that class Wallemiomycetes is an early diverging lineage of Basidiomycota as it occupies a basal position near the Entorrhizomycetidae (Matheny, Gossmann et al., 2006). Based on differences in conidial size, xerotolerance, and sequence data of the ITS rDNA, three *Wallemia* species were segregated, named *W. ichthyophaga, W. sebi* and *W. muriae*. Since the *W. ichthyophaga* differs in numerous nucleotides of the small subunit (SSU) rDNA and ITS rDNA from the other two species, existence of at least two cryptic genera not distinguishable by morphological characteristics were indicated (Zalar, de Hoog et al., 2005). Despite considerable molecular distances among *Wallemia* species,

they all exhibit unique conidiogenesis, including basauic development of fertile hyphae, segregation of conidial units more or less basipetally, and disarticulation of conidial units into mostly four arthrospore-like conidia (Z a l a r, d e H o o g et al., 2005).

Interestingly, tests of xerotolerance of the *Wallemia* spp. have shown that it represents one of the most xerophilic fungal taxa (Z a l a r, d e H o o g et al., 2005). The two halophilic *Wallemia* species, *W. muriae* and *W. ichthyophaga*, necessarily require media with low a_w, while *W. sebi* also shows growth on media without additional solutes. The narrow a_w ranges for the growths of *W. muriae* and *W. ichthyophaga* are 0.984 – 0.805 and 0.959 – 0.771, respectively. Moreover, *W. ichthyophaga* shows preferences to certain solutes for lowering a_w as it exhibited poor growth by means of prolonged growth phases and smaller colonies on the media with high concentrations of glucose compared to media with presence of high concentrations of salt (Z a l a r, d e H o o g et al., 2005; K r a l j K u n č i č, K o g e j et al., 2010). The fact that it can only thrive in the media with NaCl above 1.7 M and up to the saturation (5.3 M NaCl) makes it one of the most halophilic fungi known today. *Wallemia sebi* is capable of growth over a wider range of a_w (0.997 – 0.69) in glucose/fructose media (P i t t and H o c k i n g, 1977), but in media with NaCl as the major solute, the lowest a_w for growth was reported to be 0.80 (P i t t and H o c k i n g, 1977; Z a l a r, d e H o o g et al., 2005), corresponding to 4.5M NaCl.

Adaptations of the *Wallemia* spp. to the life at high concentrations of salt

Life in the environment with high concentrations of NaCl is stressful not only due to high osmotic pressure but also due to the toxicity of the sodium ions. As the cytoplasmatic membrane is freely permeable to water, this situation leads to subsequent dehydration and cessation of growth unless the organism has the means to adapt physiologically and morphologically to such an environment (G a l i n s k i, 1995). Mechanisms of salt-tolerance in fungi have been mostly studied in salt-sensitive *Saccharomyces cerevisiae* (B l o m b e r g and A d l e r, 1992; B l o m b e r g, 2000; H o h m a n n, 2002), halotolerant yeast *Debaryomyces hansenii* (L a r s s o n and G u s t a f s s o n, 1987; A n d r e, N i l s s o n et al., 1988; L a r s s o n, M o r a l e s et al., 1990; L a r s s o n and G u s t a f s s o n, 1993; P r i s t a, A l m a g r o et al., 1997; A l m a g r o, P r i s t a et al., 2000) and in extremely halotolerant black yeast *Hortaea werneckii* (K o g e j, R a m o s et al., 2005; K o g e j, G o r b u s h i n a et al., 2006; K o g e j, G o s t i n č a r et al., 2006; K o g e j, S t e i n et al., 2007; P l e m e n i t a š, V a u p o t i č et al., 2008). The latter is currently being a model organism for eukaryotic halophily studies. The response of eukaryotic cells to environmental stress involves complex alterations in gene expression which leads to metabolic changes and subsequent adaptation to the new conditions (Y a l e and B o h n e r t, 2001; P e t r o v i č, G u n d e - C i m e r m a n et al.,

2002; Vaupotič and Plemenitaš, 2007). An important level of adaptation is balancing the osmotic pressure of the medium by accumulating and/or synthesyzing organic compatible solutes and maintaing low salt concentration within cytoplasm (Oren, 1999). Additional adaptations at the levels of plasma membrane composition (Petrovič, Gunde-Cimerman et al., 1999; Turk, Mejanelle et al., 2004; Gostincar, Turk et al., 2008; Gostincar, Turk et al. 2009) and cell wall structure (Kralj Kunčič, Kogej et al., 2010) are required in order to prevent the damage of cells in such environments. Numerous morphological adaptations reflected in the extremophilic ecotype, characterized by meristematic growth, pigmentation and changes in colony morphology, have also been observed (Kogej, Gorbushina et al., 2006). Research of physiological and molecular adaptations of the genus *Wallemia*, especially halophilic representative *W. ichthyophaga*, to the environments with high salinity are only at an early stage.

Morphological adaptations to moderate and high NaCl concentrations of *Wallemia* spp. have been only recently studied (Kralj Kunčič, Kogej et al., 2010). The combination of light, focused-ion-beam/ scanning and transmission electron microscopy revealed an impact of high concentrations of NaCl on the cell morphology of *Wallemia* spp. Hyphal compartments of *W. sebi* and *W. muriae* were thicker and shorter and mycelial pellets were larger at high salinity.

Wallemia ichthyophaga differs from the other two *Wallemia* spp. not only from the molecular aspect, but also from the aspect of morphology, since it forms sarcina-like multicellular clumps composed of compactly packed spherical cells. The size of the cells did not respond to increased salinity, whereas multicellular clumps became significantly larger. The ability to grow meristematically, or in the form of multicellular clumps, is hypothesized to greatly enhance the survival in stressful environment (Wollenzien, de Hoog et al., 1995; Palkova and Vachova, 2006). The presence of extracellular polysaccharides (EPS) observed in all three *Wallemia* spp. is involved in the protection against desiccation in rock-inhabiting fungi (Selbmann, de Hoog et al., 2005) and might also have a protective function at high salinities. An increase in the thickness of the multilayered cell wall at higher salinities occurred in all three, but it was especially pronounced in *W. ichthyophaga*, which had extremely thick cell wall compared to *W. sebi* and *W. muriae*. The thickened cell wall of the *W. ichthyophaga* is rather an exception in the so-far-known fungal responses to extremely saline conditions (Kralj Kunčič, Kogej et al., 2010). The unique morphological adaptations of *Wallemia* spp. to high NaCl, such as increase in cell wall thickness and size of multicellular clumps or mycelial pellets, which were pronounced at high NaCl concentration (Kralj Kunčič, Kogej et al., 2010), were interestingly less apparent at high glucose concentrations (our unpublished data). To conclude, morphological phenomena observed in the above studies are believed to have an important role for successful growth in extremely saline conditions (Kralj Kunčič, Kogej et al., 2010).

As we realized from our preliminary results obtained by NMR and HPLC measurements, all three *Wallemia* spp. accumulate polyols, among which glycerol is the most significant, in response to increased salinity in the environment (our unpublished data). This is a common strategy of osmoadaptation of other fungi, as well as extremely halotolerant black yeast *H. werneckii* (Petrovič, Gunde-Cimerman et al., 2002; Kogej, Stein et al., 2007). As we have discovered from atomic absorption spectroscopy (AAS), cells of *Wallemia* spp. keep intracellular cationic (Na^+ and K^+) concentrations low in the environments with high concentrations of salt, and are, therefore, considered as Na^+-excluders (our unpublished data). So far, only halotolerant yeast *D. hansenii* has shown to maintain relatively high internal concentrations of sodium when coping with salt stress together with production and intracellular retention of compatible solutes, particularly glycerol (Prista, Almagro et al., 1997). In addition to that, a key enzyme for glycerol biosynthesis and redox balancing, Gpd1 (glycerol-3-phosphate dehydrogenase) in *W. ichthyophaga* was identified and characterized. Similarly to *S. cerevisiae* (Ansell, Granath et al., 1997), levels of mRNA of *WiGPD1* in cells adapted to different salinities showed a gradual increase in the transcript at higher salinities, with the maximum at 4.5 M (25% w/v) NaCl and responded to saline stress. Comparison of WiGpd1 and Gpd1 from *S. cerevisiae* revealed high overall amino-acid similarity, but more importantly, the N-terminal PTS2 sequence which was important for peroxisome localization (Jung, Marelli et al., 2010) was found to be lacking in the case of *W. ichthyophaga* (and *H. werneckii*) homologue. Constant cytosolic localization of the Gpd1 has appeared to be beneficial for the organisms living in extremely saline environments due to its function in osmotic stress (Lenassi, Zajc et. al., 2011, paper in press).

Bioactive potential of the genus *Wallemia*

Research of the production of biologically active compounds has been focused on mostly cosmopolitan fungi, while halotolerant and halophilic fungi, such as *W. muriae* and *W. ichthyophaga*, have been so far ignored. Toxicity of the culture filtrate of cosmopolitan *W. sebi* for HeLa cell lineage, (Saito, Ohtsubo et al., 1971) and in other biological tests was observed earlier (Wood, 1984). Two related tricyclic dihydroxysesquiterpenes were reported to be isolated from *W. sebi*, designated walleminol A and walleminol B, or walleminone that were toxic to certain cell lineages, protozoa and brine shrimps. The minimum inhibitory dose of walleminol A in the bioassays was approximately 50 micrograms/ml, which was comparable with a number of mycotoxins, such as citrinin and penicillic acid (Wood, Mann et al., 1990). Additionally, two components were identified in *W. sebi,* namely azasteroides UCA 1064-B (Chamberlin, Chaney et al., 1974) and UCA 1064-B (Takahashi, Maruta et al., 1993), that both exhibited antibacterial and antimycotic activity, while only UCA 1064-B showed anti-tumor activity.

Our recent study on screening of fungi from extreme environments, including all three *Wallemia* species for the production of haemolytic and antibacterial activities, indicated that selected halotolerant and halophilic species synthesized specific bioactive metabolites under stressful conditions. They were cultivated under controlled conditions (low concentrations of NaCl, glucose and optimal growth temperature) and under conditions with lowered a_w, high concentrations of NaCl or glucose, and at low temperature. Water and organic (acetone and methanol) extractions of the biomass were evaporated and dissolved in the appropriate solvent, water or ethanol, and used for biological assays.

Water extracts showed no biological activity, regardless of the growth conditions, suggesting that these organisms do not synthesize proteins or other polar molecules with haemolytic or antibacterial activity (S e p c i c , Z a l a r et al., 2011). The organic extracts showed haemolytic and antibacterial potential that was considerably higher if the fungi were exposed to stressful growth conditions. *W. ichthyophaga* showed higher haemolytic activity when organic extracts were obtained from the cultures grown at high concentrations of glucose, and higher antibacterial activity at high concentrations of NaCl and glucose. Interestingly the haemolytic potential of the organic extracts of *W. muriae* and *W. sebi* was considerably higher if they were exposed to low temperature (10 °C). Enhancement of this kind was not observed for antibacterial activity as it occurred at low temperature and high concentrations of glucose for *W. muriae* and for *W. sebi,* even in the case of cultivation under controlled conditions. All the active extracts exclusively inhibited growth of Gram-positive bacterium *Bacillus subtilis*, while growth of Gram-negative *Escherichia coli* remained unaffected. Taken together, low a_w induced the production of bioactive metabolites in xerophilic Wallemia spp., what may have, besides contribution to territorial competition, a yet-to-be described protective role in the adaptation to the environments with low a_w (S e p c i c , Z a l a r et al., 2011).

A novel model organism for eukaryotic halophily studies

The species of the genus *Wallemia* are able to thrive at a_w lower than most of the known fungi, and the obligative halophilic character of *W. ichthyophaga* is exceptional not only in the phylum Basidiomycota but in the whole fungal kingdom. Our studies have so far revealed some unique morphological adaptations, especially regarding the cell wall, which enable *Wallemia* spp. to thrive in extremely saline environments. Thus, it represents the potential model organism for studies of eukaryotic halophily. Additional research on the adaptations to different stress, on a molecular level, will presumably give rise to new biotechnological applications for designing salt tolerant yeasts and plants. Nevertheless, *Wallemia* spp. are also interesting due to their production of bioactive metabolites with broad spectrum of activities (antibacterial, antifungal, antitumor and haemolytic). *Wallemia* spp. are commonly involved

in the spoilage of foods with low a_w, and the presence of walleminol A has already been found in the food contaminated by *W. sebi* (Mitchell, Godfree et al., 1999). Therefore, their bioactive potential should also be considered in the food quality control.

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ХЕТЕРОФИЛНЕ ГЉИВЕ РОДА *WALLEMIA* – БИОАКТИВНИ СТАНОВНИЦИ ПРИМОРСКИХ СОЛАНА И СЛАНЕ ХРАНЕ

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Резиме

Wallemia је род космополитских, хетерофилних гљива, често укључених у разлагање посебно слатког, сланог и сувог дрвета. Доскора је из овог рода била описана само врста *Wallemia sebi*. Када је велика група узорака, сакупљених у соланама и другим еколошким нишама, анализирана на нивоу физиолошких, морфолошких и молекуларних разлика, раздвојена је нова класа базидиомицета Wallemiomycetes, која покрива ред Wallemiales и индентификовано је три врсте рода *Wallemia*: *W. ichthyophaga, W. sebi* and *W. muriae*. *W. ichthyophaga* је призната као најхалофилнији еукариот откривен до данас и као таква представља еукариотски модел за стручније анализе адаптације на хиперсалинске услове. Наше прелиминарне студије указују да све три врсте синтетишу за сада недетерминисано хемолитичко једињење, у условима изненађујуће ниске влажности. Због доскора неоткривене таксономске ситуације, до сада није било налаза о продукцији било ког биоактивног једињења код три новоописане врсте. Циљ овог чланка је да представи таксономију, екологију, физиологију и до сада описане молекуларне механизме адаптације на живот под ниским условима водне активности, као и биоактивни потенцијал рода *Wallemia,* физиолошко-генетски старог и усамљеног таксона у оквиру Basidiomycota.

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PRESENCE OF DEOXYNIVALENOL IN SMALL-GRAIN SAMPLES FROM 2009/10 HARVEST SEASON

ABSTRACT: *Fusarium* head blight (FHB) is present in all growing regions of small grains and causes significant losses in yield and grain quality. In our environmental conditions, dominant species is *Fusarium graminearum* Group 2. During 2009/10 there was a significant *Fusarium* infestation on wheat, barley and triticale. The aim of this study was to examine the contents of deoxynivalenol (DON) in cereal samples taken after 2009/10 harvest season. We analyzed 22 NS varieties of small grains from Rimski Šančevi, including 16 varieties of winter wheat, one facultative wheat variety, four varieties of winter barley and one variety of triticale.

Analytical methods based on clean-up by solid-phase extraction (SPE) columns and detection by liquid chromatography were used. Fifteen out the 22 analyzed samples were positive for the presence of DON at a mean level of 0.537 mg/kg. The highest concentration was 1.952 mg/kg. These findings were in correlation with percentage of the *Fusarium* damaged kernels.

KEY WORDS: barley, deoxynivalenol, triticale, wheat

INTRODUCTION

Fusarium head blight (FHB) is the most destructive disease of small grains which causes significant losses in yield and grain quality. In our environmental conditions the dominant species is *Fusarium graminearum* Group 2 (B a g i, 1999). During the years with higher amounts of rainfall, the frequency of *F. culmorum* is greater. Besides these two species, *F. avenaceum* is also present in our fields, but it has much lower pathogenicity.

Other *Fusarium* species do not cause typical symptoms, but can be important from the aspect of production of mycotoxins in grains (S t o j a n o v i ć et al., 1998), which have harmful effects on human and animal health. Among

toxins produced by the pathogens of the genus *Fusarium*, the most frequent and the most important ones in a great number of agricultural products are deoxynivalenol (DON) and zearalenone (ZEA) toxins (JECFA, 2001). Deoxynivalenol is a member of the trichothecene family of mycotoxins. It is among the least toxic of the trichothecenes, but it is the most frequently detected one throughout the world. Its occurrence is considered to be an indicator of possible presence of other, more toxic trichothecenes (L o m b a e r t, 2002). Consumption of contaminated feeds by livestock has been associated with a variety of adverse health effects, including feed refusal, reduced weight gain, diarrhoea and emesis (K r s k a et al. 2001; K u i p e r-G o o d m a n, 2002).

The Commission of the European Communities (Commission Regulation 1881/2006) established the following tolerance values for DON in cereals and cereal-based products: unprocessed cereals other than durum wheat, oats and maize (1250 µg/kg), unprocessed durum wheat and oats (1750 µg/kg), unprocessed maize (1750 µg/kg), cereal flour, including maize flour, maize grits and maize meal (750 µg/kg), bread, pastries, biscuits, cereal snacks and breakfast cereals (500 µg/kg), pasta (dry, 750 µg/kg), and processed cereal-based food for infants and young children and baby food (200 µg/kg). The maximum permitted level of DON has been set in our country only in feed (Official Gazzette 2010) and in complete feeding stuffs for pigs, which is 500 µg/kg.

The aim of this study was to estimate the contents of DON in cereal samples (wheat, barley and triticale) taken after 2009/10 harvest season.

MATERIALS AND METHODS

Samples

Sampling of 22 NS varieties of cereals, including 16 varieties of winter wheat, one facultative wheat variety, four varieties of winter barley and one variety of triticale was performed. Immediately after sampling, 500 g of each sample were prepared by grinding in a laboratory mill, and the sample was homogenized by mixing. Samples prepared in such a way were packed in plastic bags and stored in a freezer at -20^0C until the analysis was carried out. Prior to each analysis, the samples were allowed to reach room temperature.

Extraction and clean-up

Mycosep 225 column (Romer Labs, USA) was used for purification. Amount of 25.0 g of the sample was extracted with 100 ml of acetonitrile–water (84:16, v/v) and shaken on an Ultra Turrax (IKA, Germany) for 3 min. After filtration, through Advantec filter paper, 3.0 ml of the extract were applied to the Mycosep 225 column. The cleaned-up extract was evaporated to dryness, dissolved in 3 ml of ethyl acetate and quantitatively transferred to an

evaporation vessel by triple washing with 1.5 ml ethyl acetate. The eluate was evaporated up to the dryness.

Liquid chromatographic analysis

The equipment consisted of an LC system – Liquid Chromatograph Agilent 1200 series (Agilent Technologies, USA) with a DAD detector (Agilent Technologies, USA) and a column Hypersil ODS (100 x 4.6 mm i.d., particle size 5 µm, Agilent Technologies, USA). LC analysis of DON was performed after evaporation, the residue was redissolved in 300 µl methanol, and a 15 µl aliquot of the solution was injected into the LC system. A mobile phase consisting of a mixture of acetonitrile–water (14:86, v/v) was used at 0.8 ml/min. UV detection was performed at 220 nm. Calibration curves used for quantitative determination were constructed on the basis of the area under DON chromatographic peaks, using working standard solutions. The detection limit measured as signal-to-noise ratio (3:1) was 0.045 µg/ml for DON which corresponds to 0.018 µg/g DON in wheat. The limit of quantification was 0.15µg/ml for DON which corresponds to 0.06 µg/g DON in wheat, and it is significantly lower than the recommended maximum permitted level.

RESULTS

Presence of DON in 22 samples of small-grain cereals is shown in Table 1.

Tab. 1 – Occurrence of deoxynivalenol in small-grain cereal samples from the 2010 harvest season

Cereal	**No. of samples**	**No. of positive samples (%)**	**Concentration in samples**	
			Average (mg/kg ± SD)	**Range (mg/kg)**
Wheat	17	14 (82.4)	0.435±0.43	0.068-1.572
Barley	4	–	–	–
Triticale	1	1 (100)	1.952	1.952
Total	22	15 (68.2)	0.537±0.57	0.068-1.952

Out of 17 analyzed wheat samples, even 14 samples (82.4%) were positive for the presence of DON. Concetration range of DON was 0.068-1.572 mg/kg, with average content of 0.435 mg/kg. None of the 4 analyzed barley samples was positive on the presence of DON. The highest concentration of DON was found in a single sample of analyzed triticale, and it was 1.952 mg/kg. The overall presence of DON in all analyzed samples was as high as 68.2%, with the average DON content being 0.537 mg/kg.

Content of DON in all varieties of small-grain cereals is shown in Table 2.

Tab. 2. – Content of deoxynivalenol in small-grain varieties

No.	Variety	Cereal	Concentration (mg/kg)
1	Nov 525	Barley	ND
2	Atlas 2010	Barley	ND
3	Nonius 2010	Barley	ND
4	Nov 565 2010	Barley	ND
5	Evropa 90 2010	Wheat	0.076
6	Rusija 2010	Wheat	0.708
7	Pesma 2010	Wheat	0.240
8	Renesansa	Wheat	ND
9	Pobeda 2010	Wheat	0.852
10	Ljiljana 2010	Wheat	0.084
11	Dragana	Wheat	0.088
12	Simonida 2010	Wheat	ND
13	Arija	Wheat	0.692
14	Angelina 2010	Wheat	0.276
15	40 S 2010	Wheat	0.532
16	Etida 2010	Wheat	0.088
17	Zvezdana 2010	Wheat	ND
18	Gordana 2010	Wheat	0.068
19	Gora 2010	Wheat	0.212
20	Vojvodina 2010	Wheat	0.608
21	Nataša 2010	Wheat	1.572
22	Odisej 2010	Triticale	1.952

DISCUSSION

Established presence of DON in wheat (82.4%) can be considered extremely high. In two samples, concentrations of DON were above the maximum level adopted by the European Commission (Table 2).

During the 2010 there were two critical periods at Rimski Šančevi for infection by fungus of the genus *Fusarium* (J e v t i ć et al., 2010a). The second period was considerably longer than the first one, and coincided with the period of flowering of most varieties. It lasted from 13-25th of May, 2010. The most critical period was from 14-18th of May, the when infections occurred on most varieties.

The percentage of *Fusarium* damaged kernels was determined in 16 wheat varieties which were included in the DON content testing. The percentage ranged from 1 to 11.5% and the calculated losses in 1000 kernel weight ranged from 1.2 to 5.7%.

In seed samples of Renesansa, Simonida i Zvezdana varieties, mycotoxin DON was not determined (Table 2). Zvezdana variety had the lowest percentage (1%) of *Fusarium* damaged kernels under natural conditions of infection (J e v t i ć et al., 2010b), which was in agreement with these results. Pobeda and Gora varieties, despite the significant percentage of infected grains, did

not have greater yield losses in 2010. Based on the observations, a certain level of tolerance to Fusarium head blight was observed. However, significant differences were observed with respect to content of DON. Nataša variety, in which concentration of DON was above the maximum level, had also a very high percentage of Fusarium head blight in field conditions. In some fields, it ranged up to 33.3% of infected spikes per m^2 (J e v t i ć et al., 2010a). In this paper, the DON content of hard (durum) wheat was not examined. However, based on previous research (unpublished data) durum wheat is very sensitive on Fusarium head blight. Therefore, we assume that the DON content in this type of wheat would have high percentage.

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ПРИСУСТВО ДЕОКСИНИВАЛЕНОЛА У УЗОРЦИМА СТРНИХ ЖИТА У ЖЕТВЕНОЈ 2009/10. ГОДИНИ

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Резиме

Патогени из рода *Fusarium* су присутни у свим рејонима гајења стрних жита и наносе значајне губитке у приносу и квалитету зрна. Фузариозу класа пшенице проузрокује већи број врста из рода *Fusarium*. У нашим условима гајења доминантна је врста *Fusarium graminearum* Група 2. У току 2009/10. године дошло је до значајне појаве фузариоза на пшеници, јечму и тритикалеу. Циљ рада је био да се испита садржај DON-а на узорцима стрних жита узетих после жетве из услова природне заразе. Анализиране су 22 новосадске сорте стрних жита из локалитета Римски Шанчеви, од чега: 16 сорти озиме пшенице, једна факултативна сорта пшенице, четири сорте озимог јечма и једна сорта тритикалеа. Узорци су узети са парцела различитих површина у зависности од значаја и распрострањености гајене сорте.

Аналитичко одређивање је засновано на пречишћавању сировог екстракта анализираних узорака помоћу тзв. Mucosep колона, а затим је садржај DON-а квантитативно одређен течном хроматографијом. Од 22 анализирана узорка стрних жита чак 15 (68,2%) је било позитивно на присуство DON-а. Још већи проценат зараженост DON-ом је утврђен када је у питању само пшеница (82,4%). Просечан садржај DON-а је износио 0,537 mg/kg а највећа концентрација је утврђена у узорку тритикалеа и износила је високих 1,952 mg/kg. Од свих узорака који су били позитивни на присуство овог микотоксина, 2 су превазилазила концентрације које су прописане од стране Европске комисије. Све ово указује на високу зараженост стрних жита са наших поља из жетве 2010. Проценат фузариозних зрна код 16 испитиваних сорти пшенице кретао се од 1 до 11,5%, а губици у маси 1000 зрна од 1,2 до 5,7%. Између јачине заразе у пољу и садржаја микотоксина DON установљена је потпуна позитивна корелација код појединих сорти. Факултативна сорта пшенице Наташа је имала високу концентрацију DON од 1,572 mg/kg, при степену заразе у пољу од 33,3% заражених класова по 1 m^2. Сорта Звездана имала је најнижи проценат заразе у пољу од 1% и код ње није детерминисано присуство микотоксина DON.

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ELISA AND HPLC ANALYSES OF DEOXYNIVALENOL IN MAIZE AND WHEAT

ABSTRACT: Deoxynivalenol (DON) is a part of the family of mycotoxins called trichothecenes which are produced by a number of different *Fusarium* mold species. The presence of DON in 25 wheat and 25 maize samples was examined by Enzyme Linked Immunosorbent Assay (ELISA) and High Performance Liquid Chromatography (HPLC) methods. The presence of DON was detected and determined in 5 (20%) maize and 6 (25%) wheat samples by both of the methods. Correlation between ELISA and HPLC results was established, with the correlation coefficients (r) of 0.9691 and 0.9735 for wheat and maize samples, respectively. The results obtained by ELISA method were significantly higher than those obtained by HPLC method. This fact can be explained by the presence of conjugated or masked mycotoxins in the samples, especially DON-3-glucoside (DON-3-Glc), which could not be determined by HPLC method due to the lack of external standards. Contrary to this, being insufficiently selective towards masked DON, ELISA method measures total DON content of a sample. According to the obtained results, ELISA can be used as a reliable screening method, but the confirmation of positive results must be done by HPLC method.

KEY WORDS: Deoxynivalenol, ELISA, HPLC, maize, wheat

INTRODUCTION

The presence of mycotoxins in agricultural products, mostly grains, causes a potential hazard to the health of humans and animals (Bennett et al., 2003). Mycotoxin presence depends on several factors, such as fungal strain, climatic and geographical conditions, cultivation technique, and crop protection, particularly during storage (Lo Curto et al., 2004).

Trichothecenes are a large group of agriculturally important mycotoxins produced by many *Fusarium* species. According to their chemical structure, they have been classified into four groups: A–D types; A and B are the most frequently found in cereals (W H O , 1990).

Deoxynivalenol (DON, vomitoxin) is the main representative of trichothecene B mycotoxins, as well as the most frequently detected trichothecene. This mycotoxin occurs predominantly in grains such as wheat, barley, oats, rye and maize, and less often in rice, sorghum, and triticale.

The occurrence of DON is associated primarily with *F. graminearum* (*Gibberella zeae*) and *F. culmorum*, both of which are important plant pathogens causing Fusarium head blight in wheat and Gibberella ear rot in maize (B e y e r et al., 2006).

In contaminated crops, deoxynivalenol-3-glucoside (DON-3Glc), 3-acetyl--deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON) can co-occur in significant amounts with DON. These mycotoxins are also produced by *Fusarium* species, and they are equally or less toxic than DON (P e s t k a , 2008).

Maize and wheat cover about 1,200,000 and 570,000 ha, respectively, which makes them very important crops grown in Serbia. Due to heavy rain season (2009/2010) just before the harvest time, the expected quality of the wheat and maize crop was below average, since moisture level was up to 19 percent (H u y n h et al., 2010). Although the consumption of maize and wheat has been increased, there are no sufficient data about the contamination of crops with DON in Serbia. Moreover, the maximum permitted level of DON in food has not been set in Serbia yet.

On the other hand, allowed limits of DON in food have been established in many countries. Maximum tolerated levels of DON in food on the territory of European Union are regulated by uniform regulations for the entire European Union (EC, No. 1881/2006). According to the regulation, MTLs for different cereal products are as follows: unprocessed cereals other than durum wheat, oats and maize – 1250 μg/kg; unprocessed durum wheat, oats and unprocessed maize – 1750 μg/kg; cereal flour, including maize flour, maize grits and maize meal, pasta – 750 μg/kg; bread, pastries, biscuits, cereal snacks and breakfast cereals – 500 μg/kg; processed cereal-based food for infants and young children and baby food – 200 μg/kg.

As DON represents potential hazard to the health of humans and animals, there is a need to control the presence of the most common trichothecenes in cereals and cereal based food by sensitive and reliable methods. Several methods can be used for DON analysis: thin-layer chromatography (TLC), enzyme-linked Immunosorbent Assays (ELISA), gas chromatography (GC) and lipid chromatography with different detectors (W H O, 2001). The chosen method depends on several factors. Nowadays, ELISA and HPLC are the most ***commonly used methods*** for determination of DON in maize and wheat. Major advantages of ELISA method are minimal sample clean-up procedures, simple procedure and low prices. Major disadvantage of ELISA method is

possible cross-reactivity to similar compounds. Therefore, confirmation by HPLC based procedure is required (A n k l a m et al., 2002).

The aim of this work was to determine DON content in wheat and maize samples using ELISA and HPLC methods, and to compare the obtained results.

MATERIALS AND METHODS

Samples

Twenty five wheat and twenty five maize samples were collected in Vojvodina. Samples were collected during November 2010, and stored at 4 °C in refrigerator before analysis.

Determination of DON by ELISA

Neogen Veratox® DON 5/5 test kits were used, and the procedure provided by the manufacturer was applied for the analyses. Free DON in the samples and controls was allowed to compete with enzyme-labelled DON (conjugates) for the antibody binding sites. After washing, substrate was added, which reacted with the bound conjugate to produce blue color. More blue color meant less mycotoxin. The test was read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls formed standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of mycotoxin. According to the manufacturer's description (Veratox®, Neogen), the detection limit for DON is 0.25 mg/kg.

Sample preparation. 10 g of ground sample was extracted with distilled water and shaken for 3 minutes. After filtration, the sample was ready for testing.

Determination of DON by HPLC-DAD

Determination of DON was carried out by HPLC Agilent 1200 model equipped with an Agilent diode array detector (DAD), Chemstation Software, a binary pump, a vacuum degasser and an auto sampler. The column was an Agilent column Hypersil ODS (100 x 4.6 mm, particle size 5 μm). The mobile phase consisted of an isocratic mixture of water/acetonitrile (86:14, v/v), with a flow rate of 0.8 ml/min. Fifteen microliters of standards and samples were injected onto the HPLC column. The spectra were recorded at 220 nm. Identification of DON was done by comparing the retention times and spectra of DON from samples with those of the standards.

Sample preparation. Around 25g of maize and wheat samples was extracted with water/acetonitrile (16:84, v/v) and shaken on ultraturrax for 3

minutes. After filtration through Advantec filter paper, extract was cleaned up on MycoSep column. The purified extract was evaporated to dryness under vacuum, redissolved in 3 ml of ethyl acetate and quantitatively transferred to an evaporation vessel by triple washing with 1.5 ml ethyl acetate. The eluate was evaporated to dryness, and redissolved in 300 μl of methanol.

RESULTS AND DISCUSSION

Twenty five wheat and twenty five maize samples were investigated. The first part of this study included determination of DON content by ELISA method. DON was found in 25.0% of wheat and 20.0% of maize samples. Positive samples were further analyzed by HPLC method which confirmed the presence of DON in all of them. The contamination frequencies and average values of mycotoxin content in the examined maize and wheat samples are shown in Table 1.

Tab. 1 – Contamination frequency (CF), interval (CI), and mean (CM ± SD) of maize and wheat samples

Samples (SN)	ELISA		HPLC	
Maize (25)	CF	20	CI	0.18 – 0.62
	CI	0.35 – 1.27		
	CM	0.75 ± 0.43	CM	0.39 ± 0.21
Wheat (25)	CF	25	CI	0.69 – 1.37
	CI	1.69 – 2.00		
	CM	1.83 ± 0.14	CM	1.00 ± 0.32

SN: sample number, SD: standard deviation, CF (%), CI (mg/kg), CM (mg/kg)

Content of DON in maize samples was 0.75 ± 0.43 mg/kg and 0.39 ± 0.21 mg/kg determined by ELISA and HPLC, respectively. Referring to the obtained results, content of DON in maize samples, determined by both of the methods, was in accordance with the European regulation (1.75 mg/kg). DON content in wheat samples was 1.83 ± 0.14 mg/kg and 1.00 ± 0.32 mg/kg determined by ELISA and HPLC methods, respectively. Positive results for wheat (3 out of 25) obtained by ELISA were higher than the maximum permitted value of 1.75 mg/kg. Contrary to this, positive results determined by HPLC method were lower than the maximum level allowed by European Regulation. J a j i ć et al. (2008) analyzed DON in maize (76 samples) and wheat (18 samples) during 2004 and 2005. The number of positive samples in maize and wheat was 44.7% and 37.5%, respectively.

The obtained results (Figure 1) indicate that ELISA results are significantly higher than the results obtained by HPLC method. This can be explained by the existence of conjugated or masked mycotoxins. The name of masked mycotoxins was derived as these substances escape routine mycotoxin detection methods, but can release their toxic precursors after hydrolysis

inside the gastrointestinal tract. DON-3-Glc, 3-ADON and 15-ADON appear quite often with DON in barley, maize, wheat, and other cereals. The similar molecular structure of DON and masked mycotoxins can be the reason for their competition in the antigen–antibody reaction. This phenomenon is called cross-reactivity and it can be the reason for overestimation and false results obtained by ELISA method (C a v a l i e r et al., 2005; A u r e l i et al., 2006; R u p r i c h et al, 2008; J a j i ć , 2008). B e r t h i l l e r et al. (2005) reported DON-3-Glc as the major form of masked DON, constituting up to 12% of total content of this mycotoxin in the examined samples of wheat and maize.

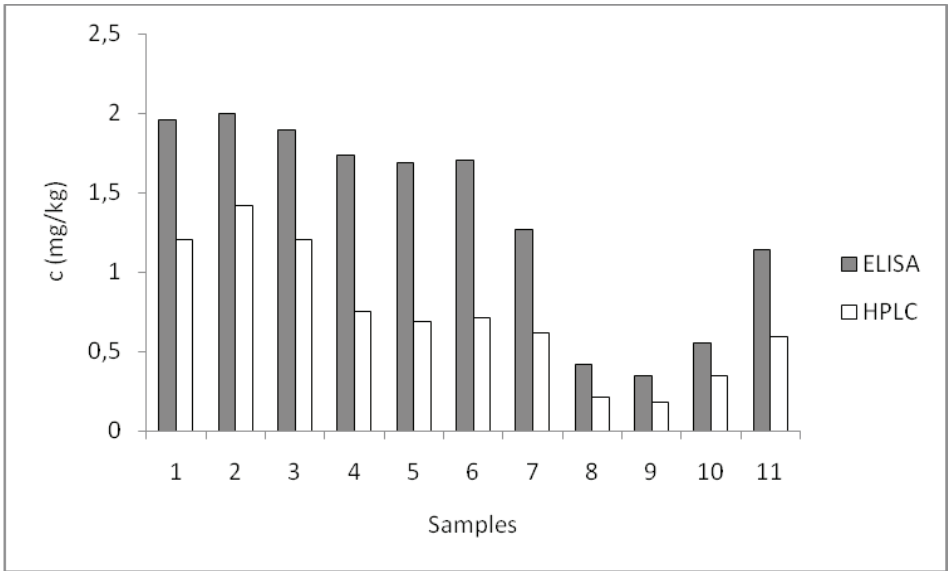

Fig. 1 – Comparison of ELISA and HPLC methods for DON determination in wheat (1-6) and maize (7-11) samples

R u p r i c h et al. (2008) found high level of cross reactivity of antibodies against DON mycotoxin with DON-3-Glc high, 82 and 98% in two repeated tests. They concluded that analytical results produced by ELISA could be interpreted as an approximate sum of DON and DON-3-Glc.

It should be emphasized that ELISA method used for determination of DON content cannot distinguish between DON, DON-3-Glc, 3-ADON, 15--ADON, and other similar compounds (J E C F A , 2001).

The correlation coefficients (r) between ELISA and HPLC data for wheat and maize samples were 0.9691 and 0.9735, respectively (Figure 2). The obtained values were in accordance with the results of other authors (A v a n t a g g i a t o et al., 2007).

Based on the given results, it can be concluded that immunoenzymatic methods are highly valuable for quantitative screening of DON presence in

food samples. Although ELISA gives overestimated results to some extent, we can fully rely on the results which are under the maximum allowed level. However, the confirmation of doubtful and/or positive ELISA results by HPLC must be done (A n k l a m et al., 2002).

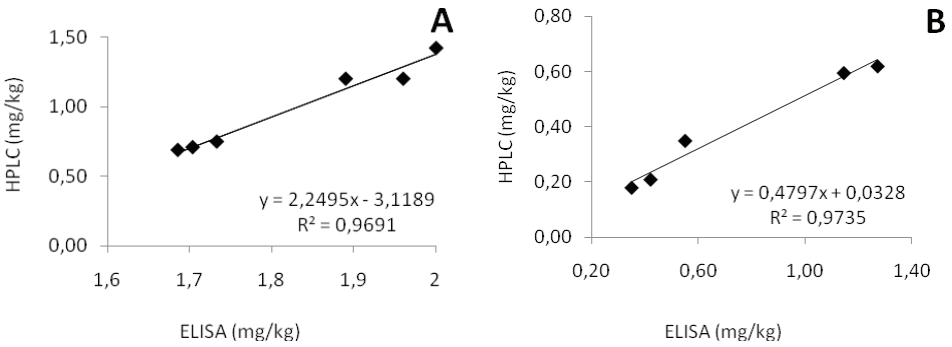

Fig. 2 – Correlation between HPLC and ELISA data for DON determination in wheat (A) and maize (B) samples

CONCLUSION

This study indicates the existence of risk related to the occurrence of DON in the food chain of Serbia, and importance of frequent monitoring of this mycotoxin. It is also important to establish maximum permitted level of DON in food at a national level.

The obtained results confirm that ELISA method can be used as a screening method for DON determination. However, confirmation of doubtful and/or positive ELISA results by HPLC method is required.

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АНАЛИЗЕ ДЕОКСИНИВАЛЕНОЛА У ПШЕНИЦИ И КУКУРУЗУ ПОМОЋУ ЕЛИСА И HPLC МЕТОДА

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Резиме

Деоксиниваленол (DON) припада групи микотоксина који се зову трихотецене, а које производе разне врсте плесни из рода *Fusarium*. Присуство DON-а у 25 узорака пшенице и 25 узорака кукуруза су испитани помоћу метода ELISA и течном хроматографијом под високим притиском (HPLC). Обе методе су потврдиле присуство DON-а у 5 узорака кукуруза (20%) и 6 узорака пшенице (25%). Утврђен је корелациони коефицијент (р) резултата добијених помоћу ELISA и HPLC метода, што је 0,9691 за узорке пшенице и 0,9735 за узорке кукуруза. Знатно виши резултати су добијени ЕЛИСА методом, у односу на HPLC метод. Ово се може објаснити присуством конјугованих микотоксина у узорцима, нарочито DON-3-глукозида (DON-3-Glc), који се не може утврдити HPLC методом због недостатка спољашњих стандарда. Насупрот томе, ELISA не прави довољну селекцију конјугованог DON-а, али мери његов укупан садржај у узорку. На основу добијених резултата утврђено је да је ELISA поуздан метод, али да се позитивни резултати морају добити HPLC методом.

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MYCOTOXINS IN HORSE FEED – INCIDENCE OF DEOXYNIVALENOL IN OAT SAMPLES FROM STUD FARMS

ABSTRACT: Reports concerning mycotoxins in horse feed are very rare and are typically restricted to fumonisins. As a non-ruminant monogastric species, horses may be more sensitive to adverse effects of mycotoxins, but the most severe effect of fumonisin B1 (FB1) in equines is that it causes fatal leucoencephalomalacia. In recent years, the European Food Safety Authority (EFSA) has evaluated several mycotoxins as "undesirable substances in animal feed" with the aim of establishing guidance values for the feed industry. In its evaluation of deoxynivalenol (DON), EFSA concluded that this toxin exhibited toxic effects in all species, but that horses were more tolerant towards this toxin than pigs. According to the available data, a systematic survey on mycotoxins in horse feed in Serbia has not been published. Therefore, the aim of this study was to investigate the incidence of mycotoxins in horse feed in Vojvodina. Samples of oats for horse consumption, collected in 2010, were analyzed by enzyme immunoassays (ELISA) for deoxynivalenol contamination. Twelve samples of oats were taken from twelve horse studs, with sport, school and hobby horses.

KEY WORDS: deoxynivalenol, feed, horse, mycotoxins

INTRODUCTION

There are two main groups of fungi that should be considered in the equine world. They are the field fungus *Fusarium*, which produces toxins such as fumonisin, deoxynivalenol (DON), zearalenone and T2, and the storage fungus *Aspergillus*, which produces toxins such as aflatoxin and ochratoxin. Important toxigenic fungus related to the horse disease is *Fusarium* because it is a potent producer of mycotoxins (Q u i n n et al., 2002).

Horses are the species which are most sensitive to fumonisin B1 (FB1) toxicity. The target organs in horses are central nervous system, liver and heart (V o s s et al., 2007). Two syndromes caused by FB1 have been described in horses, namely, the neurotoxic form of leukoencephalomalacia (ELEM) and the hepatotoxic form. These may appear independently or simultaneously. ELEM is characterized by the sudden onset of one or more of the following

signs: frenzy, incoordination, aimless circling, head pressing, paresis, ataxia, blindness, depression and hyperexcitability (Ross et al., 1991). Mortality is usually high and death may occur without clinical signs. The primary pathological feature is liquefactive necrosis of white matter of the cerebral hemispheres. To determine the levels of FB1 in feed associated with ELEM, Ross et al., (1991) studied 45 confirmed equine leukoencephalomalacia cases. FB1 concentrations ranged from <1 to 126 ppm, with the majority of the samples being above 10 ppm. The hepatotoxic syndrome occurs much less frequently than the neurotoxic form and usually takes 5–10 days from the time of onset of clinical signs to death. Clinical symptoms include loss of appetite and depression followed by oedema of the head and a prominent icterus (Voss et al., 2007). Elevated serum bilirubin concentration and liver enzyme activities are typically present. Neurotoxicity and hepatotoxicity have been reproduced experimentally in horses, ponies and donkeys by feeding them with naturally contaminated feed (Ross et al., 1993), fumonisins containing culture material (Wilson and Maronpot, 1971; Brownie and Cullen, 1987) and purified FB1 (Marasas et al., 1988; Kellerman et al., 1990). Deoxynivalenol (DON) is a member of the trichothecene family of mycotoxins. The occurrence of deoxynivalenol is associated primarily with *Fusarium graminearum* (*Gibberella zeae*) and *Fusarium culmorum*, both of which are important plant pathogens commonly found in cereals and other crops (JECFA, 2001). Although DON is among the least toxic of the trichothecenes, it is the most frequently detected one throughout the world and its occurrence is considered to be an indicator of the possible presence of other, more toxic trichothecenes (Lombaert, 2002). Although animals are differentially sensitive to DON (Pestka, 2007), reduced weight gain is universally observed in monogastric species including mice, rats, pigs, dogs and cats chronically exposed to the toxin at low doses (Forsyth et al., 1977; Hughes et al., 1999; Morrissey et al., 1985; Pestka and Smolinski 2005). The Commission of the European Communities (Commission Regulation No. 576/2006) established the tolerance values for DON in unprocessed durum wheat and oats (1750 ppb), and other unprocessed cereals (1250 ppb).

Johnson et al. (1997) investigated the effects of horse feeding by DON-contaminated grain. The five healthy adult horses were provided with barley naturally contaminated with DON for 40 days. The barley had been severely contaminated and analysis showed that the concentration of DON was between 36 and 44 ppm. No adverse effects, such as feed refusal, or altered serum chemistry or haematology, were identified during the feeding trial. The results suggested that, like ruminants but unlike other species, horses are relatively resistant to the adverse effects of DON.

In a further study (Raymond et al., 2003) horses were fed with grain naturally contaminated by Fusarium mycotoxins (DON, fumonisin, zearalenone) to investigate the effects of these mycotoxins on feed intake, serum immunoglobulin (IgA, IgG, IgM) concentrations, serum chemistry, and haematology. Such contaminated diet for horses resulted in reduced feed intake and increased serum gamma-glutamyl transferase (GGT) activities. Serum

levels of GGT were significantly higher in horses consuming contaminated grain which was sampled on the 7th and 14th day of supplementation, but not on the 21st day. The lack of difference in serum activities of GGT on 21st day implied that the horses may have adapted to the hepatotoxicity caused by the combination of Fusarium mycotoxins. According to J o h n s o n et al. (1997), serum levels of IgA, IgG and IgM were not affected by the diet.

Another study investigated the effects of feeding mature, exercised horses with grain naturally contaminated with Fusarium mycotoxins (R a y m o n d et al., 2005). In contrast to the findings of J o h n s o n et al. (1997), the results suggested a 35% decrease in feed intake, higher than the 65% reported in the previous experiment (R a y m o n d et al., 2003), possibly reflecting the increase in energy requirements due to exercise. Moreover, weight loss and unchanged serum activities of the hepatic membrane-associated enzyme, GGT, observed in horses fed with the contaminated diet as opposed to the control group (R a y m o n d et al., 2005), were in contrast with the authors' previous work (R a y m o n d et al., 2003). The results obtained by R a y m o n d et al., (2003 and 2005) suggested a relatively high degree of reduced feed intake when horses are simultaneously exposed to different fusariotoxins. However, the presence of fumonisin in the test diet (R a y m o n d et al., 2003) should have been addressed in the context of possible synergistic interactions between fumonisin and DON.

B a r n e t t et al. (1995) studied the correlation of mycotoxins with the incidence of colic in horses. Feed samples from farms experiencing possible feed related incidences of colic and control farms were analyzed. DON was found in the concentrates in 100% of the colic cases at levels ranging from 0.20 to 8.3 ppm and in 70% of the control group concentrates (0–2.5 ppm). However, the cause-effect relationship of mycotoxins and equine colic remains unclear although it should certainly be considered as a possibility, especially when a series of colic cases are observed on a single farm (N e w m a n and R a y m o n d , 2005; C a l o n i et al., 2009).

B u c k l e y et al. (2007) agreed with previous reports which suggested that zearalenone and deoxynivalenol (DON) can cause problems in horses. However, authors suggest that most horses will not eat feed contaminated with these mycotoxins unless they are starved, or they are in another feed ingredient that masks the taste and smell of mycotoxins.

There is little published information about the incidence of mycotoxins in horse feed in Serbia, but there are assumptions about their presence. Therefore, the aim of this study was to investigate the incidence of deoxynivalenol in oats for horse feeding in Vojvodina. Twelve samples of oats were taken from five private horse owners and seven equestrian ranches (horse clubs). Five samples were produced in the harvesting period in the summer of 2009 and rest (seven samples) in 2010.

MATERIALS AND METHODS

Oats for feeding horses in horse farms were kept in bulk or in bags in a dry place, and all samples were taken in a plastic bag which was sealed. Immediately after sampling, 500 g of each sample was prepared by grinding in a laboratory mill, and the sample was homogenized by mixing. Samples prepared in such a way were packed in plastic bags and stored in a freezer at -20^{0}C until analysis. Prior to each analysis, the samples were allowed to reach the room temperature. Extraction and clean-up of 20.0 g of the sample were extracted with 100 ml of distilled water and shaken on Ultra Turrax (IKA, Germany) for 3 minutes. After filtration through Whatman #1 filter paper, deoxynivalenol was analyzed by competitive direct enzyme-linked immunosorbent assays (ELISA). We used Veratox DON HS, Quantitative High Sensitivity Test (Neogen, USA) according to the manufacturer's instructions. The limit of quantification was 25 µg/kg (ppb).

RESULTS

From twelve samples used in our study, four samples (30%) were positive for DON, and we found the following concentrations: 0.005; 0.017; 0.094 and 0.150 ppm. It was remarkable, however, that none of the above mentioned symptoms of Mycotoxicosis were observed during the feeding with oats, used in our research. None of the 96 animals showed any reduced feed intake, feed refusal, reduced weight gain or clinical sign of hepatotoxicity.

DISCUSSION

Based on information received from the resident veterinarians and/or horse owners, we created a database about horses, their nutrition and care, and health. These data are discussed together with the findings of oats from the laboratory.

Our findings of DON in samples are in accordance with most studies on the occurrence of these mycotoxins in cereals (EFSA 2004 and 2005; Liesener, 2010; Buckley, 2007). Our investigation included a total of 96 horses from the equestrian clubs, and according to the category, they belonged to different breeds (Thoroughbred, Standard bred, Lipizzaner, pony and cross-breeds) and were aged between three and 20 years. The horses included 64 mares, 15 stallions and 7 castrates. Data about different equine category (mares, stallions, castrate) are important because there is a gender predisposition in relation to the occurrence of clinical symptoms in mycotoxicoses. Based on some investigations (Gonzalez-Cabrero et al., 1990) non castrated male animals (stallions) are more susceptible to this toxin than their female counterparts.

It is also important to note that the average level of DON was quite low (0.067 ppm), and that all samples met the requirements concerning guidance values for DON in cereals for animal feed industry (Commission Regulation No. 576/2006). This value for product intended for animal feed is 8 ppm. Nevertheless, the highest concentration of DON found (0.150 ppm) in oats for horse feeding in our study was below this level, and the majority of other samples was below 0.100 ppm.

Unfortunately, there are no data on no-effect or maximum level of DON in the "FSA – Opinion of the scientific panel" related only to horse feeding. There are only recommendations that other animal species, including rabbits, horses, cats and dogs, seem to have higher tolerance towards DON than pigs. On the other hand, at present, the available data do not allow the establishment of a no-effect level for pigs. The lowest reported levels with negative effect on feed intake vary between 0.35 and 0.9 ppm.

The initial adverse effect observed after the exposure to DON is reduced feed intake. These effects lead to a reduced body weight gain, particularly in growing animals. DON affects the immune response, and the release of pro-inflammatory cytokines is one of the earliest manifestations of the exposure (E F S A , 2007).

Our data showed that animals from five out of twelve horse farms (42%) were, besides forage (hay, clover, straw), fed with oats as feed concentrates. On the other seven horse studs, horses were fed with pellets, corn, barley, triticale and sugar beet pulp. Obviously, the individual toxin concentration of oat raw material would be much more important for the first group above mentioned (42%) than the added oats in the concentrated feed (pellets) and/or mixed with other cereals in the meal.

In conclusion, the results of first systematic survey demonstrate that mycotoxins are present in horse feeds in Serbia. In fact, the vast majority of these samples is considered as "food quality", since those toxins are present at the levels that are below the respective EU maximum levels for foodstuffs in the feeding industry. Although these findings are reassuring, it has to be acknowledged that very little is known about the adverse effects of other mycotoxins in horses in Serbia. Even though our study has not reported it, colic can be most common disease of the digestive system of horses in this area. In this sense, C a l o n i (2010) emphasizes that the possible cause-effect relationship between *Fusarium* mycotoxins and equine colic requires further investigation. Additionally, further extensive studies concerning mycotoxin intake from commercial and non-commercial sources of horse feed seem to be advisable.

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МИКОТОКСИНИ У ХРАНИ ЗА КОЊЕ – ДЕОКСИНИВАЛЕНОЛ У УЗОРЦИМА ОВСА СА ФАРМИ ЗА КОЊЕ

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Резиме

Извештаји о појави микотоксина код коња су веома ретки и обично се односе на фумонизин. Као моногастрична врста, а непреживари, коњи би требало да су осетљивији на деловање микотоксина, а најопаснији је фумонизин B1 (FB1) који код коња изазива леукоенцефаломалацију са фаталним последицама.

Протеклих година Европска Агенција за безбедност хране („EFSA") је испитала велик број микотоксина као "нежељених супстанци у сточној храни", делом и са циљем да успостави граничне вредности за индустрију сточне хране. У истраживањима о деоксиниваленолу (DON), у „EFSA" је закључено да он код свих врста изазива токсичне поремећаје, али да су коњи мање осетљиви на овај токсин него свиње.

На основу доступних података, до сада није било публикација о систематском истраживању микотоксина у храни за коње у Србији. Стога је циљ ове студије био да испита појаву DON-а у храни за коње у Војводини. Узети су узорци овса за исхрану коња, сакупљени у 2010. години, и анализирани помоћу ензимског имуно-везујућег теста (ELISA) на контаминацију деоксиниваленолом. Сакупљено је дванаест узорака овса из дванаест запата коња, који се користе за спорт, школу јахања и рекреацију.

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FUSARIUM SPECIES AND THEIR MYCOTOXINS IN WHEAT GRAIN

ABSTRACT: Incidence of fungi and concentration of mycotoxin deoxynivalenol (DON), zearalenone (ZON) and fumonisin (FB_1) were studied in the grain of the winter wheat collected subsequently after harvesting in 2010. In the analyzed samples the highest incidence was determined for the species of *Fusarium* (84.7%) genus, significantly lower incidence was determined for the species of genus *Alternaria* (12.35%), and especially for species of genera *Acremoniella* (2.05%), *Acremonium* (0.65%) and *Penicillium* (0.25%). *F. graminearum* (99.05%) was the most present species of *Fusarium* genus, whereas the following species *F. sporotrichioides* (0.4%), *F. subglutinans* (0.4%), *F. poae* (0.05%), *F. proliferatum* (0.05%) and *F. verticillioides* (0.05%) were only sporadic. The presence of DON, ZON and FB_1 mycotoxins was established in all studied wheat samples. DON was detected in concentrations from 123 to 393 μg kg^{-1} (average 214 μg kg^{-1}), ZON from 157.144 to 471.055 μg kg^{-1} (average 299.934 μg kg^{-1}), and FB_1 from 2.715 to 16.488 μg kg^{-1} (average 6.286 μg kg^{-1}).

KEY WORDS: *Fusarium* spp., wheat grain, incidence, mycotoxins

INTRODUCTION

Fusarium head blight (FHB) is one of the most serious diseases of cereals and causes significant losses in both yield and quality of cereals. When susceptible wheat genotypes are infected during flowering, the infected spikelets bleach prematurely. The disease progresses up and down the head and may infect all spikelets in a head when weather conditions are favorable (P a r r y et al., 1995). Frequently, only a part of the head is affected by FHB. These partly white and partly green heads are diagnostic. Additional indications of FHB infection are pink to salmon-orange spore masses of the fungus, often seen on the infected spikelet and glumes during prolonged wet weather. This causes considerable yield and quality losses and accumulation of mycotoxins in the grain (W i n d e l s , 2000).

Fusarium infected-grain often contains high concentrations of mycotoxins which are harmful to human beings and livestock. They are mainly from the group of trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), and other mycotoxins, such as zearalenon (ZON) and fumonisins (FB_1) (B a i and S h a n e r, 1994; N a k a j i m a, 2007; S t a n k o v i ć et al., 2010).

Several *Fusarium* species cause this disease, and *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae* (Schwein) Petch) is one of the most important species. *F. graminearum* can be divided into two chemotaxonomic groups, DON chemotype and NIV chemotype, based on the production of different trichothecene mycotoxins. DON is the most prevalent mycotoxin in cereals, and DON chemotypes of *Fusarium* are found worldwide. On the other hand, NIV chemotype is found in more restricted regions – this chemotype is found in Asia, Africa and Europe, but not in North America. Several countries have established legislative limits for DON in cereals. Thus, generally, greater attention is focused on DON than NIV as a trichothecene mycotoxin (N a k a j i m a, 2007).

The acute symptoms of trichothecene poisoning are characterized by skin irritation, food refusal, vomiting, diarrhoea, hemorrhage, neural disturbance, miscarriage and death (J o f f e, 1986). Chronic ingestion of small amount of trichothecenes may result in an important secondary effect, the predisposition to infectious disease through suppression of the immune system (M i l l e r and A t k i n s o n, 1987).

Zearalenone is another toxic metabolite produced by many *Fusarium* species and often co-occurs in cereals with trichothecenes. Zearalenone causes estrogenic effects and reduces reproductive capability (B i a g i, 2009).

Fumonisins are a group of toxic metabolites produced by *Fusarium* strains which contaminate cereals worldwide. Among fumonisins, fumonisin B_1 is always the most abundant. Porcine pulmonary edema is caused by fumonisins (B i a g i, 2009). Precaution should be taken to avoid inhalation of mycotoxin- containing spores and dust and direct skin contact with infected grains (T r e n h o l m et al., 1989). The control of fusarium head blight has relied on the resistant varieties and use of fungicides. Resistant cultivars are very rare and application of fungicides may be used for control of fusarium head blight (N o u r o z i a n et al., 2006).

Taking into consideration the importance of the harmful effect of *Fusarium* species and their mycotoxins in grains, especially wheat, the presence of pathogenic fungal species was studied, especially the presence of species of *Fusarium* genus, and presence of fusariotoxins, deoxynivalenol (DON), zearalenone (ZON) and fumonisin (FB_1) in wheat grains, immediately after harvesting in 2010.

MATERIALS AND METHODS

Wheat samples were collected immediately after harvesting in July, 2010. Twenty samples were selected for microbiological and 10 for mycotoxicological analysis. Collected samples were stored in refrigerator at 4ºC before analysis.

Moisture content was determined immediately after sampling. Average moisture content in the studied samples ranged from 10.75 to 15.73% (average 12.3%).

Samples of wheat grain for microbiological analysis were rinsed in tap water for one hour, disinfected in sodium hypochlorite solution (NaOCl) in the ratio of 3:1 for 5 minutes and subsequently rinsed several times in distilled water. After the grains were dried on filter paper, 100 grains of each sample were distributed in 20 Petri dishes containing 2% water agar (WA) and incubated at room temperature of 20±2ºC during 7 days. Based on morphological appearance of fungi colonies, species of various genera were identified, paying particular attention to the species of *Fusarium* genus (E l l i s, 1971; B u r g e s s et al., 1994; W a t a n a b e et al., 1994).

Based on the results of microbiological analysis of the samples, 10 samples were selected for mycotoxicological analysis. Samples were tested for presence of DON, ZON and FB_1 mycotoxins by using ELISA (Enzyme linked immunosorbent assay) methods. Samples were ground to fine powder. For the analysis of presence of ZON and FB_1 toxins, 5 g of each ground sample was mixed with 25 ml of methanol and 1 g of NaCl, whereas for the analysis of DON toxins, samples were mixed with water and 1 g NaCl. Samples prepared in this way were put in blender and mixed for 3 minutes. They were subsequently filtered through Whatman filter paper 1 and filtrate was collected. Dilution filtrate was carried out according to manufacturer instructions and differed depending on the concentration of toxin in the sample. ELISA procedure was performed following the manufacturer's recommendations (Tecna S. r. l., Trieste, Italy). Absorbance was determined using the spectrophotometer Elisa reader BioTek EL x 800^{TM} (Absorbance Microplate Reader) at 450 nm.

RESULTS

Based on microbiological analysis of wheat grains, the presence of five fungi genera was established: *Acremoniella, Acremonium*, *Alternaria, Fusarium* and *Penicillium*. Species of *Fusarium* genus were the most frequent (84.7%), followed by species from genera *Alternaria* (12.35%), *Acremoniella* (2.05%), *Acremonium* (0.65%), and *Penicillium* (0.25%) (Table 1).

Tab. 1 – Frequency of pathogenic fungi in wheat grain samples

Fungal genera	Frequency (%)
Acremoniella	2.05
Acremonium	0.65
Alternaria	12.35
Fusarium	84.7
Penicillium	0.25

F. graminearum, F. poae (Peck) Wollen., *F. proliferatum* (Matsushima) Nirenberg, *F. sporotrichioides* Sherb., *F. subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marasas, and *F. verticilliodes* (Sacc.) Nirenberg (syn. *F. moniliforme* (Sacc.)) Nirenberg (Table 2) were identified species of *Fusarium* genus. The most frequent species was *Fusarium graminearum* (99.05%), whereas other species were isolated only in 0.40% (*F. sporotrichioides* and *F. proliferatum*) and 0.05% (*F. poae, F. proliferatum* and *F. verticillioides*).

Tab. 2 – Frequency of *Fusarium* species in wheat grain samples

Fusarium species	Frequency (%)
F. graminearum	99.05
F. poae	0.05
F. proliferatum	0.05
F. sporotrichioides	0.40
F. subglutinans	0.40
F. verticillioides	0.05

The analysis of fusariotoxins showed presence of DON, ZON and FB_1 in all studied samples. ZON was detected in the highest average concentration (299.934 μg kg^{-1}) and in the widest range (157.144-471.055 μg kg^{-1}), followed by FB_1 with 6.286 μg kg^{-1} (ranging from 2.715-16.488 μg kg^{-1}) and DON with 214 μg kg^{-1} (ranging from 123-393 μg kg^{-1}) (Table 3).

Tab. 3 – Concentrations of *Fusarium* mycotoxins in wheat grain samples

Mycotoxins	Mycotoxin concentration (μg kg^{-1})	
	Average	Range
DON	214	123-393
ZON	299.934	157.144-471.055
FB_1	6.286	2.715-16.488

DISCUSSION

In these studies of mycobiota of wheat grain, the most frequent were *Fusarium* (84.7%), followed by *Alternaria* species (12.35%). Out of *Fusarium* species, the most frequent was *F. graminearum* (99.05%), whereas other species were present in very low percentage, from 0.05% (*F. poae, F. proliferatum* and *F. verticillioides*) to 0.4% (*F. sporotrichioides* and *F. subglutinans*). In similar studies, L e v i ć et al. (2008) established the presence of *F. graminearum* up to 55.5%. According to S t a n k o v i ć et al. (2007) two year results showed that the greatest number of wheat grain samples was infected with species of the genera *Fusarium* (81.8 and 65.0%), and *Alternaria* (36.3 and 17.5%) with the intensity ranging from 9.4 to 84.0% in 2005, and from 23.4 to 80.6% in 2006. Out of 13 identified species belonging to the genus *Fusarium*,

F. graminearum had the highest frequency (35.2 and 12.5%) and the intensity of up to 67.2 and 21.9% in 2005 and 2006, respectively, followed by *F. poae*, but only in 2005 (20.4%), and *F. proliferatum* in 2006 (19.7%) (S t a n k o v i ć et al., 2007).

During a two year investigation (2000-2001) of *Fusarium* spp. on wheat grains, W a a l w i j k et al. (2003) established that the species of *F. graminearum* was the most dominant in presence that was, on average, 58.4 and 58.6% in 2000 and 2001, respectively.

According to data of the Republic hydrometeorological service of the Republic of Serbia, May, 2010 was the month with tropical days, high temperatures and abundant rainfall, especially in the second and third ten days, when wheat was in the pheno stage of blooming. Such weather conditions were favorable for the development of *F. graminearum* and Fusarium head blight.

Presence of DON, ZON and FB_1 in all investigated samples of wheat grain in our research indicated that the weather conditions during the vegetation period of winter wheat in 2010 were very favorable for development of the studied fusariotoxins. ZON was detected in the highest concentration of 299.934 μg kg^{-1} (ranging from 157.144-471.055 μg kg^{-1}), followed by FB_1 with 6.286 μg kg^{-1} (ranging from 2.715-16.488 μg kg^{-1}) and DON with 214 μg kg^{-1} (ranging from 123-393 μg kg^{-1}). According to the data of J a j i ć et al. (2008), concentration of DON in samples of wheat grain ranged from 124 to 1235 μg kg^{-1}, depending on the conditions in the years of study. According to P a n c l a d i et al. (2004), grain samples, from three studied cultivars of durum wheat inoculated in the field with *F. graminearum* and *F. culmorum,* and non-treated by fungicides, had DON concentration ranging from 500 to 1.040 μg kg^{-1}.

According to K a m m o u n et al. (2009), infection levels in freshly harvested wheat grain were very low and the maximum DON level of the positive samples was 53 μg kg^{-1}. The investigation of the incidence of fusariotoxins in winter wheat in Belgium, conducted from 2002 to 2005, showed that *Fusarium* infection and DON contamination were mainly influenced by location and environmental parameters. Mean DON levels ranged from 0 to15.000 μg kg^{-1}. Seasonal and local weather conditions, before and during the flowering, along with local crop husbandry measures (crop rotation, soil preparation), seemed to be of great importance for the explanation of the variations in the obtained results. *F. graminearum* and *F. culmorum* were in general the most frequently occurring *Fusarium* spp. in Flanders over the 4 years, but the composition of the *Fusarium* population varied strongly depending on the location and year (I s e b a e r t et al., 2009).

In our study, high concentration of ZON in wheat grain samples was established (average 299.934 μg kg^{-1}). According to S t a n k o v i ć et al. (2007), ZON was determined in the range from 37 to 331 μg kg^{-1}, where 64.52% of positive samples had, on average, 133.4 μg kg^{-1}. The presence of ZON was determined in three most cultivated varieties in Serbia (Evropa-90, Pobeda and Renesansa) with an average for positive samples being 171.67, 110.50 and 114.83 ppb, respectively (S t a n k o v i ć et al., 2007). Contamina-

tion of high number of wheat grain samples (78.0%) with ZON (160-500 μg kg^{-1}) was also recorded by S t o j a n o v i ć et al. (2002).

Concentration of FB_1 in wheat grain in our investigation ranged from 2.715 to 16.488 μg kg^{-1}, which was not in concordance with lower percentage of isolated producers (0.05-0.40%). Similar results were obtained by S t a n k o v i ć et al. (2008). According to these authors, concentration of FB_1 in the studied samples of wheat grain ranged from 2.000 to 20.000 μg kg^{-1}, whereas the producers of this mycotoxin (*F. verticillioides, F. proliferatum* and *F. subglutinans*) were isolated in low percentage (0.8-8.0%).

CONCLUSION

Global climatic changes and increased warming of the atmosphere in recent years have had huge impact on an increase in the development of pathogenic and toxigenic fungi species in grains. Due to high temperatures and high air humidity during vegetation period of wheat, conditions favorable for spreading of *Fusarium* species are constantly present and recorded in Serbia. Accumulation of secondary fungal metabilites, mycotoxins, in crops, and later during the storage of grains, represents risk to human and animal health. Therefore, permanent and regular control of the quality of grains is a prerequisite for healthy nutrition of both humans and animals. Necessary measures of quality control also provide data on quantitative parameters/indicators of harmful and toxic substances in the food chain. These data generally create possibility for the development of public awareness of preventive measures against possible danger. Awareness of the danger caused by the presence of contaminants in food chain can significantly contribute to the application of measures which would ensure the required quality of food, thus preserving the quality of human and animal health.

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FUSARIUM ВРСТЕ И ЊИХОВИ МИКОТОКСИНИ У ЗРНУ ПШЕНИЦЕ

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Резиме

Учесталост гљива и концентрација микотоксина деоксиниваленола (DON), зеараленона (ZON) и фумонизина (FB_1) проучавана је у зрну озиме пшенице прикупљене непосредно након жетве у 2010. години. У испитиваним узорцима највећу заступљеност имају врсте из рода *Fusarium* (84,7%), а знатно мање врсте из рода *Alternaria* (12,35%), а посебно из родова *Acremoniella* (2,05%), *Acremonium* (0,65%) и *Penicillium* (0,25%). Од врста рода *Fusarium* најзаступљенија је врста *F. graminearum* (99,05%), док су спорадичне врсте *F. sporotrichioides* (0,4%), *F. subglutinans* (0,4%), *F. poae* (0,05%), *F. proliferatum* (0,05%) и *F. verticillioides* (0,05%). Присуство микотоксина DON, ZON и FB_1 установљено је у свим испитиваним узорцима зрна пшенице. DON је био детектован у концентрацијама од 123 до 393 μg kg^{-1} (просек 214 μg kg^{-1}), ZON од 157,144 до 471,055 μg kg^{-1} (просек 299,934 μg kg^{-1}) и FB_1 од 2,715 до 16,488 μg kg^{-1} (просек 6,286 μg kg^{-1}).

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FUMONISINS AND CO-OCCURRING MYCOTOXINS IN NORTH SERBIAN CORN

ABSTRACT: The presence of fumonisin has not been regulated in the legislation of the Republic of Serbia. Therefore, the data on contamination of cereals, especially corn, which is highly susceptible to contamination by this toxin, are not sufficient. This paper presents the results of testing the corn samples collected in the autumn 2009 on the territory of Bačka. Samples were analyzed for the contents of fumonisins and it was determined whether there is a correlation between the moisture content, total number and class of fungi, as well as the content of aflatoxin, ochratoxin and zearalenone. Using enzymatic immunoaffinity method it was discovered that the highest percentage of samples were contaminated with fumonisins, which was probably due to the presence of *Fusarium* molds as the most abundant ones. The positive samples contained fumonisin in the concentrations from 0.030 to 1.52 mg kg^{-1}. The influence of the climate and moisture content of grain on fungal contamination and mycotoxin production was analyzed in order to investigate the predictability of the presence of mycotoxins.

KEY WORDS: corn, ELISA, fumonisins, fungi, mycotoxins

INTRODUCTION

Fumonisins, secondary metabolites of fungi from the genus *Fusarium,* are mycotoxins that are most frequently found worldwide as natural contaminants in corn and corn-based products (WHO, 2000). They are important for both human and animal health. Experiments confirmed fumonisins to be causative agents of equine leukoencephalomalacia, porcine pulmonary oedema syndrome and producers of liver cancer in rats (WHO, 2000). In addition, oesophageal cancer in humans has been observed in distinct areas of the world, i.e. Southern Africa (Sydenham et al., 1990), Northern China (Chu and Li, 1994) and Northern Italy (Franceschi et al., 1990), where extremely high levels of fumonisins occur in corn and corn-based products. International

Agency for Research on Cancer (IARC) has classified *Fusarium moniliforme* toxins as possibly carcinogenic to humans (Group 2B carcinogens), similar to ochratoxin A (IARC, 2002).

Due to especially favorable climatic conditions and fertile soil in Serbia, especially in Vojvodina, its northern part, the most extensively grown crop is corn, which is mainly used as livestock feed. According to the data from the years 2005–2009, corn was planted on about 665.000 ha of arable land, with a total yield of about 3.7 million metric tons, indicating that corn growers are mainly individual agricultural producers (Statistical Office of the Republic of Serbia, 2010). Apart from somewhat lower yields, these producers face the problems concerning appropriate drying and storage, i.e. preservation of harvested corn quality, which raises questions about microbiological and mycotoxycological food and feed integrity. Namely, the data on mycologycal contamination of corn (Djilas et al., 2001; Lević et al., 2009) indicate a realistic possibility for mycotoxin production.

By studying the influence of abiotic factors (temperature, moisture content, water activity, and relative humidity) on the microflora and content of fumonisins in freshly harvested and stored corn, Orsi et al. (2000) concluded that there is a negative correlation between the presence of the genus *Fusarium* and mean temperature and air humidity, and a positive correlation between moisture content. Campa et al. (2005), by studying the effects of different factors, found that the production of fumonisins is most dependent on the location (i.e. weather conditions), insects, and finally on the hybrid type. Based on this, they developed a model that can predict fumonisin concentration using the variables such as weather conditions (daily precipitation, minimum and maximum daily temperature, relative humidity) and damages done by the insects. The weather conditions two weeks before and three weeks after the corn silking were found to be critical for the production of fumonisins.

Humidity level during the harvest period and prior to drying is important for the control of the mold growth and fumonisin production, and the data on the grain microflora may point out to the danger of mycotoxin presence. If the corn is not dried to contain less than 14% of moisture, there is a possibility of toxin production during the storage, too (Ono et al., 2002).

Out of more than 300 currently known mycotoxins, the highest effect on the health of humans and animals, apart from fumonisin, have aflatoxins, ochratoxin A, zearalenone and trichothecenes (Binder, 2007). Studies have shown that various mycotoxins present in combination have more severe effects than individual mycotoxins (Kubena et al., 1997).

The objective of this work was to investigate the contamination of corn from the area of northern Serbia with fumonisins, other mycotoxins and molds, as well as the possible influence of climatic factors on the degree of corn contamination.

MATERIALS AND METHODS

Corn samples were collected in the autumn of 2009 from different locations in Bačka. Sampling was conducted in the fields after the harvest, and immediately after, the samples were stored in the silos. After collecting, the samples were analyzed for total count of molds and their prevailing genera were determined. To this end, 1000 g of each sample were homogenized and prepared by grinding in a laboratory mill in such a way that > 93% passed through a sieve with pores 0.8 mm in diameter. After the analysis of the moisture content, samples were stored in a freezer at −20 °C for the analysis on mycotoxins. Prior to each analysis, the samples were allowed to reach room temperature.

Total counts of molds and moisture contents were determined by standard methods (The Official Gazette of SFRY, 25/80, The Official Gazette of SFRY, 15/87).

Contents of total aflatoxins, ochratoxin A, zearalenone and total fumonisins were determined by the enzymatic immunoaffinity (ELISA) method, using *Ridascreen*® test kits (Art. No. R:4701; R:1311; R:1401; R:3401, R-Biopharm, Germany), with limits of detection of 1 μg kg^{-1} for ochratoxin A, 1.75 μg kg^{-1} for aflatoxins and zearalenone, and 0.025 mg kg^{-1} for fumonisins.

The results of mycological and mycotoxicological analyses were subjected to multiple regression analysis using the software package Statistica, version 9.1 (StatSoft, Inc., 2010, www.statsoft.com).

RESULTS AND DISSCUSION

Bearing in mind the literature data on the effect of grain moisture on the mold growth and toxin production, the investigated samples were divided into two groups: in one group moisture content of the samples was ≤ 14%, and in the other it was > 14%; the results of mycological and mycotoxicological analyses were presented accordingly (Tables 1 and 2). It was found that the samples with higher moisture content were significantly more contaminated with molds (85.7%), when compared to the group of samples with lower humidity (65%), and a higher percentage of samples from the former group was contaminated with *Fusarium* spp. They were also characterized by higher frequency of positive samples and higher mean values of the content of total aflatoxins and ochratoxin A. This group of samples had also a somewhat higher mean value of the fumonisin content, although the percentage of positive samples and concentration range were the same or similar.

If all the samples are analyzed together (Table 3), although the contamination with molds (> 1000) was observed for a high percentage of samples (73.5%), only three samples had a total count of molds that exceeded the maximum allowed count predicted by the current regulations (The Official Gazette, 2010). The presence of molds of the genus *Fusarium* was confirmed in 64.7% of the samples. It can be concluded that *Fusarium* molds and

Tab. 1 – Results of mycological and mycotoxicological analyses of corn samples with moisture content ≤ 14%

LOCATION	Moisture content (%)	CFU (x1000/g)	Genus of fungi	Total Aflatoxins (ppb)	Zea-ralenone (ppb)	Total fumoni-sins (ppm)
Veternik	11.19	30	Ab	2.25	–	0.036
Čonoplja	11.77	20	Fu	–	–	0.057
Silo 3	11.98	10	Fu	–	–	0.098
Silo 1	12.03	10	Mu	–	2.43	–
Silo 4	12.27	100	Fu	–	3.02	–
Silo 6	12.34	–	–	–	3.32	0.262
Feketić	12.35	160	Fu	4.33	–	–
Silo 7	12.56	–	–	–	1.81	0.465
Silo 5	12.60	230	Fu	–	2.92	0.496
Lalić	12.81	80	Fu	–	2.24	–
Ruski Krstur	12.86	20	Fu	1.98	–	–
Silo 2	13.02	300	Ab, Fu	–	2.94	1.52
Mali Beograd	13.33	–	–	3.04	–	0.04
Savino Selo	13.40	–	–	–	–	–
Zmajevo	13.49	–	–	2.98	–	–
Lipar	13.47	–	–	–	–	–
Stepanovićevo	13.65	8	Fu, Ab	2.08	–	0.044
Temerin	13.71	220	Ab, Fu	–	–	0.143
Ravno Selo	13.71	–	–	–	–	–
Bačka Topola	13.91	10	Mu	2.58	–	–
Average	12.82	59.9		0.96	0.93	0.158
RSD	0.76	92.8		1.42	1.34	0.354
Average of positive samples		92.2		2.75	2.67	0.316
RSD of positive samples		102.0		0.81	0.53	0.457
% of positive samples		65.0	50 (on Fu)	35.0	35.0	50.0
Range of con-tamination	11.19–13.91	8–300		1.98–4.33	1.81–3.32	0.036–1.52

– not detected, Pe – *Penicillium* spp., Mu – *Mucor* spp., Fu – *Fusarium* spp., Ab – *Absidia* spp., CFU – total fungal colony count

fumonisin are present the most, but there is no significant correlation between the count of molds and contents of their toxins (Table 4), which is in agreement with the findings of M n g a d i et al. (2008). Contamination with toxins was also found in some samples with less than 1000 mold colonies. This can be explained by the fact that mycotoxins are stable compounds which can persist under the conditions that eliminate the molds that produce them (A l d r e d et al. , 2004). The survival of molds depends also on the microbiological interaction and competition, that is, it is possible that *Fusarium* spp.

Tab. 2 – Results of mycological and mycotoxicological analyses of corn samples with moisture content > 14%

LOCATION	Moisture content (%)	CFU (x1000/g)	Genus of fungi	Total Aflatoxins (ppb)	Ochratoxin A (ppb)	Zearalenone (ppb)	Total fumonisins (ppm)
Sombor	14.29	15	Fu, Ab	2.34	–	–	0.229
Rumenka	14.40	50	Fu, Ab	4.23	1.07	–	0.43
Svetozar Miletić	14.55	–		–	–	–	–
Sivac	14.23	8	Fu	–	–	–	0.257
Odžaci	15.09	25	Fu, Ab	3.93	–	–	–
Kisač	15.25	625	Ab, Pe, Fu	3.09		1.79	–
Vrbas	15.28	3	Fu	–	–	3.39	0.030
Bački Brestovac	15.68	70	Ab, Fu	–	–	–	–
Futog 1	16.54	10	Fu, Ab	–	–	–	0.036
Crvenka	16.61	–		2.34	–	–	–
Kucura	16.99	15	Fu	7.01	1.26	–	–
Kula	17.29	10	Fu	2.91	1.07	–	–
Despotovo	18.03	20	Fu, Pe, Ab	–	–	–	1.44
Futog 2	19.21	10	Fu, Ab, Pe	–	–	–	0.396
Average	15.96	61.5		1.85	0.24	0.37	0.201
RSD	1.53	163.4		2.21	0.48	0.99	0.389
Average of positive samples		71.7		3.69	1.13	2.59	0.403
RSD of positive samples		178.9		1.65	0.11	1.13	0.502
% of positive samples		85.7	78.6 (on Fu)	50.0	21.4	14.3	50.0
Range of contamination		3–625		2.34–7.01	1.07–1.26	1.79–3.39	0.030–1.44

– not detected, Pe – *Penicillium* spp., Mu – *Mucor* spp., Fu – *Fusarium* spp., Ab – *Absidia* spp. CFU – total fungal colony count.

under the conditions of lower humidity inhibit the growth of *Aspergillus* spp. (O n o et al., 2002), which have not been isolated.

Statistical analysis showed a significant correlation ($p<0.05$) between the moisture content and contamination with *Penicillium* spp. (0.55), *Absidia* sp (0.35), as well as between the moisture content and content of ochratoxin (0.35). Also, a significant coefficient of correlation (0.61) was found between the contamination with ochratoxin and aflatoxins.

In 50% of all analyzed samples, the content of fumonisins was above the limit of detection of the applied method. The mean value of fumonisin content in them was 0.352 ppm (range 0.030–1.52 ppm). Although fumonisins are present in the corn from Serbia, the total fumonisins content is below the values set by the EU regulations for the nutrition of humans (4 ppm, E C , 2007)

Tab. 3 – Average results of mycological and mycotoxicological analyses of corn samples

	Moisture content (%)	CFU (x1000/g)	Genus of fungi	Total Aflatoxins (ppb)	Ochratoxin A (ppb)	Zearalenone (ppb)	Total fumonisins (ppm)
Average	14.11	60.6		1.33	0.10	0.70	0.176
RSD	1.93	124.4		1.81	0.33	1.22	0.364
Average of positive samples		82.4		3.22	1.13	2.65	0.35
RSD of positive samples		139.3		1.33	0.11	0.61	0.46
% of positive samples		73.5	64.7 (on Fu)	41.2	8.8	26.5	50
Range of contamination		3–625		1.98–7.01	1.07–1.26	1.79–3.39	0.030–1.52

Fu – *Fusarium* spp., CFU–total fungal colony count

Tab. 4 – Distribution of total fungal content and the range of corn contamination with fumonisins

Total fungal colony count (in 1 g)	No of samples	No of positive samples on fumonisin	Range of fumonisin (ppm)
<1000	9	3	0.040–0.465
1000-200,000	21	11	0.030–1.44
>200,000	4	3	0.143–1.52

and animals (60 ppm, EC , 2006a). The contaminated samples also contained aflatoxins (41.1%), ochratoxin A (8.8%) and zearalenone (26.5%). None of the samples contained significant concentration of the investigated toxins, but chronic effect of their low concentrations should be taken into consideration.

Since fungal formation and toxin production are influenced by environmental factors during pre-harvest and harvest periods, prior to and during the harvest, the obtained results were discussed with regard to these conditions. The conditions of the vegetation season 2009 were typical of the climate in Serbia, which deviated from the average ones mostly on the territory of Vojvodina. Namely, the humidity conditions, apart from the northeast part, have the characteristics of a drought. In the middle of July, the measured temperatures (above 35°C) exceeded the optimum ones, which could have affected the course of the silking stage. The last month of the vegetation season was characterized by hot dry weather (RHZ , 2009). Such dry conditions caused less intensive production of toxins, which favor the conditions with water activity of 0.93 and humidity of even 25% (Sanchis and Magan , 2004). Lower production of fumonisins in drier ripening conditions is in agreement with our findings for the samples collected in 2001 and 2002 (Jakšić , 2004). Low fumonisin content, however, can be explained by the lack of favorable

conditions for the development of fungi due to warm and dry weather during the 2009 harvest, consequently lowering the kernel moisture.

The obtained results were compared with those obtained in Serbia in the previous year by other authors (Kokić et al., 2009; Matić et al., 2009). The results for fumonisin are similar, whereas our study gave higher frequency of samples positive on aflatoxins, as well as on zearalenone and ochratoxin, but at lower concentrations.

The obtained results were compared to those obtained in the neighboring countries – Croatia, Hungary, Romania and Bulgaria. Data from 1992 are similar to those presented in this work, regarding the frequency of positive samples, i.e. 58% for fumonisin B_1 (FB_1) and 21% for fumonisin B_2 (FB_2) in Croatia, along with 50% for FB_1 and 17% for FB_2 in Romania (Doko et al., 1995). The fumonisin content, however, was lower. Namely, the mean values of FB_1 content in the positive samples were 20 ng g^- (range 10–60 ng g^-) in Croatia, and 10 ng g^- (range 10–20 ng g^-) in Romania, while for FB_2 the value was 10 ng g^- in both countries. Conversely, according to the data obtained by Jurjević et al. (1999) and Domijan et al. (2005), the frequency of corn contamination by fumonisins (FB_1+FB_2) in Croatia, from 1996, 1997 and 2002, was significantly higher with values of 99%, 93% and 100%, respectively. The mean values for fumonisin content in the positive samples were 645 ppb of FB_1+FB_2 in the samples from 1996, 134 ppb of FB_1+FB_2 in the samples from 1997, and 459.8 ppb of FB_1, in the samples from 2002 with the concentrations of FB_2 in three positive samples (out of 49) being 68.4, 109.2 and 3084.0 ppb, respectively. The analysis of corn from 2007 (Šegvić Klarić et al., 2008) detected zearalenone in 91.9%, and ochratoxin A in 16.2 % of the samples, with mean concentrations of 318.3 ppb and 9.8 ppb, respectively.

The fumonisin content of corn flour and corn coarse meal samples has been examined in 1997 in Hungary (Fazekas, 2001). Fumonisins were detected in 67% of the samples, although the contamination levels were very low (16−58 ppb).

In Bulgaria, the analysis of corn samples on fumonisins and zearalenone under the conditions favorable for *Fusarium* molds (Manova and Mladenova, 2009) showed that only 21% of the samples were positive on zearalenone and 94.7% on fumonisin, in the range of 249−4050 ppb, with mean value being 1150 ppb, whereas according to the data from 2001 even 50% of samples contained fumonisin at a level of 0.03−6.56 ppm.

The importance of moisture content for the mold growth and fumonisin production between the harvest and drying phase was also emphasized by Ono et al. (2002). Drying immediately after the harvest and appropriate storage conditions can minimize toxin production. The results obtained by examining the corn from silos indicated the possibility of toxin production in Silo 2 because of the significant contamination, probably due to toxigenic molds, considering the already produced amount of fumonisins.

Contents of the investigated mycotoxins in 5 out of 34 samples were below the detection limit of the applied method, and 13 out of 29 positive samples contained more than one toxin. Content of aflatoxin did not exceed the

maximum tolerable level (The Official Gazette of RS, 2010; EC 2003, EC 2006b), whereas contents of other toxins were below the values prescribed/recommended by the EU legislation (EC, 2006a, 2007).

CONCLUSION

Based on all the above, it can be concluded that fumonisins are present in corn from Serbia, although in relatively low concentrations. Also, it can be concluded that their presence in corn has been confirmed in recent years and that the concentrations are similar to those found in the neighboring countries, with some deviations due to the differences in climatic conditions. It should be pointed out that such investigations indicate the indispensability of introducing the recommended limits for fumonisin in Serbia. Dry conditions, prior to harvest, and low kernel humidity lead to lower mycological and mycotoxicological contamination of corn. Further investigation should examine the effect of traditional corn storing, practiced by individual growers, on the degree of mycological and mycotoxicological contamination, encompassing trichothecene as well, due to their confirmed presence (Jajić et al., 2008).

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ПРИСУСТВО ФУМОНИЗИНА И ДРУГИХ МИКОТОКСИНА У КУКУРУЗУ СА ПОДРУЧЈА СЕВЕРНЕ СРБИЈЕ

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Резиме

Фумонизини нису обухваћени законским регулативама Републике Србије, па последично и нема довољно података о контаминацији житарица, а посебно кукуруза овим микотоксинима. У раду су приказани резултати испитивања узорака кукуруза прикупљених у јесен 2009. године са територије Бачке. Анализиран је садржај фумонизина и испитано да ли постоји корелација са садржајем влаге, укупним бројем и родом плесни, као и садржајем афлатоксина, охратоксина и зеараленона. Ензимском имуноафинитетном методом је утврђено да је, у односу на остале одређиване микотоксине, највећи проценат узорака био контаминиран фумонизинима, што је вероватно последица присуства плесни рода *Fusarium* као најзаступљенијих. У контаминираним узорцима је утврђена концентрација фумонизина у интервалу од 0,030 – 1,52 mg/kg. Анализиран је утицај климе и влажности зрна на последичну контаминацију плеснима и концентрацију микотоксина у циљу процене предвидљивости присуства микотоксина.

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INFLUENCE OF DEGREE OF WHEAT INFESTATION WITH *FUSARIUM* ON ITS TECHNOLOGICAL QUALITY AND SAFETY

ABSTRACT: Climatic conditions prior to wheat harvest 2010 were favorable for the development of field molds. The most important wheat contamination that should be determined is the presence and frequency of grain lots infected with *Fusarium*. This paper presents the results of content of fusarious kernels found in samples collected from all wheat growing regions of Serbia. Investigations were performed according to the regulations which foresee sensory determination of content of infected kernels. Determined average content of fusarious kernels was 6.01%, varying in range from 0% to 29.65 %. The obtained results, which exceeded the upper limits of permitted contents defined by national and EU regulations, pointed to the need for investigation of presence of *Fusarium* metabolic products, i.e. mycotoxins (zearalenone (ZEA) and deoxinivalenol (DON)) in wheat. Mycotoxin content was determined in average wheat samples and in wheat samples with increased content of fusarious kernels. Direct enzymatic immunoaffinity test (ELISA) was applied for determination of mycotoxin content. Although high content of fusarious kernels characterizes wheat crop in 2010, the determined quantities of two investigated mycotoxins did not exceed maximal permitted content. Consequences of unfavorable quantity and structure of total impurities in wheat crop in 2010 reflect decreased commercial and technological quality of wheat and point out to the need of necessary wheat safety control.

KEY WORDS: *Fusarium* spp., quality, mycotoxins, wheat

INTRODUCTION

Based on general opinions and facts related to the occurrence of global climatic changes in the world, and especially Europe, problem of potential impact of climatic changes on food safety has become the topic of interest for many authors. Nevertheless, specific influence on food safety is still unresolved issue. The following climatic changes were identified as relevant for agriculture and food production: temperature increase, precipitation variations, drought and increased level of atmospheric CO_2. The extent of influence of potential favorable and unfavorable effects of climatic changes on cereal crops depends on the above mentioned factors (development of molds,

production of mycotoxins, pesticide residues, impurities and pathogen microorganisms) (Miraglia et al., 2009).

Climatic conditions in wheat growing regions of Serbia, in winter and autumn of 2009/2010, were favorable for germination and protection against winterkill. In the period of kernel forming and filling, rainfall above the average resulted in delay of wheat maturation, which caused postponed beginning of the harvest and generated conditions favorable for the development of molds and germination of wheat on the ear (http://www.hidmet.gov.rs/). In the period of ear formation, under favorable weather conditions (temperature and rainfall), wheat can be infected with molds from *Fusarium* genus (B r e r a et al., 1998).

All cereals differ regarding its sensitivity to *Fusarium* infestation and accumulation of mycotoxins (M a t t h i e s et al., 2000; S i n h a and S a v a r d, 1997; F o u r b e t et al., 2001). Infestation of wheat with *Fusarium* spp. *(F. graminearum, F. culmorum, F. poae, F. avenaceum)* results in yield decrease, but even greater threat represents food and feed safety risk due to mycotoxin contamination (M a t t h i e s et al., 2000). Molds form *Fusarium* genus have the ability to produce several hundred different mycotoxins characterized by different chemical and biological properties, and extremely different toxigenic activities (carcinogenic, genotoxic, teratogenic, hapatoxic, immunotoxic) (Z i n a d i n e et al., 2006).

The aim of the research presented in this paper was to perceive the degree of infestation of wheat crop with *Fusarium* spp. in 2010, to analyze the influence of increased content of fusarious kernels on commercial and technological quality of wheat, and mycotoxin content in wheat samples.

MATERIALS AND METHODS

Commercial quality of 367 single lot wheat samples collected during the entire harvest from all wheat growing regions of Serbia was analyzed. Sampling was performed at 20 locations where harvested wheat was stored into warehouses. Composite samples were formed from single samples representing wheat from each of the involved locations, as well as the composite average wheat sample representing wheat harvested in 2010. On the basis of differences in protein and fusarious kernels contents, 13 additional composite wheat samples were formed. Commercial quality (test weight, protein content, gluten content, content and structure of impurities and Falling number) was determined for each sample, while technological quality (determination of dough properties using Brabender Farinograph, Extensograph and Amylograph) was determined for all composite samples. Fusarious and spoiled kernels were manually extracted from wheat harvested in 2010. For these samples, as well as for composite average sample of wheat harvested in 2010, quantitative determination of mycotoxins (aflatoxin (AF), ochratoxin A (OTA), zearalenone and deoxinivalenol) was conducted by direct enzymatic immunoaffinity method ELISA. Additional samples with high content of fusarious kernels were analyzed in respect to zearalenone and deoxinivalenol contents.

Moisture content, wet gluten content and sedimentation value of wheat were determined by Infratec 1241, according to the User Manual Infratec 1241 (Rev.2.3) – Foss Analytical AB, test weight was determined according to the method from the Regulation of methods of physical and chemical analysis for control of quality of wheat, milling and baking products and rapidly frozen dough (The Official Gazette SFRY 74/1988), Falling number was determined according to ICC standard No 107/1, while admixture content and structure was determined according to the Regulation about quality of agricultural products stored in public warehouses (The Official Gazette of Serbia 37/10). Determination of physical properties of wheat flour (dough) using Brabender Farinograph and Extensograph, and indirect determination of activity of cereal amylase using Brabender Amylograph were conducted according to ICC standards No. 115/1, No 114/1 and No. 126/1, respectively.

For the analysis of mycotoxins Veratox® Neogen aflatoksin (AF), ochratoksin A (OTA), zearalenone (ZEA) and deoxinivalenol (DON) test kits were applied. Readings of color intensity for quantification purposes was conducted at 650 nm using ELISA reader (Thermolabsystem, Thermo, Finland), and mycotoxin content was determined on the basis of calibration curve. According to producer's instructions, detection limits for AF, OTA, ZEA and DON are 1, 2, 25 and 250 (μg/kg), respectively. Effects of different levels of fusarious kernel content on commercial and technological quality of wheat were analyzed according to ANOVA statistical procedure from Statistica 8.0 software.

RESULTS AND DISCUSSION

Climatic conditions during the production period for wheat crop in 2010 were favorable for the development of field molds. On the basis of frequency analysis of contents of fusarious kernels determined in the samples of wheat crop in 2010, the distribution curve of fusarious kernels content was constructed (Figure 1).

Distribution curve of fusarious kernels content in wheat crop in 2010 has the shape of Gamma distribution with 6 degrees of freedom. The largest share of samples belongs to the interval of up to 10% of fusarious kernels in the sample, with the largest frequency of samples having relatively high content of fusarious kernels, from 2 to 4%. Frequency of samples with more than 10% of fusarious kernels gradually decreases towards higher contents of fusarious kernels, but samples with even more than 20% of fusarious kernels, although with low frequencies, were registered. Presented data confirm the assumption that wheat crop in 2010 had risky high content of fusarious kernels (Figure 2).

Samples with fusarious kernel content from 1.01 to 9.00% had the highest share (79%), 51% of samples of wheat crop in 2010 was with fusarious kernel content from 1.01 to 5.00%, and 28% of samples with fusarious kernels content from 5.01 to 9.00% (Figure 2). Only 4% of analyzed samples were characterized with fusarious kernel content under acceptable 1%. High share of samples with high fusarious kernel content points to the possible risk of mycotoxin

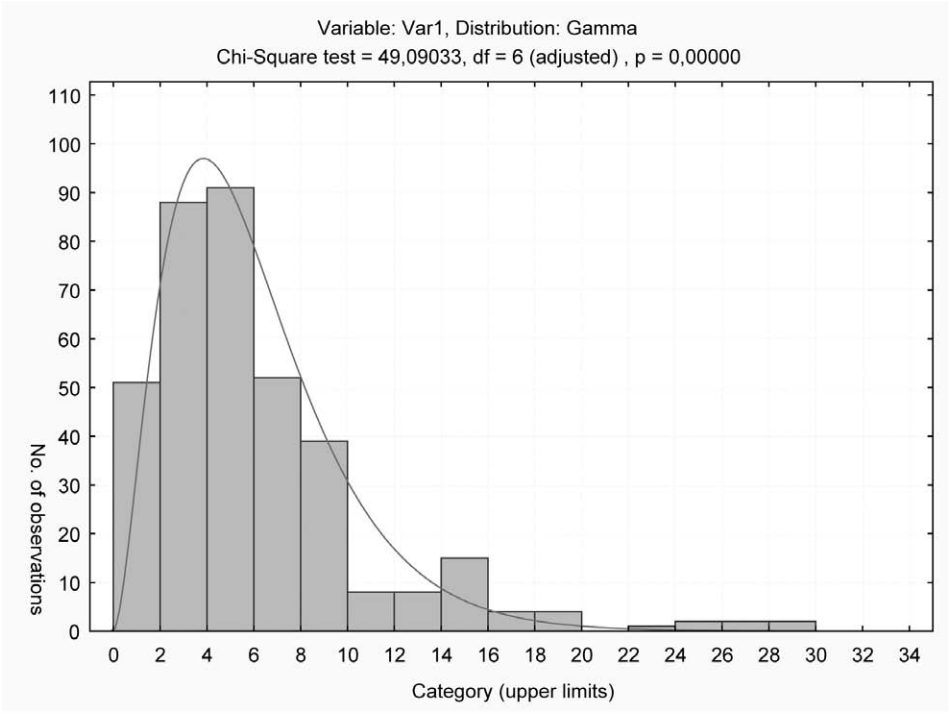

Fig. 1 – Distribution curve of fusarious kernels content in wheat crop in 2010

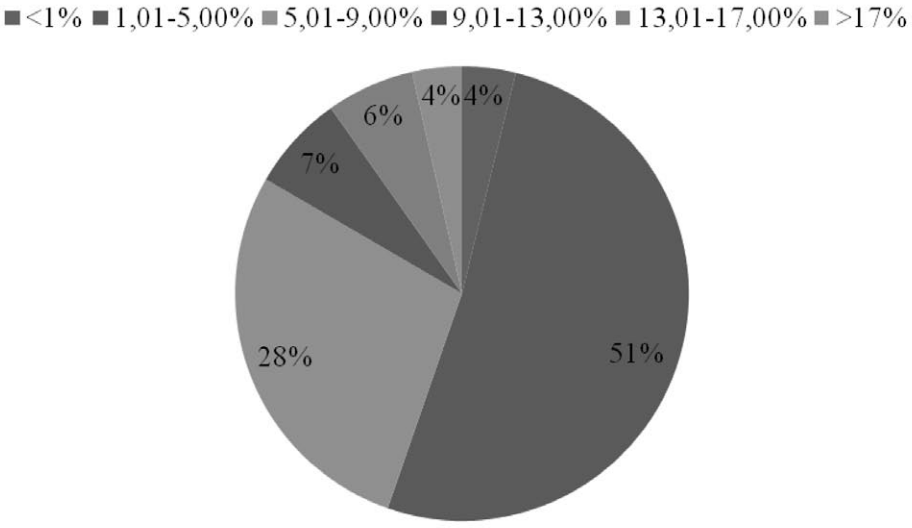

Fig. 2 – Share of samples of wheat crop from 2010 (%) depending on the range of fusarious kernel content

occurrence, as well as to the possible consequences of presence of high content of fusarious kernels that could influence commercial and technological quality of wheat.

In order to perceive the consequences of presence of different contents of fusarious kernels on commercial quality of wheat, expressed on the basis of test weight, protein content, wet gluten content, sedimentation value, falling number, content and structure of impurities, average values for samples grouped on the basis of level of fusarious kernels is presented in Table 1.

Tab. 1 – Influence of level of content of fusarious kernels on average commercial quality of wheat crop in 2010

Range of fusarious kernels content (%)	< 1	1,01-5,00	5,01-9,00	9,01-13,00	13,01-17,00	> 17,00
Number of samples	14	189	103	25	23	13
Parameters						
Fusarious kernels content (%)	$0,45^{a}$	$3,25^{b}$	$6,82^{c}$	$10,18^{d}$	$14,40^{e}$	$22,70^{f}$
Test weight (kg/m³)	$755,6^{a}$	$749,5^{ab}$	$737,4^{b}$	$735,6^{b}$	$736,2^{b}$	$739,2^{b}$
Protein content $(\%)_{dmb.}$	$13,28^{a}$	$13,06^{a}$	$13,24^{a}$	$12,79^{ab}$	$12,30^{b}$	$12,82^{ab}$
Wet gluten content (%)	$26,39^{a}$	$25,65^{ab}$	$25,88^{ab}$	$24,75^{abc}$	$23,74^{c}$	$24,47^{ac}$
Sedimentation value	41^{a}	39^{a}	39^{a}	37^{ab}	34^{b}	37^{ab}
Falling number according to Hagberg (s)	238^{a}	249^{a}	228^{a}	243^{a}	253^{a}	271^{a}
Total removable impurities content (%)	$2,08^{a}$	$2,68^{ab}$	$2,94^{bc}$	$3,05^{bc}$	$3,67^{c}$	$3,20^{bc}$
Total difficultly removable impurities content (%)	$5,95^{a}$	$9,12^{b}$	$13,26^{c}$	$17,50^{d}$	$21,05^{e}$	$28,54^{f}$
Total impurities content (%)	$8,03^{a}$	$11,80^{b}$	$16,20^{c}$	$20,55^{d}$	$24,71^{e}$	$31,74^{f}$

ANOVA statistical procedure and Duncan's test were applied for testing the significance of differences among average values of selected commercial quality parameters for samples (lots) of wheat with different level of fusarious kernel content (Table 1). Blends of wheat in which content of fusarious kernels was under 1.00%, were characterized with statistically different test weight in comparison to wheat blends with higher content of fusarious kernels. Wheat blends with content of fusarious kernels between 1.01 to 5.00% were characterized with test weight which differed from wheat with higher content of fusarious kernels, but the difference was not statistically significant.

When content of fusarious kernels in wheat is related to protein content, two groups of samples can be identified. In the first group there are wheat blends with fusarious kernel content of up to 9.00%, while the second group comprises of wheat blends with fusarious kernel content over 9.00%. The second group of samples is opposite to the findings of numerous authors (B o y a c i o g l u and H e t t i a r a c h c h y 1995; J a c k o w i a k, et al., 2005) and is characterized with lower protein content. This result was probably the consequence of the fact that wheat samples with fusarious content, originating

from all wheat growing regions of Serbia and representing different wheat varieties, were grown under different climatic conditions, and during the production different agricultural treatments were applied. The same effect could be observed in the case of sedimentation value.

Decrease trend of wet gluten content with an increase of fusarious kernel content could be noticed, but differences were not statistically significant.

Fusarious kernel content did not have statistically significant influence on Hagberg falling number, but the trend of increase of both falling number and fusarious kernels (above 9%) was noticed. It is well known that wheat varieties and growing location can significantly influence the falling number values. Nitrogen fertilization slightly increases falling number. According to the results of W a n g (2004), wheat infected with *Fusarium culmorum* is characterized with higher falling number values, indicating that falling number is not sufficiently suitable method for determination of alfa amylase activity in flours contaminated with molds.

Analysis of influence of fusarious kernel content on total content of removable impurities showed no statistically significant differences, but the trend of increase of total content of removable impurities with increase of fusarious kernel content existed. The removable impurities are composed mainly of shrunken and broken kernels which pass through 2 mm screening sieve. Wheat kernels infected with *Fusarium* in early kernel formation phases are without any doubt shrunken and can be found in removable impurities. When shrunken kernels are considered, although they cannot be directly connected to removable impurities, an indirect result is the registration of increase of removable impurities content.

Wheat blends with different levels of fusarious content differ with respect to the level of difficulty of impurity removal. Increased content of fusarious kernels in formed wheat blends results in difficult impurity removal. Fusarious kernels are considered as impurities difficult to for removal, which jeopardizes technological quality and safety of wheat due to inability of separation, resulting in transition of fusarious kernels to further processing steps. The same trend can be noticed in the case of total impurities which is higher than the tolerated limit of 8%, mainly as the consequence of impurities that are highly difficult to remove, among which fusarious kernels are predominant.

In order to comprehend the influence of fusarious kernel content in wheat blends on wheat technological quality parameters, composite wheat samples, in which rheological properties were analyzed, were grouped with respect to fusarious kernel content (Table 2).

Most of the average values of analyzed wheat, among which samples characterized with different content of fusarious kernels were present, showed no statistically significant differences in technological quality parameters. Increase of content of fusarious kernels is accompanied with trends of decrease in Farinograph quality number, dough stability and resistance, and trends of increase in dough degree of softening, Extensograph energy, resistance and ratio number (Table 2). Statistically significant differences were confirmed only in the case of Amylograph peak viscosity without confirmation based on

Tab. 2 – Influence of fusarious kernel content on technological quality of samples of wheat crop 2010

Range of fusarious kernels content (%)	≤ 5,00	5,01-10,00	≥ 10,00
broj uzoraka	6	8	4
Parameters			
Amzlograph peak viscosity (Au)	248[a]	252[a]	174[b]
Faringraph water absorption (%)	56,8[a]	56,6[a]	57,5[a]
Faringraph dough development time (min)	2,2[a]	2,0[a]	2,0[a]
Faringraph dough stability time (min)	1,5[a]	1,1[a]	0,8[a]
Faringraph dough mixing resistance (min)	3,7[a]	3,2[a]	2,8[a]
Faringraph dough degree of softening (Fu)	96[a]	98[a]	110[a]
Farinograph quality number	55,8[a]	53,8[a]	51,3[a]
Extensograph energy (cm^2)	31[a]	32[a]	34[a]
Extensograph dough resistance at 5 cm (Eu)	111[a]	121[a]	128[a]
Extensograph dough extensibility (mm)	159[a]	153[a]	155[a]
Extensograph ratio	0,68[a]	0,79[a]	0,83[a]

Falling number value (Table 1), but in accordance with the findings of some authors, W a n g et al. (2005), who discovered that the samples with extremely high content of fusarious kernels were characterized with obviously higher values of Hagberg Falling number, whereas saccharose content in flour decreased. Other authors stated that contamination of wheat with storage molds (*Aspergillus* spp. and *Penicillium* spp.) resulted in decrease of baking performance of wheat flour. It is interesting to mention that the content of mycotoxins (DON) in analyzed wheat samples was not in relation with rheological parameters and flour baking properties (A n t e s et al., 2001).

Fungal amylolytic decomposition leads to degradation of small starch granules (J a c k o w i a k et al., 2005), and due to decreased total specific area of starch granules and higher degree of damage of starch component of endosperm, in comparison to healthy kernels, fusarious kernel becomes more subjected to decomposition of starch component by fungal alpha amylase. The consequence of this phenomenon is the decrease in Amylograph peak viscosity.

B o y a c i o g l u and H e t t i a r a c h c h y (1995) confirmed that in wheat, moderately contaminated with *F.graminearum,* albumin content decreased by 33% and glutenine content by 88%, in comparison to the control sample, in spite of higher total protein content in the contaminated samples than in the healthy kernels. M e y e r et al. (1986) found out that German varieties contaminated with *F. culmorum* had worse baking properties which attributed to damaged gluten fractions. D e x t e r et al. (1996) determined that red spring wheat contaminated with *Fusarium* resulted in altered flour color, weak dough properties and unsatisfactory bread quality. Gluten originating from fusarious wheat had lower content of glutenin fraction in comparison to gluten of healthy wheat.

Determined content of fusarious kernels in wheat blends, formed on the basis of different ranges of protein content as the wheat segregation parameter applied by relatively small number of warehouses that segregate wheat in Serbia, is presented in Figure 3.

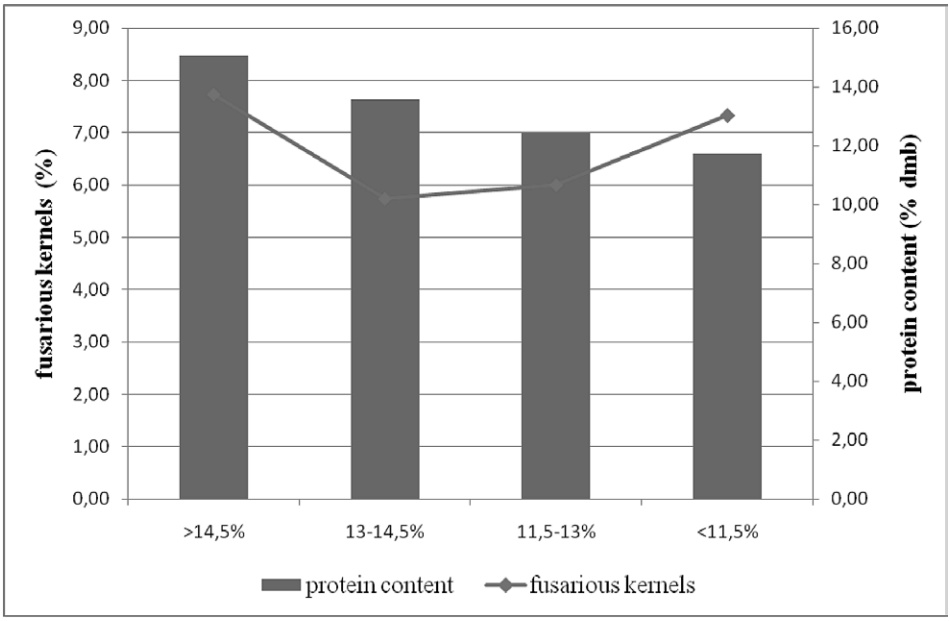

Fig. 3 – Content of fusarious kernels (%) in wheat blends formed from wheat samples from different protein content ranges

The highest content of fusarious kernels was determined in wheat samples with protein content above 14%$_{dmb}$. This result is in concordance with the findings of many authors (B o y a c i o g l u and H e t t i a r a c h c h y 1995; J a c k o w i a k et al., 2005). Higher fusarious kernels content was also determined in wheat blend obtained from samples with protein content under 11,5%$_{dmb}$. This is probably the consequence of influence of set of factors: soil composition, climatic conditions, inappropriate agricultural measures, absence of crop protection and other.

In order to obtain preliminary insight into presence of mycotoxins in wheat crop from 2010, the investigations of mycotoxin presence in the selected samples were conducted as presented in Table 3.

In average wheat sample from crop 2010 and in sample No.3 (5.65% of fusarious kernels), in spite of high fusarious kernel content (6.01%), the presence of mycotoxins AF, OTA, ZEA was not determined. Determined content of DON was under permitted limits according to the European regulations (1250 µg/kg). In the samples of fusarious kernel, content of mycotoxin ZEA was beyond both national (1 µg/kg) and European (100 µg/kg) regulations. Content of DON was determined to be above the limit of quantification of applied

Tab. 3 – Presence of mycotoxins in wheat crop from 2010 in respect to fusarious kernel content

Range od	Mycotoxins (µg/kg)			
	AFs	OTA	ZEA	DON
Determination (µg/kg) Sample	1-8	2-25	25-500	250-2000
Average	-	-	-	1060
Fusarious kernels (100%)	-	-	242	>2000
Spoiled kernels (100%)	-	-	103	1720
Sample 1 (15,18% fusarious kernels)	*	*	340	>2000
Sample 2 (8,83% fusarious kernels)	*	*	328	1630
Sample 3 (5,65% fusarious kernels)	*	*	-	380

– under limit of quantification
* not analyzed

method and above European regulations (1250µm/kg) in the same samples. In the sample of spoiled kernels, as well as in the sample No. 2 (8.83% of fusarious kernels) determined contents of ZEA and DON were above the limits of European regulations. Having in mind the possibility of false positive results which can be obtained by applying ELISA method, it is important to check the obtained results with a confirmative method. G u y et al. (2004) proved that dark bread obtained from wheat after cleaning and intensive scrubbing contains about 40-50% of starting quantities of OTA, independently of level of starting contamination of wheat. Starting contamination of 20-30% was determined in white bread. There are not many results concerning the content of DON in products of wheat processing. DON is stable and results in contamination of corn even under extreme pH values and temperature. As for ZEA, it was determined that 60% of starting contamination remained in bread and 50% in pasta.

Results from multiple research confirm that fungal breeds of the same species, even their isolates from the same region, express variability in mycotoxin production (B l a n e y and D o d m a n, 2002; L o r e n s et al., 2004b; V o g e l g s a n g et al., 2008a; K o k k o n e n et al., 2010). All presented facts point at actual risk of presence of mycotoxins (ZEA, DON) in wheat crop from 2010 in the quantities close to limits permitted by European and national regulations.

CONCLUSIONS

Wheat crop from 2010 is characterized with high content of fusarious kernels. Most samples are characterized with fusarious kernel content at the level of 1 to 9%. Only 4% of samples had content of fusarious kernels that was under 1%, content permitted by the regulations.

Increased content of fusarious kernels resulted in decrease of commercial (decreased test weight and protein content and increased content of impurities) and technological quality (trends of decrease of Farinograph quality number, stability and resistance of dough, Amilograph peak viscosity and trend of increase of Farinograph dough degree of softening) of wheat.

Increased content of fusarious kernels in wheat crop from 2010 resulted in increased risk of occurrence of mycotoxins in wheat and consequently in wheat products. On one hand, this emphasizes the need of application of agricultural measures for prevention of development of fusarium in agricultural production, while on the other hand, it also points to the need for introduction of contemporary wheat cleaning methods which enable separation of fusarious kernels from wheat prior to processing.

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УТИЦАЈ СТЕПЕНА ЗАРАЖЕНОСТИ ПШЕНИЦЕ МИКОТОКСИНОМ *FUSARIUM* НА ЊЕН ТЕХНОЛОШКИ КВАЛИТЕТ И ИСПРАВНОСТ

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Резиме

Климатски услови пред жетву 2010. године били су повољни за развој плесни. Најважнији аспект код загађења пшенице је одређивање присуства и учесталости микотоксина из рода *Fusarium* на парцелама пшенице. У раду су представљени резултати истраживања количине фузариозних зрна у узорцима узетим из делова Србије у којима се узгаја ова житарица. Истраживање је обављено у складу са прописима који предвиђају одређивање садржаја заражених зрна. Утврђена количина фузариозних зрна је у просеку била 6,01%, варирајући од 0% до 29,65 %. Добијени резултати су прелазили горње дозвољене границе које су утврђене европским регулативама, што указује на неопходност даљег истраживања метаболичких производа који су заражени врстама из рода *Fusarium*, тј. микотоксинима (зеараленоне (ZEA) и деоксиниваленол (DON)). Садржај микотоксина је установљен у узорцима пшенице просечних карактеристика, као и у узорцима пшенице са повећаним садржајем фузариозних зрна. Садржај микотоксина је утврђен помоћу ELISA теста. Иако усев пшенице из 2010. године има присутан велик број фузариозних зрна, утврђене количине два испитивана микотоксина нису прелазиле максимално дозвољену границу. Последица неповољне количине и структуре нечистоћа које су утврђене у пшеници из 2010. године је смањен тржишни и технолошки квалитет пшенице, што указује на неопходну контролу квалитета ове житарице.

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MYCOPOPULATIONS AND OCHRATOXIN A – POTENTIAL CONTAMINANTS OF *PETROVSKÁ KLOBÁSA*

ABSTRACT: *Petrovská klobása* is traditionally produced dry fermented sausage from the area of Bački Petrovac (Vojvodina Province, Serbia) that has been protected with designation of origin (PDO) according to Serbian legislation. Contamination of this kind of sausage casings by different mould species often occur during the production process, mainly during the ripening and storage. The aim of this study was to isolate and identify moulds that contaminate ingredients used for *Petrovská klobása* production and its casings during different phases of ripening and storage. Sampling was done during the production process and after 2, 6, 9, 11, 14, 34, 65, 90, 120, 217 and 270 days. Total mould counts in components ranged from 1.60 (mechanically mixed filling) to 4.14 (red hot paprika powder) $\log_{10}$ CFU/g, while the number of moulds isolated from sausage casing surfaces ranged from 0.01 (C_3 sausage, 217th day) to 1.60 (C_1 sausage, 270th day) $\log_{10}$ CFU/cm^2. After total mould counts were determined, isolates were identified and classified in five genera for components (*Penicillium* – 7 species; *Fusarium* – 2 species; *Aspergillus* – 1 species; *Alternaria* – 1 species; *Verticilium* – 1 species) and 3 genera for casings surfaces (*Penicllium* – 3 species; *Aspergillus* – 1 species; *Eurotium* – 1 species). It was appointed that 83.33% of isolated species are potential producers of toxic metabolites.

The analyses of ingredients and sausages on the presence of ochratoxin A, following the ELISA method, gave the negative results.

KEY WORDS: fermented sausage, mould contamination, ochratoxin A

INTRODUCTION

Fermented dry sausage is defined as a mixture of seasoned, raw meat, fat, salt and various spices, which is stuffed into casings, subjected to fermentation and then allowed to dry (Leroy et al., 2006; Ammor and Mayo, 2007). Distinctive environmental and climatic conditions, as well as cultural and social backgrounds of the populations in different geographical regions, determine a great variety of fermented sausages, produced in European coun-

tries (Ikonić et al., 2010). *Petrovská klobása* is one of the most representive Serbian fermented sausages. It is a traditional dry fermented sausage, produced in small household enterprisses in the municipality of Bački Petrovac (Vojvodina province, Serbia). It is produced from pork meat and fat, with addition of spices, salt and sugar. The traditional procedure exludes the addition of any aditives and starter cultures (Petrović et al., 2010; Jokanović et al., 2010; Tasić et al., 2010). Due to its unique sensory profile and recognizable quality, *Petrovská klobása* has been protected with designation of origin (PDO) according to Serbian legislation (Petrović et al., 2007).

Mould growth on traditional dry fermented sausages can be observed during ripening and storage. It can be a quality problem, because if extensive growth has occurred, sausages may become spoiled due to visible mould colonies on the surface and off-flavors they produce. Moreover, mould growth may represent a health risk because some of the fungal species associated to meat products, such as *Penicillium*, *Aspergillus* and *Fusarium* species, are able to produce mycotoxins (Frisvad and Thrane, 2002). Mycotoxins are extracellular metabolites and they diffuse into substrate after synthesis in fungal cells, so removing of mouldy sausage casings before consumption wouldn't remove the toxins. Mould growth on fermented sausage is therefore an important issue, as it may present a significant economic problem, as well as risk for human health (Papagianni et al., 2007; Castellari et al., 2010).

The most effective way to prevent contamination of fermented sausages with mycotoxins is to avoid growth of mycotoxigenic fungi (Muñoz et al., 2010). The first step so would be isolation of moulds from raw material and moulds that contaminate fermented sausages in different phases of ripening and storage, followed by identification of possible toxigenic species among the isolates.

The aim of this work was to isolate and identify mould contamination of the raw material used for dry fermented *Petrovská klobása* production, and its surface mycobiota in different periods of ripening and storage. Also, all components used for the sausage production and sausages sampled in different phases of ripening and storage, were tested on the presence of ochratoxin A.

MATERIALS AND METHODS

In the present study, mycological and mycotoxicological examinations of *Petrovská klobása* during processing, ripening and storage were carried out.

Sausage production: Sausages were produced in December 2009, in Bački Petrovac, from cold pork meat and fat, salt, red hot paprika powder, garlic and caraway, using traditional procedure. The filling was prepared by two different methods – in the first case, the components were mixed manually, and in the second case they were mixed using mixing machine. The first filling was divided in two parts – one was stuffed into pig natural casings (samples C_1) and other was stuffed into plastic casings (samples C_2). The second filling was stuffed in plastic casings (samples C_3).

Ripening and storage: Produced sausages were left to ripen in chambers in Bački Petrovac (1^{st}-11^{th} day) and Kucura (11^{th}-65^{th} day). On the 65^{th} day they were moved and stored in an industrial facility in Novi Sad.

Mycological investigations: In order to evaluate mould contamination, a sow carcass was swabbed after barking, covering four locations at the carcass (ham, back, belly and jowl). Pig natural casings were also swabbed. Examined parameter was total mould count per square centimeter, expressed as $\log_{10}$ CFU/cm^2. Wet swabs were placed in tubes containing 9 ml of sterile saline solution, a series of decimal dilutions were prepared and 1 ml of each dilution was placed in sterile Petri dish in duplicate. Plates were subsequently poured with 12-15 ml of warm (45 ± 1°C) Sabouraud-maltose agar containing 2% of chloramphenicol. Medium was mixed well with inoculum; the mixture was allowed to solidify and incubated at the 25°C during 7 days. Sampling of the two fillings and the components (chopped meat, salt, garlic, caraway and paprika) was carried out parallel with manufacturing process, and total mould count per gram was determined, using dilution method by Koch (Harrigan, 1998). Inoculated Petri dishes were incubated at the 25°C during 7 days. Results were expressed as $\log_{10}$ CFU/g. Sampling of sausage casings surfaces was taken on 2^{nd}, 6^{th}, 9^{th}, 11^{th}, 14^{th}, 34^{th}, 65^{th}, 90^{th}, 120^{th}, 217^{th} and 270^{th} day of ripening and storage. The surfaces of sausage casings were swabbed using wet swabs, and isolation and identification of moulds were performed as described above.

The identification of isolated fungal species was performed according to the patterns described by Ellis (1971), Nelson et al. (1983), Samson and Frisvad (2004) and Samson et al. (2004).

Ochratoxin A analyses: All sampled components and sausages, which were stored frozen until the analyses were carried out, were tested on the presence of ochratoxin A using ELISA method. Screening method for analysis was done using Neogen Veratox® testing kits with limits of detection of 1 µg/kg (ppb) for ochratoxin A. The test itself is a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Free mycotoxins in the samples and controls are allowed to compete with enzyme-labelled mycotoxins (conjugates) for the antibody binding sites. After a wash step, substrate is added, which reacts with the bound conjugate to produce blue color. More blue color means less mycotoxin. The test is read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of mycotoxin (Kokić et al., 2009).

RESULTS AND DISCUSSION

Results for total mould counts per cm^2 (TMC/ cm^2) of carcass and casings surfaces, expressed as $\log_{10}$ CFU/cm^2, are shown in Table 1. Total mould count determined in the swab samples of belly, jowl and natural pig casings was very low (in range -0.3-0.51 $\log_{10}$ CFU/cm^2), while the mould contamination of ham and back did not occur.

Tab. 1 – Total mould count per cm^2 of carcass and casings surfaces [log_{10} CFU/cm^2]

	Swab	TMC/cm^2
Carcass	Ham	0
	Back	0
	Belly	0.44 ± 0.01
	Jowl	-0.30 ± 0.06
Casings	Natural pig casing	0.51 ± 0.02

Results for total mould count per gram of sausage components (TMC/g), expressed as log_{10} CFU/g, are presented in Table 2. As it can be seen, all components used for sausage production were contaminated by moulds. The highest mould contamination of spices was detected in the sample of red hot paprika powder, followed by caraway, garlic and salt. As expected, manually mixed filling contamination was considerably higher than contamination of mechanically mixed filling.

Tab. 2 – Total mould count per gram of sausage components [log_{10} CFU/g]

Component	TMC/g
Chopped meat	2.71 ± 0.002
Salt	2.32 ± 0.002
Garlic	2.65 ±0.004
Red hot paprika powder	4.14 ± 0.001
Caraway	3.90 ±0.001
Manually mixed filling	2.34 ±0.002
Mechanically mixed filling	1.60 ± 0.020

Table 3 and Table 4 show the results obtained from identification of isolated moulds. Mould species isolated from the swab samples are listed in Table 3, and results for mould species isolated from the components and two sausage fillings are presented in Table 4. Tables also present the moiety of species in mycopopulations isolated from specific sample.

Tab. 3 – Fungal species isolated from carcass and natural casings

Swab	Species	Isolated (%)
Carcass – belly	*Penicillium spinulosum*	100
Carcass – back	*Verticilium lecanii*	100
Natural pig casings	*Penicillium aurantiogriseum*	100

Tab. 4 – Fungal species isolated from components used for *Petrovská klobása* production

Component	Species	Isolated (%)
Chopped meat	*Penicillium roqueforti*	100
Salt	*Penicillium janthinellum*	100
Garlic	*Fusarium subglutinans*	100
Red hot paprika powder	*Aspergillus flavus* *Penicillium aurantiogriseum*	33.33 66.67
Caraway	*Alternaria citri* *Penicillium glabrum* *Penicillium italicum*	10 70 20
Manually mixed filling	*Fusarium sporotrichioides* *Fusarium subglutinans* *Penicillium roqueforti* *Penicillium aurantiogriseum*	50 30 10 10
Mechanically mixed filling	*Penicillium decumbens*	100

Isolated moulds belong to 5 genera and 12 different species. Genera are *Aspergillus, Alternaria, Fusarium*, *Penicillium* and *Verticilium,* and species are: *Aspergillus flavus* Link*, Alternaria citri* Ellis & Pierce apud Pierce, *Fusarium sporotrichioides* Sherb.*, F. subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marass, *Penicillium spinulosum* Thom*, P. aurantiogriseum* Dierckx*, P. janthinellum* Biourge*, P. glabrum* (Wehmer) Westling*, P. roqueforti* Thom, *P. italicum* Wehmer*, P. decumbens* Thom and *Verticilium lecanii* (Zimm.) Viégas. *Penicillium* genus was presented with 7 different species, which makes 58.33% of all isolated species. Carcass belly, natural pig casings, four components (chopped meat, salt, paprika, caraway) and both fillings were contaminated with some of *Penicillium* species, which makes 66.67% of all tested samples. The most abundant was *P. aurantiogriseum,* which was isolated from 25% of tested samples.

Fusarium genus was presented with two species and it was isolated from two different samples (16.67%). Garlic was significantly contaminated with *Fusarium subglutinans*, while both isolated species – *F.subglutinans* and *F. sporotrichioides*, were isolated from manually mixed filling.

Aspergillus, *Alternaria* and *Verticillium* genera were presented with one species each. *Aspergillus flavus* was isolated from paprika powder, *Alternaria citri* from caraway and *Verticilium lecanii* from carcass back swab. Picture 1 shows the presence of different mould genera in mycopopulations isolated from raw material used for *Petrovská klobása* production.

Comparing total mould counts isolated from components with maximum allowed count according to Rule Book for Microbiological Accuracy of Foodstuffs on the Market (Official Journal FRY, 26/93, 53/95, 46/02) and results obtained by several authors (Dimić et al., 2008; Hashem and Alamri, 2010), it can be concluded that meat and spices used for *Petrovská klobása* production were of acceptable quality. However, some of the isolated species, such as *Penicillium aurantiogriseum*, *Aspergillus flavus, Fusarium subglutinans, F.sporotrichioides* etc., are reported to be toxigenic (Ožegović and

Pepeljnjak, 1995; Samson et al., 2004; Marasas et al., 1984), so they posses a high risk for human health.

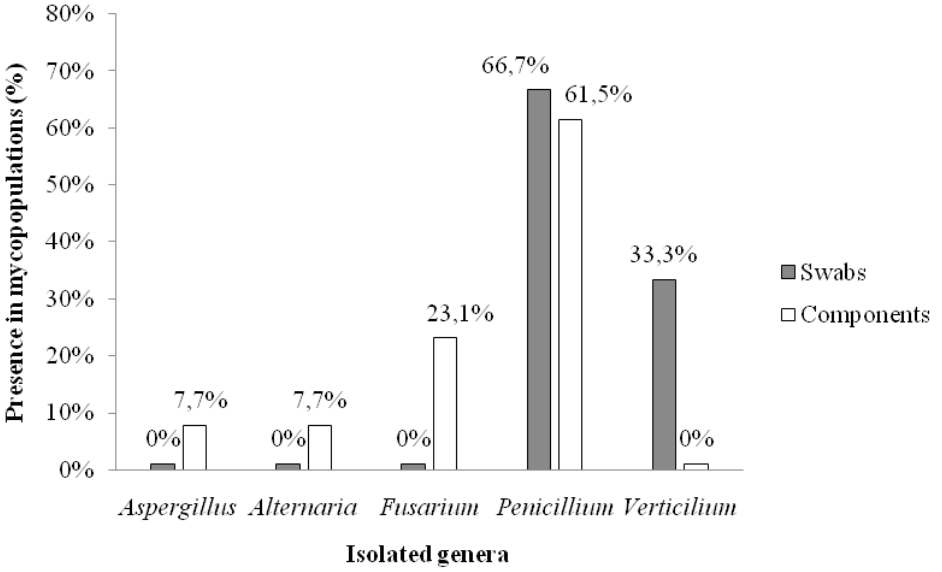

Fig. 1 – Presence of different mould genera in mycopopulations isolated from raw material used for *Petrovská klobása* production.

As has been reported by a number of authors (Škrinjar 2008; Vesković-Moračanin et al., 2009; Iacumin et al., 2009), mould contamination of fermented sausage casings often occur during the production process, mainly during the ripening and storage. Therefore, the surface of sausages C_1, C_2 and C_3 were swabbed in a few different periods of ripening and storage, in order to examine the presence of moulds on the sausage casings and identified possible toxigenic species.

During the first 90 days of ripening and storage, none of the tested samples was contaminated by moulds. The first mould growth was noticed on the 120^{th} day on the casings of sausage C_1. On the 217^{th} day, the moulds were isolated from the surface of C_1 and C_3 sausages. On the 270^{th} day, all three types of sausages were contaminated by moulds. Results for total mould counts isolated from different sausages on different days of ripening and storage, species and percentage of species in isolated mycopopulations, are shown in Table 5.

The moulds isolated from sausage casings belong to three genera: *Eurotium, Penicillium* and *Aspergillus. Eurotium* and *Aspergillus* genera were presented with one species each – *E.herbariorum* (Wiggers) Link and *A. versicolor* (Vuill.) Tiraboschi, and *Penicillium* with three – *P. aurantiogriseum* Dierckx, *P.chrysogenum* Thom and *P.olsonii* Bainier and Sartory.

Tab. 5 – Total mould count per cm^2 [log_{10} CFU/cm^2] and fungal species isolated from *Petrovská klobása* casings during ripening and storage

Sausage	Day of sampling	TMC/cm^2	Species	Isolated (%)
C_1	120	0.69 ± 0.01	*Eurotium herbariorum*	100
C_1	217	0.95 ± 0.01	*Eurotium herbariorum*	100
C_3	217	0.01 ± 0.01	*Penicillium olsonii*	100
C_1	270	1.60 ± 0.002	*Eurotium herbariorum* *Penicillium aurantiogriseum* *Penicillium chrysogenum* *Aspergillus versicolor*	37.5 25 25 12.5
C_2	270	0.65 ± 0.01	*Penicillium chrysogenum*	100
C_3	270	0.78 ± 0.005	*Penicillium aurantiogriseum* *Penicillium chrysogenum*	50 50

In the mycopopulations isolated from sausage C_1 casings, the species *Eurotium herbariorum* dominated. This species was the only contaminant of sausage C_1 casings after 120 and 217 days of ripening and storage, and makes 37.5% of total mould count isolated from this sausage casings on the 270th day. *Penicillium aurantiogriseum* makes 25% of isolated moulds on the last day of sampling, as well as *P.chrysogenum*, while the species *Aspergillus versicolor* makes 12.5% in isolated mycopopulations.

The mould growth on the sausage C_2 casings was detected only on the last day of sampling, and it was contaminated with one species, *P. chrysogenum*.

Swabs of sausage C_3 casings showed the presence of moulds on the 217th and on the 270th day of ripening and storage. In the first case, it was just one species, *Penicillium olsonii*, while the mycopopulations isolated after 270 days consisted of two species, *P.aurantiogriseum* and *P.chrysogenum*, with equal moiety.

The 83.33% of isolated species has been reported (Samson et al., 2004; Marasas et al., 1984) as potentially toxigenic, with the ability to synthesize different types and amounts of mycotoxins, under specific conditions. Considering their possible harmful effect on people's health, this percentage can be regarded as very dangerous.

The tests of components and *Petrovská klobása* samples on the presence of ochratoxin A, gave the negative results. It can be concluded that biosynthesis of this toxin didn't occur, even some of the isolated moulds are potentially toxigenic. Other explanation of the negative results, considering samples of sausages, might be the possible interaction between mycotoxigenic fungi and some lactic acid bacteria presented in sausage filling. It has been reported by a number of authors lately that some lactic acid bacteria can inhibit the biosynthesis of mycotoxins, but they are also capable of binding, detoxifying and/or degrading already synthesized toxins (Schnürer and Magnusson, 2005; Shetty and Jespersen, 2005; Dallé et al., 2010).

CONCLUSION

This study has identified mould species that cause contamination of traditional dry fermented sausage, *Petrovská klobása*. Isolated moulds can originate from contaminated raw material used for sausage production, or could come from environment, facilities and/or handling, as secondary contamination. A high percentage, 83.33%, of the species isolated at high frequencies are capable of producing mycotoxins. All tested samples of *Petrovská klobása* were ochratoxin A free, however, the presence of potential toxigenic moulds posses a high risk of the product contamination with mycotoxins. Care therefore has to be taken during production and storage, and a raw material of high quality has to be used.

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МИКОПОПУЛАЦИЈЕ И ОХРАТОКСИН А – МОГУЋИ КОНТАМИНЕНТИ ПЕТРОВАЧКЕ КОБАСИЦЕ (*PETROVSKÁ KLOBÁSA*)

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Резиме

Petrovská klobása је на традиционалан начин произведена сува ферментисана кобасица из околине Бачког Петровца (Војводина, Србија), која је заштићена ознаком географског порекла, према закону Републике Србије. Контаминација ове врсте кобасица различитим врстама плесни се веома често дешава током процеса производње, посебно током зрења и складиштења. Циљ овог рада је био да се изолују и идентификују плесни које контаминирају састојке коришћене у производњи Петровачке кобасице и њен омотач, у различитим фазама зрења и складиштења. Узорковање је обављено током производње и након 2, 6, 9, 11, 14, 34, 65, 90, 120, 217 и 270 дана. Након одређивања укупног броја плесни, изолати су идентификовани и сврстани у пет родова, изолованих из компонената (*Penicillium* – 7 врста; *Fusarium* – 2 врсте; *Aspergillus* – 1 врста; *Alternaria* – 1 врста; *Verticilium* – 1 врста) и три рода изолованих са површине омотача (*Penicllium* – 3 врсте; *Aspergillus* – 1 врста; *Eurotium* – 1 врста). Показано је да 83.33% изолованих врста спада у потенцијалне произвођаче токсичних метаболита.

Анализом састојака и готових кобасица на присуство охратоксина А, коришћењем ELISA методе, добијени су негативни резултати.

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EFFECT OF *ORIGANUM HERACLEOTICUM* L. ESSENTIAL OIL ON FOOD-BORNE *PENICILLIUM AURANTIOGRISEUM* AND *PENICILIUM CHRYSOGENUM* ISOLATES

ABSTRACT: Molds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes. The difficulty of controlling these undesirable molds, as well as the growing interest of the consumers in natural products, have been forcing the industry to find new alternatives for food preservation. The modern trends in nutrition suggest the limitation of synthetic food additives or substitution with natural ones. Aromatic herbs are probably the most important source of natural antimicrobial agents. *Origanum heracleoticum* L. essential oil has been known as an interesting source of antimicrobial compounds to be applied in food preservation. In the this work, we have investigated the effect of essential oil obtained from *O. heracleoticum* on growth of six isolates of *Penicillium aurantiogriseum* and four isolates of *Penicillium chrysogenum* isolated from meat plant for traditional Petrovacka sausage (Petrovská klobása) production.

The findings reveal that the essential oil of *O. heracleoticum* provides inhibition of all of fungal isolates tested. *O. heracleoticum* L. essential oil exhibited higher antifungal activity against the isolates of *P. chrysogenum* than the isolates of *P. aurantiogriseum*. *O. heracleoticum* essential oil showed a MIC value ranging from 25 to 100 μL/mL. The fungi cultivated in the medium with higher concentration of essential oil showed certain morphological changes. The alterations included lack of sporulation and loss of pigmentation.

KEY WORDS: antifungal activity, essential oil, *Origanum heracleoticum*

INTRODUCTION

Molds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes (Carmo et al., 2008). Mold contamination is often associated with unpleasant appearance, odor and changes in taste and nutritional value of foods (Papagianni et al., 2007).

Some molds are able to produce mycotoxins, which apart from the toxic effects, frequently have degenerative, toxinogenic or carcinogenic effects (Milićević et al., 2010).

The difficulty of controlling these undesirable fungi, as well as the growing interest of the consumers in natural products, have been forcing the industry to find new alternatives for food preservation. One of the possibilities is the usage of essential oils as antifungal additives. Many naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against food borne pathogens (Škrinjar and Nemet, 2009; Škrinjar et al., 2009). Their systematic screening may result in the discovery of novel effective antimicrobial compounds.

Among the aromatic plant species from *Lamiaceae* (*Labiateae*) family, genus *Origanum* occupies a special position. Essential oils from genus *Origanum* are known to exhibit antimicrobial activities against bacteria and fungi (Skandamis et al., 2001; Džamić et al., 2008). This is basically due to their major components, carvacrol and thymol (Govaris et al., 2010) which have potential to be used as food preservatives (Burt, 2004). Biological activity of essential oils depends on their chemical composition, which is determined by genotype and influenced by environmental and agronomic conditions (Burt, 2004).

Chemical analysis of the oregano (*O. heracleoticum* L.) essential oil revealed the presence of several ingredients, most of which have important antioxidant activity (Tsimogiannis et al., 2006), and antimicrobial properties (De Martino et al., 2009). Carvacrol and thymol, two main phenolic compounds that constitute about 78–85% of oregano oil, are principally responsible for the antimicrobial activity of the oil (Kokkini et al., 1997; Govaris et al., 2010). In addition, other minor constituents, such as monoterpene hydrocarbons γ-terpinene and p-cymene, also contribute to antimicrobial activity of the oil (Burt, 2004).

Thymol and carvacrol do not exhibit adverse effects on human health, and are proved to cause neither significant nor marginal toxic effects at cellular level. Also, the concentrations at which they exhibit antimicrobial activity do not reach the possible genotoxic level (Stammati et al., 1999; Burt, 2004). One limitation on the use of essential oils or their constituents in foods is their herbal aroma; in fact carvacrol and thymol are permitted food flavorings in the U.S. and Europe (CFSAN, 2006).

The aim of this study was to investigate antifungal effects of various concentrations of essential oil *O. heracleoticum* on the six isolates of *Penicillium aurantiogriseum* and four isolates of *Penicillium chrysogenum* isolated from meat plant for traditional Petrovacka sausage (Petrovská klobása) production.

MATERIALS AND METHODS

Plant Material: Aerial parts of *O. heracleoticum* L. (*O. vulgare* L. ssp. *hirtum*) were collected during blooming stage (August 2009) from the locality Kamendol near Smederevo, Serbia. The plant material was dried under laboratory conditions (20-25 ºC). Institute of Medicinal Plant Research Dr. Josif Pančić identified the plants, and voucher specimens were stored in the herbarium of the Institute of Medicinal Plant Research Dr. Josif Pančić.

Isolation of essential oil: The essential oils were isolated from dried plant material by hydro-distillation according to the standard procedure reported in the Sixth European Pharmacopeia (European pharmacopoeia, 2008). Distillation was performed using Clevenger type apparatus for 2.5 hours. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4ºC. The oil solution (1%) in ethanol was used for chromatographic analysis.

Chemical analysis of the essential oil: Gas chromatography-mass spectrometry (GC-MS) analyses were carried out using Agilent 5975C Series GC-MSD system (7890A GC and 5975C inert MSD), equipped with a HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.50 μm). 1 μl of diluted essential oil sample was injected in split mode (50:1), and inlet temperature was held at 250 ºC. Helium was used as carrier gas in constant flow mode at 1 ml/min. The oven temperature was programmed as follows: 70 °C increased to 180 °C (2 °C/min) without holding, and then to 200 °C (4 °C/min) which was held for 10 min. Ion source was operated at 70 EV, and mass spectra were acquired in scan mode in the 50-550 m/z range.

Identification of the essential oil components: Essential oil components were indentified by comparing their retention indices and mass spectra with those published by Adams (2007) and with Wiley and NIST/NBS mass spectral libraries. Experimental values of retention indices were calculated using calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1., DTRA/NIST, 2002).

Microorganisms: The antifungal activity of essential oil was evaluated using six isolates of *Penicillium aurantiogriseum* and four isolates of *Penicillium chrysogenum* isolated from Petrovačka sausage (Petrovská klobása) processing unit. Test cultures belonged to the culture collection from the Laboratory for Food Microbiology, Faculty of Technology in Novi Sad. The cultures were maintained on Sabouraud maltose agar (SMA) slants and were stored at +4 °C. The fungal isolates used in the experiments are shown in Table 1.

Antifungal assay: Evaluation of the effectiveness of oregano essential oil against ten isolates of *P. aurantiogriseum* and *P.chrysogenum* was carried out *in vitro* by disc-diffusion method. The suspension of the fungal isolates was prepared from 7-day-old cultures. Spores were taken by adding 10 ml of sodium chloride solution containing 0.5 % tween 80 onto slant, scraped with sterile loop and aseptically transferred into sterile test tubes. Determination of total mold count per 1ml of suspension was performed using the standard Koch's method. Final concentration of spore suspension was approximately 1×10^8 spores/ml. One ml of suspension was inoculated in sterile Petri dishes

Tab. 1 – *P. aurantiogriseum* and *P. chrysogenum* isolated from traditional Petrovačka sausage (Petrovská klobása) processing unit

Species	Sign	Origin
P. aurantiogriseum	PS-1	Air in the ripening chamber
P. aurantiogriseum	PS-2	Air in the ripening chamber
P. aurantiogriseum	PS-3	Air in the ripening chamber
P. aurantiogriseum	PS-4	Pepper
P. aurantiogriseum	PS-5	Swab of sausage casing
P. aurantiogriseum	PS-6	Air in the ripening chamber
P. chrysogenum	PS-7	Air in the ripening chamber
P. chrysogenum	PS-8	Swab of sausage casing
P. chrysogenum	PS-9	Swab of sausage casing
P. chrysogenum	PS-10	Air in the ripening chamber

poured with about 15 mL of Sabouraud maltose agar (SMA) tempered at 47 °C. Essential oil was diluted in dimethylsulphoxide (DMSO) to the test concentrations of 250, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 µl/mL. The filter paper discs (7 mm in diameter) were impregnated with 10 µl of the oil dilution in the concentration range from 250 to 1.56 µl/mL and placed on the inoculated agar. Negative controls were prepared using the same solvents to dissolve the essential oil – dimethylsulphoxide (DMSO). The plates were incubated at 25 °C for seven days. At the end of the incubation period, the minimal inhibitory concentration (MIC) was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm. Diameters of the inhibition zones were measured in millimeters. All analyses were performed in duplicate, and the mean values with the standard deviations (SD) are reported.

RESULTS AND DISCUSSION

It is well known that both environmental and genetic factors have effects on the observed variations among *O. heracleoticum* accessions with high accuracy (J e r k o v i ć et al., 2001). Because of this, yield and chemical composition of essential oil can vary among the populations of the same species from different localities.

From the collected plant material of *O. heracleoticum* L. total of 2.05% (v/w) of essential oil has been isolated by the process of hydro-distillation. The oil was intensively yellow, with characteristically strong and pleasant odor. The results of chemical analysis of *O. heracleoticum* essential oil are presented in Table 2.

Tab. 2 – Chemical constituents of the essential oil of *O. heracleuticum* L. [%]

No.	Constituents	*KIL	%	No.	Constituents	*KIL	%
Monoterpenes hydrocarbons				18	Thymol	1289	7.94
1	α-Thujene	924	0.20	19	Carvacrol	1298	69.00
2	α-Pinene	932	0.65	20	Carvacrol acetate	1370	0.18
3	Camphene	946	0.19	∑			**79.21**
4	β-Pinene	974	0.30	**Sesquiterpenes hydrocarbons**			
6	β-Myrcene	988	0.60	21	β-Caryophyllene	1417	1.52
7	α-Phellandrene	1002	0.11	22	Aromadendrene	1439	0.06
8	Δ3-Carene	1008	0.05	23	α-Humulene	1452	0.23
9	α-Terpinene	1014	0.99	24	β-Bisabolene	1505	1.01
10	p-Cymene	1020	10.50	25	*δ*-Cadinene	1522	0.15
11	β-Phellandrene	1025	0.11		∑		**2.97**
12	γ-Terpinene	1054	2.86	**Oxygenated sesquiterpenes**			
	∑		**16.56**	26	Caryophyllene oxide	1582	0.51
Oxygenated monoterpenes				∑			**0.51**
13	Terpinolene	1086	0.14	**Other**			
14	Linalool	1095	0.21	5	1-octen-3-ol	974	0.07
15	Borneol	1165	0.31	∑			**0.07**
16	Terpinen-4-ol	1174	0.73	∑			92.86
17	Carvacrol methyl ether	1241	0.70				

*KIL: literature values (A d a m s , 2007)

Twenty six components (92.86 %) were identified as constituents of this essential oil by GC/ MS analyses. The major components were carvacrol (69.00%), p-cymene (10.50%), thymol (7.94%) and γ-terpinene (2.86%). Except β–caryophyllene (1.53%) and β–bisabolene (1.01%), the amount of all remaining oil components was less than 1%.

Aromatic alcohol carvacrol was also dominant compound in *O. heracleoticum* oil analyzed by other authors (D ž a m i ć et al., 2008; G o v a r i s et al., 2010).

The predominant group of compounds in the oil were monoterpenes (95.77 %), with significantly more oxidized compounds (79.21 %) than hydrocarbons (16.56 %). Sesquiterpenes were present in low percentage in the oil (3.48 %).

Results for antifungal activity of *O. heracleoticum* L. essential oil obtained by the disc diffusion method are shown in Table 3. The results show that the essential oil of *O. heracleoticum* provides inhibition of all of the fungal isolates tested. *O. heracleoticum* L. essential oil exhibited higher antifungal activity against isolates of *P. chrysogenum* than isolates of *P. aurantiogriseum*. Isolate of *P. chrysogenum* (PS-9) shows complete inhibition (Figure 1) at the highest tested concentration (250 μL/mL) of *O. heracleoticum* L. essential

oil. The oil also exhibited high antifungal activity against other tested isolates of *P. chrysogenum*. The zones of inhibition tested isolates of *P. chrysogenum* were in the range from 54.0 mm (PS-7) to 87.6 mm (PS-10). For the same concentration of *O. heracleoticum* L. essential oil isolates of *P. aurantiogriseum* achieved lower inhibition zones ranging from 16.2 mm (PS-4) to 38.0 mm (PS-2) (Table 3). At the highest tested concentration (250 µl/mL) most susceptible to the effects of essential oil was isolate PS-2 (38.0± 3.11) (Table 3, Figure 2, a, b). Essential oil did not have any influence on the tested fungal isolates at concentration lower than 25 µl/mL.

Tab. 3 – Effect of various concentrations of essential oil of *Origanum heracleoticum* L. on the growth of fungal isolates

Isolate sign	Concentration (µl mL^{-1})			
	250	**100**	**50**	**25**
PS-1	26.4* ± 0.28**	15.2 ± 4.24	9.7± 0.14	7.87 ± 0.25
PS-2	38.0± 3.11	13.6 ± 0.84	10.2 ± 1.09	7.75 ± 0.27
PS-3	19.2 ± 0.84	15.4 ± 2.82	9.3± 1.27	-
PS-4	16.2± 1.69	13.4 ± 2.26	8.2 ± 0.28	-
PS-5	22.1± 4.10	19.6 ± 0.28	8.6 ± 0.54	-
PS-6	31.8 ± 1.09	24.4 ±0.81	8.0 ± 0.0	7.87 ± 0.25
PS-7	54.0 ± 4.24	22.4 ± 4.80	12.4 ± 3.67	8.3 ± 0.14
PS-8	67.8 ± 2.26	19.2 ±1.41	10.8 ±0.84	8.7 ±0.42
PS-9	c.i	c.i	12.4 ± 0.28	11.0 ± 1.41
PS-10	87.6 ± 1.15	52.0 ± 7.07	12.8 ± 0.84	9.5 ± 1.64

* diameter of inhibition zone (mm) including disc diameter of 7mm
** standard deviation (SD)
c.i. – complete inhibition

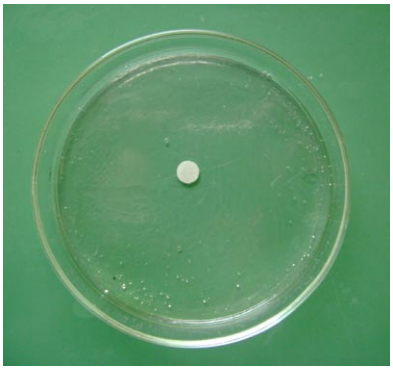
a)  b) 

Fig. 1 – a) Complete inhibition of *P. chrysogenum* (PS-9) by *Origanum heracleoticum* L. essential oil (250 µL/mL) obtained by disc-diffusion method; b) Blank sample

a)  b) 

Fig. 2 – a) Diameter of inhibition zone for *Origanum heracleoticum* L .essential oil (250 µL/mL) against *P. aurantiogriseum* (PS-2) obtained by disc-diffusion method; b) Blank sample

Minimal inhibitory concentration (MIC) was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm. *O. heracleoticum* essential oil showed MIC value of 50 µL/mL against isolates of *P. chrysogenum*, with the exception of PS-9 isolates which showed MIC value of 25 µL/mL.

MIC value of essential oil against isolates of *P. aurantiogriseum* was higher. Essential oil showed MIC value of 100 µL/mL, against tested isolates of *P. aurantiogriseum*, with the exception of PS-2 isolates that showed MIC value of 50 µL/mL (Figure 3). These results are in agreement with the findings of Carmo et al. (2008). They found the MIC values of *O. vulgare* essential oil ranging from 20 to 80 µL/mL against *Aspergillus* species. Obtained results are in contrast with data published by Džamić et al. (2008) who reported lower MIC values. Their results concerning the essential oil of *O. heracleoticum* obtained by broth microdilution assay exhibited fungicidal characteristics with MIC and MFC of 0.1–1 µL/mL.

This disparity in results could be related to a great extent to the volatility of the essential oil in an open air system when using the solid medium diffusion technique (Duarte et al., 2005). However, it has been established that the antifungal activity of different essential oils ranges from narrow to wide spectrum depending on the assayed essential oil, its concentration and fungal target (Burt and Reinders, 2003).

The applied disk diffusion method can be used only for preliminary screening of antimicrobial substances, since the easily volatile components of essential oils evaporate over a period of incubation together with the solvent, while poorly dissolved components do not pass through the medium (Griffin and Markham, 2000).

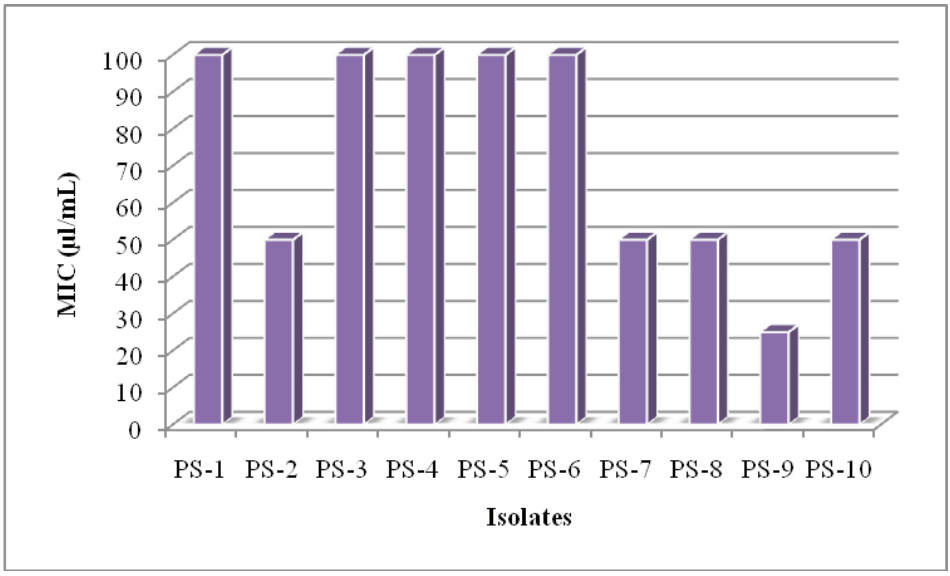


Fig. 3 – The MIC values (µl/mL) of the of *Origanum heracleoticum* L. essential oil against isolates of *P. aurantiogriseum* and *P. chrysogenum* by the disc – diffusion method

The fungi cultivated in the medium with concentration of essential oil higher than 50 µl/mL showed certain morphological changes. The alterations included lack of sporulation and loss of pigmentation (Figure 2. a, b). Previous studies have reported that essential oils are able to cause morphological changes in *Aspergillus* species including lack of sporulation, loss of pigmentation, aberrant development of conidiophores (flattened and squashed), and distortion of hyphaes (De Billerberk et al., 2001; Rasooli and Abyaneh, 2004; Sharma and Tripath, 2008). These findings suggest that the mode of antifungal activity of essential oils could include an attack on the cell wall and retraction of the cytoplasm in the hyphae ultimately resulting in the death of the mycelium.

Velluti et al. (2003) suggested that the antimicrobial activity of the essential oil depends on the chemical structure of their components. Carvacrol and thymol, phenolic compounds known as major constituents of *Origanum* essential oil, have their antimicrobial property attributed to the presence of an aromatic group that is known to be reactive and to form hydrogen bonds with active sites of target enzymes (Souza et al., 2007). However, it is also suggested that the effectiveness of complete essential oils is higher than the activity of each separated compound (Miloš et al., 2000). Based on all our results, essential oil of *O. heracleoticum* L. may be used against molds, but at higher concentration.

CONCLUSION

Results obtained by disk diffusion method showed that the essential oil of *O. heracleoticum* provides inhibition of all tested fungi isolates. *O. heracleoticum* L. essential oil exhibits higher antifungal activity against isolates of *P. chrysogenum* than isolates of *P. aurantiogriseum*. At the highest concentration tested (250 µL/mL), *O. heracleoticum* L. essential oil shows complete inhibition of the *P. chrysogenum* (PS-9) isolate. The oil also exhibited high antifungal activity against other tested isolates of *P. chrysogenum*. The inhibition zones of tested *P. chrysogenum* isolates were in the range from 54.0 mm (PS-7) to 87.6 mm (PS-10). For the same oil concentration used, *P. aurantiogriseum* isolates were less inhibited, with inhibition zones ranging from 16.2 mm (PS-4) to 38.0 mm (PS-2). For concentration lower than 25 µl/mL, essential oil has not influenced any of the tested fungal isolates. The fungi cultivated in the medium with concentration of essential oil higher than 50 µl/mL showed certain morphological changes. The alterations included lack of sporulation and loss of pigmentation.

Future research will be focused on determination of MIC and MFC using more precise broth microdilution method and examination of the influence of *O. heracleoticum* oil on the production of mycotoxins.

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УТИЦАЈ ЕСЕНЦИЈАЛНОГ УЉА *ORIGANUM HERACLEOTICUM L.* НА ИЗОЛАТЕ *PENICILLIUM AURANTIOGRISEUM* И *PENICILIUM CHRYSOGENUM* ПОРЕКЛОМ ИЗ ХРАНЕ

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Резиме

Плесни су широко распрострањене у природи и њихове споре се могу наћи у атмосфери чак и на врло великим висинама. Тешкоће у контроли ових непожељних микроорганизама, као и све већа потражња за природним производима, приморавају индустрију да пронађе нове алтернативе у конзервисању хране. Модерни трендови предлажу ограничену примену синтетичких адитива и њихову замену природним. Ароматично биље је вероватно најважнији извор природних антимикробних агенаса. Есенцијално уље *Origanum heracleoticum* L. је познато као интересантан извор антимикробних компонената у погледу примене у конзервисању хране. У овом раду испитивали смо утицај есенцијалног уља добијеног из *O. heracleoticum* на раст шест изолата *Penicillium aurantiogriseum* и четири изолата *Penicilium chrysogenum*, изолованих у погону за производњу традиционалне Петровачке кобасице (*Petrovská klobása*).

Резултати су показали да есенцијално уље *O. heracleoticum* инхибира све испитане изолате. Јачи антифунгални ефекат уље је показало према изолатима *P. chrysogenum* него према изолатима *P. aurantiogriseum*. Минимална инхибиторна концентрација уља кретала се у опсегу од 25 до 100 µl/ml. Плесни гајене на подлози са вишом концентрацијом есенцијалног уља показују одређене морфолошке промене. Те промене укључују смањену спорулацију и одсуство пигментације.

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MYCOTOXICOLOGICAL TESTS FROM THE ASPECT OF THE HACCP SYSTEM AND LEGISLATION

ABSTRACT: Mycotoxins, as secondary metabolic products of molds, are common contaminants of raw feed materials and compound feeds. Depending on the agro-meteorological and storage conditions, molds can contaminate grains and produce mycotoxins in the field, before and after harvest and during storage. Way of preventing animal mycotoxicoses and transfer of mycotoxins to humans through food chain is regular inspection on mycotoxicological feed safety. This paper presents the results of examination of aflatoxin, ochratoxin and zearalenone in 89 samples tested under laboratory conditions during a one year period. The analyses of types of samples, contamination, and their origin demonstrated the need for preventive control of mycotoxin content, primarily in grains. The results of testing the content of aflatoxin in milk indicate the existence of risks to human health. In order to protect humans and animals from mycotoxicological contamination, we propose the use of the system hazard analysis and critical control point (HACCP), which has been proved to be an effective strategy in food safety control. The basic principles of HACCP approach in the production of feed and foodstuffs, as well as the compliance and harmonization of legislations with those in the European Union, in the field of mycotoxicological tests, are the best prevention against mycotoxin effects on health and economy. Implementation of HACCP system currently presents one of the basic preconditions for the export of our products and their placement on the European market.

KEY WORDS: animal feed, HACCP, mycotoxins, regulations

INTRODUCTION

Raw feed materials are suitable substrates for the growth of mold and toxin production. Depending on the type of feed, growing and maturing conditions, as well as the conditions of storage, different toxins can be present in various feed materials (Table 1).

Tab. 1 – Feedstuffs and their associated mycotoxins (P e t t e r s s o n , 2004)

Feed raw material	Mycotoxins produced	
	In field during culturing	Preservation and storage
Barley	DON, NIV, Zea, HT-2, T-2	OTA, Afla, Cit
Maize	DON, Fum, Zea	Zea, Afla
Oats	DON, NIV, HT-2, T-2	OTA, Afla, Cit
Rice	Ergot	OTA
Wheat	DON, NIV, Zea, Ergot	OTA, Afla, Cit
Soya bean meal	–	Afla
Silages	Afla, DON	Zea

Afla − aflatoxins; Cit − citrinin; DON − deoxynivalenol; Ergot − ergotamin;
Fum − fumonisin; NIV − nivalenol; OTA − ochratoxin A; Zea − zearalenone.

Mycotoxins are rarely present in food at high concentrations. More common problem is chronic mycotoxicosis caused by low toxin concentrations that are consumed by animals for a longer period (P e t t e r s o n , 2004). The negative effect of mycotoxins results in a decline of productivity and their presence in food chain is of great economic importance. For the purposes of health safety and avoiding economic losses it is very important to prevent the mold growth and toxin production in cereals.

HACCP (Hazard Analysis Critical Control Point) is a food managment system, designed to prevent the safety problems, including food poisoning. It has been developed by NASA and US army with the aim of providing ”absolutely safe food“ for US astronauts, and implemented in the food industry. Today, it presents a global food safety standard. HACCP has been proven as an effective way to prevent the presence of hazardous substances that rarely occur in food, by eliminating the need for complicated analyses of final products. HACCP approach involves detailed analysis of each step in food processing using seven clearly defined principles. The presence of mycotoxins in final product is often the result of circumstances which occurred much earlier in the production chain. In addition, mycotoxins are very stable compounds that are difficult to remove once formed. The analyses are complicated, time consuming and expensive (A l d r e d et al., 2004). Therefore, prevention is more economical and convenient way of fight against mycotoxins. For this reason modern technology aims to introduce low-cost rapid tests for mycotoxicological control of the raw materials in order to avoid expensive and complicated analyses of the final product.

On the basis of the results obtained in mycotoxin examinations, the objective of this paper is to point to the necessity of prevention in the fight against mycotoxins and mycotoxicoses by introducing novelties in science and by harmonization with the legislation of the European Union (EU).

MATERIALS AND METHODS

The feed samples obtained from the feed producers, from the farms where health problems were detected or death cases occured, as well as the samples submitted by the inspection for the purpose of monitoring safety, were analyzed.

Raw milk samples were taken from five commercial dairy farms. A total of 50 samples of raw milk were examined.

Part of the animal feed (45 samples) was analyzed by thin layer chromatography (TLC, The Official Gazette of SFRY, No. 15/87), and part of the samples (44 samples) were analyzed by immunoaffinity enzymatic method (ELISA) using *Ridascreen*® kits (Art.No. R:4701; R:1311; R:1401, R-Biopharm, Germany). For the analysis of milk samples ELISA kit *Tecna*, Italy, was used.

RESULTS AND DISCUSSION

The results of feed examining obtained by TLC are presented in Table 2. In only one raw feed material sample aflatoxins were detected, and ochratoxin A was detected in two samples, but in a concentration lower than the maximum prescribed (The Official Gazette of RS, 2010). In the tested feedingstuff samples, toxin concentrations were not above the detection limit of the methods used.

Tab. 2 – The examination results of animal feed samples using TLC

Sample	No. of samples	No. of contaminated samples	Limits of detection (ppm) Range of contamination (ppm)		
			Aflatoxins	Ochratoxin A	Zearalenone
Raw feed material	9	3	<0.025 0.025	<0.02 0.02–0.1	<0.05 –
CF for broilers	15	–	<0,005	<0,02	<0.25
CF for layers	3	–	<0.01	<0.05	<0.25
CF for poultry	2	–	<0.01	<0.2	<0.25
CF for piglets	1	–	<0.005	<0.02	<0.05
CF for piglets from 1–15 kg	1	–	<0.01	<0.02	<0.05
CF for fattening pigs	5	–	<0.01	<0.04	<0.1
CF for sows	5	–	<0.01	<0.04	<0.05
Other CF	4	–	<0.005	<0.02	<0.05

CF – Complete feedingstuffs

As it can be seen from Table 3, mycotoxins were observed in a large number of the samples tested by ELISA, but no significant contamination was

Tab. 3 – Content of mycotoxins in feed samples using ELISA

Sample	No. of samples	No. of contaminated samples Range of contamination (ppb)		
		Aflatoxins	Ochratoxin A	Zearalenone
Raw feed material	23	17 (2.1–35.6)	14 (1.2–900)	21 (2.0–241)
CF for poultry	11	3 (2.6–5.6)	4 (1.1–4.6)	11 (14.2–102.2)
CF for pigs	8	5 (5.4–13.9)	7 (1–9.8)	8 (6.2–42.9)
CF for cattle	2	0 –	1 2.42	2 (10.6–20.8)

(CF – Complete feedingstuffs; Limits of detection: 1 ppb for ochratoxin A, 1.75 for total aflatoxins and zearalenone)

detected. However, in one maize sample originating from a pig farm, ochratoxin A was detected in the quantity of 900 μg/kg. This result was also confirmed by TLC method. Although the current regulation on the quality of animal feed (The Official Gazette of RS, 2010) does not prescribe the maximum amount of ochratoxin A in maize, having in mind the maximum permissible concentrations in complete feedingstuffs for piglets and pigs, this corn is not safe for the consumption. By analyzing and comparing the existing regulations in Serbia and those in the EU, it can be concluded that there are significant differences in the types of samples encompassed by the legislation, but also in the prescribed maximum permissible concentrations (Table 4). In order to comply with the EU regulations, it is necessary to update and amend the current regulations in Serbia which regulate only aflatoxins in the raw material. New directions, based on the recommendations of the European Commission are to be given for other toxins in the raw materials used for feed production. It is necessary to introduce monitoring of fumonisins in corn in Serbia because of the confirmed presence of these toxins (Jakšić, 2004; Kokić et al., 2009). The current regulation related to the trichothecenes should be defined more precisely.

When analyzing the types of tested samples, the number of complete feedingstuffs (57) significantly exceeds the number of raw feed materials (32). The number of requests made by the manufacturer makes 55% of the samples, the number of the requests by the inspections makes 37%, and the number of the requests by the veterinary services from the farms with health problems makes 8%.

Out of 50 tested samples of raw milk, aflatoxin M_1 was detected in two samples taken from different farms. The amount of aflatoxin was 7.5 ng/kg and 10 ng/kg which was significantly lower than the maximum permissible concentration (0.5 μg/kg) according to the Regulation (The Official Gazette, No. 5/92; 11/92; 32/02). The limit values, set by the EU, were not exceeded (0.05 μg/kg), but the results confirmed the possibility of excretion into milk.

Tab. 4 – Legislation on mycotoxins in animal feed and milk in the EU and Serbia (EC, 2003, 2006a, b, The Official Gazette RS, No. 4 / 2010, the Official Gazette SRY, No. 5 / 92, 11/92, 32 / 02)

Mycotoxin	Sample	Maximum content (ppm) EU	Serbia
Aflatoxin B_1	Feed materials; com. and compl. feedingstuffs for cattle, sheep and goats	0.02	0.05
	Com. feedingstuffs for dairy animals	0.005	0.01 (and compl.)
	Com. feedingstuffs for calves and lambs	0.01	0.01
	Com. and compl. feedingstuffs for pigs and poultry	0.02	0.03
	Other com. feedingstuffs	0.01	0.01
	Other compl. feedingstuffs	0.005	0.01
Aflatoxin M_1	Milk and milk products	0.05×10^{-3}	0.5×10^{-3}
		Guidance values (ppm)	**Maximum content (ppm)**
DON	Feed materials, cereals with exception of maize by-products	8	–
	Maize by-products	12	–
	Compl. and com. feedingstuffs with the exception of:	5	–
	for pigs	0.9	0.5
	for calves, lambs and kids	2	–
Zearalenone	Feed materials, cereals with exception of maize by-products	2	–
	Maize by-products	3	–
	Compl. and com. feedingstuffs for piglets and gilts	0.1	0.5
	Compl. and com. feedingstuffs for sows and fattening pigs	0.25	1.0
	Compl. and com. feedingstuffs for calves, dairy cattle, sheep and goats	0.5	3.0
Ochratoxin A	Feed materials, cereals	0.25	–
	Compl. and com. feedingstuffs for pigs	0.05	0.1 pigs (0.2 fattening and sows)
	Compl. and com. feedingstuffs for poultry	0.1	1 (0.25 for layers)
Fumonisin	Feed materials, maize and maize by-products	60	–
	Compl. and com. feedingstuffs:		
	for pigs, horses, rabbits and pet animals	5	–
	for fish	10	–
	For poultry, calves, lambs and kids	20	–
	For ruminants and mink	50	–
Trichothecenes	Feedingstuffs for broilers, pigs, calves	–	0.30
	Feedingstuffs for sows, cattle, poultry	–	0.60
T_2, DAS and derivates	Com. feedingstuffs for pigs and poultry	–	1.0
	Com. and compl. feedingstuffs for broilers, piglets and calves	–	0.5

Com. – complete; Compl. – complementary; DON – deoxynivalenol, DAS – diacetoxyscirpenol, − not legislated

Our results point to the following data: the analyzed number of samples of feed raw material is smaller in comparison to complete feedingstuffs, feed raw material is contaminated by the mycotoxins not enompassed in the regulations, there is a suspicion of mycotoxicosis on the farms, and the excretion of toxins in milk has been proven. Therefore, it can be concluded that well organized preventive mycotoxic control is needed.

Combining Good Agricultural Practice, Good Manufacturing Practice, Good Storage Practice, and applying the seven HACCP principles whithin the framework of the quality systems such as ISO 9000, it is possible to introduce *the most cost-effective prevention* of mycotoxicosis (F A O , 2001). Although a large number of mycotoxins has been discovered so far, only around ten have been proven to have impact on human health, and are marked as hazardous. The critical control points are extreme conditions during crop growth and harvesting, the conditions in distribution, processing and storage of food (A l d r e d et al., 2004). Critical limits may be maximum values of mycotoxins prescribed by the regulations, or simply measurable parameters that influence the production of toxins, such as temperature, humidity and toxin producing molds. The procedures for monitoring critical control points in this case are the methods for determining the parameters. The corrective actions include implementation of all available measures for combating mold and preventing toxin production, which includes implementation of appropriate agricultural practice in the field, and use of feed additives like adsorbents, antioxidants, vitamins or enzymes. The verification procedures are also necessary within the HACCP system applied in the control of mycotoxins, as a way of checking that the system works in all control points. Finally, all the stages that describe monitoring procedures and corrective actions, as well as routine records of parameters, must be documented.

We believe that the information obtained by monitoring of mycotoxins in Serbia is very important to obtain a complete picture of the frequency and amounts of certain toxins in some samples, in the agrometeorological climatic condition of this area. All the results obtained by monitoring are to be taken into account when assessing risk and compliance with European regulations.

The methods for mycotoxin determination have significant role in monitoring and therefore must meet certain standards. Due to different chemical structures, a large number of mycotoxins, and matrix heterogeneity it is difficult to choose fast, simultaneous and precise method for routine determination. To assure the quality of analysis, it is necessary to validate and verify test methods according to the European requirements (E C , 2006c, 2010), to accredit the methods and carry out interlaboratory exchange programs, and to use certified reference materials.

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МИКОТОКСИКОЛОШКА ИСПИТИВАЊА СА АСПЕКТА НАССР СИСТЕМА И ПРАВНЕ РЕГУЛАТИВЕ

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Резиме

Микотоксини, као секундарни метаболички продукти плесни, чести су контаминенти биљних хранива и смеша за исхрану животиња. У зависности од агрометеоролошких услова и услова чувања, плесни могу да контаминирају житарице и продукују микотоксине на пољу пре жетве, као и након жетве у току складиштења. Основни начин превенције микотоксикозе животиња и преношења микотоксина ланцем исхране до људи је редовна контрола микотоксиколошке исправности хране за животиње. У раду су приказани резултати испитивања афлатоксина, охратоксина и зеараленона у 89 узорака хране за животиње пристиглих у лабораторију у току једне године. Анализом врсте узорака, контаминираности, као и анализом порекла узорака, доказана је потреба за превентивном контролом садржаја микотоксина, пре свега у биљним хранивима. Резултати испитивања садржаја афлатоксина у млеку показују постојање ризика по здравље људи. У циљу заштите људи и животиња од микотоксиколошке контаминације, предложена је примена система анализе опасности и контроле критичних тачака (НАССР), која се показала као ефикасна стратегија за контролу исправности хране. Основни принципи НАССР приступа у производњи хране за животиње и намирница, поштовање и усклађивање законских прописа у области микотоксиколошких испитивања са Европском унијом, најбоља су превенција здравствених и економских последица микотоксина. Имплементација НАССР система је тренутно један од основних предуслова за извоз наших производа и освајање европског тржишта.

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THE ROLE OF *PAECILOMYCES LILACINUS* (THOM) SAMSON AND OTHER FUNGAL SPECIES IN BIODEGRADATION OF OCHRATOXIN A

ABSTRACT: Nine isolates of fungi of genera *Aspergillus, Fusarium*, *Paecilomyces* and *Penicillium* were cultured on the modified Vogel's medium with the addition of crude ochratoxin A (OTA) extract. This crude OTA extract was derived from a natural solid substrate on which *Aspergillus ochraceus* strain CBS 108.08 was cultivated. OTA was isolated, partially purified, dried by evaporating and dissolved in ethanol (1 mg ml^{-1}), and added to the test medium up to the final concentration of 10 μg ml^{-1}. The presence of OTA residues was determined after 7 and 14 day cultivation of fungi in the test medium at 27±1°C. The *Paecilomyces lilacinus* isolate (Inf. 2/A), which completely degraded OTA (150 μg) after only seven days, was selected for further studies. Wet sterile rice grains (50 g + 25 ml distilled water) were inoculated with individual isolates of fungi *A. ochraceus* (strain CBS 108.08) and *P. lilacinus* (isolate Inf. 2/A), and with their combination. In the case of *P. lilacinus* monoculture, 0.9 mg of crude OTA was also added into cultivation substrate. Each test was done in three replications. After the four week cultivation of individual and combined fungi at 27±1°C, inoculated rice grains were dried to the constant weight and pulverized. OTA was determined in these samples by the application of standard TLC method for fodder analysis.

OTA in the amount of 61.310 μg kg^{-1} dry matter (DM) was determined only in the samples inoculated with a producer of ochratoxin A (*A. ochraceus*, strain CBS 108.08). On the other hand, a much smaller amount of OTA (80 μg kg^{-1} DM) was detected in samples inoculated with combined cultures of *A. ochraceus* and *P. lilacinus* isolates. Gained results indicate that *P. lilacinus* degraded, on average, 99.8% of OTA. After four week cultivation, the same fungal isolate in the samples of wet sterile rice kernels with the addition of 0.9 mg of crude OTA, completely degraded added crude OTA (<8 μg kg^{-1}).

KEY WORDS: biodegradation, ochratoxin A, *Paecilomyces lilacinus*

INTRODUCTION

Ochratoxin A (OTA) is considered to be one of the most toxic mycotoxins, whose presence has been established in food, feed and commercial feed

mixtures. In order to reduce the presence of this mycotoxin in food, different methods for the inhibition of the growth of OTA producing fungi (*Aspergillus alliaceus,* T h o m and C h u r c h*, A. Carbonarius,* B a i n, *A. Ochraceus,* W i l h e l m*, A. steynii,* F r i s and S a m s o n*, A. westerdijkiae,* F r i s and S a m s o n*, Penicillium nordicum,* D r a g o n i and C a n t o n i, and *P. Verrucosum,* E i e r c h x) and for the prevention of this mycotoxin production are applied (A b r a n h o s a et al., 2010). Biological methods have been considered as an alternative to physical and chemical treatments.

Numerous microorganisms capable of degrading, absorbing and detoxifying OTA and ochratoxin B (OTB) have been reported in the literature. Besides several protozoan species, yeasts and bacteria, filamentous fungi are also believed to have these abilities. It is presumed that two biochemical pathways may be involved in this process (K a r l o v s k y, 1999). First, OTA can be biodegraded through the hydrolysis of amide bond to the non-toxic compounds of L-*β*-phenylalanine and OTα. Secondly, a more hypothetical process, involves OTA being degraded via the hydrolysis of lactone ring, although in this case, the final degradation product is an opened lactone form of OTA, which is of similar toxicity to OTA when administered to some laboratory animals (A b r a n h o s a et al., 2010).

By the application of ochratoxin biodegradation it is possible to avoid toxic effects, primarily nephrotoxicity and carcinogenicity of ochratoxin, when found in food and feed in the amounts that can be toxic to both humans and animals. Therefore, finding new candidates of microorganisms, especially fungi of various geographical and agroecological origins, which will be more efficient in biodegradation of ochratoxins and bioremediation of food, is of a great interest. Accordingly, the aim of this study was to determine the role of some fungi, originating from Serbian region and not expressing toxigenic properties, in the biodegradation of OTA.

MATERIALS AND METHODS

Microorganisms. Nine isolates of fungi belonging to species *Aspergillus flavus* (Bain. and Sart.) Thom and Church (2), *A. fumigatus* Fres. (1), *A. ochraceus* Wilhelm(1), *Fusarium poae* (Peck) Wollenw. (1), *Fusarium* sp. (1), *Paecilomyces lilacinus* (Thom) Samson (1) and *Penicillium* spp. (2) were selected as test organisms. With an exception of the culture of *A. ochraceus* strain CBS 108.08, which is a known ochratoxin A producer (B o č a r o v-S t a n č i ć et al., 2009b), tested fungi originated from Serbian samples of livestock feed and their components not contaminated by mycotoxins, or were isolated from air in the course of a regular sterility control of premises in which microbiological analyses were carried out. The fungal identification was performed after D o m s h et al. (1980) and 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). The fungal cultures were kept on potato dextrose agar (PDA) at 4-6ºC.

Crude toxin production. Crude ochratoxin A (OTA) was produced by the isolate of *A. ochraceus* strain CBS 108.08 using a procedure described in detail

in the previous manuscript written by B o č a r o v-S t a n č i ć et al. (2009b). Inoculated Roux bottles containing 50 g of sterilized wheat kernels, wetted with 50 ml of sterile water, were cultivated at 30±1ºC for four weeks. Samples obtained after the cultivation were dried for 24 h or more at 60ºC until constant weight. After the pulverization of dried samples, crude OTA was obtained by the use of Serbian official methods for sampling and fodder analyzing (The O f f i c i a l G a z e t t e of S F R Y, issue 15/87). When the chloroform extract of OTA was evaporated, dry residue of this mycotoxin was dissolved in 96% ethanol (1 mg ml^{-1}) and stored until used at 4-6ºC.

Cultivation conditions. Test fungi were cultured on the modified Vogel's medium N (pH 6.3) with the addition of crude OTA extract for 14 days at 27±1ºC. OTA was added to the test medium immediately before its pouring into Petri dishes (15 ml per dish), and its final concentration in the medium amounted to 10 μg ml^{-1}. A test microorganism was applied with an inoculating loop to the central part of the solidified medium. The modification of Vogel's minimal medium (V o g e l, 1956) consisted of excluding the solution of biotin and sucrose, and addition of peptone (1 g l^{-1}) and yeast extract (2 g l^{-1}).

Mycotoxicological studies. Fungal capacity to produce and degrade OTA was preliminary studied by a rapid screening method described by F i l t e n b o r g et al. (1983), and modified by B o č a r o v-S t a n č i ć et al. (2009a, 2010). Vogel's minimal medium without addition of mycotoxins was used only in case of studies on fungal ability to biosynthesize OTA.

The second part of the experiment encompassed the study on the capability of the selected *Paecilomyces lilacinus* isolate (Inf. 2/A) to biodegrade OTA. Wet sterile rice grains (50 g + 25 ml distilled water) were inoculated with individual isolates of fungi *A. ochraceus* (strain CBS 108.08) and *P. lilacinus* (isolate Inf. 2-A) and with their combination. In the case of *P. lilacinus*, crude ochratoxin A (0.9 mg) was added to the cultivation substrate. After the four week cultivation of individual and combined fungal cultures at 27±1°C, inoculated grains were dried to the constant weight (dry residue) and pulverized. OTA in these samples was determined by the application of standard thin layer chromatography (TLC) method for the fodder analysis (T h e O f f i c i a l G a z e t t e of S F R Y, issue 15/87). All tests were performed in three replications.

Thin layer chromatography was done in saturated system of the benzene-acetic acid mixture (9:1, v/v). OTA was visually detected under long wave UV rays (366 nm) after TCL plates were sprayed with $NaHCO_3$ solution in ethanol and heated for 10 min at 130°C. The limit of detection (LOD) of the applied TLC method amounted to 8 μg kg^{-1}.

RESULTS AND DISCUSSION

Out of nine fungal isolates tested by a rapid screening method (F i l t e n b o r g - a et al., 1983; B o č a r o v-S t a n č i ć et al., 2009a, 2010), only the isolate of *A. ochraceus* (CBS 108.08) produced OTA in Vogel's minimal me-

dium without the addition of this mycotoxin (Table1). In previous studies, this isolate proved to be a good producer of OTA (B o č a r o v-S t a n č i ć et al., 2009b).

Tab. 1 – Capability of biosynthesis (control test for toxigenicity) and microbiological degradation of OTA by means of fungi

Ord. no.	Species	Isolate origin	Isolate design.	Biosynthesis[a] Degradacija[a]		Degradation[b]	
				7 d.	14 d.	7 d.	14 d.
1.	*Aspergillus flavus*	Soya bean grits	675/09	no	no	no	no
2.	*A. flavus*	Air	D-2	no	no	no	no
3.	*A. fumigatus*	Air	D-3	no	no	no	no
4.	*A. ochraceus*	CBS	108.08	no	yes	no	no
5.	*Fusarium poae*	Wheat kernel	598/09-8	no	no	no	no
6.	*Fusarium* sp.	Air	Inf. 3	no	no	no	no
7.	*Paecilomyces lilacinus*	Air	Inf. 2/A	no	no	yes	yes
8.	*Penicillium* sp.	Air	Inf. 2/B	no	no	no	no
9.	*Penicillium* sp.	Wheat kernel	598/09-7	no	no	no	no

[a] Vogel's minimal medium without addition of OTA,
[b] Vogel's minimal medium with addition of OTA.

P. lilacinus isolate (Inf. 2/A) was a single isolate that biotransformed the total amount of crude OTA (150 µg per Petri dish) after only seven days under given laboratory conditions (Table 1). Although numerous available literature data show that fungi, such as *Aspergillus clavatus* Desm., *A. ochraceus, A. versicolor* (Vuill.) Tirab., *A. wenti* Wehmer (A b r a n h o s a et al., 2002), *A. niger* Tiegh., *A. japonicus* Saito (B e j a o u i et al., 2006), *Rhizopus microsporus* Teigh., *R. homothallicus* Hesseltine & Ellis, *R. oryzae* Went and Prinsen-Geerligs (V a r g a et al., 2005) and others, can biotransform up to 95% of the initial OTA amounts, in this study, our own isolates of the genus *Aspergillus* were not capable of degrading this mycotoxin. On the other hand, we could not find in literature at our disposal that *Paecilomyces* spp. was capable of OTA detoxification.

It is interesting to point out that the fungus *P. lilacinus* (Inf. 2/A), as well as *A. flavus* (D-2), *A. fumigatus* (D-3), *Fusarium* sp. (Inf. 3) and *Penicillium* sp. (Inf. 2/B), was isolated after screening ambient air above working areas in the Department of Microbiology of the Bio-Ecological Center in Zrenjanin. Although all these isolates grew well on Vogel's minimal medium, to which crude OTA had been added, only *P. lilacinus* isolate (Inf. 2/A) had capability to biodegrade this mycotoxin.

The amount of OTA (150 µg per Petri dish), used in this experiment for the growth of the test organisms, was significantly higher (Table 2) than the common natural contamination of different substrates with this mycotoxin. In similar experiments of OTA biodegradation, other authors used 40 µg (H w a n g

and D r a u g h o n, 1994) or 50 μg of this mycotoxin (B ö h m et al., 2000), which is three-fold lower amount than that used in our experiment. The obtained preliminary results point out that our *P. lilacinus* isolate (Inf. 2/A) has an excellent potential for OTA biotransformation.

After the completed cultivation of *P. lilacinus* (Inf. 2/A) on wet sterile rice grains, the result obtained on Vogel's minimal medium was confirmed – this fungi was not toxigenic because it had no ability to biosynthesize OTA (Table 2).

Tab. 2 – Amount and percentage of OTA biodegradation (average values) after the four week cultivation on wet sterile rice grain

No.	Species	Isolate/isolates	Dry residue (g)	Amount of OTA (μg kg^{-1})	Degraded OTA (%)
	P. lilacinus	Inf. 2/A	33.13	n.d. (<8)	0
	P. lilacinus[a]	Inf. 2/A	32.95	n.d. (<8)	100.0
	P. lilacinus + *A. ochraceus*	Inf. 2/A+ CBS 108.08	27.00	80	99.8
	A. ochraceus	CBS 108.08	16.13	61,310	–

[a] wet sterile rice grain with the addition of 0.

It was not possible to detect OTA residues (<8 μg kg^{-1}) in the samples of wet sterile rice grains to which 0.9 mg of crude OTA was added, and which were inoculated only with *P. lilacinus* isolate (Inf. 2/A), because this isolate completely biotransformed the added amount of crude OTA. If this mycotoxin was not degraded, the expected amount of it would be 27.160 μg kg^{-1} dry matter (DM).

V a r g a et al. (2005) observed effective degradation of OTA in wheat kernels that were inoculated with *Rhizopus stolonifer* (Ehrenb.) Lind. The same authors also observed that this and other species of the same fungal genus (*R. microsporus, R. homotallicus* and *R. oryzae*) were capable to biotransform OTA in the liquid medium (up to 95% of initial quantity). However, the biodegradation process in the liquid medium was much faster than in wheat grain (max. 16 days in comparison to four weeks).

In the samples of wet sterile rice kernels inoculated only with *A. ochraceus* (CBS 108.08), detected amount of dry residue (16.13 g) was lower than in the medium inoculated with *P. lilacinus* (33.13 g) (Table 2). The addition of 0.9 mg of crude OTA into the rice grain used for *P. lilacinus* monoculture did not significantly affect the amount of dry residue in *P. lilacinus* (32.95 g). After the four week cultivation of both fungi combined on the given substrate, the amount of dry residue was 27.0 g. The reduction of the dry residue was not surprising considering the competition for the same substrate between *A. ochraceus* and *P. Lilacinus* (Table 2).

Significantly lower average amount of OTA (80 μg kg^{-1}DM) was established in the samples of wet sterile rice kernels inoculated with combined cultures of *A. ochraceus* and *P. lilacinus*, than that detected in *A. ochraceus*

monoculture (61.310 μg kg^{-1} DM). These results indicate that *P. lilacinus* isolate degraded, on average, 99.8% OTA during the four week cultivation on the solid natural substrate.

The determined percentage of biotransformation of OTA in the present study was significantly higher than that established by E n g e l h a r d t (2002), who observed capability of degradation of OTA and OTB in three fungal species cultured on barely kernels. According to this author, the white rot fungus *Pleurotus ostreatus* (Jacq. ex Fr.) Kumm. was ranked first as it degraded 77 and 97% of the initial amount of OTA and OTB, respectively, after the four week cultivation. Achieved results point out that our *P. lilacinus* isolate has an excellent potential for biotransformation of OTA when grown not only in minimal Vogel's medium but also in rice grain substrate.

CONCLUSION

Only *P. lilacinus* isolate (Inf. 2/A), out of nine fungal isolates tested by the rapid screening method, showed capability to biotransform OTA when grown during seven days at 27±1°C on Vogel's minimal medium with the addition of crude OTA (150 μg per Petri dish).

After four week cultivation at 27±1°C, the same isolate in samples of wet sterile rice kernels with the addition of 0.9 mg of crude OTA, completely degraded initially added ochratoxin (<8 μg kg^{-1}).

P. lilacinus (Inf. 2/A) grown together with a good OTA producer, *A. ochraceus* CBS 108.08, biodegraded 99.8% of OTA after the four week cultivation on wet sterile rice kernels at 27±1°C.

The isolate of fungus *P. lilacinus* (Inf. 2/A) has a significant potential for biotransformation of OTA, hence further studies will be aimed at finding the mode to use this isolate in safe decontamination of cereals and their products intended to be used as food and feed.

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УЛОГА *PAECILOMYCES LILACINUS* (THOM) SAMSON И ДРУГИХ ВРСТА ГЉИВА У БИОДЕГРАДАЦИЈИ ОХРАТОКСИНА А

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Резиме

Девет изолата гљива из родова *Aspergillus, Fusarium*, *Paecilomyces* и *Penicillium* гајено је на модификованој Вогеловој подлози са додатком сировог екстракта охратоксина А (ОТА). Сирови екстракт ОТА је добијен из чврстог природног супстрата на којем је гајен сој *Aspergillus ochraceus* CBS 108.08. Изолован и делимично пречишћен ОТА, упарен до сувог остатка и растворен у етанолу (1 mg ml^{-1}), додат је у тест подлогу до финалне концентрације 10 μg ml^{-1}. Након седам и 14 дана гајења култура гљива у тест подлози на 27 ± 1°C детерминисано је присуство резидуа ОТА применом модификоване методе F i l t e n b o r g -a и сар. (1983).

Од девет тестираних изолата за даља испитивања је одабран изолат *Paecilomyces lilacinus* (Inf. 2/A), који је већ после седам дана у потпуности разградио иницијалну количину ОТА (150 μg).

У другом делу експеримента влажно стерилно зрно пиринча (50 g + 25 ml дестиловане воде) засејано је са појединачним изолатима *A. ochraceus* (CBS 108.08) и *P. lilacinus* (Inf. 2-A), као и комбинацијом оба изолата. У случају монокултуре *P. lilacinu*s у подлогу је додат и сирови ОТА (0,9 mg). Сваки од тестова је урађен у 3 понављања. Након четири недеље гајења монокултура и мешаних култура гљива на 27±1°C, инокулисана зрна су осушена до константне тежине и самлевена до финог праха. У овим узорцима извршена је детерминација ОТА применом стандардне методе танкослојне хроматографије за анализу сточне хране.

У узорцима који су били засејани само са продуцентом ОТА (*A. ochraceus*, сој CBS 108.08) детектован је ОТА у просечној количини од 61.310 μg kg^{-1} сувог остатка. У узорцима који су били засејани комбинованим културама изолата *A. ochraceus* и *P. lilacinus* утврђена је знатно мања просечна количина ОТА (80 μg kg^{-1}). Ови резултати указују да је изолат *P. lilacinus* разградио просечно 99,8% ОТА присутног у подлози за култивацију. У узорцима влажног стерилног зрна пиринча са додатком 0,9 mg сировог ОТА исти гљивични изолат је после четири недеље култивације комплетно биоразградио додат сирови ОТА (<8 μg kg^{-1}).

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ANTIFUNGAL ACTIVITY OF INDIGENOUS *BACILLUS* SP. ISOLATE Q3 AGAINST MARSHMALLOW MYCOBIOTA

ABSTRACT: Marshmallow is a host of a number of saprophytic and parasitic fungi in Serbia. The seeds of marshmallow are contaminated with fungi from different genera, especially *Alternaria* and *Fusarium*, which significantly reduced seed germination and caused seedling decay. In this study we investigate antagonism of indigenous *Bacillus* sp. isolate Q3 against marshmallow mycopopulation. *Bacillus* sp. Q3 was isolated from maize rhizosphere, characterized by polyphasic approch and tested for plant growth promoting treats. *Bacillus* sp. Q3 produced antifungal metabolites with growth inhibition activity against numerous fungi in dual culture: 61.8% of *Alternaria alternata*, 74.8% of *Myrothecium verrucaria* and 33.6% of *Sclerotinia sclerotiorum*. That effect could be caused by different antifungal metabolites including siderophores, hydrolytic enzymes, organic acids and indole acetic acid (IAA). Suppression of natural marshmallow seed infection by Q3 isolate was observed. The seeds were immersed in different concentrations of bacterial suspension during 2h and their infections by phytopathogenic fungi were estimated. The results showed significant reduction of seed infection by *Alternaria* spp.

The presented results indicate possible application of this isolate as promising biological agent for control of marshmallow seed pathogenic fungi.

KEY WORDS: *marshmallow, Alternaria, Bacillus sp., antagonism, mycopopulation*

INTRODUCTION

Marshmallow as medicinal plant is among the most economically significant herb in Serbia. Its cultivation has been started in Serbia because of medical properties of root, leaves and flowers.

Marshmallow is the host of many fungal species (Pavlović and Stojanović, 2001, Pavlović et al., 2002, 2006). Fourteen species from 10 genera were identified on the seed, leaf, stem and root of marshmallow (Pavlović et al., 2007). Fungus from the genera *Alternaria* and *Fusarium*, were dominant on the seed. In smaller percentage, fungus from the genera *Phoma*,

Epicoccum, *Cladosporium*, *Penicillium*, *Aspergillus* and *Rhizopus* were present. *Alternaria alternata* was permanently present on the seed. A significant pathogen of the leaves was *Puccinia*. Very destructive pathogens on the stem and root were *Fusarium* species and *Sclerotinia sclerotiorum* causing tissue necroses and white rot of root and stem (P a v l o v i ć and et al., 2006).

Pathogenic fungi decrease the yield and quality of herbal raw material. The use of chemicals is not allowed in plantation of marshmallow. Therefore, alternatives in plant protection are very important. The biological control of soil-born pathogens with antagonistic bacteria has received special attention because of the dural role of these bacteria in plant-growth promotion (PGPR) and disease control (Z e h n d e r et al., 2001). PGPR enhance the adaptive potential of their hosts through a number of mechanisms, such as the mobilization of recalcitrant soil nutrients, the fixation of molecular nitrogen, the control of phytopathogens and the synthesis of phytohormones and vitamins. Direct promotion by PGPR occurs by providing the plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. Indirect promotion of plant growth occurs when PGPR lessens or prevents the deleterious effect of one or more phytopathogenic microorganisms.

Bacillus spp. are well known rhizosphere residents of many crops and usually show plant growth promoting activities that include biocontrol capacity against some phytopatogenic fungi. Recently, intensive agricultural production has been paying greater attention to crop protection from pathogens that lessen yields, as well as to the microbial quality of these crops as raw materials. From the initial implementation of sustainable agriculture, the availability of alternative protective strategies has been reassessed and consequently, the development of environment-friendly and food-hygienically-safe plant-protecting methods based on biological agents has been greatly emphasized (W a r r i o r, 2000).

In order to find effective biocontrol agents which act through the combination of several different mechanisms, the procedures that allowed selection of strain, positive for more than one antagonistic mechanism, were used. Isolate *Bacillus* Q3 seems to be more promising biocontrol agent then other *Bacillus* isolates from soil rhizosphere. The results of testing of cultural, biochemical and PGPR traits of Q3 isolate, as well as the estimations of its antifungal activity to *A. alternata* on seed are presented in this paper.

MATERIALS AND METHODS

Influence of different cultivation conditions on growth rate of indigenous *Bacillus* sp. – isolate Q3. Influence of different nutrient agar media on *Bacillus* Q3 growth was tested: Waksman agar (B e r g, 2002), Yeast Mannitol agar – YMA (V i n c e n t, 1970), Nutrient agar, King B, Potato Dextrose agar – PDA and Triple sugar Iron (Biomedics, Spain). O/F basal medium supplemented with different carbon sources was used for testing the utilization of carbohy-

drates as only carbon source. In order to test the effect of NaCl, temperature and pH on *Bacillus* Q3 growth, the isolate was grown in nutrient broth after inoculation with $5x10^6$ cells. Growth was measured at 600 nm using Shimadzu UV-160 spectrophotometer.

Enzymatic activities assay. Catalase, lysine decarboxylase, gelatinase and urease were detected as recommended by S m i b e r t and K r i e g (1994).

Enzymatic activities for protease, chitinase, pectinase, amylase, and cellulase were identified by clear zone formation around the cell (S m i b e r t and K r i e g, 1994) after incubation for 3 days at 28°C. Protease production was assayed using skim milk agar, chitinase by using Waksman agar supplemented with colloidal chitin, pectinase by using M9 medium supplemented with pectin, cellulase by using M9 with carboxymethyl cellulose (CMC), amylase using M9 with starch.

Estimation of main PGPR trait of *Bacillus* Q3. Phosphate solubilization ability of isolate Q3 was tested on Pikovskaya agar plates (P i k o v s k a y a, 1948) with 0.5% tricalcium phosphate [Ca_3 $(PO4)_2$] and identified by clear halo zones around the colonies.

The production of indole acetic acid (IAA) by Q3 strains and the effect of L-tryptophan on IAA production were assayed according to the Salkowski method (G l i c k m a n and D e s s a u x , 1995). The bacteria were inoculated into the nutrient broth containing L-tryptophan in the concentrations of 2 and 5 mM.

Production of siderophore was determined by Chromazurol Sulphonate (CAS) agar method (A l e x a n d e r and Z u b e r e r, 1991). After incubation at 28°C for 5 days, siderophore production was assayed by the change in medium color, turning from blue to orange.

Hydrogen cyanide production was assayed according to the method suggested by C a s t r i c (1977). Bacteria were streaked onto Nutrient agar plates supplemented with glycine. Petri plates were inverted and a piece of filter paper impregnated with 0.5% picric acid and 2% of sodium carbonate was placed on the lid. Petri plates were sealed with parafilm and incubated at 28°C for 96 h. Discoloration of the filter paper from orange to brown after incubation was considered as microbial production of cyanide.

Sampling and fungal isolation. Marshmallow plant parts expressing pathological changes were collected during the period 2008-2010 at the localities of Pančevo, Ruma and Zrenjanin. Seed samples were collected from the plantation intended for marshmallow seed production in Pančevo.

Isolation of the collected samples of leaves, leaf stalks, stems and roots was conducted in in a common manner, by taking the fragments from the zones between healthy and diseased tissues. Before transferring to PDA, plant material was sterilized with 2% of NaOCl solution for 2 minutes, washed with distilled water and incubated for 7-10 days at 25°C. Isolation of fungi from the seeds was carried out according to the procedure supplied by ISTA (M a t h u r and K o n g s d a l, 2003), using the incubation methods that include filter paper and nutritive medium. After the incubation of seeds for 5-10 days, formed mycelia were transferred to PDA. Twelve isolates were obtained from marshmallow (Table 1).

Tab. 1 – Fungi isolated from marshmallow used in this study

Fungal	Reference of source
Alternaria alternata	Seed
Aspergillus niger	Seed
Aspergillus flavus	Seed
Myrothecium verricaria	Seed
Fusarum oxysporum	Seed, root
F. verticillioides	Seed, root
F. proliferatum	Seed, root
F. semitectum	Stalk
F. sporotrichoides	Seed
F. equiseti	Seed
F. solani	Root
Sclerotinia sclerotiorum	Collar root

Antifungal activities of Bacillus Q3 isolate against phytopathogenic fungi. Antifungal activities were tested with 15 phytopathogenic fungi (Table 1.) A dual plate method was used for *in vitro* screening of biocontrol PGPR. Percentage of growth inhibition was calculated using the formula proposed by V i n c e n t (1947): I = (c- T)/c x 100, where I is the percentage of growth inhibition; C the growth of fungi in control; T the growth of fungi in treatment.

Marshmallow's seed treatment with *Bacillus* sp. – isolate Q3. Marshmallow seeds were suface sterilized with 70% ethanol for 5 min, and rinsed five times with destilled water. The sterilized seeds were submerged into the culture solutions of *Bacillus* Q3 isolate (concentration $5x10^4$, $5x10^5$ and $5x10^6$ CFU ml^{-1}) for 2 hours. Four replications (with 100 seeds per replication) were placed in filter paper in Petri dishes. The seeds immersed in distilled water were applied as negative control. The seeds were incubated at 25°C and the percentages of infected seeds were recorded. The obtained results were analyzed by Duncan test.

RESULTS AND DISCUSSION

Recent developments have encouraged the research into PGPR commercialization. These developments include the need for alternatives to soil fumigants to control soil borne plant pathogens. Current fumigants are being banned or restricted in use or are too costly for annual crop producers. Medicinal plants are to be targeted for PGPR application as these producers may replace costly soil fumigants with as likely costly PGPR.

Influence of different cultivation conditions on growth rate of indigenous *Bacillus* sp. – isolate Q3. On the basis of cultural, morphological and biochemical characteristics, the isolate Q3 was presumptively identified as *Bacillus* strain. Growth on different carbon sources added in O/F basal medium, on different nutritive agar media and at different conditions is presented in Table 2.

Tab. 2 – Effect of different nutritive media, carbon sources, temperature, saline and pH conditions on *Bacillus* Q3 growth

Growth on nutritive agar media		Growth on carbon sources (in O/F basal medium)		Growth in Nutrient broth at pH 7		Growth in Nutrient broth at 37°C			
Waksman	+	glycerol	+	t° C		% NaCl		pH	
Yeast Mannitol	+	mannitol	+	4	-	1	+	5	-
Potato Dextrose	+	lactose	+	10	±	3	+	5.5	±
Triple Sugar Iron	+	sucrose	+	26	+	5	+	7	+
Nutrient	+	glucose	+	37	+	7	+	8	+
King B	-	maltose	+	41	±	8	±	8.5	±

Bacillus Q3 showed optimal growth at pH 7-8 in temperature range 26-37 °C. We observed tolerance to saline concentration up to 8%.

Enzymatic activities. Enzymatic activities of catalase and lysine decarboxylase were observed, while gelatinase and urease activities were not detected (Table 3). The absence of clear zone formation around the cells on media for chitinase, pectinase, amylase and celulase were identified suggesting negative results for enzyme activities. Protease activity was poor and zones on skim milk agar were observed, but with not clear appearance. L e o n et al. (2009) reported that several enzymatic activities (proteolytic, chitinolytic and cellulolytic) of *B. amyloliquefaciens* BNM340 were detected.

Estimation of PGPR traits of *Bacillus* Q3 isolate. Phosphate solubilizing microorganisms (PSM) play a significant role in making phosphorus available to plants by bringing about favorable changes in the soil microenvironment and leading to solubilization of inorganic phosphate sources. Solubilization ability of isolate *Bacillus* Q3 was detected as clear halo zones around the colonies on Pikovskaya agar plates supplemented with 0.5% tricalcium phosphate. The solubilization of inorganic phosphate has been attributed to the production and release of organic acids or some additional mechanisms as it is confirmed by weak or even lack of linear correlation between pH and the amount of P-solubilized (A l a m et al., 2002).

Tab. 3 – Enzymatic activities and main PGPR traits of *Bacillus* Q3 isolate

		PGPR traits			
Enzymatic activity				Solubilization of insoluble phosphates	±
katalase	+	Protease	±	Antifungal activity	+
lysine decarboxilase	+	Pectinase	-	Production of	
gelatinase	-	Cellulase	-	indole acetic acid	+
urease	-	Chitinase	-	siderophores	+
amylase	-			HCN	±

Another important trait of PGPR – production of low-molecular-weight compounds called siderophores was observed. Indigenous isolate *Bacillus* Q3 showed large amount of siderophores production detected by the color change intensity of the CAS medium from blue to orange. Siderophores may indirectly influence the plant growth and health. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a serious competition (L o p e r and H e n k e l s, 1997). Under iron-limiting conditions, PGPR produces siderophores in order to acquire ferric ion (W h i p p s, 2001). They bind to the available form of iron (Fe^{3+}) in the rhizosphere, thus making it unavailable for the phytopathogens and protecting the plant's health. Although various bacterial siderophores differ in their abilities to sequester iron, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (L o p e r and H e n k e l s, 1999).

Indigenous isolate *Bacillus* Q3 was inoculated into the nutrient broth containing L-tryptophan in the concentrations of 2 and 5 mM and production of indole acetic acid (IAA) was observed. IAA production by microorganisms promotes the root growth by directly stimulating plant cell elongation or cell division (I d r i s et al., 2004). *B. amyloliquefaciens* BNM340-inoculated soybean plants were protected from a high *P. ultimum* infestation, since only 30% of seedlings emerged in the control treatments (L e o n et al. 2009). This strain was able to produce auxins, as well as excreted surfactin and some iturin-like lipodepsipeptides, such as iturin A. These mechanisms have been previously correlated with antifungal activity (Z e h n d e r et al., 2001; I d r i s et al., 2004).

Bacillus Q3 showed very light discoloration of the filter paper from orange to brown, and it was considered to be a poor cyanide producer. Hydrogen cyanide is a general biocide forming stable compounds with divalent ions and inhibiting cytochrome oxidase of many organisms (V o i s a r d et al., 1994). A h m a d et al. (2008) published that three strains of *B. subtilis* were unique in their characteristics, being antagonistic to *C. falcatum,* deficient in HCN production and producers of surfactin lipopeptide only. Inability of strains to produce HCN will make them biocontrol agents of choice, since HCN imposes negative effects on plant growth (S c h i p p e r s et al., 1990).

Antifungal activities of Bacillus Q3 isolate against phytopathogenic fungi. Biocontrol activity of *Bacillus* strains against multiple plant pathogens have been widely reported and well documented (L e o n et al., 2009; K l o e p p e r et al., 2004). Indigenous isolate *Bacillus* Q3 showed hyphal deformation, inhibition of hyphal elongation and different percent of growth inhibition of tested marshmallow pathogenic fungi (Table 4). Maximum inhibitory zone and antifungal activity was observed against *Myrothecium verrucaria* (about 75%). Bacillus Q3 isolate caused high percent of inhibition (61.75) on *Alternaria alternata* growth. We assume that inhibitory effect may be caused by different antifungal metabolites including siderophores, organic acids, IAA and antifungal antibiotics. These results are in agreement with earlier report on *Bacillus* sp. producing antifungal metabolites with activity against a number of mycelial fungi (R a m i r e z et al., 2004; C a z o r l a et al., 2007).

Tab. 4 – The inhibitory effects of the bacterial isolates *Bacillus* Q3 on tested pathogenic fungi

Phytophatogenic fungi	Hyphal deformation	Inhibition of hyphal elongation	Percent of growth inhibition
Alternaria alternata	+	+	61.75 ± 2.70
Aspergillus niger	+	±	ns
Fusarium solani	+	±	ns
F. verticillioides	+	±	ns
F. semitectum	+	±	ns
F. sporotrichioides	+	±	ns
F. equiseti	+	+	ns
Sclerotinia sclerotiorum	+	+	33.63 ± 2.50
Myrothecium verrucaria	-	+*	74.80 ± 4.91
A. flavus	-	-	-
Fusarium oxysporum	-	-	-
F. proliferatum	-	-	-

(–) no inhibition; (±) inhibition during 5 days; (+) inhibition longer than 7 days; ns- not significant, less than 10%; *- hyphal elongation in opposite direction of bacteria;

VVV reported that *B. circulans* MTCC 8983 also showed antifungal activity against *Rhizoctonia solani, Fusarium oxysporum, Sclerotinia sclerotiorum.*

Efficacy of indigenous *Bacillus* sp. – Q3 isolate against marshmallow seeds mycoflora. The average percentage of seed infection with *Alternaria althernata* was 34% in control, but only 6.5% in the variant treated with *Bacillus* sp. isolate in the concentration of $5x10^6$ CFU ml^{-1} for 2 hours. The obtained results showed that isolate Q3 reduced infection of seeds by *A. althernata* for more than five times. The same results were obtained by using bacterial concentration of $5x10^5$ CFU ml^{-1} and $5x10^4$ CFU ml^{-1} for 2 hours.

Our results coincided with the results of K a u r et al., (2007) who found that some bacterial isolate from rhizosphere, such as *Pseudomonas* spp., could inhibit *Aspergilus* and *Fusarium*. U m e c h u r u b a (2004) studied antagonistic activity of *Bacillus subtilis* against *Alternaria* spp. isolated from seed and found the inhibitory effect of 26-58%.

CONCLUSION

Indigenous *Bacillus* sp. – isolate Q3 showed strong *in vitro* antagonistic activity against *Myrothecium verrucaria, Alternaria alternata* and *Sclerotinia sclerotiorum* that continued for eight days. It has now been confirmed that a single PGPR – indigenous *Bacillus* Q3 has several modes of action. So far, this has been the first report of a *Bacillus* sp. isolated from maize rhizosphere in Serbia with phosphate solubilizing ability simultaneously producing siderophore, IAA and antagonistic activity against marshmallow mycobiota. This study confirmed the potential of rhizoplane and rhizosphere to protect medicinal plants, in this case marshmallow, from some diseases.

Bacillus sp. isolate Q3, appeared to be very promising biocontrol agent against *Alternaria* spp., a predominant marshmallow seeds pathogen.

Identification of key antimicrobials produced by *Bacillus* Q3 can be exploited for the testing of antifungal activity against other medicinal plant pathogenic fungi. Also, *Bacillus* Q3 could be further exploited both as a biofertilizer and an effective biocontrol agent.

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АНТИФУНГАЛНА АКТИВНОСТ АУТОХТОНОГ ИЗОЛАТА *BACILLUS* SP. Q3 НА МИКОПОПУЛАЦИЈУ БЕЛОГ СЛЕЗА

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Резиме

Бели слез гајен у Србији је домаћин многим сапрофитним и паразитским гљивама. Семе је заражено гљивама из различитих родова, нарочито *Alternaria* и *Fusarium*, које значајно редукују клијавост семена и изазивају сушење. У овом раду испитиван је антагонизам аутохтоне бактерије *Bacillus* sp. изолата Q3 и микопопулације белог слеза. *Bacillus* sp. Q3 је изолован из ризосфере кукуруза, карактеризација је извршена полифазном методологијом и тестиране су особине одговорне за стимулацију раста биљака. *Bacillus* sp. Q3 продукује антифунгалне метаболите са израженом активношћу против фитопатогених гљива са различитим процентом инхибиције раста у двојној култури: 61.75% код *Alternaria alternata*, 74.80% код *Myrothecium verrucaria* и 33.63% код *Sclerotinia sclerotiorum*. Овај ефекат је последица продукције различитих антифунгалних метаболита, укључујући сидерофоре, хидролитичке ензиме, органске киселине и индолсирћетну киселину (IAA). Установљено је сузбијање природне инфекције семена белог слеза применом изолата *Bacillus* Q3. Семена су потапана у различите концентрације бактеријске суспензије током 2 h и праћен је степен инфекције фитопатогеним гљивама. Резултати су показали значајан степен редукције инфекције семена белог слеза гљивом *Alternaria* spp.

Ови резултати указују на могућу примену овог изолата као потентног биолошког агенса за контролу инфекције семена белог слеза фитопатогеним гљивама.

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THE EFFECT OF FUNGICIDE TREATMENT ON MYCOTOXIN CONTENT AND YIELD PARAMETERS OF WHEAT

ABSTRACT: Effects of treatment with triazole fungicide were evaluated on 14 wheat genotypes with respect to mycotoxin (aflatoxins, ochratoxin A and deoxynivalenol), yield, 1000 kernel weight and hectoliter weight. Mycopopulation of seed samples was also determined. According to the results, fungicide treatment can reduce the level of mycotoxins in seed samples in order to improve the quality parameters and reduce the level of fungal contamination.

KEY WORDS: fungicide treatment, mycotoxins, *Fusarium*

INTRODUCTION

Agro-climatic conditions in Serbia are favorable for the development of fungus *Fusarium graminearum* (Schwabe) which is the main causal agent of wheat head blight. Disease incidence of wheat head blight in years with average precipitation level is about 5% (B a g i, 1999). Besides *F. graminearum*, wheat seed can be colonized by a number of other *Fusarium* species, including fungi from genera *Alternaria, Aspergillus, Penicillium* and other. Fungal contamination causes significant yield decrease, but the losses are even greater because of mycotoxins produced by these fungi (H a v l o v a et al., 2006). Application of proper agronomic measures, i.e. fungicide treatment, during or immediately after wheat flowering can significantly reduce Fusarium head blight infections and also control leaf diseases of wheat (H a i d u k o w s k i et al., 2005). The aim of this work was to investigate the impact of fungicide treatment in the control of Fusarium head blight, as well as fungicide effect on seed fungal contamination and mycotoxin content.

MATERIALS AND METHODS

The trial was conducted at the locality of Vrbas (Serbia) during 2009. The following commercial wheat varieties were included into the investigation: NS 40S, Dama, Rapsodija, Astra, Milijana, Srma, Isidora, Janja, Barbara, Arija, Pobeda, Kruna, Vizija and Toplica. Plots of every treated wheat variety with its untreated control were established randomly in four replications. Elementary parcel for every treatment was 256 m^2. During vegetation, standard agrotechnical measures were applied. The previous crop was maize, which is important source of *Gibberella zeae* (Schwein.) Petch. ascospore infections. The level of precipitation during flowering and at the beginning of seed formation was lower than 30-year average (46.2 mm in May during 2009, while the 30-year average for May was 56.9 mm), which moderately favored head blight infections. Fungicide Prosaro EC 250 (tebuconazole 125 g/l + protioconazole 125 g/l) was applied by ground application during the end of the flowering phase in a dosage of 1 l/ha using widely exploited Twin Jet 60 sprayers. The amount of used water was 400 l/ha. Fusarium head blight severity was evaluated by calculating the number of infected ears/m^2 during early milk stage of wheat. Immediately after the harvest, representative seed samples were taken for the analyses of mycotoxin and quality parameters, as well as for the determination of mycopopulation. Yield, 1000 kernel weight and hectoliter weight were measured for every variety and treatment. Thousand kernel weight was determined by Perten SK CS 4100 (Kernel Hardness Tester, Perten Instruments, Reno, Nevada, USA). The hectoliter weight was measured by dusting the procedure and equipment Schopper scale. Level of fungal infection was determined by Petri dish test according to P i t t and H o c k i n g (1985). Determination of fungi to the level of genus was performed on the basis of morphological and cultural characteristics. Mycotoxins, aflatoxin (B1+B2+G1+G2), ochratoxin A and DON, were determined in both treated and untreated wheat cultivars by ELISA (enzyme-linked immunosorbent assay) method. Screening method for analysis was done using Neogen Veratox® testing kits with limits of detection of 1 µg/kg for aflatoxins ((B1+B2+G1+G2) 337-1-V alfa HS 0303), 2 µg/kg for ochratoxin A (A 2-V-Ochra 1205) and 0.25 mg/kg for DON (342 Veratox DON-a 5/5). Each sample was extracted by confinable solvents. All data were processed using Statistica 9.1 statistical software (StatSoft Inc., Tulsa, Oklahoma). Statistically significant differences between the means were detected by Tukey's test after variance analysis (ANOVA). Correlation analysis was performed for each combination of the investigated parameters.

RESULTS

During this research, incidence of Fusarium head blight was within the average values based on disease incidence in several previous years, and depending on cultivar, it varied from 0-5 diseased ears/m^2. In most cultivars, the

fungicide treatment significantly reduced the incidence of head blight when compared with untreated control (Table 1). Significant differences between treated and untreated variants, regarding the incidence of Fusarium head blight, were present only in cultivars Milijana, Astra and Srma, probably due to lower infection level in untreated control (1-2 ears/m^2). Besides head blight, applied fungicide was also efficient against leaf rust, Septoria leaf blotch and powdery mildew (data not presented).

All examined genotypes had higher yield in treated than untreated variants, and most of them (9 out of 14 cultivars, 64.2%) showed statistically sig-

Tab. 1 – Impact of chemical control on yield parameters and Fusarium head blight infection

Cultivar	Fungicide treatment	Yield kg/ha	1000 kernel weight (g)	Hectoliter weight (kg/hl)	FHB /m^2	Aflatoxin (µg/kg)	Ochratoxin A (µg/kg)	DON (mg/kg)
Rapsodija	Control	8.938 b	39.6 jk	78.7 bcd	4.0 ab	<1	2.85 a	<0.25 d
	treatment	9.388 a	40.1 j	79.0 bcd	0.5 fg	<1	<2 d	<0.25 d
Astra	Control	8.480 cd	46.1 de	79.1 bcd	1.5 defg	<1	2.32 b	<0.25 d
	treatment	8.780 bc	46.8 bc	80.2 ab	0.0 g	<1	<2 d	<0.25 d
NS – 40 S	Control	8.566 c	39.4 k	75.6 g	4.0 ab	<1	2.37 b	0.45 a
	treatment	8.966 b	39.6 jk	76.2 fg	0.0 g	<1	<2 d	<0.25 d
Dama	Control	7.855 f	44.1 h	78.4 bcd	4.0 ab	<1	2.81 a	<0.25 d
	treatment	8.505 cd	44.2 h	79.0 bcd	0.5 fg	<1	<2 d	<0.25 d
Vizija	Control	8.439 cd	46.8 bc	79.0 bcd	5.0 a	<1	2.08 c	0.34 c
	treatment	8.789 bc	46.9 bc	79.2 bcd	0.5 fg	<1	<2 d	<0.25 d
Kruna	Control	8.507 cd	40.0 jk	80.3 ab	2.5 bcde	<1	<2 d	<0.25 d
	treatment	8.950 b	41.2 i	81.4 a	0,0 g	<1	<2 d	<0.25 d
Arija	Control	7.890 ef	46.0 e	75.0 g	3.0 bcd	<1	<2 d	<0.25 d
	treatment	8.210 de	45.8 ef	76.4 fg	0.25 g	<1	<2 d	<0.25 d
Milijana	Control	7.715 fg	41.4 i	78.5 bcd	1.0 efg	<1	<2 d	<0.25 d
	treatment	8.194 de	41.6 i	79.2 bcd	0.0 g	<1	<2 d	<0.25 d
Barbara	Control	7.094 ijkl	47.0 bc	79.3 bcd	2.0 cdef	<1	<2 d	<0.25 d
	treatment	7.486 gh	47.2 b	80.0 abc	0.25 g	<1	<2 d	<0.25 d
Pobeda	Control	7.070 ijkl	46.4 cde	78.5 bcd	4.0 ab	<1	<2 d	<0.25 d
	treatment	7.392 ghi	46.8 bc	79.2 bcd	0.5 fg	<1	<2 d	<0.25 d
Isidora	Control	6.958 jkl	45.1 g	76.1 fg	2.0 cdef	<1	<2 d	<0.25 d
	treatment	7.386 ghi	45.8 ef	76.6 efg	0.0 g	<1	<2 d	<0.25 d
Srma	Control	6.880 kl	44.0 h	78.2 cde	2.0 cdef	<1	<2 d	<0.25 d
	treatment	7.215 hijk	45.2 fg	79.0 bcd	0.5 fg	<1	<2 d	<0.25 d
Janja	Control	6.770 l	46.7 bcd	77.5 def	4.0 ab	<1	<2 d	0.41 b
	treatment	7.278 hij	48.2 a	78.4 bcd	0.5 fg	<1	<2 d	<0.25 d
Toplica	Control	6.324 m	45.8 ef	76.3 fg	3.5 abc	<1	2.73 a	<0.25 d
	treatment	6.741 l	46.0 e	76.5 efg	0.5 fg	<1	<2 d	<0.25 d

Yield LSD 0.05= 0.3208; TWG LSD 0.05 = 0.5972; HlW LSD 0.05 = 1.6110; FHB LSD 0.05 = 1.3506; Ochra A LSD 0.1484; DON LSD 0.0238; Mean values in the same column followed with different letters are significantly different ($p<0.05$).

nificant differences (Table 1). In the case of 1000 kernel weight and hectoliter weight, treated cultivars had higher values, but of no significance (Table 1). Correlation coefficient between untreated control and treated variant was significant for all cultivars on the level of $p<0.05$. As for yield, 1000 kernel weight and hectoliter weight, it amounted $r = 0.99$ ($y = 436.82+0.99 x$), $r = 0.98$ ($1.144 + 0.9854 x$) and $r = 0.97$ ($y= 1.1173 + 0.9946 x$), respectively. Also, correlation coefficient between Fusarium head blight intensity in untreated control and treated variants was also significant ($p<0.05$) $r =0.64$.

The presence of mycotoxin DON in three wheat cultivars (NS 40S, Janja and Vizija) was above the detection limit. These amounts were detected in untreated control of all three cultivars with disease incidence of 4-5 infected ears/m^2 (Table 1). Infection of kernels of these three cultivars after harvest with *Fusarium* sp. was very high, between 20 and 24% (Table 2).

The level of ochratoxin A was above 2 μg/kg in untreated control of 6 wheat varieties (NS40S, Dama, Rapsodija, Astra, Vizija and Toplica). These genotypes were colonized more than others by fungi from genera *Penicillium* and *Aspergillus*: 10-16% and 3-5%, respectively (Table 2). None of these samples had level of aflatoxin exceeding 1 μg/kg.

Blotter method was used to determine the percentage of pathogenic and saprophytic fungi present on the seeds of tested cultivars, both treated and untreated with fungicide. Representatives of genera *Fusarium, Alternaria, Penicillium* and *Aspergillus* were found on wheat kernels. Detection of *Fusarium* species was 2-24 % (Table 2). All tested cultivars had higher disease intensity in untreated control than in treated variants. Species from genus *Fusarium* were identified using selective CLA medium and they included *F. graminearum, F. culmorum, F. avenaceum, F. poae* and *F. sporotrichioides*. Species from genera *Alternaria*, *Penicillium* and *Aspergillus* were present on wheat kernels in the percentage of: 1-18%, 1-16% and 0-5%, respectively. Without exception, fungi from these genera caused higher disease intensity in untreated control than in variants treated with fungicide, although differences were not always significant (Table 2).

DISCUSSION

In Serbia, protection of wheat with fungicides is carried out only in the fields where high yields are expected due to previously applied good agrotechnical practice and fertilization. Fields that are not expected to produce high yield are usually not chemically treated against fungal diseases, which causes Fusarium head blight to occur in high intensity in the years with favorable weather conditions, and consequently increases the risk of mycotoxin contamination. Earlier research showed that fungicide treatment increases yield both quantitatively and qualitatively (B a l a ž et al., 2006). Ground application using spray nozzle enables better ear coverage with fungicides (L e h o c z k i - K r s j a k et al., 2008). Regarding the choice of fungicides, a number of authors have stated that triazole fungicides decrease intensity of

Tab. 2 – Impact of chemical control on wheat seed fungal contamination (%)

		Fusarium	Alternaria	Penicillium	Aspergillus
Rapsodija	untreated	10 bcd	18 a	16 a	5 a
	treated	5 bcde	4 fg	5 cde	1 bc
Astra	untreated	12 b	6 defg	15 a	3 abc
	treated	9 bcde	2 g	4 cde	0 c
NS – 40 S	untreated	20 a	5 efg	10 abc	4 ab
	treated	3 de	14 abc	3 de	2 abc
Dama	untreated	10 bcd	13 abcd	16 a	4 ab
	treated	4 cde	2 g	4 cde	1 bc
Vizija	untreated	23 a	8 bcdefg	10 abc	4 ab
	treated	5 bcde	4 fg	3 de	1 bc
Kruna	untreated	4 cde	8 bcdefg	5 cde	1 bc
	treated	4 cde	7 cdefg	2 de	0 c
Arija	untreated	9 bcde	4 fg	5 cde	0 c
	treated	3 de	4 fg	2 de	0 c
Milijana	untreated	7 bcde	6 defg	6 bcde	2 abc
	treated	6 bcde	2 g	2 de	0 c
Barbara	untreated	11 bc	12 abcde	4 cde	1 bc
	treated	8 bcde	4 fg	6 bcde	0 c
Pobeda	untreated	3 de	3 fg	3 de	1 bc
	treated	2 e	6 dedg	1 e	0 c
Isidora	untreated	10 bcd	8 bcdefg	4 cde	1 bc
	treated	3 de	10 bcdef	3 de	0 c
Srma	untreated	7 bcde	4 fg	2 de	1 bc
	treated	5 bcde	2 g	3 de	0 c
Janja	untreated	24 a	13 abcd	8 bcd	2 abc
	treated	4 cde	15 ab	6 bcde	0 c
Toplica	untreated	9 bcde	5 efg	12 ab	4 ab
	treated	6 bcde	1 g	2 de	0 c

Fusarium LSD 0.05 = 6.2420; *Alternaria* LSD 0.05 = 6.1207; *Penicillium* LSD 0.05 = 5.5290; *Aspergillus* LSD 0.05 = 2.9986

Fusarium head blight and content of DON (S i m p s o n et al., 2001; H a i d u k o w s k i et al., 2005). According to H a u s e r H a h n et al. (2008), combination of triazoles (tebuconazole + protioconazole) effectively controls Fusarium head blight and significantly decreases mycotoxin contamination.

Dominant causer of Fusarium head blight in Serbia is *F. graminearum* (B a g i, 1999), while *F. culmorum* appears only in the years with high humidity. However, besides these two, other *Fusarium* species, as well as fungi from genera *Penicillium* and *Aspergillus* that produce mycotoxins, may be detected on wheat kernels. In the last decade, the most dominant fungus was *Alternaria* spp. (B a g i et al., 2004). All these fungi that are found on wheat kernel after harvest are important toxin producers (A n d e r s e n and T h r a n e, 2006).

During the evaluation of resistance of genotypes to Fusarium head blight, fungicide efficacy and mycotoxin accumulation, attention was turned to DON content in wheat kernel (S i m p s o n et al., 2001; P i r g o z l i e v et al., 2002). Deoxynivalenol is mycotoxin from trichothecene group and it is considered to be the most frequent contaminant of wheat with high intensity of Fusarium head blight. It is produced by *F. graminearum* and *F. culmorum*. From the aspect of healthy and safe food production, toxins produced by other fungi that colonize wheat kernel are also important. For example, ochratoxin A, which was detected in post harvest wheat samples in this research, is formed by species from genera *Penicillium* and *Aspergillus*, among which are *P. verrucosum* and *A. ochraceus*. Additionally, production of aflatoxin is connected to *Aspergillus flavus* (A n d e r s e n and T h r a n e , 2006). A number of authors detected ochratoxin A in wheat samples in their research (B o u d r a et al., 1995; J a n s s o n et al., 1997). The presence of aflatoxin in wheat samples is characteristic for regions with warm and humid climate (M u t h o m i et al., 2008).

During 2009, DON was detected in 21.5% of untreated wheat genotypes, while this percentage for ochratoxin was 42.8%. On the other hand, in cultivars treated with fungicide, mycotoxin content was below detection limit. According to the presented data, appearance of mycotoxins creates health hazard even in the years with average precipitation, while fungicide treatment presents one of the preventive measures against mycotoxin contamination and it leads to an improvement of technological quality of seed.

In this investigation 14 genotypes used in wheat production were involved. Differences within cultivars were observed regarding Fusarium head blight intensity and mycotoxin content. However, in order to determine closely their level of susceptibility, research ought to be conducted with artificial inoculation. Testing wheat cultivars under the conditions of artificial inoculations, together with determination of mycotoxin content, is suggested to be a part of the process of wheat genotype registration in EU (R o d e m a n n and R e n t e l, 2010). Additionally, standardization of the procedure for artificial inoculations is required in order to obtain more reliable results (M e s t r e h a z y et al., 2010).

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УТИЦАЈ ЗАШТИТЕ ПШЕНИЦЕ ФУНГИЦИДИМА НА САДРЖАЈ МИКОТОКСИНА И ПАРАМЕТРЕ ПРИНОСА

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Резиме

Испитиван је утицај заштите фунгицидима из групе триазола код 14 сорти пшенице на принос, масу хиљаду зрна, хектолитарску тежину и садржај микотоксина (афлатоксин, охратоксин А и деоксиниваленол). Такође, одређена је и микопопулација семена како у третираној, тако и у нетретираној – контролној варијанти. Утврђено је да хемијска заштита препаратом на бази комбинације активних материја тебуконазол 125 г/л + протиоконазол 125 г/л утиче на повећање количине и квалитета приноса, док се садржај микотоксина и зараженост семена фитопатогеним гљивама смањује у односу на нетретирану варијанту.

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MYCOFLORA OF COMMERCIAL MAIZE SEED IN 2010

ABSTRACT: Ear and kernel rots can reduce yield, quality and feed value of grain. Toxins produced by the fungi in corn can also have serious implications on the end use of the grain. Various fungi cause ear and kernel rots. Fungi belonging to the genus *Fusarium* are the most significant fungi which can cause corn ear and kernel rots.

The aim of this paper is to test health of mercantile maize seed belonging to different hybrids. Seed health testing was done using filter paper and nutritive media (PDA) method.

Fungi from genera *Fusarium*, *Penicillium*, *Aspergillus* and *Alternaria* were isolated from tested corn seed by both methods. Two species from the genus *Fusarium* were found in the tested corn samples *F. graminearum* and *F. monilirome.* Tested hybrids that belonged to different FAO maturity groups showed differences in susceptibility to ear and kernel rot.

KEY WORDS: corn seed, mycoflora, *Fusarium* spp., meteorological conditions

INTRODUCTION

Corn is susceptible to a number of ear and kernel rots, some of which are widely distributed. These rots can cause considerable damage in humid areas, especially when rainfall is above normal from silking to harvest. The prevalence of rots can be increased by insect and bird damage to ear and stalk. Ear and kernel rots can reduce yield, along with the quality and feed value of grain. Toxins produced by the fungi in corn can also have serious implications on the end use of the grain.

Various fungi can cause ear and kernel rots. Fungi belonging to the genus *Fusarium* are capable of causing ear rots. For *Fusarium* ear rot, the species that cause infection include *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. Rot caused by *Gibberella zea* (asexual state *F. graminearum*) is often called *Gibberella* or red ear rot. (P a y n e, 1999). These fungi can also cause stalk rot. Other fungi that can cause ear and kernel rots include species of

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Penicillium, *Diplodia, Aspergillus*, *Nigrospora, Botryosphaeria, Cladosporium, Rhizoctonia,* and *Rhizopus*.

The aim of this paper is to test the health of mercantile maize seed belonging to different hybrids from different maturity groups.

MATERIAL AND METHOD

Corn seed samples

Hybrid corn seeds of different FAO maturity groups were used PR37N01 (FAO 370), PR36K67 (FAO 530) and PR34N43 (FAO 690). Hybrid corn seeds were treated with insecticides: Gaucho 600 FS (a. m. imidacloprid 600 g/l), Cruiser 350 FS (a. m. thiamethoxam 350g/l) and Force zea 280 FS (a.m. thiamethoxam 200 g/l + tefluthrin 80 g/l). Prior to the insecticide treatment and during seed processing, seeds were treated with fungicide Maxim XL 035 FS (a. m. fludioxonil 25 g/l). Seeds were sown in the field at Sombor locality. Harvest was carried out on 15th October, 2010. From each treatment 25 ears were taken in 4 replicates. After harvest, the seed moisture content was measured by Dickey Jones apparatus. Seed sub samples were made and seed health testing was performed.

Seed health testing

Seed health testing was done using filter paper and nutritive media (PDA) method. One hundred seeds were taken from each sample and the health test was done in four replicates. For seed health test on PDA, 5 seeds in 4 replicates were used. Seeds were sterilized in 1% NaOCl and then incubated for seven days on filter paper and PDA at 25°C. The seed health was determined based on percentage of fungi present in the seeds. *Fusarium* spp. were isolated on carnation leaf medium (CLA) (F i s h e r et al.,1982) and identification of the isolates was performed according to N e l s o n et al. (1983), and Burgess (1994).

RESULTS AND DISCUSSION

Total rainfall amount for Sombor during vegetation period (April-October) was 744 mm. This amount was almost double the amount of multi annual average in Serbia, which is 415 mm. Average temperatures were at the level of multi-annual average for that period. Rainfall amounts recorded in Sombor in May and June were three times higher than multi annual average, while in September the amount of rainfall was doubled (108 mm) in comparison to the multi annual average. In July, the amount of rainfall was at the

level of 76% for this month. During September, air temperature at Sombor locality was 1°C lower than the multiannual average and it was 16°C. October was characterized with colder weather (temperature was 2-3°C lower than multiannual average) (Republic Hydrometeorological Service of Serbia, 2010). Weather during the vegetation period was quite variable and there were significant differences between the amounts of rainfall.

F. moniliforme – spread and development of the disease are favored by dry warm weather. Cool wet weather within three weeks of silking favors development of red ear rot caused by *F. graminearum* (A l m a š i et al., 2002). These authors also confirmed there were clear differences in corn hybrid susceptibility to causal agents of ear rots.

The moisture content in tested seed samples ranged from 18.17-20.43% (Table 1). Such high percent of moisture in seed was caused by extremely wet weather in the harvest period. These moisture values are significantly higher than maximum permitted for corn seed, which is 14% (T h e O f f i c i a l G a z e t t e, 1987).

Tab. 1 – Seed moisture content in tested corn seed samples

Hybrid+insecticide	Seed moisture content
PR 34N43+Cruiser	20.43
PR 34N43+ (Maxim) control	20.25
PR 34N43+Gaucho	20.13
PR 36K67+Cruiser	19.85
PR 34N43+ Force zea	19.8
PR 37N01+Gaucho	19.05
PR 36K67+Force zea	19.03
PR 36K67+Gaucho	18.98
PR 36K67+ (Maxim) control	18.33
PR 37N01+Force zea	18.25
PR 37N01+Cruiser	18.33
PR 37N01+ (Maxim) control	18.17

Fungi from genera *Fusarium*, *Penicillium*, *Aspergillus* and *Alternaria* were isolated from tested corn seed by filter paper method. Fungi from genera *Fusarium* and *Penicillium* were noticed in all tested seed samples. Fungi from genus *Aspergillus* were noticed in 4 samples (3 samples are hybrid PR 34 N43). *Alternaria* was observed in low percent only in two samples. Appereance of species from *Fusarium* genus 4 days after seed incubation was from 0.5-4.75%. Seven days after incubation on filter paper, number of seeds infected with *Fusarium* increased in some samples but it still ranged from 0.75-4.75% (Table 2). Fungi were noticed on mechanically damaged seed, on seed with discoloration and on seed without symptoms. Infected seed had brittle kernel and its cavity was filled with mycelium.

Seeds treated only with fungicide and seeds treated with both fungicide and insecticide showed no significant difference in the number of infected seeds.

The highest number of infected seeds was noticed in the hybrid PR 36 K 67, followed by PR 37 N 01, while the lowest infection was observed in the hybrid PR 34 N 43.

Tab. 2 – Occurrence of fungi in tested corn seed (filter-paper method)

Occurrence of fungi in tested corn seed (%)								
HYBRID Number of days after incubation	*Fusarium* sp.		*Penicilium* sp.		*Aspergillus* sp.		*Alternaria* sp.	
	4th day	7th day	4th day	7th day	4th day	7th day	4th day	7th day
PR 36 K 67 Control	4	5	0.75	1.25	–	–	0.25	0.25
PR 36 K 67 Gaucho	4.75	4.75	2	3.5	–	–	–	–
PR 36 K 67 Cruiser	3	4.5	3.75	7.75	–	–	–	–
PR 36 K 67 Force zea	3	3.5	1.25	2	–	–	–	–
PR 34 N 43 Control	2.75	3.75	3.75	5.5	0.25	0.25	–	–
PR 34 N 43 Gaucho	1.75	4.5	1.5	4	0.75	1.25	–	–
PR 34 N 43 Cruiser	0.5	0.75	2.5	4	–	–	0.25	0.25
PR 34 N 43 Force zea	1	3.5	2.25	4	0.75	0.75	–	–
PR 37 N 01 Control	2	3.25	2.75	2.75	–	–	–	–
PR 37 N 01 Gaucho	2.75	3.25	2	2	0.25	0.25	–	–
PR 37 N 01 Cruiser	2	2.25	0.5	0.5	–	–	–	–
PR 37 N 01 Force zea	1.75	2.25	1.25	1.25	–	–	–	–

Fungi that developed in the tested corn seed on filter paper developed in the nutritive media as well. Seed infection was the highest with *Fusarium* species. The lowest number of infected seeds was noticed in the hybrid PR 34 N 43 (5.6), then in the hybrid PR 36 K 67 (7.2), while the highest number of infected seeds was in hybrid PR 37 N 01 (9.7). Number of infected seeds was mostly uniform within a hybrid, except in the case of hybrid PR 37 N 01, where significantly higher number of infected seeds was observed in the control (Table 3). Such a number of infected seeds indicates that the tested hybrids are, to a certain extent, susceptible to *Fusarium* species.

Two species from the genus *Fusarium* were found in the tested corn samples, *F. graminearum* and *F. moniliforme* (Figure 1). These two species are probably the most widespread causes of ear rot disease in maize. According to numerous authors, the causal agent of maize ear and kernel rot is caused by *Gibberella zeae* (*Fusarium graminearum*). *Fusarium verticilioides* syn. *Fusarium moniliforme*, *F. proliferatum* and *F. subglutinans*.

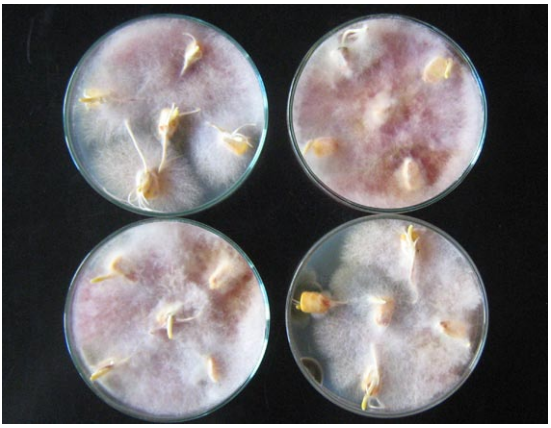

Fig. 1 – Occurrence of *F. graminearum* and *F. moniliforme* in tested corn seed on PDA

Tab. 3 – Occurrence of fungi in tested corn seed (PDA)

Occurrence of fungi in tested corn seed (%)						
HYBRID	*Fusarium* sp. (total)	F. *graminearum*	F. *moniliforme*	*Penicillium* sp.	*Aspergilus* sp.	*Alternaria* sp.
PR 36 K 67 Control	6.25	1.25	5.00	2.50	-	-
PR 36 K 67 Gaucho	6.25	0	6.25	1.25	1.25	1.25
PR 36 K 67 Cruiser	7.50	3.75	3.75	6.25	-	0.75
PR 36 K 67 Force zea	8.75	6.25	2.5	3.75	-	-
PR 34 N 43 Kontrola	2.50	2.5	0	16.25	-	0.75
PR 34 N 43 Gaucho	6.25	1.25	5.00	1.25	-	1
PR 34 N 43 Cruiser	5.00	2.5	2.50	0	1.25	0.25
PR 34 N 43 Force zea	8.75	3.75	5.00	5.00	--	0.5
PR 37 N 01 Kontrola	21.25	12.50	8.75	1.25	-	0.25
PR 37 N 01 Gaucho	2.50	0	2.50	3.75	1.25	0.75
PR 37 N 01 Cruiser	11.25	6.25	5.00	1.25	-	0.25
PR 37 N 01 Force zea	3.75	2.5	1.25	13.75	-	0.25

At harvest time there were a lot of cobs with visible *Fusarium* infection. *Fusarium* cob rot caused by *F. verticillioides*, *F. proliferatum* and *F. subglutinans* is characterized by pale orange mycelium, covering either individual kernels or, in serious cases, the entire ear. If infection is not so severe the streaks or white lines run across the kernels and are most likely to appear on some kernels on ear every year without being noticed. Seed infected with *Fusarium* species can be without visible symptoms (latent infection). Gibberella ear rot (caused by *Gibberella zeae*) usually begins as a reddish mold at the tip of the ear. Infected kernels have pinkish to reddish color.

The most important toxigenic fungi occurring in moderate climatic zones of North America and Europe are *Fusarium* fungi (K o s et al., 2003).

Zearalenone, deoxynivalenol (DON) and fumonisins are more prevalent mycotoxins that occur in grain (S c h a a f s m a et al., 1998). The presence of these mycotoxins can affect various animals so they must be monitored and managed. The first step in that process is monitoring the hybrid susceptibility in the field and also health testing of seed lots. Maize seed monitoring from 2009 harvest (65 samples) in Serbia showed that two samples were contaminated with zerealenone above the established maximum level adopted by European Commission for unprocessed maize (J a j i ć et al., 2010).

Penicillium rot on corn seed is usually evident as discrete tufts or clumps of a blue-green or gray-green mold of individual kernels. The fungi appearance is more common in broken kernels. *Penicillium* appears in the form of small, discrete colonies of mold growth with a dusty or powdery appearance. (C i m m y t, 2011). The attack of fungi from genus *Penicillium* in tested corn seeds ranged from 1.25-16.25%. Only one sample did not show the presence of these fungi. Number of infected seeds varied between hybrids, as well as within the hybrid (Table 3).

Fungi from genus *Aspergillus* was observed in 3 samples in very low percent (Table 3). The reason for such a low percent of infection may be in the fact that *Aspergillus* species favor high temperatures and dry conditions. *Aspergillus* ear rot is typically associated with drought stress.

CONCLUSION

One of the main factors influencing kernel health is the amount of rainfall, especially in the harvesting period. Susceptibility of hybrids to ear and kernel rots is also very important factor in the disease development. However, if ear and kernel rots developed in the field, it is important to harvest the field in a timely manner and to store the grain under the best possible conditions. It is very important to dry the grain up to 15% of moisture as quickly as possible, and to monitor the grain on a regular basis throughout its storage life in order to ensure that the moisture and temperature are maintained at correct levels. If the infection potential is present the optimal storage condition can prevent severe seed contamination.

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МИКРОФЛОРА МЕРКАНТИЛНОГ СЕМЕНА КУКУРУЗА У 2010. ГОДИНИ

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Резиме

Плеснивост клипа и семена кукуруза могу довести до смањења приноса и утицати на квалитет и вредност зрна. Токсини које продукују гљиве могу имати значајан утицај на крајње коришћење зрна. Различите гљиве могу проузроковати трулеж клипа и зрна. Гљиве из рода *Fusarium* су најзначајнији проузроковачи трулежи клипа и семена кукуруза.

Циљ овог рада је био да се испита здравствено стање меркантилног семена различитих хибрида који припадају различитим FAO групама зрења. Здравствено стање семена је утврђено методом филтер папира и на хранљивој подлози.

Са семена кукуруза применом обе методе изоловане су гљиве из родова *Fusarium*, *Penicillium*, *Aspergillus* и *Alternaria*. На семену су идентификоване две врсте *Fusariuma – F. graminearum* и *F. moniliforme*. Испитивани хибриди који припадају различитим FAO групама зрења показали су различит ниво осетљивости према гљивама проузроковачима трулежи семена.

Укупна количина падавина за локалитет Сомбор током вегетације је била 744 mm што је скоро двоструко више у односу на вишегодишњи просек за Србију који износи 415. Овакви метеоролошки услови су утицали на значајнију појаву гљива проузроковача трулежи клипа и зрна кукуруза.

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EFFECTIVENESS OF FUNGICIDE-FREE APPROACHES TO THE PROTECTION OF POTATO AND TOMATO AGAINST LATE BLIGHT

ABSTRACT: The article describes fungicide-free ecologically tolerant technologies for the protection of potato and tomato against *Phytophthora infestans* (Mont.) de Bary, agent of late blight of these crops. It has been proved that potato cultivars with field resistance (Lugovskoy) provide a protection level comparable to the effectiveness of modern fungicides. Plant extracts from Southern plants can be used as biopesticides in order to additionally suppress the development of late blight. For example, extracts of four species from Yemen (fam. Asteraceae: *Psiadia arabica* Jaup and Spach; fam. Lamiaceae: *Lawsonia inermis* L ; *Dodonea viscos* L ; *Euryops arabicus* Steud. ex Jaub and Spach) were capable of suppressing late blight development on potato.

On the contrary, plant growth regulators, Novosil (active compound triterpene acids), Larixin (active compound dihydroquerticin), and Terpenol (active compound triterpene acids), in spite of the periods of high efficiency against late blight, do not provide satisfying effect since these substances could sometimes even promote a pathogen development. Also, vaccination of tomato with attenuated strain of tobacco mosaic virus (*TMV*) could improve viability and immune status of plants under normal conditions, and in the case of stress situation it promoted weakness of plants and *P. infestans* infection.

In Eastern Europe, fungicide-free technologies for the protection of potato and tomato against late blight, corresponding to the ecological criterion, should be additionally elaborated and tested for potato and tomato cropping.

KEY WORDS: late blight, *Phytophthora infestans*, potato protection, fungicide-free technologies, plant growth regulators, attenuated viruses

INTRODUCTION

Currently, application of fungicides is a true method for the protection of harvest in all areas producing potato and tomato. However, at least two problems already strongly disturb large-scale development of this. First of all, we

are facing an increase in the resistance of populations of late blight agent oomycete *Phytophthora infestans* (Mont.) de Bary to both systemic and even protective fungicides (S m i r n o v, 2010). Other problem is connected to agroecology. Fungicide residuals in agricultural production can be dangerous for human generation though we have no true information on the relation between fungicide application and frequency of the most important human diseases.

Of course, anti-resistant strategies and development of decision support systems (DSS) are very helpful for fungicide application (S c h e p p e r s , 2005). However, they do not fully solve aforementioned problems. Additional measures need to be elaborated for potato and tomato ecologically tolerant cropping.

Objective of this study is to test some fungicide-free approaches and technologies for the protection of potato and tomato against late blight. The role of field resistance of potato cultivars, plant growth regulator, plant extracts, and viral immunization was elucidated.

MATERIALS AND METHODS

Determination of field resistance of potato cutivars. The investigation was conducted in Moscow Region (Russia).

Disease frequency (DF) and index of disease severity (DS) was calculated as follows (P o p k o v a, B i c h e n k o v a, 1969, with additions):

$DF = n \cdot 100\% / N;$

$DS = \sum(a_i \cdot b_i)100\% / 5N$, where:

n – number of affected plants, a_ib_i – sum of magnifications of number of affected plants (a_i) at corresponding range (b_i): [the smallest range – **0** (absence of affection), **1** – 0.1-10% of plant was affected, **2** – 11-25% of plant was affected, **3** – of plant was affected, **4** – of plant was affected, **5** (the biggest range) – 90-100% of plant was affected], N – total number of affected and healthy plants.

Index of presence of zoosporangia (IZ) was calculated as described in Smirnov, Kuznetsov (2009).

Index of aggressiveness (IA) of field *P. infestans* population at certain cultivar was calculated as follows:

$IA = (DF \cdot DS \cdot IZ) / 10\,000.$

For all plants in a plot of a certain cultivar index of field resistance of cultivar (IFRC) was calculated as follows:

$IFRC = (1/IA) \cdot 100$

IFRC<10.0 – cultivar in general susceptible; 10.1-25.0 – cultivar weakly resistant; 25.1-50.0 – cultivar moderately resistant; 50.1-100.0 – cultivar resistant; > 100.0 – cultivar hyperresistant. According to the Latvian method of V. A. G a u e r (1965) biological effectiveness of application of cultivar with field resistance (BECFR) was calculated as follows:

BECFR = $((IA_s-IA_{fr})/ IA_s)\cdot 100$, where:

IA_s and IA_{fr} – indexes of aggressiveness for susceptible and resistant cultivars, respectively.

Biological effectiveness of fungicide application (BEFA) was calculated as follows:

BEFA = $((IA_c-IA_f)/ IA_s)\cdot 100$, where:

IA_c and IA_f – indexes of aggressiveness for control variant (without treatment) and variant including treatment with fungicide, respectively.

Values BECFR and BEFA can also be calculated in the same way on the basis of features $DS_s – DS_{fr}$, $IZ_s – IZ_{fr}$; $IZ_c – IZ_f$, $IZ_c – IZ_f$.

Determination of effectiveness of plant growth regulators and plant extracts. Three plant growth regulators (Table 1) and twenty plant extracts from Yemen (Table 2) (Al-Saadi et al., 2009) were used in this study.

Tab. 1 – Characteristics of plant growth regulators

Name	Active compound	Preparative form	Concentration	Danger for humans
Novosil	triterpene acids	water emulsion	100 ml/hectare	not dangerous
Laryxin	dihydroquerticine	water emulsion	100 ml/hectare	not dangerous
Terpenol	triterpene acids	water emulsion	200 ml/hectare	not dangerous

Tab. 2 – Botanical characteristics of plant species from Yemen (Al-Saadi et al., 2009).

Plant species	Family	Morphological characteristics	
		Living form	Maximal altitude, m
Argemone mexicana L.	Papaveraceae	Annual grass	1.0
Withania somnifera (L.) Dun	Solanaceae	Semishrub	1.0
Calotropis procera Ait .	Asclepiadaceae	Shrub	2.5
Lawsonia inermis L.	Lytheraceae	Shrub	2.0
Ocimum basilicum L.	Lamiaceae	Annual grass	0.8
Meriandra benghalensis (Hamit.) Benth.	Lamiaceae	Shrub	2.0
Rumex nervosus Vahl.	Polygonaceae	Shrub	2.0
Aloe tomentosa Deflers.	Aloeaceae	Perennial grass	0.4 (till flowering) 1.0 (flowering plant)
Gnidia somalensis Gilg.	Thymeliaceae	Annual grass	0.5
Kelinia odora A.Berger	Asteraceae	Dwarf semishrub	1.0
Psiadia arabica Jaup & Spach	Asteraceae	Dwarf shrub	1.0
Euryops arabicus Steud. ex Jaub & Spach	Asteraceae	Shrub	0.5
Dodonea viscose L.	Sapiandaceae	Shrub	4.0
Alkanna orientalis L.	Boraginaceae	Annual grass	0.5
Chenopodium ambrosoides L.	Chenopodiaceae	Annual grass	0.9
Azadirachta indica A. Juss	Meliaceae	Tree	8.0

In order to estimate the efficiency of plant growth regulators and plant extracts against *P. infestans,* 6 components of aggressiveness were used for potato tuber discs, already described in previous investigations (T o o l e y et al., 1986; K a d i s h and C o h e n, 1988; S m i r n ov, 2010): Infection Frequency (IF), Lesion Area (LA), Sporulation Capacity (SC), Incubation Period (IP), Latent Period (LP), and Composite Aggressiveness Index (CAI). CAI was calculated as follows:

$$CAI = (IF \cdot LA \cdot SC) / (IP \cdot LP)$$

CAI was determined for all variants (treatment with tested substance or without it).

For plant growth regulators, all tests indicated suppression of *P. infestans* (strong or weak), absence of effect, and stimulation of *P. infestans* (strong or weak) were calculated and summarized in the table. For this, growth of mycelium (vegetative stage), formation of zoosporangia and zoospores, CAI (asexual stage), and formation of oospores (sexual stage) were estimated.

Determination of effectiveness of viral immunization of tomato. Two field trials and one laboratory test were carried out.

In the field trial of 2008 suppressed tomatoes preliminary vaccinated (inoculated) with attenuated strain of tobacco mosaic virus (*TMV*), as well as those that were not vaccinated, were planted in the plot. The weather conditions were wet and favorable for the late blight development.

In the field trial of 2010 normal tomatoes preliminary vaccinated (inoculated) with attenuated strain of tobacco mosaic virus (*TMV*) and not vaccinated tomatoes were planted in the plot. The weather conditions were abnormally hot and dry, which caused suppression of late blight development.

For late blight development estimation in all variants, indexes IZ, IO (S m i r n o v and K u z n e t s o v, 2009), DF, DS, and IA (current investigation) were calculated.

In the laboratory test, for leaflets from preliminary vaccinated (inoculated) tomatoes with attenuated strain of tobacco mosaic virus (TMV), and for leaflets from non vaccinated plants, aforementioned features IF, LA, SC, IP, LP, and CAI were determined.

Statistical analysis. Comparison of variants was conducted according to the LSD_{05} value that was determined by means of software STRAZ, developed at the Russian Agrarian University.

RESULTS

Effectiveness of field resistance of potato cultivar in comparison with fungicides. Majority of tested cultivars did not manifest any level of field resistance to late blight (Table 3). Almost all Dutch and many Russian cultivars were susceptible. It was interesting that cultivar Sante moderately resistant in Western Europe was highly susceptible to late blight under conditions of Moscow Region. A few cultivars demonstrated different level of field resistance,

Tab. 3 – Resistance of different potato cultivars to late blight

Cultivar	Country of origin	Year of testing	IFRC	Range of field resistance
Granola	Netherlands	2000	93.5	Resistant
Sante	Netherlands	2003	6.6	Susceptible
		2004	4.9	Susceptible
Lugovskoy	Ukraine	2003	25.6	Moderately resistant
		2004	4.2	Susceptible
Nevsky	Russia	2004	23.6	Weakly resistant
		2004	3.7	Susceptible
Latona	Netherlands	2004	2.5	Susceptible
Scarlett	Netherlands	2004	7.9	Susceptible
Vetraz	Belarus	2004	102.0	Resistant
Nikulinsky	Netherlands	2004	3.8	Susceptible
		2006	62.5	Resistant
Lina	Russia	2006	2.5	Susceptible
Udacha	Russia	2006	32.7	Moderately resistant
		2006	2.8	Susceptible
		2006	12.3	Weakly resistant
		2008	2.5	Susceptible
Ledy Rosetta	Netherlands	2006	5.5	Susceptible
Ausonia	Netherlands	2006	3.0	Susceptible
Vodoley	Russia	2006	40.0	Moderately resistant
Geran	Russia	2006	35.7	Moderately resistant
Resurs	Russia	2006	4.1	Susceptible
Utenok	Russia	2006	2.0	Susceptible
Russian Souvenir	Russia	2006	4.6	Susceptible
Charodey	Russia	2006	3.7	Susceptible
Ilynsky	Russia	2008	1.4	Susceptible

such as Russian cultivars Udacha and Lugovskoy. Some cultivars (Vetraz from Belarus) were resistant (Table 3).

Fungicides Shirlan and Rydomil gold MZ were effective against late blight, with effectiveness of about 80-100% on susceptible cultivar Sante (Table 4). However, the effectiveness of field resistance of cultivar itself was about 70%. It was less than in the case of Shirlan and Rydomil gold MZ variants, but much more than in the case of variants with other fungicides, Copper oxichloride and Amistar. Also, it should be emphasized that the effectiveness of fungicide application on the resistance of cultivar Lugovskoy was low in comparison with field resistance and had no principal role for its protection against late blight (Table 4).

Effectiveness of plant growth regulators. In the majority of tests no true effect of plant growth regulators Laryxin, Novosil, and Terpenol was revealed. Also, in many cases suppression of *P. infestans* development (especially formation and activity of zoospores) was observed (Table 5). On the other hand,

Tab. 4 – Biological effectiveness of field resistance of cultivar and fungicide application against late blight on the basis of features DS, IZ, and IA on the plots of Laboratory of Crop Protection of Russian Agrarian University-Moscow Timiryazev Agricultural Academy in 2003.

	Cultivar Sante (Susceptible)			Cultivar Lugovskoy (with field resistance)		
	DS	IZ	IA	DS	IZ	IA
	–	–	–	40.9	51.8	74.1
Shirlan	86.4	92.9	99.4	28.3	11,3	21,3
Copper oxichloride	31.8	-25.0	45.8	-17.8	-103.6	-44.1
Rydomil gold MZ	86.4	-42.9	94.7	36.0	-122.1	18.0
Amistar	22.7	-16.1	-14.1	5.3	0.2	7.7

Tab. 5 – Number of tests indicated different kinds of influence of plant growth regulators on the formation of different stages of *P. infestans* living cycle.

Stages of *P. infestans* living cycle	Tests indicated					
	Total number	Strong suppression	Weak suppression	Neutral effect	Weak stimulation	Strong stimulation
Vegetative growth of mycelium	53	0 0%	23 43.4%	14 26.4%	16 30.2%	0 0%
Formation of zoospo-rangia	27	5 18.5%	4 14.8%	2 7.4%	5 18.5%	11 40.7%
Formation of zoospores	36	26 72.2%	4 11.1%	6 16.7%	0 0%	0 0%
Mobility of zoospores	22	20 90.9%	0 0%	2 9.1%	0 0%	0 0%
Aggressive-ness (CAI)	143	37 25.9%	22 15.4%	79 55.2%	1 0.7%	4 2.8%
Formation of oospores	185	65 35.1%	4 2.2%	105 56.8%	0 0%	11 5.9%
In total	466	153 32.8%	57 12.2%	208 44.6%	22 4.7%	26 5.6%

some cases revealed that after treatments with plant growth regulators vegetative growth and formation of zoosporangia became more intensive.

Effectiveness of plant extracts. Extracts of only four species from Yemen (fam. Asteraceae: *Psiadia arabica*; fam. Lamiaceae: *Lawsonia inermis*, *Dodonea viscos*, *Euryops arabicus*) were capable of suppressing effectively late blight development on potato tuber slices but in a large initial concentration (Figure 1). Extracts from other species were fully, or almost ineffective at suppression of late blight development on potato tuber slices.

Effectiveness of viral vaccination. In 2008, both vaccinated and control tomato plants were strongly affected with late blight. Vaccinated plants were much weaker and *P. infestans* developed on them even more than in untreated control plants (Table 6).

Fig. 1 – Variants with inoculated *P. infestans* isolate of potato tuber discs preliminarily treated with extracts of different plant species from Yemen.
Left column – control (without treatment).
Rows 1-4 which are to the right of the control: treatment with *P. arabica* extract (the top raw), *D. viscose* (the second row from the top), *L. inermis* (the third row from the top), *E. arabicus* (the bottom row). Treatment with extract of every aforementioned species at concentration 1:1 (the right column after control), 1:2, 1:4 (intermediate columns), 1:8 (the right column).

Tab. 6 – Late blight development features on tomato plants vaccinated with Tomato Mosaic Virus (*TMV*) and control plants (not vaccinated).

Variant	Features of late blight			
	Disease Frequency (DF)	Disease Severity (DS)	Index of formation of zoosporangia (IZ)	Index of aggressiveness (CIA)
Control	100	73.3	50.0	40.0
Control	100	54.3	43.6	15.3
With *TMV*	100	80.0	100	73.3
With *TMV*	100	35.0	100	54.3

In 2010, due to abnormally dry and hot weather, late blight was suppressed in all variants. Vaccinated plants developed normally and looked even better than the tomato plants in control. Development of early blight was slightly suppressed on vaccinated plants but it was a very short-termed effect.

In laboratory tests preliminary vaccination with *TMV* virus did not prevent late blight development on tomato leaflets after artificial inoculation with *P. infestans* isolates (Table 7). In comparison with the unvaccinated leaflets, vaccination led to increased Incubation Period, as well as increased Composite Aggressiveness Index after the inoculation.

Tab. 7 – Components of aggressiveness for tomato leaflets (cultivar Bely Naliv) preliminary vaccinated with tobacco mosaic virus (*TMV*) and not vaccinated control leaflets inoculated with *P. infestans* isolates TL and MP.

Variants	Incubation period (IP), days	Latent period (LP), days	Infection frequency (IF), scores	Lesion area (LA), scores	Sporulati-on capacity (SC), scores	Composite aggressive-ness index (CAI), scores
TL (control)	3.03	4.83	7.84	2.20	1.93	1.20
MP (control)	3.00	4.63	9.08	2.23	1.93	1.13
TMV + TL	3.30	4.83	8.41	2.33	1.67	1.54
TMV+MP	3.80	4.13	10.00	2.76	2.23	1.93

LSD_{05} for all factors – 0.48

DISCUSSION

Our trials and experiments have estimated a possible role of fungicide-free ecologically tolerant technologies for the protection of potato and tomato against late blight. However, they are not that effective as fungicides. In order to provide at least diversification of protective measures in large-scaled potato cropping by means of ecologically tolerant technologies, it is necessary to use biopesticides of plant origin based on plant extracts. They are also less effective than chemical fungicides. Furthermore, an additional link, which could along with field resistance provide a sufficient protective effect in order to get a harvest, should be made.

On the other hand, not all tested technologies were effective for the suppression of *P. infestans*. Application of plant growth regulators is appealing but it does not always guarantee desirable effect. In spite of proved cases of *P. infestans* suppression, pathogen development stimulation has rarely been detected. Neutral effects can also be shifted. It is related to the fact that terpene and flavonoid substances participate in different ways in chains of a secondary metabolism. For example, there are some metabolism activities which can promote synthesis of sterines needed for *P. infestans*, while others can block its synthesis. This is the main reason why plant growth regulators do not provide certain effect for potato protection against late blight (D e r e v y a g i n a et al., 2007).

Vaccination of tomato with tobacco mosaic virus did not provide protection sufficient enough for tomato late blight. Virus definitely promoted a cascade of protective measures, such as synthesis of PR proteins, proteinase in-

hibitors, and phytoalexines (D y a k o v et al., 2001). In general, viability level of tomato plant increased. However, it took a lot of energetic potential. In the case of absence of stress factors the situation is normal and the virus can be in a latent form. But in the case of a pathogen attack or plant weakness related to stress the tomato plant is not be able to react in a proper way and virus becomes rather harmful for the plant. This explains all our obtained results (especially high values of CAI and IA for vaccinated tomato leaflets) on viral preliminary vaccination directed at late blight suppression. Therefore, it is probably better to use elicitors obtained from virions instead of non attenuated viral strains.

Thus, it is necessary to carry out the elaboration of fungicide-free approaches to the protection of potato and tomato against late blight, and combination of cultivars with field resistance and biopesticides should be a promising decision for the countries of Eastern Europe. This technology, which is in accordance with ecological criterion, should be additionally elaborated and tested for potato and tomato cropping.

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ПРОДУКТИВНОСТ ЗАШТИТЕ КРОМПИРА И ПАРАДАЈЗА ОД ПЛАМЕЊАЧЕ БЕЗ УПОТРЕБЕ ФУНГИЦИДА

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Резиме

Описане су еколошки прихватљиве технологије, без примене фунгицида, за заштиту кромпира и парадајза од *Phytophthora infestans* (Mont.) de Bary, агенса који проузрокује пламењачу ових култура. Потврђено је да сорте кромпира поседују ниво отпорности (Lugovskoy) који се може упоредити са модерним фунгицидима. Екстракти јужних биљака се могу употребити као биопестициди како би се додатно сузбио развој пламењаче. На пример, екстракти четири врсте биљака из Јемена (породица Asteraceae: *Psiadia arabica* Jaup и Spach; породица Lamiaceae: *Lawsonia inermis* L; *Dodonea viscos* L; *Euryops arabicus* Steud. Jaub и Spach) су успеле да сузбију развој пламењаче на кромпиру.

Насупрот томе, регулатори раста биљака, Новосил (активно једињење тритерпенске киселине), Larixin (активно једињење дихидрокверцетин) и Терпенол (активно једињење тритерпенске киселине), упркос периодима високе ефикасности у спречавању пламењаче, не дају задовољавајући резултат из разлога што ове супстанце понекад изазивају развој патогена. Вакцинацијом парадајза дуванским мозаичним вирусом могло би се побољшати имунолошко стање биљака у нормалним условима, док у стресним условима биљке слабе и могу да буду заражене са *P. infestans*.

У источној Европи, технологије без примене фунгицида, које се примењују за заштиту парадајза и кромпира од пламењаче, а у складу са еколошким критеријумима, требало би додатно проучити и тестирати на овим културама.

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ELF ELECTRO-MAGNETIC FIELDS AS STRESS FACTORS IN SOME YEASTS AND MOLDS

ABSTRACT: The possibility of species targeted growth inhibition of three yeast (*Candida albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*) and one mold species (*Aspergillus fumigatus*) by electromagnetic fields of certain characteristics was investigated. Cultures were exposed to sinusoidal 50 Hz fields, and 10, 40 and 70 mT magnetic components and 20 V/m electric component, for 30 minutes. Cell density in yeast cultures and germination time and rate in mold cultures were investigated.

KEY WORDS: yeasts, molds, electromagnetic stress, inhibition

INTRODUCTION

The impact potential of extremely low frequency (ELF, 50 or 60 Hz) electromagnetic fields (EMFs) has been evaluated worldwide in various biological systems, but not a single organism insensitive to those fields has been found yet. There are many results that point to specific and effective action of certain electromagnetic fields (EMF) of extremely low frequency on the functions of individual organisms, organs and cells. During many years of research on the influence of ELF of electromagnetic fields on different organisms, we found the frequency "window" dependence, within which the EMFs affect microorganisms, in the range from stimulation to extreme inhibition (Galonja – Coghill et al., 2009).

For the inhibition of certain yeasts and allergenic, pathogenic and toxigenic molds, as well as those causing food spoilage, chemotherapeuticals (antibiotics), radiation, temperature control, water removal etc. have been used. Some of these methods can result in creating resistant forms, and are often not cost effective. We examined the possibility of species targeted growth inhibition of three yeasts (*Candida albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*) and one mold species (*Aspergillus fumigatus*) by

EMFs of certain characteristics. All four species are widespread. Although *Saccharomyces* is commonly used in bread baking, alcohol fermentation and beer brewing, scientists noticed its frequent appearance in immunocompromised patients, thus raising questions about its survival and virulence in humans. After the SSD1 gene was disabled, the virulence of some *Saccharomyces* strains largely rose (W h e e l e r et al., 2003). *Cryptococcus neoformans* is clinically important pathogenic yeast, belonging to *Hymenomycetes*, commonly occupying woody substrates. It can cause life-threatening infections, such as cryptococcosis, meningoencephalitis and meningitis, particularly in immunocompromised patients (rate in AIDS patients goes up to 30%), entering the body by inhaling. The species has asexual (anamorph) and sexual (teleomorph) state (B o e k h o u t et al., 2001). *Candida albicans* is another common yeast capable of causing systemic infection in mammals with low neutrophil cell count, with overgrowing populations in various organs, having possible lethal effect. This overgrowing process is supported by germination and filamentation that enable *Candida* to bind to the respiratory tract (K u r n a t o w s k i et al., 2003). *Aspergillus fumigatus* is an important airborne pathogen causing life-threatening infections in immunocompromised patients, by inhaling its conidia (S e g a l and R o m a n i, 2009). Their phagocytes are incapable of removing the spores while neutrophils cannot destroy the hyphae development (M c C or m i c k et al., 2010). When growing on some building materials *A. fumigatus* can produce mycotoxins (e.g. gliotoxin). It plays an important role in carbon and nitrogen cycles in nature.

MATERIALS AND METHODS

Fungal cultures were all obtained from Albright laboratory, Abergavenny, UK and cultivated in YMB (Yeast Mold Broth, Difco, BD – Diagnostic Systems, USA), except for *A. fumigatus* whose asexual spores (conidia) were placed into YMB for the purpose of germination analyses.

Yeasts

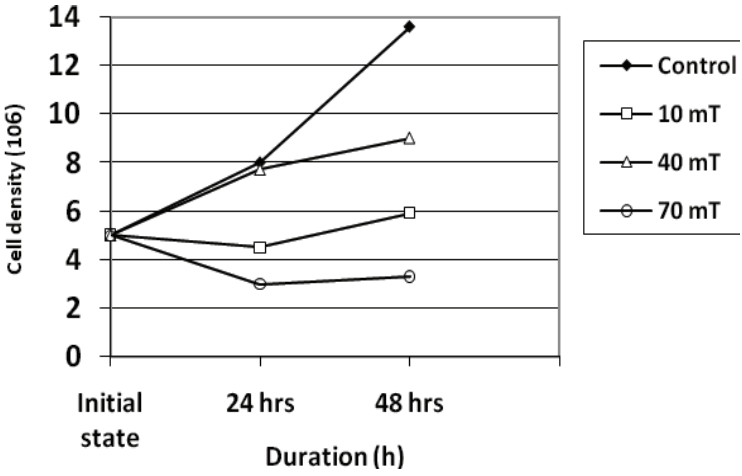
Candida albicans, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were cultivated separately in YMB for seven days. Each species culture cell density was adjusted to $5x10^6$/ml liquid medium. This was done by comparing hemocytometer counts and numbers derived from LeeHatrwell table after spectrophotometrical determination of cultures' optical density (OD_{660}) (B u r k e et al., 2000). Pure growth medium was used to zero the absorbance. All three species cultures were split into eight erlenmeyer flasks (25 ml each), two of which were kept at ambiental EMFs (control samples), and others were exposed in pairs for 30 minutes to sinusoidal 50 Hz EMFs, 20 V/m electric component and three variants of magnetic component: 10, 40 and 70 mT. Cell density in the samples was determined spectrophotometrically 24 and 48 hours after the exposure.

Molds

Although it is assumed that *A. fumigatus* exercises virulent behaviour at temperatures around 37є C only, we preferred the experimental approach based on measuring the conidial germination time and rate, under the influence of electric and magnetic fields, rather than measuring the apical growth rate of hyphae as a function of colony diameter. The conidia were placed in Yeast Mold Broth and stirred. Quantity of 200 ml of YMB at spore concentration of about $1x10^4$/ml was split into eight erlenmeyer flasks (25 ml each). Two of them were kept at ambiental EMFs (control samples), and others were exposed in pairs for 30 minutes to sinusoidal 50 Hz EMFs, magnetic component being 10, 40 and 70 mT and electric component being 20 V/m. During the experiment, samples were kept at 30º C. After the initial isotropic growth phase, spores enter the polar growth phase when germ tubes are created (R h o d e s, 2006). Since it was uncertain whether the germination might occur early under the influence of applied EMFs, we monitored germination microscopically every hour for 10 hours.

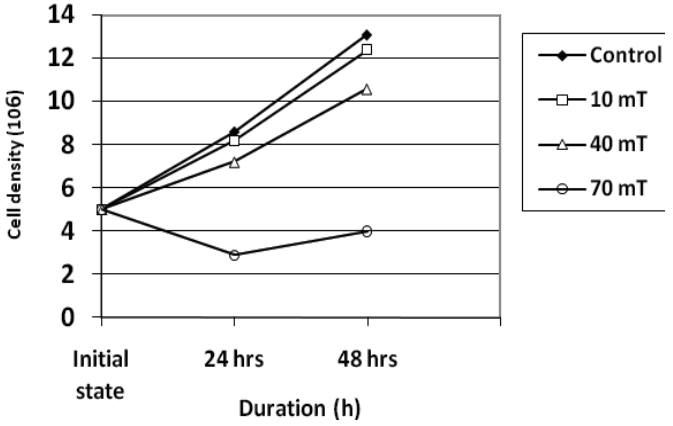
RESULTS

Candida albicans populations decreased after the exposure to all EMFs applied. Strongest inhibitory effect showed 70 mT EMF, reducing cell numbers well below the initial state, even 48 hours after the exposure (Chart 1). The least inhibitory was 10 mT field, showing no significant effect 24 hours after the exposure, yet 48 hours after the exposure differences between the controls ($13.6x10^6$ cells per ml) and exposed samples ($5.9x10^6$ cells per ml) were significant.



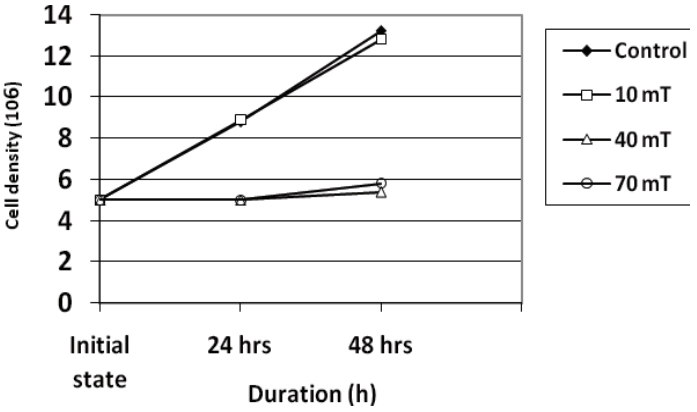
Graph. 1 – Changes in *C. albicans* cell density 24 and 48 hours after 30 minute exposure to 10, 40 and 70 mT, 20 V/m, 50 Hz electromagnetic fields.

Cryptococcus neoformans only slightly reacted to 10 and 40 mT EMFs, while 70 mT fields caused significant decrease in cell density. It appears that *C. neoformans* cells experienced those as a stress, particularly during the first 24 hours, after which cell density started to increase slowly (Chart 2). However, there is a huge difference between cell densities in the control samples ($13.1x10^6$) and samples exposed to 70 mT ($4 x10^6$).



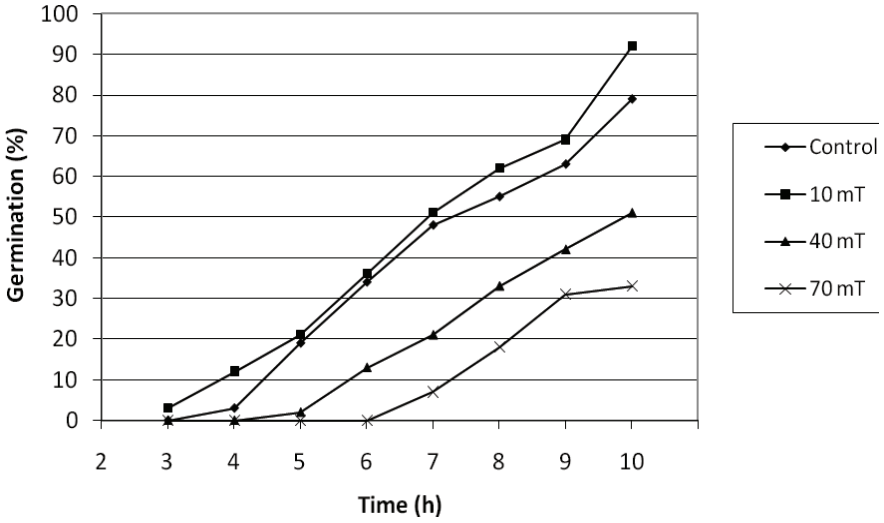
Graph. 2 – Changes in *C. neoformans* cell density 24 and 48 hours after 30 minute exposure to 10, 40 and 70 mT, 20 V/m, 50 Hz electromagnetic fields.

Cell densities of *Saccharomyces cerevisiae* populations were kept at the exact initial state value for the first 24 hours after the exposure to 40 and 70 mT fields, and at almost the same value during the following 24 hours (5.4 and 5.8 million cells per ml, respectively), while 10 mT fields did not cause any changes during the first 48 hours after the exposure, with respect to the control samples (Chart 3).



Graph. 3 – Changes in *S. cerevisiae* cell density 24 and 48 hours after 30 minute exposure to 10, 40 and 70 mT, 20 V/m, 50 Hz electromagnetic fields.

First signs of *A. fumigatus* spores germination in the control samples were observed four hours after being placed into YMB. Germination rate was intensifying until the seventh hour, when about 48% of conidia germinated. During the eighth and ninth hour, germination rate slightly decreased, and ten hours after being placed into YMB 81% of germinated conidia was recorded. Comparative review of *A. fumigatus* germination under various EMFs applied is given in Chart 4.



Graph. 4 – *Aspergillus fumigatus* conidial germination time and rate after 30 minute exposure to 10, 40 and 70 mT, 20 V/m, 50 Hz electromagnetic fields.

The results show that even conidia are susceptible to EMFs, resulting in changes in both time and rate of germination. Samples exposed to 10 mT EMFs germinated one hour earlier than the controls. By comparison with control samples, it appears that 40 mT EMFs caused one hour germination delay, while under the influence of 70 mT EMFs this delay was for four hours. The applied fields seemed to decrease the germination rate, which was at about the same percentage four hours after the germination started (33% at 70 mT, 33% at 40 mT, 36% at 10 mT in comparison to 48% in the control samples). However, samples exposed to 10 mT showed this increase rate later, ten hours after placing the conidia into YMB, being 13% higher in comparison to the control samples. The obtained results show great potential of electromagnetic fields to cause targeted and highly selective effects on organisms without using any chemical compounds.

CONCLUSIONS

There are obviously certain patterns according to which various external stimuli, among which are electric and magnetic fields, affect and modulate fungal physiology, development and growth. This experiment shows how easily those processes can be managed without using chemicals and at levels incapable of causing DNA damage. It seems that there is some dose-response behaviour, referring to 70 mT fields showing greatest inhibitory effect in all four species cultures tested. There is no doubt that this is done by interfering with intracellular signalling. However, subtle language of each cell and all its components is yet to be decrypted.

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ELF ЕЛЕКТРО-МАГНЕТСКА ПОЉА КАО СТРЕС ФАКТОРИ НЕКИХ КВАСАЦА И ПЛЕСНИ

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Резиме

Испитивана је могућност species-специфичне и циљане инхибиције раста три врсте квасаца (*Candida albicans*, *Cryptococcus neoformans* и *Saccharomyces cerevisiae*) и једне врсте плесни (*Aspergillus fumigatus*) путем електромагнетских поља одређених карактеристика. Културе су изложене синусоидалним пољима фреквенције 50 Hz, магнетске компоненте поља 10, 40 и 70 mT и електричне компоненте 20 V/m, током 30 минута. Испитивана је густина ћелија у културама квасаца, као и време и интензитет герминације у култури плесни.

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MYCOTOXIGENIC MOLDS IN SPICES FROM MACEDONIAN STORES

ABSTRACT: Twenty-six samples of spices most frequently occurring in the stores of the Republic of Macedonia were examined for their fungal contamination and the incidence of *Aspergillus* and *Penicillium* species and their teleomorphs. It included mainly commercial packages most frequently occurring in the stores of the Republic of Macedonia. According to the relative frequency of each of the isolated species, the typical mycoflora of these samples includes *A. niger, A. flavus, A. fumigatus, P. chrysogenum* and *Eurotium* sp.

Fungal counts varied from $\log_{10_1} < 2$ CFU g^{-1} (DG18 at 25°C; DRBC at 25°C), for a sample of paprika, to $\log_{10}$ 6.17 CFU g^{-1} (DG18 at 25°C), for a sample of bay leaf.

During the experiment, *A. flavus* was detected in 17 samples, out of which 7 isolates were capable of producing Af-B1, and 4 isolates produced Af-B2. All isolates of *A. nomius* and *A. parasiticus*, in the experimental conditions, produced Af-B_1, Af-B_2, Af-G_1 and Af-G_2.

KEY WORDS: spices, *Aspergillus, Penicillium, Eurotium,* aflatoxins

INTRODUCTION

Molds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes, carried and disseminated by wind and air currents, or spread by insects, rodents, and other animals (C a r m o, 2008). Mycotoxins are considered to be unavoidable additional poisonous contaminants in susceptible food and feed crops. It is not possible to predict their presence or to prevent their occurrence entirely during pre-harvest, storage, and processing operations by current agronomic practices. Levels of mycotoxins and mycotoxicogenic molds, which can cause risk to population, are refused by consumers and many countries have set regulations for various agricultural foods. The consumption of moldy products can cause human or animal mycotoxicoses, and more importantly, some mycotoxins are potent carcinogens (K a w a s h i m a and S o a r e s, 2006; R a s o o l i and A b y a n e h, 2004; W a n g i k a r et al., 2005).

Recent reports indicate that mycotoxins continue to pose health concern via human exposure to contaminated spices. Furthermore, average mold counts in the examined peppers range from 200 to 3.400.00 mold spores g^{-1}, where 49% of *Aspergillus flavus* isolates are found to be toxigenic, out of which 79% produce Af-B_1 (L l e w e l l y n et al. 1992). Levels of Af-B_1 in *Coriandrum sativum* exceeded 75ppb and sampling of commercial spices and herbs in Thailand revealed Af-B_1 contamination within the range of 40-160 ppb (L l e w e l l y n et al., 1992). Investigations of ethnic foods in UK revealed that out of 121 samples the highest mycotoxin levels were found in chilli powder, curry powder and ginger, and that the most common contaminants were trichotecenes and ochratoxin A (P a t e l et al., 1996). In a similar investigation in UK, out of 157 samples of imported herbs and spices, it was discovered that nearly 95% contained aflatoxin levels below 10 ppb and only nine samples contained higher levels (MAFF, 1994). Similarly, during his study on herbal drugs of Indian pharmaceutical industries, C h o u r a s i a (1995) found that the crude drug samples and their finished products contained Af-B_1 beyond the tolerance level (20 μg kg^{-1}) set by the World Health Organization, and that this incidence of mycotoxins in finished herbal drugs was influenced by the use of mycotoxin contaminated crude drug samples.

On the other hand, H i t o k o t o et al. (1980) reported that cloves, star anise seeds, thyme and some other spices inhibit fungal growth and toxin production, possibly due to the major component in the essential oils. Similar results were obtained by A b d e l h a m i d et al. (1985) in an investigation of the preserving power of some herbs and spices.

Although many analytical methods have been developed to detect mycotoxins in agricultural products, it is not possible to assay all commodities for all mycotoxins. Existing methods for aflatoxin detection and quantification in spices are not very sensitive and are somewhat complex (B a n e r j e e et al., 1993). In order to reduce this problem to a manageable level, it is necessary to determine the fungi characteristically present in agricultural products from specific localities, and mycotoxins that can be expected (A b r a m s o n and C l e a r, 1996).

The aim of this study was to screen the isolates of *A. flavus, A. nomius* and *A. parasiticus* for the production of aflatoxins, to enumerate the fungi in spices most frequently occurring in Macedonian stores, and to identify species of *Penicillium* and *Aspergillus* and their telemorphs.

MATERIALS AND METHODS

Samples

Twenty-six samples of spices most frequently occurring in the stores of the Republic of Macedonia were examined. It included consumer packs of 17 samples of paprika, pepper, caraway, cinnamon, oregano, bay leaf and samples of other products containing particular spice or spice mixture: “Bukovec”

and "food additive". One sample of "Bukovec" and one sample of sweet and hot paprika, purchased at the open-air market in Skopje, were also included in the analysis. The so called "Bukovec" consists mainly of hot paprika mixed with salt, sodium glutaminate, dried vegetable, carbohydrates, ribonucleotides and spice mixture (not specified). Except for one sample of spice mixture, all samples were marketed by domestic producers; only few of the producers indicated the country of origin on the package.

Isolation and identification of the contaminant mycoflora

The isolation of mold flora of the examined spices was in accordance with the recommendations of the Second International Workshop on Standardization of Methods for Mycological Examination of foods (S a m s o n et al., 1990).

DRBC agar (pH 5.6 = 0.2; K i n g et al., 1979) supplemented with 100mg chloramphenicol and DG18 (pH 6.5; H o c k i n g and P i t t, 1980) prepared according to the author's directions, were used to enumerate fungi in all spice samples. Sterile (121 °C, 15 min.) molten (ca. 50°C) agar was poured (ca. 20 ml) into 90 mm glass Petri dishes and allowed to solidify overnight in the dark before using.

The dilution scheme consisted of combining 10 g of the spice sample with 90 ml of 0.1% peptone, and homogenization with top driven blander (Delonghi, for home use) for 30 seconds. Additional dilutions of 1:10 (1+9, v+v) were made in 0.1% peptone also. Inoculated plates (0.1 ml) were incubated in the dark in an upright position at 25 and 35 °C for five days, and when necessary, for seven days. Plates supporting the development of 10-100 fungal colonies were chosen for enumeration and calculation of fungal populations (CFU g-1).

The plates were examined under stereo microscope after fifth and seventh day of incubation, and all colonies of *Penicillium, Aspergillus* and their teleomorphs were subcultured and eventually purified. The isolates were further identified after P i t t (1979; 1991) and S a m s o n and P i t t (1989) for *Penicillium,* and after R a p e r and F e n n e l (1965), S a m s o n and P i t t (1989), D o m c h and G a m s (1993) and K l i c h and P i t t (1994) for *Aspergillus* and their teleomorphs.

Qualitative examination of aflatoxins

The qualitative examination of aflatoxin produced by *A. flavus, A. nominus* and *A. parasiticus* isolates was carried out after A b r a m s o n and C l e a r (1996). Their method is given below in details.

Yeast agar with 0.5% magnesium sulfate (F r i s v a d et al., 1990) was steam sterilized and 5.0 ml was added to 50 ml Erlenmeyer flasks. The flasks were capped and set aside to cool. Spore suspensions from fungi cultured

on MEA were made in sterile distilled water. The flasks were inoculated with 0.5 ml.

After 21 days in the dark at 22 °C, the flasks were opened and 2.5ml of methylene chloride/formic acid 25:1 was added. Each flask was tightly re-capped with foil and, after 60 minutes, filled with 10ml disposable glass capillary pipette that was inserted through the foil. The pipette contents were applied to the origin of the TLC plates. Identification of the Af-B1 was done by comparison with Af-B1 standard (Fluka,USA). Silica gel plates (Merck, Darmstad) were developed with the following solvent: chloroform/acetone 9:1. Mycotoxins were visualized by their natural fluorescence at 365 nm and 254 nm. Identification was confirmed by spraying the plates with 25% sulfuric acid (L e i t a o et al., 1987).

RESULTS

Fungal counts of the spice samples and their related products on both DRBC and DG18, are given in Table 1 for two temperatures, 25 and 35 ºC. The number of colonies forming units (CFU) is given as Log_{10} values. Despite the fact that only two samples of "food additives" were examined, it seems that these products typically have lower mold counts. With few exceptions, the mold counts of the plates incubated at 25 ºC were slightly greater than those incubated at 35 ºC. In general, the fungal counts on DRBC and DG18 were similar.

The plates were examined under stereo microscope after the fifth and seventh day of incubation; all colonies of *Penicillium, Aspergillus* and their teleomorphs were subcultured and eventually purified.

A. fumigatus was the dominant species in fourteen samples, *Eurotium* spp. in twelve samples, *Penicillium* spp. in seventeen samples, *Aspergillus niger* in nineteen samples, *Aspergillus* spp. in one sample, *A. niger* and *A. flavus* were dominant mycoflora in two samples, *A. niger* and *Eurotium* sp. dominated in the mycoflora in one sample, and *A. niger* and *A. terreus* dominated in the mycoflora in one sample.

As determined by the TLC method, 7 out of 17 isolates of *A. flavus* (41%) produced Af-B1 in culture. All isolates of *A. nomius* and *A. parasiticus* produced Af-B1 and Af-G1 (Table 2).

DISCUSSION

Because of tropical origin, spices are frequently heavily contaminated with xerophilic fungi capable of rapid growth at low water activities (0.77 aw) (P i t t and H o c k i n g, 1985). H i t o k o t o et al. (1980) found that in most of the 49 samples examined, the mycoflora was dominated by species of *Aspergillus* and *Penicillium* genera, and only in few samples species of genera *Mucor, Rizopus, Cladosporium* and *Aureobasidium* were found. S a m s o n et

Tab. 1 – Fungal count ($log_{10}g^{-1}$) of spice samples after incubation at 25 and 35°C on DRBC and DG18

Product	DG18-25°C	DG18-35°C	DRBC-5°C	DRBC-5°C
Bukovec 1	3.58	3.69	3.84	4.15
Bukovec 2	5.73	5.43	5.65	5.6
Cinnamon 1	4.1	4.04	4.22	4.15
Cinnamon 2	2.3	2.3	2.3	2.6
Cinnamon 3	4.8	4.66	4.5	4.59
Food add. 1	3	2.78	2.7	2.7
Food add. 2	2.8	2	3.3	2.3
Caraway 1	3.89	3.18	3.62	3
Caraway 2	5.98	4.6	5.88	4.57
Bay leaf 1	4.28	3.84	4.23	3.4
Bay leaf 1	6.17	5.61	6.3	5.78
Oregano 1	4.25	3.57	4.12	3.4
Oregano 2	4.52	2.78	4.32	2.78
Oregano 3	5.95	5.43	6.02	5.59
Pepper 1	3.78	3.66	3.83	3.62
Pepper 2	2.6	3.68	3.25	3.61
Pepper 3	3.9	4.1	3.95	2.78
Pepper 4	5.84	5.08	5.78	3.63
Paprika 1	5.72	5.71	5.75	5.59
Paprika 2	4.65	4.5	4.7	4.61
Paprika 3	2	2.48	2	3
Paprika 4	5.54	5.54	5.63	5.65
Paprika 5	3.84	4.23	4	4.38
Paprika 6	4.36	4.15	4.18	3.84
Paprika 7	5.73	4.9	5.7	5.11
Paprika 8	5.41	5.43	5.3	5.53

al. (1995) listed *Aspergillus flavus, A. tamari, A. niger, A. candidus, A. versicolor, Eurotium* spp., *Walemia sebi, Penicillium islandicum, P. purpurogenum* and *P. cirinum* as important for spices. Most of the spices investigated by A k e r s t a n d (1992) had similar mycoflora with *Aspergillus* and *Eurotium* species being predominant.

Black and white peppers, some of the most common spices, are notorious for carrying high microbial populations. In their collaborative study on dilution schemes for enumerating (mold counts on DRBC, 25 ºC) fungi in ground pepper, B e u c h a t and H w a n g (1996) found that the mold counts varied between Log_{10} 5.55 CFU g^{-1} and Log_{10} 5.88 CFU g^{-1}. A k e r s t r a n d (1992) found (mold counts on DG18, 25 ºC) less than Log_{10} 1.00 CFU g^{-1}, and Log_{10} 4.20 CFU g^{-1}, for two samples of black pepper, and Log_{10} 4.60 CFU g^{-1} for the sample of white pepper. F r i s v a d et al. (1990) found (mold counts on DG18, 25 ºC) Log_{10} 5.12 CFU g^{-1} for the sample of black pepper, and Log_{10} 3.99 CFU g^{-1},

Tab. 2 – Isolates proved to be aflatoxigenic in pure culture

Species	FNS-FCC*	Product	Toxins
A. flavus	6	Caraway	AfB_1, AfB_2
A. flavus	7	Black pepper	AfB_1
A. flavus	8[c]	Bukovec	AfB_1
A. flavus	9[c]	Bukovec	AfB_1, AfB_2
A. flavus	10	Paprica	AfB_1
A. flavus	12[a]	Black pepper	AfB_1
A. flavus	13[b]	Bukovec	AfB_1, AfB_2
A. flavus	14	Nutmeg	AfB_1, AfB_2
A. flavus	15	Dried carrot	AfB_1
A. flavus	16[a]	Black pepper	AfB_1
A. flavus	17[b]	Bukovec	AfB_1, AfB_2
A. nomius	43	Caraway	AfB_1, AfB_2, AfG_1 AfG_2
A. nomius	44	Cinnamon	AfB_1, AfB_2, AfG_1 AfG_2
A. nomius	45	Orchis morio	AfB_1, AfB_2, AfG_1 AfG_2
A. parasiticus	54	Origano	AfB_1, AfG_1
A. flavus	ex type		AfB_1
A. parasiticus	ex type		AfB_1, AfG_1

* isolates followed by the same letter come from the same sample

and Log_{10} 5.19 CFU g^{-1} for two samples of white pepper. These results are comparable with those obtained in our examination. C h o u r a s i a (1995), in his study on herbal drugs of Indian pharmaceutical industries, found that maximum numbers of fungi were recorded from the fruits of *Piper longum*, *P. nigrum* and *Elettaria cardamomum,* whereas the lowest incidence was on *Carum ajmoda* roots and barks of *Cinnamomum zeylanicum*. Our results are comparable with those obtained in these examinations (Table 1).

Comparatively high mold counts were also detected for oregano, caraway and bay leaf. On the other hand, when processed properly paprika is only moderately contaminated (D e a k and F a b r i, 1984). Main mold counts of a large number of samples of paprika investigated in Hungary during 1974, 1978, 1980 and 1982 varied between Log_{10} 2.00 CFU g^{-1} and Log_{10} 3.71 CFU g^{-1} (D e a k and F a b r i, 1984). A k e r s t r a n d (1990) found less than Log_{10} 1.00 CFU g^{-1} in one sample examined. As it can be observed from Table 1, in our investigation fungal counts (DG18, 25 °C) for the samples of paprika varied between less than Log_{10} 2.00 CFU g^{-1} and Log_{10} 5.73 CFU g^{-1}. However, most of the samples had a surprisingly high count which indicated improper sanitary practice during the harvest or processing.

Similarly, comparatively high fungal counts were detected for the two samples of "Bukovec", which consisted mainly of paprika.

The highest frequency was recorded for *A. flavus, A. fumigatus, A. niger* and *Eurotium* spp. The only isolated *Penicillium* species was *P. Chrysogenum,* which had comparatively high frequency. This finding differs from the conclusion of S a m s o n et al. (1990) which indicated that *P. islandicum, P. purpurogenum* and *P. citrinum* are some of the important species that could be found in spices. Nevertheless, this can eventually be supported by the findings of W i l l i a m s (1989).

Among 17 isolates of *A. flavus* tested for the potential of aflatoxin production, 7 were found to be Af-B1 positive, which is 41%, and 4 produced Af-B2 as well. C h o u r a s i a (1995) and L i e w e l l y n et al. (1992) found that nearly 50 and 49% of *A. flavus* isolates, respectively, were Af-B1 positive. Morphologically, toxigenic and non-toxigenic strains of *A. flavus* were not possible to distinguish. All isolates of *A. nomius* and *A. parasiticus* produced Af-B1 and Af-G1 (Table 2).

CONCLUSIONS

Over the past few decades it has been well documented that mold-contaminated food and foodstuffs are often responsible for animal mycoses after ingestion of mycotoxins. Since some of the spices are not free from such contamination, the risk of mycotoxicoses in consumers of these products may also be suspected. Our results support the previous findings which indicate the incidence of potentially mycotoxigenic species of *Penicillium* and *Aspergillus* is very high in some spices, and that about 40% of *A. flavus* isolates are Af-B1 producers. Elimination of all traces of mycotoxins in spices should not be expected. However, there is a need for simple and rapid sensitive method for the detection of Af-B1 in spices which could prevent the presence of highly contaminated crude spices or/and commercial spices.

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МИКОТОКСИГЕНЕ ПЛЕСНИ КОД РАЗЛИЧИТИХ ЗАЧИНА У МАКЕДОНСКИМ МАРКЕТИМА

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Резиме

Двадесет и четири примерка комерцијалних паковања млевене паприке, буковца, црног бибера, оригана, цимета, ловоровог листа, кима, додатака за јела, као и три примерка с отворених пијаца у Скопљу, љута и слатка млевена паприка и буковац, били су анализирани на присуство и степен контаминације с микотоксигеним плеснима из родова *Aspergillus* и *Penicillium* као и њихови телеоморфи. Исто тако били су тестирани изолати из *A. flavus, A. nomius* и *A. parasiticus* за продукцију афлатоксина. Ови тестови су рађени помоћу TLC методе коришћењем стандардних раствора.

Број плесни у испитиваним примерцима варирао је од Log_{10} 2 CFU g^{-1}, код паприка до Log_{10} 6,17 CFU g^{-1} у ловоровом листу. Међу видове који се најчешће јављају као део контаминантне микрофлоре у овим продуктима убрајају се *A. niger, A. flavus, Eurotium sp.* и *P. chrysogenum*. Релативно често јављају се *A. terreus, A. ochraceus, A. versicolor, A. sydowii, Emericela nidulans, E. repens* и *E. rubrum*.

Од укупно 17 издвојених и тестираних изолата *A. flavus*-а, у експерименталним условима, 7 је било способно за продукцију Af-B1, од чега 4 изолата за продуцију Af-B2. Сви изолати *A. nomius*-а и *A. parasiticus*-а у експерименталним условима продуцирају Af-B1 и Af-G1.

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ANTIFUNGAL ACTIVITY OF OREGANO EXTRACT AGAINST *A. VERSICOLOR, E. NIDULANS AND EUROTIUM* SPP. – PRODUCERS OF STERIGMATOCYSTIN

ABSTRACT: The paper presents the influence of oregano extract (*Origanum vulgare* L.) on growth of *Aspergillus versicolor*, *Emericella nidulans*, *Eurotium herbariorum*, *E. amstelodami*, *E. chevalieri* and *E. rubrum* – producers of sterigmatocystin (STC) isolated from salads. Antifungal tests were performed by agar plates method. The composition of the active component of extract was determined by GC-MS method and the major components were: carvacrol (34.20%), triacetin (22.91%), carvone (18.05%), p-cymene (8.05%) and thymol (3.74%).

The examined extract showed the ability to reduce mold growth at all applied concentrations. Minimum inhibitory concentrations (MIC) for *E. nidulans*, *E. chevalieri* and *E. amstelodami* were 2.5% (v/v) and over 2.5% (v/v) for *A. versicolor.* At 1.5% (v/v) concentration the extract completely inhibited the growth of *E. rubrum*, whereas higher dose of 2.5% (v/v) was fungicidal against *E. herbariorum*. Besides its sensory role in food products, the examined oregano extract also exhibits antifungal activities against producers of STC.

KEY WORDS**:** antifungal activity, oregano extracts, producers of sterigmatocystin

INTRODUCTION

A. versicolor, E. nidulans, E. herbariorum, E. amstelodami, E. chevalieri and E. rubrum are classified in the group of xerophilic fungi. They are frequent contaminants of food low in free water content (water activity (a_w) below 0.80) such as spices, stored grains, dried fruits, vegetables, etc. (Pitt and Hocking, 1997; Kocić – Tanackov et al., 2007; Dimić et al., 2008a). These microorganisms deteriorate food by their metabolic activity causing enormous economic damage. Moreover, species possessing genetic base for mycotoxin production can biosynthesize sterigmatocystin (STC), nidulotoxin, dechloronidulin, penicillin, physcion, and echinulin in the substrate under favorable conditions. STC is a biological precursor of aflatoxin B1 (AB1) which is less

toxic than AB1 (about 1/50th as potent as AB1) (Terao, 1983), but shows similar toxic effects, since it is excreted at higher quantities. Consumption of food contaminated with mycotoxins might cause serious disorders in humans and animals, such as hepatome, kidney lesions, and myocardial heart necrosis, and there are indications that STC is a potent mutagenic agent, too.

The occurrence of mold growth in raw materials and food products, as well as the potential for mycotoxin production, can be reduced by numerous measures: thermal process, dehydratation, filtration, acids, smoke, sanitation improvement during processing, and most often, usage of synthetic preservatives. However, the demand for safe products without added preservatives and improvers has been increasing. Consequently, extensive investigations have been carried out on the application of essential oils, extracts and oleoresins, extracted from spices and aromatic herbs. These alternatives, natural preservatives have great potential for the prolongation of food shelf-life (Nielsen and Rios, 2000; Selvi et al., 2003; Hsieh et al., 2001; Guynot et al., 2003; Benkeblia, 2004; Rasooli and Abyaneh, 2004; Vagi et al., 2005; Rasooli et al., 2006; Lopez–Malo et al., 2007; Omidbeygi et al., 2007; Pereira et al., 2006; Fung and Zheng, 2007; Dimić et al., 2008b; Viuda–Martos et al., 2007, 2008; Kordali et al., 2009; Škrinjar and Nemet, 2009; Tzortzakis, 2009; Reddy et al., 2009; Tatsadjieu et al., 2009; Kocić–Tanacov et al., 2009). Major advantage of these preparations is the absence of microorganisms which are abundantly present in natural spices (Karan et al., 2005; Kocić–Tanacov et al., 2009). Their antimicrobial activity is caused by the presence of many active phytochemicals, including flavonoides, terpenoides, carotenoides, coumarins and curcumines (Ceylon and Fung, 2004; Tepe et al., 2005; Tajkarimi et al., 2010).

Oregano is a widely used spice in the food industry. It is mainly used for its aromatic properties with a primary role of enhancing the food taste and aroma.

This paper aims at investigating its other role, regarding antifungal activity of commercial oregano extract against some xerophilic fungi: *A. versicolor, E. nidulans, E. herbariorum, E. amstelodami, E. chevalieri* and *E. Rubrum,* producers of STC isolated from salads.

MATERIALS AND METHODS

Oregano extract. For the purpose of antifungal activity testing, commercially available food grade oregano (*Origanum vulgare* L.**)** extract was provided from ETOL Tovarna arom in eteričnih olj d.d., Celje, Slovenia.

Determination of extract composition. GC-MS analysis was carried out on a Varian T2100 GC-MS instrument equipped with data processor. A fused silica capillary columnVF-5MS (30 m x 0.25 mm i.d., 0.25 μm film thickness, Varian) was used for the separation of the sample components. The carrier gas, ultra pure helium, was passed through moisture and oxygen traps with

constant flow rate of 0.62 cm^3min^{-1}. The following temperature program was used: injector temperature of 230 °C, initial temperature of 40 °C (held for 5 min), temperature increase of 5°C min^{-1} to 200 °C and held at this temperature for 25 minutes. The mass spectrometer was operated in the electron ionization mode. Data acquisition was carried out in the scan mode (range 50-550 m/z). Injection volume was 1 µl.

The compounds were identified by matching the mass spectra with NIST Mass Spectra Library stored in the GC-MS database.

Fungal strains used. As testing microorganisms, the following xerophilic fungal species were used: *Aspergillus versicolor* (Vuill.) Tiraboschi*, Emericella nidulans* (Edam) Vuill.*, Eurotium herbariorum* Link*, E. amstelodami* L. Mangin*, E. chevalieri* L. Mangin and *E. rubrum* Jos. K ö n i g et al. supplied from the Laboratory of the Food microbiology Department at the Faculty of Technology, University of Novi Sad, Serbia. The fungi were isolated from salads and maintained on Potato Dextrose Agar (PDA) at 4°C.

Antifungal assay. Antifungal activity of the extract was tested according to the agar plate method. PDA was used as a basic medium for antifungal activity testing. Equal volumes of medium (150 ml) were poured into 250-ml Erlenmeyer flasks and autoclaved for 15 minutes at 121ºC. After sterilization, the oregano extract was added to the cooled PDA medium at following concentrations: 0, 0.35, 0.7, 1.5 and 2.5% (v/v). The PDA medium was then poured into Petri plates (Ø 9 cm). Each plate was added with 12 ml of the medium.

Suspensions of conidial spores were prepared in a medium which contained 0.5% Tween 80 and 0.2% agar dissolved in distilled water, and they were adjusted to provide initial spore count of 10^6 spores/ml by using haemocytometer. For each extract dose and fungi species, including the controls, triplicate plates were centrally inoculated by spreading 1 µl of spore suspension (10^3 spores/ml) using inoculating loops. After inoculation, the Petri plates were closed with Parafilm.

Inhibition of the fungal growth was determined by daily measurements of diameter of radial colony growth during 14 days of incubation at 25 ±2ºC. The Parafilms were removed from the Petri dishes in which no colony growth was observed after 14 days, and the plates were left till the 30^{th} day to check the fungicidal effect (MFC) of the extracts. The inhibitory effect of oregano extract after 14 days was calculated following the formula (P a n d e y et al., 1982):

$$I\ (\%) = (C\text{-}T)/C \cdot 100$$

where I is inhibition (%), C is colony diameter on the control plate (cm) and T is colony diameter on the test plate (cm).

Changes in macroscopic and microscopic features of fungi were also observed and compared to the controls. The macroscopic features were observed using a binocular, magnifying glass Technival 2, Carl Zeiss, whereas the microscopic features by using a microscope Aristoplan, Leitz.

*Statistical analysis***.** Determination of the effect of oregano extract on the growth of tested fungi was carried out in 3 series and 2 replications. MS Sta-

tistica 4.5 was used to calculate the means. Significant differences between values of inhibition were determined by Duncan's multiple range test ($p<0.05$), following one-way ANOVA.

RESULTS

The inhibitory effect of oregano extract on the colony growth of tested fungal species after 14 days of incubation is presented in Table 1. Growth rate and colony growth inhibition of tested fungi in the presence of oregano extract are presented in Figures 1-6.

Tab. 1 – Inhibitory effect (%) of oregano extract on colony growth after 14 days of incubation

Fungi	Colony growth inhibition (%)			
	0.35% (v/v)	**0.7% (v/v)**	**1.5 % (v/v)**	**2.5% (v/v)**
A. versicolor	32.78 ± 0.0387^{Aa}	39.34 ± 0.0372^{Aa}	58.36 ± 0.0235^{Ab}	78.03 ± 0.0190^{Ac}
E. nidulans	29.19 ± 0.0180^{Aa}	41.37 ± 0.0257^{Ab}	52.64 ± 0.0207^{Bc}	100.00 ± 0.000^{Bd}
E. herbariorum	20.61 ± 0.0030^{Aa}	43.29 ± 0.0228^{Ab}	84.53 ± 0.0123^{Cc}	100.00 ± 0.000^{Bd}
E.rubrum	23.07 ± 0.000^{Aa}	36.53 ± 0.0271^{Ab}	100.00 ± 0.000^{Dc}	100.00 ± 0.000^{Bc}
E. chevalieri	34.24 ± 0.0127^{Aa}	36.16 ± 0.0007^{Aa}	53.97 ± 0.0220^{Bb}	100.00 ± 0.000^{Bc}
E. amstelodami	10.90 ± 0.0486^{Aa}	40.00 ± 0.0411^{Ab}	63.63 ± 0.0093^{Ec}	100.00 ± 0.000^{Bd}

Values followed by the same small letter (a-d) within the same row are not the significantly different ($p>0.05$) according to Duncan's multiple rang test
Values followed by the same big letter (A-E) within the same column are not the significantly different ($p>0.05$) according to Duncan's multiple rang test

At the lowest tested concentration, oregano extract inhibited the growth of *E. chevalieri* (34.24%), *A. versicolor* (32.78%) and *E. nidulans* (29.19%), whereas lower inhibitory effect was exhibited against *E. amstelodami* (10.90%). Values of inhibition at 0.35% (v/v) concentration of oregano extract were not significantly different ($p>0.05$). The inhibition of colony growth ranged from 36.16% for *E. chevalieri* to 43.29% for *E. herbariorumi* ,at 0.7% (v/v) concentration of oregano extract. Values of inhibition were not significantly different either ($p>0.05$). Significant differentiation between values of inhibition could be observed at the concentration of 1.50% (v/v) of oregano extract ($p<0.05$). *E. nidulans* (52.64%) and *E. chevalieri* (53.97%) exhibited the least significant inhibition. During 14 days of incubation, 2.5% (v/v) extract inhibited the growth of *A. versicolor* by 78.03% and completely inhibited the growth of other species. *A. versicolor* showed significantly higher resistance at high concentrations (2.5%) (v/v) ($p<0.05$). Depending on the concentration, different inhibitions of each fungus was observed. Exceptions were *A. versicolor* and *E. chevalieri*, at lower concentrations (0.35%(v/v) and 0.70% (v/v)). All in all, significant inhibitory effects of oregano extract statistically differentiated at high concentrations (1.50% (v/v) and 2.50% (v/v)) (Table 1).

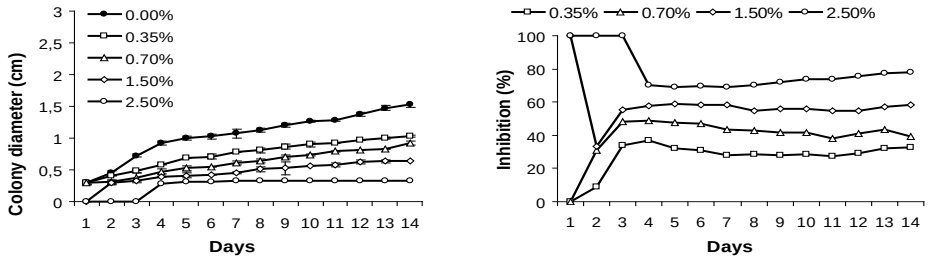

Fig. 1 – Growth rate and colony growth inhibition of *A. versicolor*

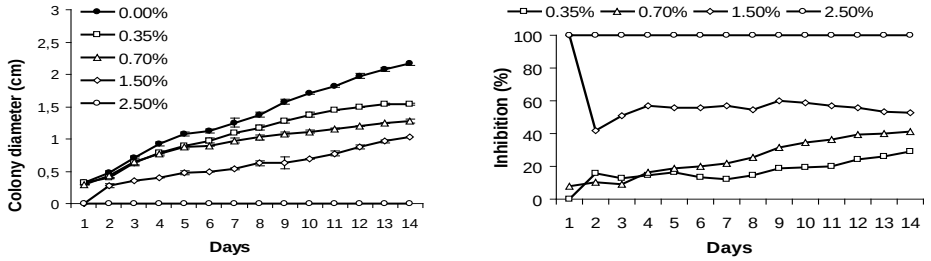

Fig. 2 – Growth rate and colony growth inhibition of *E. nidulans*

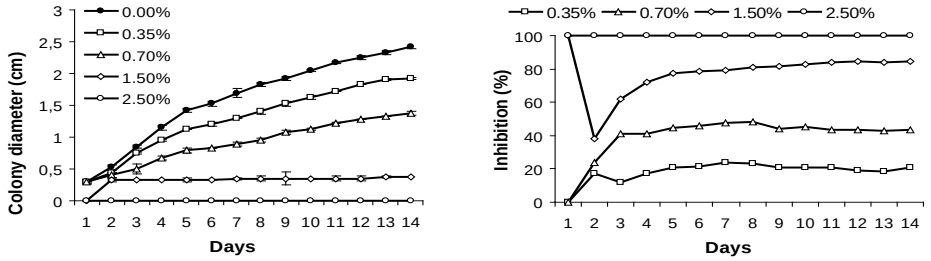

Fig. 3 – Growth rate and colony growth inhibition *E. herbariorum*

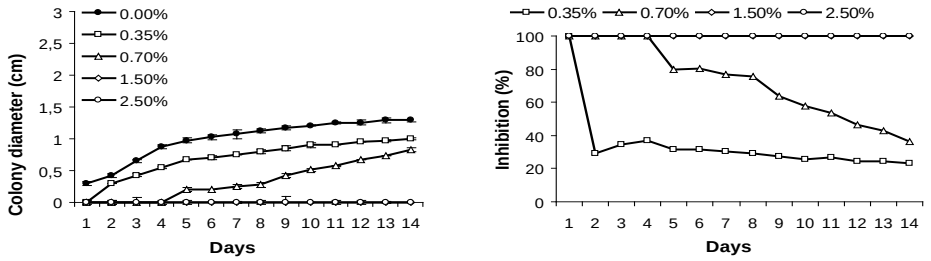

Fig. 4 – Growth rate and colony growth inhibition *E. rubrum*

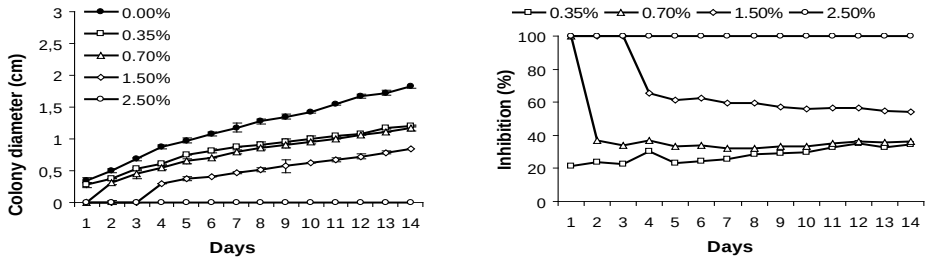

Fig. 5 – Growth rate and colony growth inhibition *E. chevalieri*

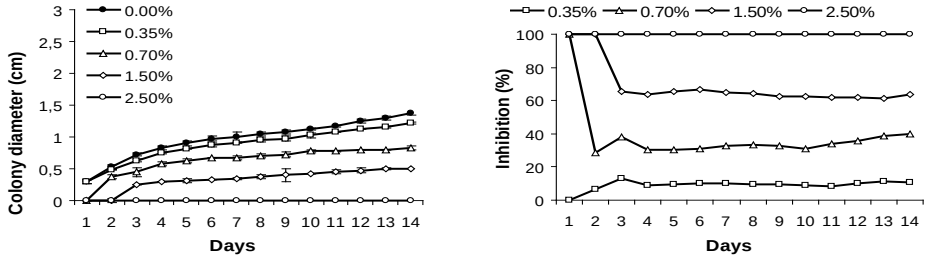

Fig. 6 – Growth rate and colony growth inhibition *E. amstelodami*

The increasing concentrations of oregano extract caused absence or delay in germination of tested fungi, showing various inhibitory effects on the growth rate reduction. The lowest tested concentration (0.35% (v/v)) delayed the growth of *E. rubrum* for one day, whereas the colony growth of other fungi was observed already on the first day. The extract concentration of 0.7% (v/v) delayed the growth of *E. rubrum* for 4 days, and the growth of *E. chevalieri* and *E. amstelodami* for one day. At 1.5% (v/v) concentration, colony growth delay of one day was observed for *A. versicolor*, *E. nidulans* and *E. herbariorum*, of two days for *E. amstelodami*, and of three days for *E. chevalieri*. The growth of *E. rubrum* was completely inhibited at 1.5 and 2.5% (v/v) doses during 30 days of incubation. Moreover, it was noted that 2.5% (v/v) extract concentration completely inhibited the growth of *E. herbariorum* during 30 days of incubation. At this concentration, the following delays in the colony growth were observed: three days for *A. versicolor*, 19 days for *E. nidulans* and *E. chevalieri,* and 25 days for *E. amstelodami* (Figures 1-6).

Twenty components were identified in oregano extract: carvacrol (34.20%), triacetin (22.91%), carvone (18.05%), p-cymene (8.05%), thymol (3.74%), limonene (3.36%), γ-terpinene (2.35%), dill ether (1.69%), linalool (1.51%), α-phellandrene (0.97%), β-caryophyllene (0.90%), camphene (0.31%), myrcene (0.31%), α-terpineole (0.29%), α-pinene (0.28), dihydrocarvone (0.24%), α-thujene (0.23%), borneole (0.22%), α-terpinene (0.20%) and 4-terpineole (0.05%).

DISCUSSION

The oregano extract at the tested doses showed a capacity to reduce or inhibit the growth of tested fungi. Inhibitory effect of extract increased proportionally with dose and was also affected by treatment duration. The efficiency of inhibition depended on the fungi resistance to the applied extract. According to Duncan's multiple range test, all concentrations of oregano extract had significantly different values of inhibition ratio in comparison to the control. Significant differences between the tested fungi were found, and partially between the concentrations of oregano extract. Low concentrations (0.35% (v/v) and 0.75%(v/v)) showed no significant differences in inhibition, while high concentrations (1.50% (v/v) and 2.50%(v/v)), in contrast, showed significant differences of inhibition.

The strongest inhibitory effect was expressed against *E. rubrum*. *A. versicolor* was the least sensitive fungus. Significant antifungal effect was scored fot the concentration of the extract above 0.7% (v/v). The oregano extract at 1.5% (v/v) showed fungicidal effect (MFC) against *E. rubrum*. Fungicidal effect was also registered against *E. herbariorum* at 2.5% (v/v), whereas minimal inhibitory effect (MIC) was observed against *E. nidulans*, *E. chevalieri* and *E. amstelodami*. Concentrations over 2.5% (v/v) exhibited MIC against *A. versicolor.*

Earlier studies also reported strong inhibitory effect of oregano on some molds, food contaminants. Akgul and Kivanc (1988) reported that, among ten spices investigated, only oregano extract showed antifungal effect against nine tested fungi. The addition of grinded oregano and thymian into the growth medium reduced the production of aflatoxin by *Aspergillus parasiticus* (Salmeron et al., 1990), whereas its extract at 2% completely inhibited the growth of this species during 10 days of incubation at 30°C (Özcan, 1998). Essential oil of oregano was described as strong growth inhibitor of fungi. Paster et al. (1995) demonstrated the antifungal activity of oregano essential oil at concentration of 2 and 2.5 µL/L on the mycelium and spores of *A. niger, A. flavus* and *A. ochraceus*. The volatile oil *Origanum syriacum,* that has carvacrol and thymol as the major components, completely inhibited the mycelial growth of *A. niger*, *Penicillium* spp., and *F. oxysporum* at the concentration of 0.1 µL/mL in extract surcose broth (YES broth) (Daouk et al., 1995). At 1000 ppm, essential oils of oregano and mint inhibited the growth of *A. ochraceus* and ochratoxin A production in YES broth in up to 21 days (Basilico and Basilico, 1999). Baratta et al. (1998), Bouchra et al. (2003) and Vuida–Martos et al. (2007) implied that essential oil of oregano showed stronger fungicidal effect on *A. niger* and *A. flavus* than those of rosemary, sage, thyme and clove.

Antifungal activities of tested oregano extract depended on the content of its major and minor components. The majority of researchers named carvacrol and thymol as the main compounds associated with the antifungal activity of oregano (Arnold et al., 2000; Sokovic et al., 2002; Veres et al., 2003; Lopez et al. 2005; Vuida–Martos et al., 2007; Lukas et al., 2009). Thymol and carvacrol, at doses of 0.0025 and 0.05% (w/v) and with pH 5.5,

were found to completely inhibit the growth of *A. flavus, A. niger, Geotrichum candidum, Mucor* spp., *P. roqueforti* and *Penicillium* spp. on PDA medium. p-cymene and γ-terpinene were evaluated as weaker antimicrobial agents in comparison to carvacrol and thymol, even though they are their bioprecursors (Sivropolou et al., 1996; Dorman and Deans, 2000).

Besides the growth inhibitory effect, oregano extract caused changes in the macro and micro morphology of the tested fungal species. At higher concentrations (0.7, 1.5 and 2.5% (v/v)), non-characteristic, markedly convex colonies with firm, granular structure were formed (*A. versicolor, E. rubrum*), with central depression (*E. nidulans*), and diminished conidiation. In addition, at the same extract concentrations, examination of microscopic preparation showed deformation of hyphae with frequent occurrence of fragmentations and thickenings, rare vesicles and conidia typical for these species, and the absence of fruiting bodies (*E. rubrum, E. amstelodami, E. chevalieri* and *E. nidulans*). Deformed ascomata without ascus were observed with *E. herbariorum*. These morphological changes indicated the possibility that changes on cellular levels had occurred (destruction of fungal cells, decrease in the oxygen uptake, reduction in the cellular growth, inhibition of the synthesis of lipids, proteins and nucleic acids, changing in the lipid profile of the cell membrane and inhibiting the synthesis of the fungal cell wall) (Adetumbi et al., 1986; Ghannoum, 1988; Gupta and Porter, 2001; Corzo–Martinez et al., 2007). Investigations of cellular ultrastructures in *A. niger* demonstrated that aetheric oils of thyme and ajoene, extracted from garlic, caused the absence of surface ornaments, thinning of cell walls, abruption of cell membrane from cell wall, surface yielding of hyphae and destruction of cellular organelles (Yoshida et al., 1987; Rassoli et al., 2006). It can be assumed that active components of essential oils like thymol, eugenol and carvacrol damage the biological membrane by bonding to essential enzymes integrated with cellular membranes (Knobloch et al., 1989; Davidson, 2001).

CONCLUSION

Finally, it can be concluded that oregano extract, apart from enhancing food palatability, can be used as a potential antifungal agent against investigated xerophilic molds – producers of sterigmatocystin. Therefore, further investigations should be conducted on the profiles of other food contaminant fungi from genera *Penicillium*, *Aspergillus*, *Fusarium*, *Cladosporium*, etc. Also, the evaluations of tested extract concentrations in real food systems should be continued.

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АНТИФУНГАЛНА АКТИВНОСТ ЕКСТРАКТА ОРИГАНА (*ORIGANUM VULGARE* L.) НА РАСТ *ASPERGILLUS VERSICOLOR, EMERICELLA NIDULANS* И *EUROTIUM* SPP. – ПРОДУЦЕНТЕ СТЕРИГМАТОЦИСТИНА

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Резиме

У раду је приказан утицај екстракта оригана (*Origanum vulgare* L.) на раст *A. versicolor, E. nidulans, E. herbariorum, E. amstelodami, E. chevalieri* и *E. rubrum* – продуценте стеригматоцистина (STC), изолованих из салата. Антифунгални тестови су изведени методом агар плоча. Састав активних компонената екстракта је одређен GC-MC методом и као главне компоненте одређене су: карвакрол (34,2%), триацетин (22,91%), карвон (18,5%), p-цимен (8,05%) и тимол (3.74%).

Испитивани екстракт је на свим примењеним концентрацијама показао способност редукције раста плесни. Минимална инхибиторна концентрација екстракта (MIC) за *E. nidulans*, *E. chevalierii* и *E. amstelodami* је била 2.5% (v/v), а већа од 2.5% (v/v) за *A. versicolor.* При концентрацији од 1.5% (v/v) комплетно је инхибиран раст *E. rubrum*, док је концентрација од 2.5% (v/v) била фунгицидна према *E. herbariorum*. Испитивани екстракт оригана је поред основне, сензорне улоге коју има у прехрамбеним производима, истовремено показао и антифунгалну активност према испитиваним продуцентима STC.

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PARASITIC MYCOBIOTA OF YELLOW GENTIAN (*GENTIANA LUTEA* L.)

ABSTRACT: Mycopopulation of yellow gentian growing in plantations was studied in 2008 and 2009. Fourteen species of fungi were registered at seed, out of which five were pathogenic. The most common species was *Alternaria alternata* (72-74 %). Species of the genus *Fusarium* (*F. oxysporum*, *F. solani* and *F. equiseti*) were present in a small percentage (2-6 %). These species, as well as *F. verticillioides,* were isolated from root, and *Fusarium* sp. was isolated from the flowers. *Alternaria alternate, Epicoccum purpurescens*, *Phoma* sp. and *Alternaria* sp. were regularly present on the leaves and stems.

KEY WORDS: *Gentiana lutea*, yellow gentian mycobiota, pathogens

INTRODUCTION

Yellow gentian is a perennial herb on mountain meadows and pastures that are between 800 and 2500 meters of altitude. It grows also in sparse woods and on rocky ground. The root of yellow gentian is used for various galenic preparations such as teas, tinctures, extracts, medicinal wines and spirits. Recent studies indicate that gentian leaf and flower are more interesting drug than the root (Menković et al., 2000).

The uncontrolled exploitation of natural gentian habitats raised the question of its survival. In recent years European countries have banned the collection of wild gentian from nature, which caused serious shortages throughout the world. Its collection in Serbia is, limited and controlled by the order of the Ministry of Environmental Protection (The Official Gazette RS, 50/93). Gentian has been grown on plantations in Serbia over the last six years in order to increase the yield of uniform quality and to preserve biodiversity of wild gentian.

There is little evidence about the disease of yellow gentian. In Serbia, it is a host of 16 fugal species (Pavlović et al., 2006).

MATERIALS AND METHODS

The pathogenic mycrobiota of gentian was studied on the plantation of the Institute of Medicinal Plant Research, Belgrade, in the National Park "Tara" at Tara Mountain in 2008 and 2009. The samples of diseased plant were collected from April to October. Isolation of pathogens from plant material was performed by standard procedure (Kiraly et al., 1970; Dingra and Sinclair, 1986). Fragments of diseased plants were rinsed with tap water, surface sterilized with 2 % NaOCl for two minutes, rinsed again with sterile distillate water and planted on potato dextrose agar (PDA). Isolation from gentian seeds was performed by ISTA method (2003). Four hundred seeds from each seed lot was surface sterilized in 2 % NaOCl for two minutes, rinsed with sterile distillate water and transferred to wet filter paper in Petri dishes. Also, fifty seeds taken from each lot after surface sterilization were transferred to Petri dishes with PDA. Seeds were incubated for 10 days at 25ºC.

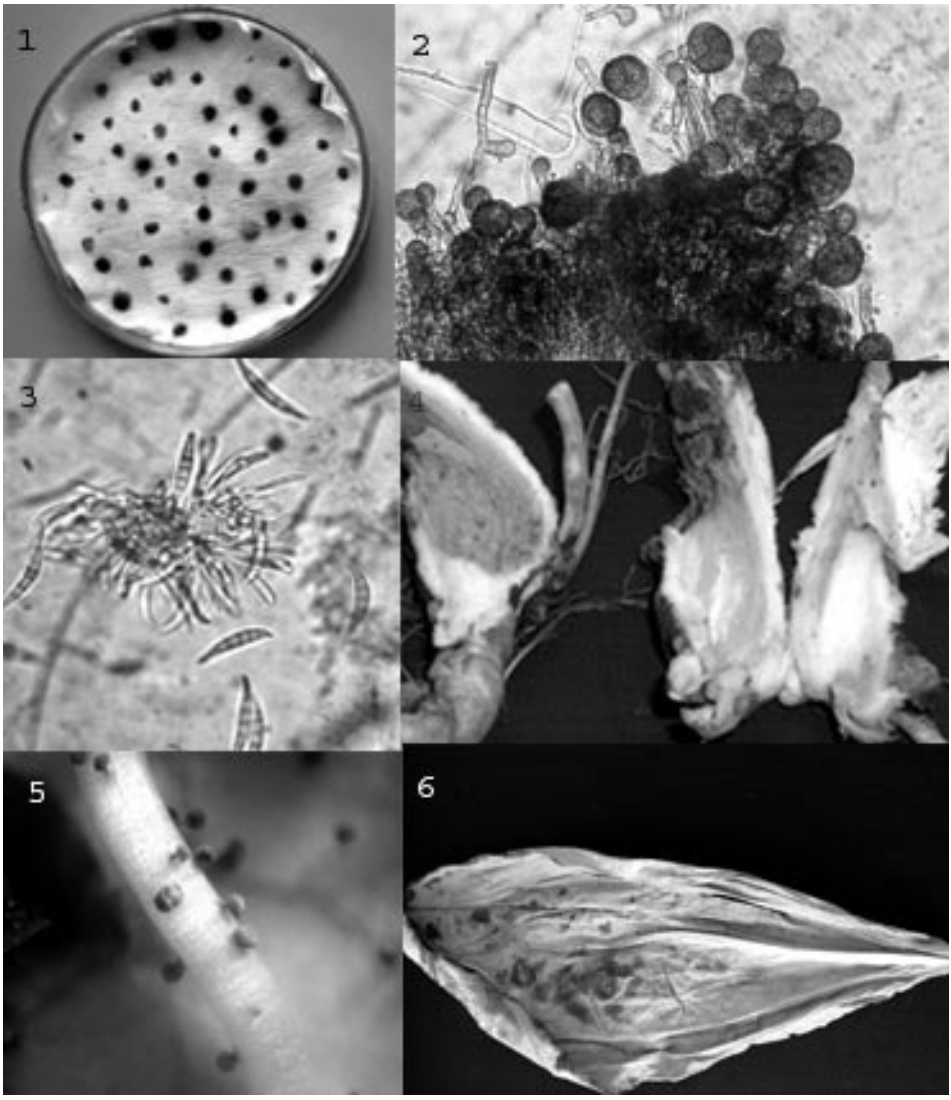
Determination was based on morpho-physiological characteristics and the cultivation of fungi tested (Sutton 1980; Gerlach and Nirenberg, 1982; Nelson et al., 1983; Joffe, 1986; Leslie and Summerell, 2006; Simons, 2007).

RESULTS AND DISCUSSION

Yellow gentian is a host of numerous parasitic and saprophytic fungal species in Serbia (Table 1 and 2). The most common species on seed is *Alternaria alternata* (>70 %). In most cases, seeds infected with this fungus do not germinate. This species is also registered on seeds of balm, sage, tansy, valerian, St.John's wort, *Echinacea* spp., and camomile (Kostić et al., 1999; Pavlović and Dražić, 2000; Pavlović et al. 2000a; Pavlović, 2001; Kostić et al., 2003; Pavlović, 2003). Seeds in small percentage (2-6 %) were infected with fungal species of the genus *Fusarium* (*F. oxysporum*, *F. solani* and *F. equiseti*), but they are very destructive causing rotting and decay of seedlings. Other registered species do not present any danger to the gentian seed.

Three species of *Fusarium* (*F. oxysporum*, *F. solani* and *F. verticillioides*) caused wet root rot. *Alternaria* sp. and *Epicoccum purpurescens,* and *Alternaria alternata* and *Phoma* sp., respectively, were isolated from stem and leaves with spot symptoms, while *Fusarium* sp. was isolated from flower only.

In general, medicinal plants, as well as weeds, can be classified into a group of plants with the most numerous mycopopulation. However, small number of plant pathologists studied the mycobiota of medicinal plants. This is understandable, because the attention of researchers is focused primarily on the mycopopulation of most important cultivated field and vegetable crops, fruit trees and grape vines.



Seeds of marshmallow severely infected by *Alternaria alternata* (Fig. 1). Conidia of *Epicooccun purpurescens* on seed (Fig. 2). Conidial cells and macroconidia of *Fusarium equiseti* (Fig. 3). The wet root rot of yellow gentian caused by mix infection of *Fusarium* spp. (Fig. 4). *Physarum notabile* on the seedling (Fig. 5). The leaf spots of yellow gentian caused by *Phoma* sp.

Tab. 1 – Incidence of fungi (%) on the seeds of yellow gentian in 2008 and 2009

Pathogen	Year	
	2008	2009
Alternaria alternata	72	74
Aspergillus flavus	2	2
Aspergillus niger	3	3
Botrytis cinerea	4	2
Cephalosporium spp.	2	2
Epicoccum purpurascens	2	4
Fusarium oxysporum	2	6
Fusarium solani	3	2
Fusarium equiseti	2	2
Penicillium spp.	3	3
Physarum notabile	2	3
Physarum sp.	1	-
Mucor sp.	3	2
Rhyzopus spp.	3	3

Tab. 2 – Mycobiota of yellow gentian during vegetation

Plant part	Fungus	Time of isolation
stem	*Alternaria* spp.	Jun-July
	Epicoccum purpurescens	July
Leaf	Alternari alternate	September
	Phoma spp.	August
Flower	*Fusarium* spp.	August
Root	Fusarium oxysporum	September
	Fusarium solani	October
	Fusarium verticillioides	October

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ПАТОГЕНА МИКОБИОТА ЖУТЕ ЛИНЦУРЕ (*GENTIANA LUTEA* L.)

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Резиме

Жута линцура је вишегодишња зељаста биљка планинских ливада и пашњака на надморским висинама између 800 и 2500 метара. Расте и у ретким шумама и на камењарима. Као сировина за фармацеутску индустрију користи се првенствено корен, а у новије време лист и цвет. Да би се сачувао биодиверзитет и повећао принос уједначеног квалитета, линцура се све више плантажно гаји у свету, а код нас последњих шест година. Плантажним гајењем жуте линцуре створени су услови за појаву већег броја патогених гљива у различитим фенофазама развића.

Проучавање патогене микофлоре жуте линцуре испитивано је на плантажи Института за проучавање лековитог биља, Београд, у националном парку "Тара" током 2008. и 2009. године. Преко 70% семена жуте линцуре било је нападнуто врстом *Alternaria alternata.* У већини случајева, семе заражено овом гљивом уопште не клија. Врсте из рода *Fusarium* (*F. oxysporum*, *F. solani* и *F. equisti*), заступљене су у мањем проценту (2-6%) и проузрокују у првом реду смањење енергије клијања и клијавости семена, а затим и трулење и пропадање клијанаца. На корену је констатовано присуство мешане инфекције са *Fusarium oxysporum*, *F. solani* и *F. verticillioides*. Са надземних делова линцуре изоловане су *Alternaria* spp., *Alternaria alternata, Epicoccum purpurescens, Phoma* ps. и *Fusarium* sp.

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PATHOGENIC, MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS OF *ALTERNARIA TENUISSIMA* FROM SOYBEAN

ABSTRACT: During 2008 and 2009 phytopathological isolations were done from soybean plants and seed samples from several localities in Serbia. A total of 19 isolates of *Alternaria* spp. were isolated, 13 from the seed and 3 from both leaf and stem. In order to determine and characterize isolates, cultural, morphological, molecular and pathogenic characteristics were thoroughly investigated.

The slowest growth of the examined isolates was noted on Malt agar (MA) with average colony diameter of 42.9 mm after 7 days of incubation. On other two media (V8 and PCA), colony growth was uniform and faster, with average diameter of 66.8 mm and 66.1 mm, respectively.

Isolates of fungi form unbranched or poorly branched conidial chains on short unbranched conidiophores. Conidia are dark in colour, multicellular with transverse and longitudinal septae. They are of different size regarding the place of formation in the chain. Based on these characteristics, the tested isolates were determined as *Alternaria tenuissima*.

Molecular identification with sequencing of ITS1-5.8S-ITS2 rDNA verified that investigated isolates belong to *Alternaria tenuissima* group.

Pathogenicity test proved that all isolates were more or less virulent to soybean seed (12.5% to 40% of rotten seeds), while pathogenicity on plants was poorly expressed.

KEY WORDS: *Alternaria tenuissima*, soybean, pathogenicity, morphology, ITS1-5.8S-ITS2 rDNA sequence

INTRODUCTION

It is well known that the majority of *Alternaria* species are saprophytic fungi. However, some species may appear as significant parasites of cultivated plants (T h o m m a , 2003). Soybean is infected by species from this genus, most frequently by *Alternaria alternata* and *A. tenuissima* (S i n c l a i r and B a c k m a n , 1989; B a i r d et al., 2001). These species most frequently cause pathological changes on seed, and rarely on leaf and stem (S i n c l a i r and B a c k m a n , 1989). However, these parasites are of low economical significance (B a i r d et al., 2001). Additionally, *Alternaria* spp. are described as facultative parasites of soybean seed in Serbia (M e d i ć et al., 2007), but there have not been detailed investigations.

During the inspection of soybean seed health originating from different regions of Serbia, fungi from genus *Alternaria* were frequently isolated. It was noticed that seed infected with *Alternaria* spp. does not germinate and it rots completely. Therefore, under certain conditions, these species may be considered as important seed pathogens and should be investigated more thoroughly. Due to previously mentioned, species from genus *Alternaria* originating from soybean seed were determined according to cultural, morphological and molecular characteristics, and their pathogenicity on seed and adult soybean plants was examined.

MATERIALS AND METHODS

Isolation of fungi

During 2008 and 2009 a soybean seed health was observed through analyses of a large number of samples from different regions in Serbia. Phytopathological isolations on PDA (Potato Dextrose Agar) were carried out from 100 randomly selected seeds. The isolations were also performed from necrotic spots on leaf and stem. For further research, 19 monosporial isolates were chosen – 13 from the seed and 3 from both leaf and stem (Table 1.).

Cultural and morphological characteristics

For the analysis of cultural characteristics of *Alternaria* spp. isolates, three nutritive media were used: Malt Agar (MA), V8 Agar and Potato Carrot Agar (PCA), whereas for morphological features only PCA was used.

Isolates were cultured in Petri dishes (diameter 90 cm) at room temperature (around 24 °C) for 5 days in the dark. Afterwards, they were constantly exposed to the fluorescent light for 2 days, following the recommendations of S i m m o n s (2007). Colony growth was determined after 2, 5 and 7 days by measuring colony diameter. After 7 days, morphology of the colonies on used nutritive media was described (color, structure, aerial and substrate mycelia).

Tab. 1 – Isolates from soybean from different localities in Serbia and the year of isolation

Isolate	Organ of isolation	Locality	Year of isolation
A118	Stem	Rimski Šančevi	2008
A129	Seed	Karavukovo	2008
A130	Seed	Karavukovo	2008
A131	Seed	Karavukovo	2008
A132	Seed	Karavukovo	2008
A133	Seed	Vrbas	2008
A134	Seed	Vrbas	2008
A135	Seed	Vrbas	2008
A136	Seed	Zrenjanin	2008
A137	Seed	Laćarak	2009
A138	Seed	Sombor	2008
A139	Seed	Sombor	2008
A140	Stem	Kisač	2009
A141	Seed	Karavukovo	2008
A142	Seed	Karavukovo	2008
A143	Stem	Rimski Šančevi	2009
A144	Leaf	Jaša Tomić	2009
A145	Leaf	Jaša Tomić	2009
A146	Leaf	Jaša Tomić	2009

Microscopic examinations of the morphological characteristics of reproductive organs of isolates (conidia, conidiophores, chain formation etc.) were carried out after 7 days of colony development on PCA. In six isolates (4 from seed and one from both leaf and stem) dimensions of 100 conidia were measured.

Molecular characteristics

Molecular characteristics of the studied isolates were determined by DNA extraction from 100 mg mycelia using ”Plant DNeasy Mini Kit” (Qiagen). ITS1-5.8S-ITS2 region of ribosomal DNA was amplified using specific primers for fungi ITS1F (G a r d e s and B r u n s , 1993) and ITS4 (W h i t e et al, 1990). PCR reaction was performed in 30 µl of total volume following the protocol described by W h i t e et al. (1990). Prior to sequencing, DNA was purified using QIAquick PCR Purification Kit (Qiagen) following the instructions of the producer.

Phylogenetic analysis

Analysis of ITS sequences was performed using CLUSTAL W software (L a r k i n et al., 2007). Sequences were compared with a total of 18 ITS1-5.8S--ITS2 sequences obtained from GeneBank: 7 *Alternaria* species that belong to *A. tenuissima* group, as well as 6 sequences from 3 other *Alternaria* species. Accession numbers of all the sequences are given in the Table 2.

Pathogenicity test

Pathogenicity test of chosen isolates was performed on soybean seed and plants.

Inoculum for artificial inoculations of seed and plants was prepared on PDA, where isolates were grown for 7 days. In Petri dishes with colonies, 50 ml of sterile water was added in order to acquire conidial suspension. Concentration of conidia was not determined; however, their ability to germinate was tested prior to the inoculation.

Seed inoculation

Soybean seeds, cv. Sava, were surface sterilized in 1% sodium hypochlorite solution, for three minutes, rinsed two times in sterile water and then kept soaked in conidial suspension for two hours. Afterwards, seeds were placed on filter paper in 90 mm diameter Petri dishes. For every isolate, 4 Petri dishes were used with 10 seeds in each (four replications). Germination was done in incubator at 25 °C. After 7 days, a percentage of germinated healthy and rotten seeds were calculated. The controls were sterilized seed soaked in sterile water for two hours (K1), and unsoaked, dry, surface sterilized seed (K2).

Inoculation of plants

Adult plants, cv. Sava, were grown in vegetation shed in Micherlich's pots. Inoculation was performed by spraying the plants in their flowering stage (phenophase R_2) with conidial suspension using small hand sprayer. Isolates A118, A134, A136 and A147 were used. After inoculation, plants were covered with wet polyethylene bags for 4 days. Plants used for control were sprayed only with water. Symptom development was monitored and reisolations on PDA were carried out.

Tab. 2 – Molecular identifications of *Alternaria* spp. isolated from soybean in Serbia and other isolates of a different origin with GenBank accession numbers for ITS1-5.8S-ITS2 rDNA sequences that were used for the Clustal W comparison

No	Name of the isolate	Accession number	Plant material	Geo-graphic origin	Sequence homology (%)			
					A118	A134	A136	A145
1	*Alternaria* sp. strain A118*	HQ025971	*Glycine max*, steam	Serbia	-	99%	99%	94%
2	*Alternaria sp.* strain A134*	HQ025969	*Glycine max*, seeds	Serbia	99%	-	99%	94%
3	*Alternaria sp.* strain A136*	HQ025970	*Glycine max*, seeds	Serbia	99%	99%	-	94%
4	*Alternaria* sp. strain A145*	HQ025968	*Glycine max*, steam	Serbia	94%	94%	94%	-
5	*Alternaria mali* strain AMM	AF314582	/	China	99%	98%	98%	94%
6	*Alternaria alternata* strain SS-L6	GU797144	*Spiranthes sinensis*	China	99%	99%	99%	94%
9	*Alternaria alternata* strain 23	DQ156340	*Helianthus annuus*	India	99%	98%	98%	94%
10	*Alternaria brassicae*	FJ869872	*Lepidium draba*	USA	99%	98%	99%	94%
11	*Alternaria gaisen* strain EGS90-0512	AY762944	-	China	99%	99%	99%	94%
12	*Alternaria tenuissima* XSD-93	EU326181	*Huperzia whangshanensis*	China	99%	99%	98%	94%
13	*Alternaria tenuissima* isolate SMBA02	EU816392	Macrotermitinae fungus comb	France	99%	99%	99%	94%
14	*Alternaria tenuissima* strain IA287	AY154712	*Prunus avium* (leaves)	Iran	99%	99%	99%	94%
15	*Alternaria longipes* isolate T3664	EF104215	*Trifolium pratense*	USA	99%	99%	99%	94%
19	*Alternaria arborescens* isolate UASWS0332	EU098120	*Malus domestica*	Switzerland	94%	95%	94%	89%
20	*Alternaria solani*	HM242287	*Solanum lycopersicum*	India	93%	93%	93%	89%
21	*Alternaria solani* strain P4853	GU983658	-	USA	93%	93%	93%	89%
22	*Alternaria brassicicola*	AF201964	*Brassica* species	Thailand	93%	93%	93%	88%
24	*Alternaria carotiincultae* strain BMP0064	EU136641	*Daucus carota*	USA	96%	96%	96%	91%
26	*Leptosphaeria maculans*	AY531688	*Brassica napus*	Italy	81%	81%	81%	77%

Data analysis

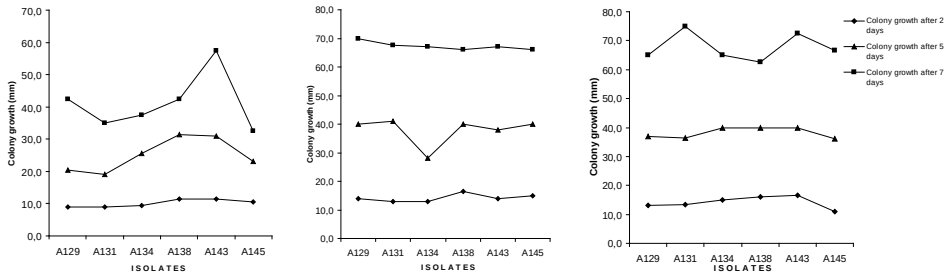
Data on pathogenicity of the tested isolates were analyzed with Statistica 9.1 using ANOVA and Duncan's Multiple Range Test (D u n c a n, 1955) with significance level of 1 and 5%.

RESULTS

Cultural characteristics

Development and the appearance of colonies of all 19 isolates were observed on Malt Agar, V8 Agar and Potato Carrot Agar. Some differences regarding the isolate growth intensity were observed (Chart 1 a, b and c). The weakest growth was noted on MA, where the average colony diameter was 42.9 mm after 7 days. On other 2 media, colony growth was more or less even and faster than on MA (average diameter on V8 and PCA was 66.8 mm and 66.1 mm after 7 days, respectively).

Graph. 1 – Growth of isolates on different nutritive medium after 2, 5 and 7 days



a) Malt Agar b) V8 Agar c) Potato Carrot Agar

Some differences were observed in colony appearance on different media. On MA, aerial mycelium was less abundant with less obvious zones. Colonies had loose aerial mycelia with two differently colored, poorly distinguished zones, dark olive grey to black in the centre, and lightly colored on margins. Substrate mycelium was radial and similarly colored as aerial with two weakly notable zones (Figure 1a).

Colonies formed abundant and compact mycelia with 2-4 dark and light concentric zones on PCA. Central part of the colony was greenish black, while the margin was grey (Figure 1b). Substrate mycelium was well developed and radial. Clearly observable concentric zones were formed, from dark olive greenish black in the center to greyish on the edge.

On V8, isolates form dense and compact aerial mycelia with 3-4 differently coloured concentric zones. Colour of mycelia varies from greenish black in centre to gray and black on margin (Figure 1c). The substrate mycelia is weakly developed with weakly distinguished zones dark gray in centre and lightly gray on margins.

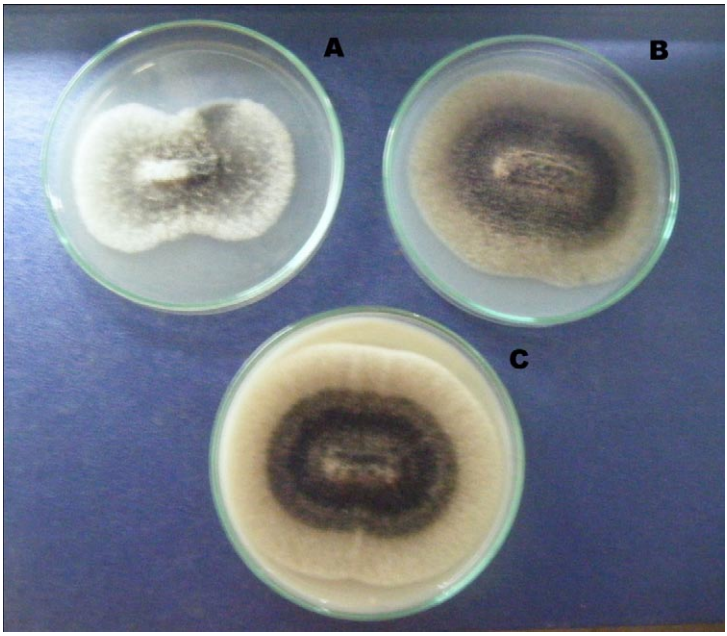


Fig. 1 – Appearance of colonies (isolate A138) on different nutritive medium
a) MA; b) PCA; c) V8

Morphological characteristics

Morphological characteristics of reproductive organs were observed through microscopic observations of isolates grown on PCA. Morphology of conidiophores, conidia and the structure of conidial chains were described. All isolates formed conidia in chains, which were mostly unbranched, only rarely sympodially branched (predominantly one lateral branch with 3-5 conidia in chain). There were 5-12 conidia in one chain (Figure 2).

Apical conidia in chain are smaller, oval without articulated beak and have no septae while they are young. In time, 2-3 transverse septae, and very rarely longitudinal, may form. Conidia that are closer to the base and conidiophores are elongate to elliptic with conspicuous beak. They most frequently have 2-7 transverse septae and rarely few longitudinal septae. Conidia that are in the middle of the chain are also elongate to elliptic, large with more transverse and longitudinal septae (Figure 3).

Some conidia have punctulate ornamental surface of the cell wall. Conidia are formed on simple, non-branched conidiophores of various lengths. Dimensions of conidia of 4 isolates from soybean seed and one isolate from both leaf and stem are shown in Table 3. Conidia from leaf are much smaller in size in comparison to conidia from seed and stem.

Tab. 3 – Dimensions of conidia of *Alternaria tenuissima*

Isolate	Organ of isolation	Dimensions (min-max)	Average size
A129	Seed	27.75-57.35 ×11.40-22.02	41.21 × 16.57
A131	Seed	24.17-54.03 × 10.35-18.95	38.14 × 15.31
A133	Seed	34.32-76.13 × 14.56-18.39	47.20 × 16.00
A134	Seed	36.00-75.64 × 13.71-17.71	47.80 × 14.00
A138	Seed	23.86-75.80 × 10.75-17.95	46.17 × 13.63
A143	Stem	23.84-67.34 × 11.87-18.81	41.28 × 14.82
A145	Leaf	14.92-44.15 × 7.30-14.76	26.19 × 10.85

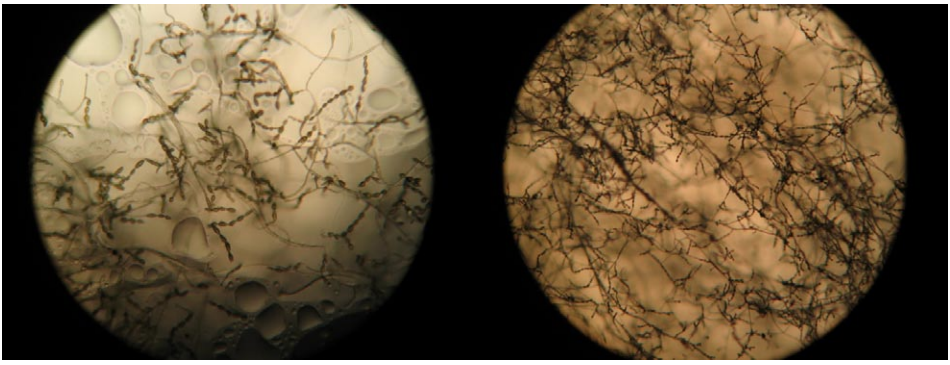


Fig. 2 – Appearance of conidial chains (isolate A-118); conidial chains (A-118) detail

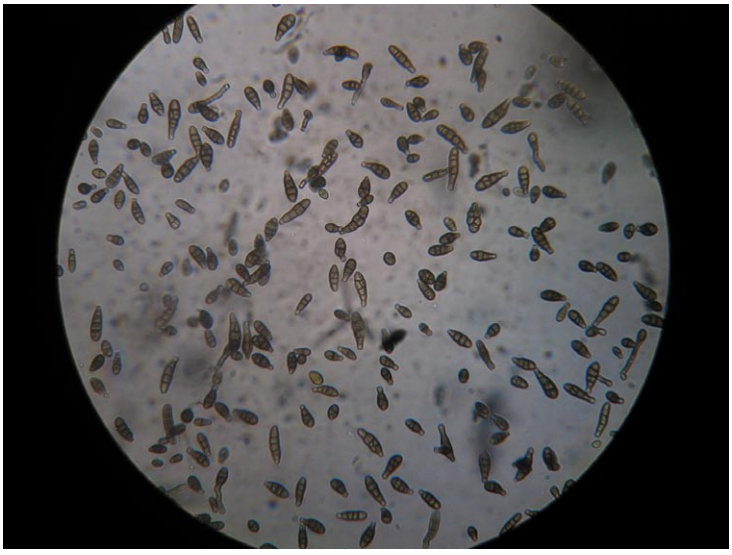


Fig. 3 – Conidia of *A. tenuissima* (different shapes and sizes)

Based on cultural and morphological characteristics, it can be concluded that the examined isolates are *Alternaria tenuissima*.

Molecular characteristics

Sequencing of the ITS1-5.8S-ITS2 rDNA gave products that ranged from 566 to 576 base pairs. Sequence homology of tested *Alternaria tenuissima* isolates was 99% with isolates that belong to *A. tenuissima* group (Table 2). In order to confirm this, isolate 147 should also be 99%, which cannot be observed from the table.

Pathogenicity test

Pathogenicity test on filter paper proved that all examined isolates were pathogenic to soybean seed (Table 4). Pathogenicity was manifested in the form of seed rot (Figure 4).

The percent of rotten seeds varied from 12.5 to 40.0% (Table 4) depending on *A. tenuissima* isolate. Isolates A118, A143 from stem, A139, A130, A131, A133, A134, A137, A138, A141 from seed and A144 from leaf caused significantly higher pathogenicity than the control K2 (surface sterilized, unsoaked, dry seed), while lower number of isolates expressed highly significant statistical differences in comparison to K2 (A118, A129, A130, A137, A138, A141, A144). The results from Table 4 show that there is a statistically significant difference in pathogenicity between the isolates.

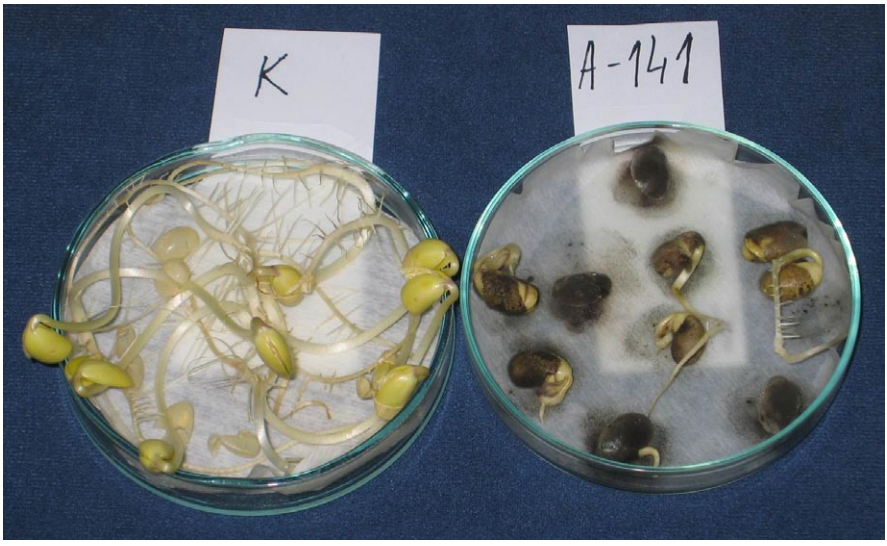


Fig. 4 – Pathogenicity test on filter paper

Tab. 4 – Pathogenicity of *Alternaria tenuissima* isolates on soybean seed

Isolate	Tissue of isolation	Average No of healthy seeds	Average No of rotten seeds	Average %	Rank	
					1%	5%
K1	-	9.0	1.0	10.0	bc	ab
K2	-	10.0	0.0	0.0	c	a
A118	Stem	6.8	3.3	32.5	ab	bc
A129	Seed	6.0	4.0	40.0	a	c
A130	Seed	7.0	3.0	30.0	ab	bc
A131	Seed	7.3	2.8	27.5	ab	abc
A132	Seed	8.0	2.0	20.0	abc	abc
A133	Seed	7.8	2.3	22.5	ab	abc
A134	Seed	7.3	2.8	27.5	ab	abc
A135	Seed	8.3	1.8	17.5	abc	abc
A136	Seed	8.8	1.3	12.5	bc	abc
A137	Seed	7.0	3.0	30.0	ab	bc
A138	Seed	6.8	3.3	32.5	ab	bc
A139	Seed	8.0	2.0	20.0	abc	abc
A140	Stem	8.0	2.0	20.0	abc	abc
A141	Seed	6.0	4.0	40.0	a	c
A142	Seed	8.8	1.3	12.5	bc	abc
A143	Stem	7.8	2.3	22.5	ab	abc
A144	Leaf	7.0	3.0	30.0	ab	bc
A145	Leaf	8.3	1.8	17.5	abc	abc
A146	Leaf	8.0	2.0	20.0	abc	abc

Tested isolates expressed low level of pathogenicity on the inoculated soybean plants. Symptoms, such as small necrotic spots, were observed on some leaves. Spots were round, 2-3 mm in diameter with light grey center and reddish to brown margin (Figure 5). Only few spots were formed on leaves, and they were not merging or causing leaf decay. Symptoms were not observed on stem and pod.

DISCUSSION

Fungi from *Alternaria* genus are frequently isolated from a large number of plant species. Most commonly, they are saprophytes or facultative pathogens (T h o m m a, 2003). More intensive occurrence of these fungi over the last couple of years could be explained by the fact that these pathogens attack weak plants, which have already been stressed (drought and high temperatures) under the influence of climatic changes (T h o m m a 2003; J e v t i ć and J a s n i ć, 2007; M i h a i l o v i ć et al., 2009). Soybean is also common host for the species of this genus. The most frequent species are usually *A. alternata* and *A. tenuissima*, which are considered as parasites of leaf, pods and soybean seed (S i n c l a i r and B a c k m a n, 1989; B a i r d et al., 2001;

Fig. 5 – Symptom on soybean leaf (artificial inoculation)

B r o g g i et al., 2007). In Serbia, species from *Alternaria* genus were isolated in high percentage from soybean plants, particularly seeds (M e d i ć-P a p et al., 2007; P e t r o v i ć and V i d i ć, 2010). However, determination of the isolated species has not been done so far, nor has detailed investigation on their cultural morphological, molecular or pathogenic characteristics been performed.

In this paper, cultural, morphological and molecular characteristics of collected *Alternaria* spp. isolates were studied and species was determined based on acquired results. Also, pathogenicity of isolates was tested on seeds and soybean plants.

The slowest growth was noted on Malt agar (MA), where colony diameter was 42.9 mm on average after seven days of incubation. On other two media (V8 and PCA), colony growth was uniform and faster with average diameter of 66.8 mm and 66.1 mm after 7 days, respectively. It was determined that examined isolates form unbranched, or rarely and poorly branched conidial chains, on short non-branched conidiophores. Conidia are dark in colour, multicellular with transverse and longitudinal septae, they are of different size and shape regarding the age and the place of formation in the chain. Classification of *Alternaria* species is mostly based on the type of conidial chain formation, while the size of conidia is less relevant due to great variability (T h o m a , 2003). Regarding all the tested characteristics, and in comparison with the results of other authors engaged in the field of taxonomy of genus

Alternaria (B a i r d, 2001; P r y o r and M i c h a i l i d e s, 2002; R a j a et al., 2005; S i m m o n s, 2007), it was concluded that the tested isolates from soybean seed and plants belonged to *Alternaria tenuissima*.

Correct identification of plant pathogenic *Alternaria* species demands combining classical methods that comprise morphological and cultural characteristics, and modern methods, such as molecular, which enable molecular systematization of the target population. Analysis of ITS rDNA proved that investigated isolates belonged to *A. tenuissima* group. ITS sequencing was used for determination of *Alternaria* isolates from pistachio, and considering the minimal variation in ITS sequences among some *Alternaria* spp., it was suggested that this region was inappropriate for taxonomic resolution of those species (P r y o r and M i c h a i l i d e s, 2002). However, previous research supported the use of ITS sequence analysis in resolving the genus *Alternaria* into species-groups correlating in morphology and differentiating among closely related species within groups (P r y o r and G i l b e r t s o n, 2000). Besides ITS sequencing, *Alternaria tenuissima* was determined by using AFLP (G a n n i b a l et al., 2007), microsatellite fingerprints (P r y o r et al., 2003) and RAPD-PCR (S h a f i q u e et al., 2009). However, all the analyses were conducted along with morphological, cultural and pathogenic characterization of the tested isolates, which proved these classical analysis to be inevitable for accurate determination of *Alternaria tenuissima* isolates.

As it was already mentioned, fungi of *Alternaria* spp. are present in high percentage in soybean seed in Serbia (M e d i ć - P a p et al., 2007; P e t r o v i ć and V i d i ć, 2010). However, their harmfulness in seed production is still unknown. The results obtained in this research show that *A. tenuissima* isolates in laboratory tests cause significant and highly significant seed rot. Variability within the population of this pathogen is presented. Degree of pathogenicity is not geographically related, nor does it depend on the location of isolation from the plant (soybean seed, stem or leaf). The tested isolates, regardless of their origin, manifest very low pathogenicity on leaf. Based on our observations from several years, in field conditions, symptoms on leaf are rare and have no economical significance. Obtained results indicate that, in favourable weather conditions, *A. tenuissima* could have economic significance in seed crop production (reduction of number of plants) and could influence decreased technological value of mercantile seed.

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ПАТОГЕНЕ МОРФОЛОШКЕ И МОЛЕКУЛАРНЕ ОСОБИНЕ *ALTERNARIA TENUISSIMA* СА СОЈЕ

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Резиме

Из биљака и семена соје током 2008. и 2009. године вршене су фитопатолошке изолације гљива из већег броја локалитета у Србији. На бази ових испитивања утврђено је да су у изолацијама највише заступљени били представници рода *Alternaria*. За даља проучавања одабрано је 19 изолата овог рода и то 13 са семена и по три са листа и стабла соје.

У циљу детерминације и карактеризације одабраних изолата детаљно су проучене одгајивачке морфолошке, молекуларне и патогене карактеристике.

Ради испитивања одгајивачких и морфолошких особина изолати су гајени на три храњиве подлоге: подлози од малц-агара (MA), V8 подлози и подлози од кромпир-шаргарепе-агара (PCA). Изглед колонија и репродуктивних органа гљиве описани су после седам дана инкубације на температури од 25°C.

Најслабији пораст проучавани изолати имају на подлози од малц-агара (MA) са просечним пречником колоније од 42,9 mm након седам дана инкубације. На остале две коришћене подлоге (V8 и PCA) пораст код свих изолата је био уједначен и бржи са просечним пречником од 66,8 mm kod V8 и 66,1 mm код PCA подлоге.

Утврђено је да проучени изолати формирају неразгранате или ретко слабо разгранате конидијске ланце на кратким неразгранатим конидиофорама. Конидије су крупне, тамно обојене, вишећелијске, издељене попречним и уздужним преградама, различитог облика и величине у зависности од старости и места формирања у ланцу. На бази одгајивачких и морфолошких особина (формирање и изглед конидијских ланаца, конидиофора и конидија) извршена је детерминација испитиваних изолата и утврђено је да ови изолати припадају врсти *Alternaria tenuissima*. Молекуларном идентификацијом применом секвенционирања ITS2-5. 8S-ITS2 потврђено је да изолати припадају *Alternaria tenuissima* групи.

Патогеност на семену је испитана помоћу теста на филтер папиру потапањем семена у суспензију конидија и на биљкама у фази цветања прскањем суспензијом конидија. Утврђено је да су сви испитивани изолати испољили патогеност на семену соје, која је износила 12,5-40,0 % трулих зрна. Неки изолати су показали статистички значајну или врло значајну патогеност у односу на контролу К2, док код мањег броја изолата није испољена статистички значајна разлика. Патогеност на листовима била је слабија и испољава се у виду ситних некротичних пега.

Добијени резултати указују да би *A. tenuissima* при повољним условима спољне средине могла да има економски значај у семенској производњи (редукција склопа биљака) и да умањи технолошку вредност меркантилног семена.

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MOLECULAR AND MORPHOLOGICAL DETERMINATION OF *COLLETOTRICHUM TRIFOLII* ISOLATES ORIGINATING FROM ALFALFA

ABSTRACT: *Colletotrichum trifolii* is a fungal pathogen responsible for anthracnose disease in alfalfa. The isolates of *C. trifolii* were obtained from diseased alfalfa stems collected from field in Serbia. It was determined by pathogenicity examination that four isolates (Luc-7, Luc-17, Luc-27, Luc-33) can cause stem lesions on inoculated alfalfa plant. Our isolates were compared using reference isolates of *C. trifolii* (CBS 158.83). Isolates on MA and CDA developed olive green to grey colonies with white margin, while the substrate got dark olive green color. Conidiophores were hyaline, varied in length and produced a succession of conidia apically. Conidia were hyaline, straight, rounded at the ends, and non-septated with average size 7.85x3.87 μm. Average sizes of appressoria were 7.5-16.5 x 5.5 x 8.9 μm. The polymerase chain reaction (PCR) with one set of specific primers was used for the detection of examined isolates of *Colletotrichum trifolii*. Amplification of desired DNA fragment (590 bp) was determined using specific primer pair TB3-F/TB3-R. Achieved results of amplification indicated that the isolates Luc-7, Luc-17, Luc-27, as well as CBS 158.83, had traits of *C. trifolli*. Amplification and band on about 430 bp appeared in isolate Luc-33, which also belonged to *C. trifolli* species.

KEY WORDS: *Colletotrichum trifolii*, alfalfa, isolates, specific primers

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most important and widely grown forage legume worldwide. One of the most desirable traits of alfalfa is its high nutritive quality as animal feed. Alfalfa is rich in proteins, vitamins and minerals, making it highly favorable for hay production and pasture for livestock, especially dairy cows. *Colletotrichum trifolii* is a fungal pathogen which causes anthracnose disease in alfalfa. Anthracnose causes significant losses in alfalfa

crops in Serbia (V a s i ć, 2007). Infected plants manifest symptoms on stems: straw-colored, brown-bordered, and diamond-shaped lesions in which black acervuli develop (S t u t e v i l l e and E r w i n, 1990). Under favorable conditions, these lesions become enlarged, coalesce, girdle, and kill one or more stems. The fungi then spread internally from lesions on stem bases into crown tissues. A bluish-black discoloration of invaded tissue characterizes the crown rot phase of the disease. Symptoms also include blackening and killing of petioles and formation of a shepherd's crook when the stem wilts and dies suddenly (Figure 1).

Fig. 1 – "Shepherd's crook" symptoms

As alfalfa is a perennial, the fungi can persist in stems and crowns of alfalfa grown in warmer areas and re-infect the surrounding plants when conditions are favorable. Anthracnose limits alfalfa production by affecting plant growth, plant vigor, forage yield and quality. Severe infection in susceptible alfalfa varieties can cause 25-30% losses in forage yield (B a r n e s et al., 1969).

MATERIALS AND METHODS

Isolates of *C. trifolii* originated from lesions found on stems of alfalfa sampled Serbia. Several isolates of fungi were obtained out of which four were selected for further observation. The fifth determined isolate, CBS 158.83, originated from the Netherlands. Five selected isolates of *Colletotrichum* spp. (Luc-7, Luc-17, Luc-27, Luc-33 and CBS 158.83) were submitted to intensive observation on nutritive media by methods of B a x t e r et al. (1983). Culture traits were compared on Malts Agar (MA) and Czapek-Dox Agar (CDA). Five replicates of each isolate per medium were incubated at 25 ^{0}C and described

on the tenth day of incubation. Linear increase in colony diameter was recorded as the mean of measurements along two diametrical axes, measured from the third to the tenth day. Texture of aerial mycelium, color of colony edges, zonation and formation of appressoria were described by H a w k s w o r t h and G r a h a m (1974). Presence or absence of seta in culture was determined according to the methods by S m i t h and B l a c k (1990). Possibilities of teleomorphic stage forming in the examined isolates were determined according to the method by B a x t e r et al. (1983).

Final determination of the tested *Colletotrichum trifolii* isolates was done by the molecular methods (PCR). DNA from mycelia of tested isolates was extracted by the method of D a y and S h a t t o c k (1997).

Necessary components were added to DNA extract. Detection of *Colletotrichum trifolii* species was carried out with the primers designed by C h e n and D i c k m a n (2002). Used primers were: TB3-F (5'-CGGAATTCATTAGTGCCAGCGGGTTG) and TB3-R (5'-CGGGATCCACGGAAGTTGTTGTCG).

Standard linear DNA (Fermentas, Lithuania) of the fragment size (in bp) (from the bottom: 100; 200; 300; 400; 500; 600; 700; 800; 900; 1031; 1200; 1500; 2000 and 3000) was used. Visualization of amplified products was performed by UV transillumination in the chamber. The appearance of bands in the gel at the appropriate position was considered as a positive reaction.

RESULTS

Morphological characteristics

All colonies on MA medium grew relatively fast, attaining a diameter of 45-77 mm in ten days. Margins were mildly sinuated, marginal zones were closely apressed with pale olive green color, becoming subfelty and alternating with concentric farinaceous bands towards the center, dark olive becoming mouse gray to blackish mouse gray (Figure 2). On the reverse side of the colonies, marginal zones were pale olive-buff becoming iron gray and dark olive-gray in alternating concentric zones in the center.

On CDA, colonies attained diameter of 35-71 mm in ten days and frequently radially folded. Margins were irregular, farinaceous, hair brown towards the central zone which was cinnamon – buff alternating with light cinnamon-drab segments, and benzo brown in the center (Figure 2). Reverse side of the colonies marginal zones were cartridge buff, becoming chaetura drab to chaetura black in segments, alternating with segments of benzo brown to hair-brown in the central zone.

Conidiomata on MA and CDA were small, irregular in size, usually pulvinate, brown to black, setose, formed in concentric bands, and 100-250 µm in diameter. Scattered sclerotia-like aggregations of submerged mycelium also occurred.

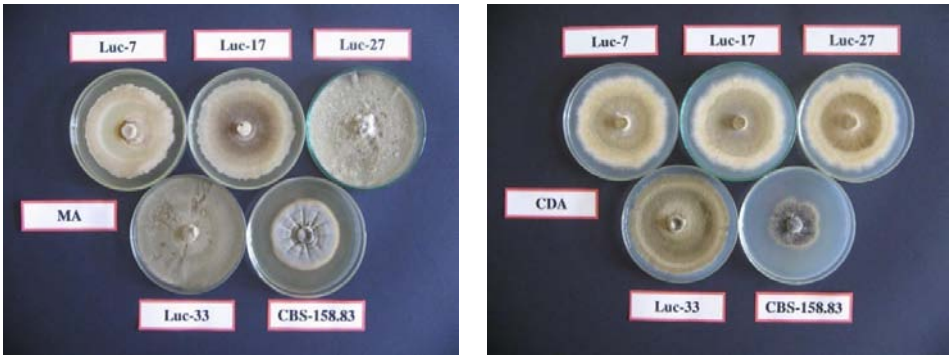


Fig. 2 – Colony morphology of *C. trifolii* isolates on MA and CDA media

Conidiogenus cells were robust, cylindrical, hyaline, becoming pale brown (17-21.5) – 25 x (4-4.1) – 4.5 µm. Conidia were short, cylindrical, relatively broad, both ends obtused mostly 7.85 x 3.87 µm (Figure 3). These traits were similar on both media (MA and CDA).

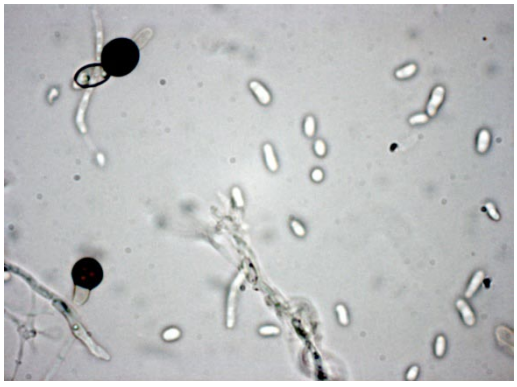


Fig. 3 – Colonies of isolate Luc-7

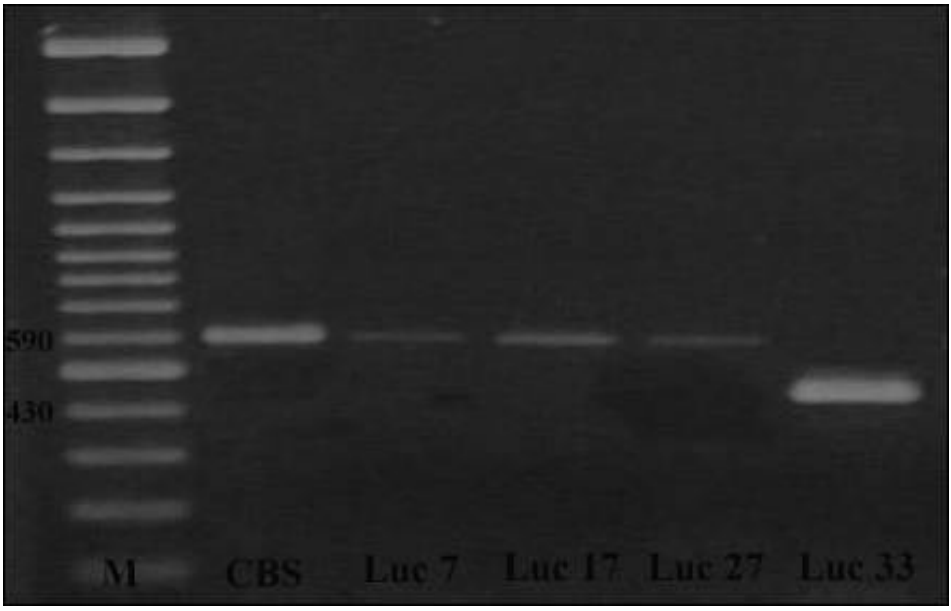
Initial hyphae often germinate on the apex, or their arms form apresoria or conidium. Apresories are ovoid to obovoid, at first luminous brown or hyaline; oil globes are formed in time, and outer walls are calloused and brown colored. Average dimension of apresoria is 7.5 -16.5 x 5.5 – 8.9 µm.

It was determined that the isolate Luc-17 and reference isolate CBS-158.83 formed setae in cultures, which were septated with 1-3 septs with dimensions 45.5 – 65.45 x 3.2 – 5 µm.

All the observed isolates of *Colletotrichum* spp. originated from Serbia, and the control isolate CBS 158.83 originated from the Netherlands and it did not form perithecia.

Molecular detection of the examined isolates of Colletotrichum trifolii

PCR method was performed for the final determination of the examined isolates of *Colletotrichum trifolii*. After the extraction of total DNA of tested isolates, PCR was performed with a pair of specific primers (TB3-F/TB3-R) designed for *Colletotrichum trifolii.* Visualization of the obtained products was carried out using electrophoretic separation in 1.5% agarose gel, staining with ethidium bromide, and the observation under UV light was performed on transilluminator. The results are shown in Figure 4.



Legend: M – length standard DNA (in bp from the bottom: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000 and 3000), CBS 158.83 – reference isolate *C. trifolii*; Luc-7, Luc-17, Luc 27 and Luc-33 – examined isolates.

Fig. 4 – Visualization of amplicons of size 590 bp and 430 bp, obtained using TB3-F and TB3 R primers on 1.5% agarose gel.

Amplification of the desired nucleic acid fragment of about 590 bp PCR with the primer pair TB3-F/TB3-R was done in four examined isolates Luc-7, Luc 17, Luc-27 and CBS 158.83. Pair of designed sequence-specific primers was applied for the amplification of *C. trifolii* in the ITS region C h e n and D i c k m a n (2002). Obtained results of amplification indicated that the isolates Luc-7, Luc 17, Luc-27 and the control isolate CBS 158.83 showed traits corresponding to the descriptions of *C. trifolii* in literature, thus proving to belong to this species.

Amplification and the appearance of bands at about 430 bp were observed in the isolates of Luc-33, which, according to their morphological and other

properties belong to type *C. trifolii*. This reaction could be caused by insufficient specificity or other lack of applied primers. By using other molecular techniques and genome sequencing, this reaction could be explained. In addition, determined molecular traits of Luc-33 isolates, which differ from the other isolates, may indicate the variability of isolates within individual species of the genus *Colletotrichum,* and are described in the literature (B a x t e r et al., 1983; F r e e m a n et al., 1998).

E l g i n and O s t a z e s k i (1982) reported the existence of two pathogenic races of *Colletotrichum trifolii*. A r i s s and R h o d e s (2007) described four races in the United States. The existence of differences between the members of the same species may have caused so many doubts over the historical development of taxonomy of the genus *Collerotrichum*.

DISCUSSION

Based on morphological traits of the tested isolates grown on MA and CDA media, it can be concluded that these isolates belong to *Colletotrichum trifolii*, which corresponds to the studies of B a x t e r et al. (1983), S t u t v e i l l e and E r w i n (1990), B a i l e y and J e g e r (1992).

Results obtained by amplification indicate that the isolates Luc-7, Luc 17, Luc-27, as well as the control isolate CBS 158.83, exhibit the characteristics that are in accordance with the descriptions of *C. trifolii* in literature, and that they, as such, really belong to this species.

Amplification and the appearance of bands at about 430 bp were present in isolates of Luc-33, which by all morphological and other properties belongs to type *C. trifolii*.

This reaction could be caused by insufficient specificity or other lack of applied primers. By using other molecular techniques and sequencing of the genome, this reaction could be explained. In addition, the determined molecular traits of isolate Luc-33, which to some extent differ from the other examined isolates, may indicate the variability of isolates within individual species of genus *Colletotrichum*. This has already been described in the literature (B a x t e r et al., 1983; F r e e m a n et al., 2000).

Three races of *C. trifolii* were determined in America and Australia: races 1, 2 and 4 (A r i s s and R h o d e s , 2007).

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МОЛЕКУЛАРНА И МОРФОЛОШКА ДЕТЕРМИНАЦИЈА ИЗОЛАТА *COLLETOTRICHUM TRIFOLII* ПОРЕКЛОМ СА ЛУЦЕРКЕ

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Резиме

Colletotrichum trifolii је фитопатогена гљива која проузрокује антракнозу на луцерки. Изолати *C. trifolii* коришћени у овом раду добијени су изолацијама са стабљика оболелих биљака луцерке пореклом из Србије. У тесту патогености изолати *C. trifolii* (Luc-7, Luc-17, Luc-27, Luc-33) су проузроковали типичне симптоме стабљичних лезија на тестираним биљкама. На основу морфолошких и молекуларних особина изолати из Србије поређени су са референтним изолатом (CBS 158.83) из Холандије који је детерминисан као врста *Colletotrichum trifolii*.

На MA и CDA подлогама испитивани изолати формирају ваздушну мицелију маслинасто зелене боје са рубом крем беле боје, док је у супстратном делу мицелија тамно маслинасто зелене боје. Конидиофоре су безбојне, различите дужине и формирају апикално конидије. Конидије су безбојне, праве са заобљеним крајевима, несептиране, димензија 7.85 x 3.87 µm. Димензије апресорија су 7.5-16.5 x 5.5 x 8.9 µm.

За молекуларну детерминацију испитиваних изолата *C. trifolii* коришћена је метода ланчане реакције полимеразе (PCR) са једним паром специфичних прајмера.

Амплификација жељеног фрагмента нуклеинских киселина величине око 590 bp вршена је применом PCR методе са TB3-F/TB3-R паром прајмера. Добијени резултати амплификације показују да испитивани изолати Luc-7, Luc-17, Luc-27 поређењем са изолатом CBS 158.83 припадају врсти *Colletotrichum trifolli*. Амплификација фрагмента нуклеинске киселине око 430 bp забележена је код изолата Luc-33, који такође припада врсти *C. trifolii*.

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THE INFLUENCE OF MINERAL AND BACTERIAL FERTILIZATION ON THE NUMBER OF FUNGI IN SOIL UNDER MAIZE

ABSTRACT: The aim of this study was to evaluate the influence of an application of different rates of mineral fertilizers and their combination with associative N-fixing Klebsiella planticola and Enterobacter spp., and sampling period on the number of fungi in Cambisol and grain yield of maize. The investigation was conducted on Mladenovac experimental station and in the Laboratory of Institute of Soil Science, Belgrade, during 2006. Unfertilized soil was used as the control soil. Each of the studied variants was carried out in three replications. The effect of the studied fertilizers was determined three times during the maize growing season, the number of fungi being determined by indirect dilution method on Czapek nutritive medium. The results of the study showed that all fertilization variants studied influenced, more or less, fungal growth in the study soil. However, the applied high content of mineral nitrogen, phosphorus and potassium, as well as their combination with bacterial inoculants brought about the highest increase in the number of fungi during all studied vegetation periods of maize. The highest increase in the number of soil fungi was registered in the second sampling period. The highest increase in the grain yield of maize was obtained by combined application of microbial inoculants and high rates of mineral NPK fertilizers.

KEY WORDS: fungi, maize, N-fixing bacteria, NPK fertilizers, soil

INTRODUCTION

The studies in the field of fertilization are mostly focused on the increase of the crops yield whereas the traits of the cumulative effect of fertilizers (the change of biological and chemical soil properties, the content of biogenic elements and heavy metals etc.) have often been disregarded. Regardless of their major role in crop productivity and soil fertility, the application of mineral fertilizers (particularly nitrogen) may induce a series of negative consequences from microbiological, economic and ecological aspects (Acosta – Martinez and Tabatabai, 2000). The problems concerned can be overcome by partial replacement of these fertilizers by application of microbial inoculants,

in order to inhibit or stimulate certain cellular processes, including mineralization ones, thus leading to the improvement of physico-chemical and biological soil properties (M i l o š e v i ć et al., 2003).

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, except for fungi (J e m c e v and Đ u k i ć , 2000; P e š a k o v i ć , 2007). With regard to the predominance of fungi in acid soils, it has also been suggested that their population number rises with more intensive application of the stated fertilizers. A large number of authors addressing this issue account for this rise in population density and activity of the majority of microorganisms in soil by limiting 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 favor of soil fungi (A c o s t a – M a r t i n e z and T a b a t a b a i , 2000; Z h a n g and W a n g , 2006; P e š a k o v i ć et al., 2008).

Fungi perform important functions in the soil related to nutrient cycling, disease suppression and water dynamics, all of which help plants become healthier and more vigorous. Along with bacteria, fungi are important decomposers of hard to digest organic matter. They use nitrogen in the soil to decompose residues rich in woody carbon and low in nitrogen, and to convert the nutrients in the residues into forms that are more accessible for other organisms (J e n k i n s , 2005).

Given the fact that soil fungi have evolved a complex enzymatic system that helps them transform chemical compounds that are not easily degradable (M a n d i ć, 2002), the underlying assumption of this study was that a change in their number may be used as a reliable indicator of soil biogeny.

Having in mind the above mentioned, the aim of this investigation was to examine the influence of different rates of mineral fertilizers [composite NPK (15:15:15)] and their combination with selected soil bacterial inoculants, and sampling period on the number of fungi in Cambisol and grain yield of maize.

MATERIALS AND METHODS

The investigation was conducted on Mladenovac experimental station of Institute of Soil Science, located 55 km from Belgrade in Serbia, during 2006. Mean monthly temperature and precipitation sum for the investigated period are presented in Table 1.

The studied soil type was Cambisol. The experiment was set up in a randomized block design with three replicates, based on the following variants: control (Ø, non-fertilized soil); 60 kg·ha^{-1} N and P_2O_5, and 40 kg·ha^{-1} K_2O (N1); 120 kg·ha^{-1} N, P_2O_5 and K_2O (N2); *Enterobacter* sp. strains + 60 kg·ha^{-1} N and P_2O_5, and 40 kg·ha^{-1} K_2O (ES+N1); *Enterobacter* sp. strains + 120 kg·ha^{-1} N, P_2O_5 and K_2O (ES+N2); *Klebsiella planticola* + 60 kg·ha^{-1} N and P_2O_5, and

Tab. 1 – Mean monthly temperature and precipitation sum for the study year (Belgrade Weather Bureau)

Month	Year 2006		Mean 1990-2006	
	Temperature (°C)	Precipitation (mm)	Temperature (°C)	Precipitation (mm)
January	-0.5	43.2	1.8	41.9
February	1.9	59.1	3.7	36.8
March	6.5	104.4	8.0	42.8
April	13.7	97.0	12.8	54.6
May	17.4	42.3	18.2	51.4
June	20.2	137.8	21.6	94.8
July	24.7	23.3	23.2	66.1
August	20.9	120.6	23.1	60.1
September	19.2	24.3	17.6	63.8
October	15.2	20.9	13.1	53.8
November	8.9	24.5	7.4	55.6
December	4.3	51.9	2.3	61.5
Mean	12.8	-	12.7	-
Total	-	749.3	-	683.2

40 kg·ha^{-1} K_2O (KP+N1); *K. planticola* + 120 kg·ha^{-1} N, P_2O_5 and K_2O (KP+N2). Maize (hybrid ZP-341, FAO 300) was used as a test plant.

Nitrogen fertilizer was applied in the form of urea with 46% N, phosphorus – in the form of monoammonium phosphate with 52% P_2O_5 and 11% N, and potassium – as 40% potassium salt (KCl).

The pure culture of an associative N-fixing bacterium *K. planticola* was obtained from the stock culture of the Microbiology Laboratory of Faculty of Agronomy (Čačak, Serbia), while the *Enterobacter* strains (KG-75 and KG-76) were obtained from the stock culture of the Microbiology Laboratory in the Center for Small Grains (Kragujevac, Serbia), where they have been isolated from the rhizosphere of wheat.

Pure liquid inoculums of *K. planticola* and *Enterobacter* spp. were made using fermentors with suitable nutrient broth and incubated with aeration for 48 h at 28°C ± 2. The inoculation of the soil under young formed plants of maize with 2-3 leaves was carried out using plastic haversack sprinkler with 300.00 cm^3/m^2 of diluted liquid bacterial inoculum, previously made by adding the tap water into pure bacterial liquid inoculum.

The preliminary observation of the soil studied included the analyses of the following soil chemical parameters: soil acidity – potentiometrically, using glass electrode pH meter; available phosphorus and potassium – spectrophotometrically and flame-photometrically, respectively, using Al-method by Egner-Riehm; humus content, after Tiurin's method, modified by S i m a k o v (Dž a m i ć et al., 1996); soil total N, using elemental CNS analyzer, Vario model EL III (N e l s o n and S o m m e r s, 1996).

The samples subjected to microbiological analyses were taken three times during the vegetation period of maize (intensive plant growth stage – 7-8 leaves, milk-waxy maturity stage, full grain maturity stage), from the plough soil layer (0-15 cm), using method of the scattered sampling according to V o j i n o v i ć et al. (1966).

The number of soil fungi was determined on Czapek nutritive medium, using indirect dilution method, by inoculation of the nutritive medium with decimal dilution of certain amount of soil suspension (S a r i ć, 1989). The duration of incubation was 5 days at 28°C ± 2. The analyses were performed in three replications, whereby the number of fungi was calculated on 1.0 g of absolutely dry soil.

The obtained microbiological data were analyzed by the method of variance analysis, using SPS Statistica Software. The significance of the differences between the study factors was compared by the LSD test at $P < 0.05$ and $P < 0.01$. The grain yield of maize was calculated at 14% moisture.

RESULTS AND DISCUSSION

1. Chemical properties of the study soil

The main chemical characteristics of the study soil are presented in Table 2. The soil is characterized by acid reaction, high available potassium and medium available phosphorus, humus and total nitrogen supply.

Tab. 2 – Main chemical characteristics of the studied Cambisol

Parameter		Mean	Standard deviation	Range
pH	nKCl	4.06	0.05	4.00-4.10
	H_2O	4.90	0.03	4.87-4.92
P_2O_5 (mg 100 g^{-1})		15.73	0.31	15.51-16.09
K_2O (mg 100 g^{-1})		25.30	0.30	25.08-25.65
Humus (%)		2.19	0.01	2.18-2.19
Total N (%)		0.136	0.005	0.132-0.141

2. Studying the effect of applied fertilizers on average number of soil fungi

The obtained experimental data on the average number of soil fungi inferred that the presence of this group of microorganisms in Cambisol depended on the fertilization variant used, as well as the studied sampling period (Table 3). It was determined that all fertilization variants studied stimulated the growth of fungi in the study soil. The highest and statistically highly significant ($P < 0.01$) stimulation of the growth of soil fungi was determined in the variant with high rates of NPK nutrients (N2) during all studied vegetation periods of maize, as well as in the variants where combination of the microbial inoculants and high rates of NPK fertilizers (variants ES+N2 and KP+N2) were applied. This trend

was notably observed in the second sampling period of the maize growing season, which was characterized by higher temperatures and moisture rates (Table 1). This was also determined by other authors (J e m c e v and Đ u k i ć , 2000). The fertilization variant x vegetation period interaction (A x B) during the study year showed that statistically highly significant stimulating effects of the variants with high rates of NPK nutrients on the growth of soil fungi did not significantly varied between the studied vegetation periods ($P > 0.05$), although they were more pronounced in the second sampling period.

Tab. 3 – The effect of fertilization variant (A) and sampling period (B) on average number of fungi (10^4 g^{-1} of an absolutely dry soil) in Cambisol under maize

Variant (A)		Ø	N1	N2	KP+N1	KP+N2	ES+N1	ES+N2	X̄B
Sampling period (B)	I	13.97	30.45	52.55	24.18	38.67	24.27	37.91	31.71
	II	15.73	32.48	54.88	27.24	38.94	26.70	38.48	33.49
	III	13.64	30.00	50.03	20.24	33.61	19.12	34.88	28.79
X̄A		14.45	30.98	52.49	23.89	37.07	23.36	37.09	31.33
LSD			A		B			A x B	
0.05			5.94		3.10			10.29	
0.01			7.90		4.13			13.69	

Generally speaking, the rise in fertilizer rate was accompanied by the rise in the number of soil fungi, which, to a 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 (M a n d i ć et al., 2004) and the incidence of toxic fungi (M i l o š e v i ć et al., 1993), whereby *Penicillium* species assume predominance (M a n d i ć , 2002; P e š a k o v i ć , 2007; P e š a k o v i ć et al., 2009).

3. Studying the effect of applied fertilizers on the yield of maize

The analysis of the grain yield of maize (Figure 1) showed that the highest increase was obtained by using combined application of bacterial inoculants and high rates of mineral NPK fertilizers, although it should be noted that with combined usage of bacterial inoculants and low rates of mineral NPK fertilizers higher yields were obtained, in comparison to the application of lower rates of mineral NPK fertilizers in the conditions of agricultural production typical for this study. Similar results for both constatations were also obtained in previous researches (D o b b e l a e r e et al., 2001; D a l l a S a n t a et al., 2004).

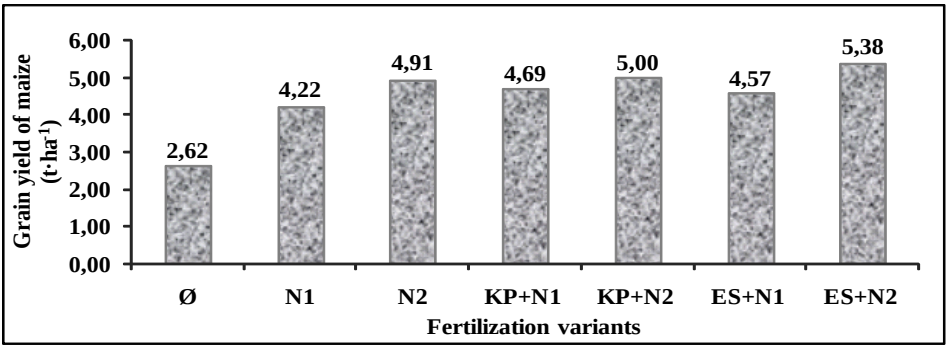

Fig. 1 – The effect of the fertilization variants on the grain yield of maize ($t \cdot ha^{-1}$)

CONCLUSIONS

The results of the study on the effects of an application of different rates of mineral fertilizers and their combination with associative N-fixing *Klebsiella planticola* and *Enterobacter* spp. on the number of fungi in Cambisol and grain yield of maize infer the following:

- the number of the studied group of microorganisms depended on the type and rate of fertilizers used, as well as the sampling period studied;
- the applied fertilizers brought about an increase in the number of fungi, particularly in the variants that included high content of nitrogen, phosphorus and potassium;
- the highest number of soil fungi was registered in the second sampling period;
- the highest increase in the grain yield of maize was obtained by combined application of bacterial inoculants and high rates of mineral NPK fertilizers.

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УТИЦАЈ МИНЕРАЛНИХ И БАКТЕРИЈСКИХ ЂУБРИВА НА БРОЈНОСТ ГЉИВА У ЗЕМЉИШТУ ПОД УСЕВОМ КУКУРУЗА

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Резиме

Циљ истраживања је био да се испита утицај примене различитих доза минералних ђубрива и њихових комбинација са асоцијативним азотофиксирајућим бактеријама *Klebsiella planticola* и *Enterobacter* spp., као и фазе узимања узорака на бројност гљива у земљишту типа гајњача и принос зрна кукуруза. Истраживања су извођена на експерименталном Огледном пољу Института за земљиште у Младеновацу, и у Лабораторији Института за земљиште у Београду, током 2006. године. Као контрола коришћено је неђубрено земљиште. Свака од анализираних варијаната била је заступљена у три понављања. Ефекат примењених ђубрива одређиван је три пута током вегетације кукуруза, а праћен је путем утврђивања бројности гљива индиректном методом агарних плоча на Чапековој хранљивој подлози. Резултати истраживања су показали да су све испитиване варијанте ђубрења у мањој или већој мери стимулисале развој гљива у проучаваном типу земљишта, али је примењена висока доза минералног азота, фосфора и калијума, као и њена комбинација са тестираним бактеријским инокулантима, условила највеће повећање бројности гљива током целог вегетационог периода кукуруза. Највећа бројност земљишних гљива је утврђена у другој фази узимања узорака. Највеће повећање приноса зрна кукуруза забележено је на варијантама на којима је примењена комбинација бактеријских инокуланата и високих доза NPK ђубрива.

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MICROBIOLOGICAL AND MYCOTOXICOLOGICAL CORRECTNESS OF PROTEIN FEED INGREDIENTS IN VOJVODINA

ABSTRACT: During 2009 and 2010, the microbiological tests of a total of 40 samples of protein feed ingredients (sunflower meal, soybean, soybean cake, soybean grits and soybean meal) originating from Vojvodina were carried out. The most of the samples (57.5%) matched the Serbian regulations on feed. Microbiologically, there was not adequate quality of protein ingredients, which was a consequence of the presence of pathogenic bacteria: *Proteus* spp. in 12 samples of sunflower meal, 1 sample of soybean meal and 2 samples of soybean cake, and *E. coli* in 2 samples of soybean meal. The highest total number of bacteria (1×10^7 g^{-1}) and the highest number of yeasts and molds (148.000 g^{-1}) was identified in one sample of sunflower meal.

Mycological analysis of protein feed established the dominance of species from the genera *Aspergillus* (*A. flavus, A. fumigatus, A. niger* and *A. ochraceus*), *Fusarium* (*F. solani, F. subglutinans* and *F. verticillioides*) and *Mucor* (*Mucor hiemalis* f. *hiemalis* and *M. racemosus* f. *racemosus*).

The study of biochemical characteristics of 10 fungal isolates from sunflower meal, soybean grits and cake has established that: a) 2 cultures of *Aspergillus* spp. possessed antagonistic activity against other fungal species, b) 1 isolate *F. solani* biosynthesized T-2 toxin, c) 1 culture of *F. subglutinans* produced zearalenone, d) 4 isolates of *Mucor* spp. showed the ability to degrade one or both trichothecenes of type A (diacetoxyscirpenol – DAS and T-2 toxin).

Mycotoxicological studies that included 24 samples of protein ingredients showed the absence of mycotoxins (aflatoxin B1, ochratoxin A, zearalenone, deoxynivalenol, DAS and T-2 toxin) in all 15 samples of sunflower meal. In the samples of soybean and its products (meal and cake) only T-2 toxin was detected in 3 analyzed samples. The amount of this fusariotoxin did not exceed 375 μg kg^{-1} .

KEY WORDS: protein ingredients, microbiological and mycotoxicological quality

INTRODUCTION

The most important objective in animal husbandry is the improvement of livestock production potential of domestic animals, and obtainment of high

quality products. The quality of fodder is one of the basic conditions for achieving these goals. It does not only involve the optimal nutritional and food safety (microbiological quality), but also the absence of harmful substances.

One of the main components of feed mixtures, protein ingredients, such as soybean products and sunflower meal, represent suitable substrates for growth of mycobiota and biosynthesis of their toxic metabolites, mycotoxins. Inadequate quality of feed components leads to inadequate quality of feed mixture. Reason for this lies in the fact that, besides the strict use of determined fodder recipes, sometimes the satisfactory quality of the mixture cannot be obtained and, therefore, desired growth and animal health cannot be achieved (Đ o r đ e v i ć and P e t r e v s k i , 2008). Accordingly, the aim of this study was to investigate the microbiological and mycotoxicological correctness of protein feed ingredients originating from the region of Vojvodina, as well as to test the specific biochemical characteristics of fungal isolates from the same feed samples (antagonism, the ability to biosynthesize mycotoxins and the ability to biotransform mycotoxins).

MATERIALS AND METHODS

Samples. Total number of 40 samples of protein feed ingredients was analyzed in the accredited laboratory of "Bio-Ecological Center" Ltd. in Zrenjanin from the second half of 2009 and during 2010. All tested samples (17 samples of feed components based on soybean, and 23 of sunflower meal) originated from the region of Vojvodina (Serbia).

Microbiological investigations were performed according to the *Regulations on maximal quantity of harmful substances and ingredients in fodder* (T h e O f f i c i a l G a z e t t e o f S R Y , No. 2/1990) and *Regulations on the quality of feed* (T h e O f f i c i a l G a z e t t e o f R S , No. 4/2010). Total counts of bacteria, molds and yeasts, as well as identification of pathogenic microorganisms (*E. coli,* coagul. positive *Staphylococcus* spp., *Proteus* spp., *Salmonella* spp*.,* sulphite-reducing *Clostridium* spp.) were done in accordance with the *Regulations on methods of microbiological analysis and superanalysis of foodstuffs* (T h e O f f i c i a l G a z e t t e o f S F R Y , No. 25/80). Identifications of fungi were performed according to D o m s h et al. (1980), and 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).

Mycotoxicological investigations. The presence of aflatoxin B1 (AFL B1), ochratoxin A (OTA) and zearalenone (ZON) was determined according to the *Regulations on sampling methods and methods of physical, chemical and microbiological analysis of fodder* (T h e O f f i c i a l G a z e t t e o f S F R Y , No. 15/1987), while deoxynivalenol (DON), 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).

Analyses of biochemical properties of 10 selected fungal isolates from protein feed ingredients included testing of ability for mycotoxin biosynthesis and degradation of certain mycotoxins *in vitro.*

Fungal potentials for the production of fusariotoxins (DAS, T-2 toxin, DON and ZON) were tested according to the rapid screening procedure by Filtenborg-a et al. (1983), modified by Bočarov-Stančić et al. (2009). Cultivations of microorganisms were performed on the media: YESA (2% yeast extract, 15% sucrose and 2% agar, pH 6.5), PPSA (2% peptone-1, 15% sucrose and 2% agar, pH 6.5), and PDA (potato-dextrose agar, pH 6.9) for fourteen days at 27±1°C.

Potentials of mycobiota for degradation of OTA, DAS and T-2 toxin were tested by a simple screening method described by Filtenborg et al. (1983), modified by Bočarov-Stančić et al. (2010). Test fungi were grown on modified Vogel's medium N (pH 6.3) during 14 days at 27±1 °C (Vogel, 1956). The medium was supplemented with the extracts of crude toxins (final concentration 0.02 mg mL^{-1}) as the sole sources of C atoms. The modification of the minimum Vogel's medium consisted of the exclusion of the solution of biotin and addition of peptone (1 g L^{-1}) and yeast extract (2 g L^{-1}).

RESULTS AND DISCUSSION

The majority of tested samples (57.5%) had satisfactory microbiological quality which was in accordance with the *Regulations on maximal quantity of harmful materials and ingredients in fodder* (The Official Gazette of SRY, No. 2/1990) and *Regulations on the quality of feed* (The Official Gazette of RS, No. 4/2010)**.** The maximum number of bacteria – BCFU (1 x 10^7 g^{-1}), as well as yeasts and molds – MYCFU/g (148,000 g^{-1}), was detected in one sunflower meal sample, while the lowest number of microorganisms (120 BCFU g^{-1} and 10 MYCFU g^{-1}) was recorded in a soybean grit sample.

Tab. 1 – Microbiological quality of protein feed ingredients

Sample type	No. of samples	BCFU/g (aver. value)	MYCFU/g (aver. value)	Adequate quality (%)
Soybean	1	3.700	5.200	100.0
Soybean cake	7	146.000	160	71.4
Soybean grits	8	23.000	480	62.5
Soybean meal	1	107.000	3.400	100.0
Sunflower meal	24	3.400.000	13.000	47.8
TOTAL	40	120 – 10.000.000	10 – 148.000	57.5

Microbiologically inadequate quality of protein feed ingredients was a consequence of the presence of pathogenic bacteria. *Proteus* spp. were found in 12 samples of sunflower meal, 1 sample of soybean grits and 2 of soybean cake, while *E. coli* was identified in 2 samples of soybean meal.

Samples of protein feed ingredients, especially sunflower meal, that were analyzed in the second half of 2009 and in 2010 (Table 1), had higher total count of microorganisms (BCFU g^{-1} and MYCFU g^{-1}) and frequent presence

Tab. 2 – Dominant mycobiota in protein feed ingredients

Ord. no.	Fungal species	Soybean feed	Sunflower pellet
1.	*Aspergillus flavus*	40	60
2.	*A. fumigatus*	20	20
3.	*A. ochraceus*	20	20
4.	*A. niger*	-	20
5.	*Aspergillus* spp.	20	60
6.	*Fusarium solani*	10	-
7.	*F. subglutinans*	20	20
8.	*F. verticillioides*	40	20
9.	*Fusarium* spp.	-	20
10.	*Mucor hiemalis* f. *hiemalis*	10	-
11.	*M.racemosus* f. *racemosus*	10	-
12.	*Mucor* spp.	40	60
13.	*Penicillium* spp.	20	60
14.	*Rhizopus stolonifer*	20	20
15.	*Scopulariopsis* sp.	-	20

of pathogenic bacteria was observed, more frequent than during the first part of 2009 when our previous research of feed ingredients and fodder mixtures was done (Miljković et al., 2009).

Mycological analysis of protein feed ingredients (Table 2) established the dominance of mold species belonging to the following genera: *Aspergillus* (*A. flavus, A. fumigatus, A. niger* and *A. ochraceus*), *Fusarium* (*F. solani, F. subglutinans* and *F. verticillioides*) and *Mucor* (*Mucor hiemalis* f. *hiemalis, M. racemosus* f. *racemosus* and *Mucor* spp.).

The presented results are in agreement with our previous research that has revealed the dominance of the same fungal genera in the sunflower seeds and sunflower meal (Bočarov, 1983; Bočarov-Stančić et al., 2006) and protein ingredients (Miljković et al., 2009), as well as with researches done by other authors from Vojvodina (Škrinjar and Karlović, 2001; Škrinjar et al., 2007).

According to foreign authors, the predominant species in the sunflower seeds in Spain were *Penicillium* spp., *Aspergillus niger*, *A. flavus*, *A. glaucus* and *Rhizopus* spp. (Jimenez et al., 1991). In soybeans from Egypt, El-Kadi and Youssef (2007) noted most often the presence of the representatives of the genera *Aspergillus*, *Penicillium*, *Mucor* and *Rhozopu*s.

The study of biochemical characteristics of 10 fungal isolates from sunflower meal, soybean meal and cake established that: a) 2 cultures of *Aspergillus* spp. possessed antagonistic activity against other fungal species, b) 1 isolate *F. solani* biosynthesized T-2 toxin, c) 1 culture of *F. subglutinans* produced zearalenone, d) 4 isolates of *Mucor* spp. showed the ability to degrade one or both trichothecenes of type A (DAS and T-2 toxin).

Tab. 3 – Biochemical characteristics of fungal cultures isolated from protein feed ingredients

Ord. no.	Fungal species	Isolate origin	Isolate design.	Biochemical characteristics
1.	*Aspergillus flavus*	SG	675/09	Do not degrade OTA
2.	*Aspergillus* sp.	SC	366/09	Antagonist of other fungi
3.	*Aspergillus* sp.	SC	419/09	Antagonist of other fungi
4.	*Fusarium solani*	SG	162/09-A	Producer of T-2 toxin
5.	*F. subglutinans*	SC	134/09-B	Producer of ZON
6.	*F. verticillioides*	SP	134/09-A	Do not produce T-2, DAS, DON ZON
6.	*Mucor hiemalis* f. *hiemalis*	SP	1216/09	Biotransform DAS
8.	*M. racemosus* f. *racemosus*	SP	1215/09	Biotransform DAS and T-2
9.	*Mucor* sp.	SP	46/10	Biotransform DAS and T-2
10.	*Mucor* sp.	SP	675/10	Biotransform DAS

Legend: SG – soybean grits; SC– soybean cake; SP – sunflower meal

Mycotoxicological studies of 24 samples of protein feed ingredients showed the absence of mycotoxins (AFL B1, OTA, ZON, DON, DAS and T-2 toxin) in all 15 tested samples of sunflower meal. In the samples of soybean and its products (meal and cake) only T-2 toxin was detected in 2010, in 3 cases, in a quantity not exceeding 375 μg kg^{-1}. All samples that were tested satisfied the *Regulations on maximal quantity of harmful materials and ingredients in fodder* (The Official Gazette SRY, No. 2/1990) and *Regulations on the quality of feed* (The Official Gazette of RS, No. 4/2010)**.**

If we compare the obtained micotoxicological results with the results presented in Table 3, it is obvious why most of the tested samples showed absence of mycotoxins. All isolates of the genus *Mucor* from sunflower meal had the ability to biotransform one or both types of A trichothecenes. On the other hand, the only fungal isolate (*Fusarium solani* 162/09-A), that biosynthesized T-2 toxin, was derived from sunflower meal. This is why it was not surprising that the same fusariotoxin was found only in the samples of soybean products (Table 3).

In contrast to the reported results, our previous studies carried out in the period 1991-2000 (Bočarov-Stančić et al., 2001) showed a significantly higher contamination of protein feed ingredients from Vojvodina region with T-2 toxin (25% of soybean meal samples and 50% of sunflower meal samples). In addition to the frequent presence of T-2 toxin and ZON, Lević et al. (2004) reported that in some years soybean, sunflower seed and their products could be significantly contaminated with other mycotoxins, such as aflatoxin and ochratoxin A. On the other hand, Valenta et al. (2002) found 50 samples of soybean cake from Germany with very low concentrations of AFL B1 (0.41 μg kg^{-1}), OTA (max. 1 μg kg^{-1}) and ZON (max. 1μg kg^{-1}).

CONCLUSIONS

Most of the tested samples of protein feed ingredients (57.5%) had satisfactory microbiological quality, and all 100% had satisfactory mycotoxicological quality (having in mind the maximum allowed concentrations of harmful substances) according to the *Regulations on maximal quantity of harmful materials and ingredients in fodder* (The Official Gazette of SRY, No. 2/1990) and *Regulations on the quality of feed* (The Official Gazette of RS, No. 4/2010)**.**

Microbiologically inadequate quality of 42.5% of protein feed samples was a consequence of the presence of pathogenic bacteria *Proteus* spp. and *E. coli*.

Biochemical testing of fungal isolates from protein feed ingredients has shown that 2 isolates of *Aspergillus* spp. were the antagonists of other fungal species, 2 representatives of the genus *Fusarium* biosynthesized mycotoxins (*F. solani* – T-2 toxin, and *F. subglutinans*- ZON), and that all 4 cultures of *Mucor* spp. were able to biotransform DAS and/or T-2 toxin.

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МИКРОБИОЛОШКА И МИКОТОКСИКОЛОШКА ИСПРАВНОСТ ПРОТЕИНСКИХ ХРАНИВА У ВОЈВОДИНИ

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Резиме

Током 2009. и 2010. године микробиолошким испитивањима је било обухваћено укупно 40 узорака протеинских хранива (сунцокретова сачма, сојино зрно, сојин гриз, сојина погача и сојина сачма) пореклом из Војводине. Хигијенска исправност је констатована код 57,5% тестираних узорака. Микробиолошка неисправност протеинских хранива је била последица присуства патогених бактерија: *Proteus* spp. код 12 узорака сунцокретове сачме, 1 сојиног гриза и 2 сојине погаче, односно *E. coli* код 2 узорка сојине сачме. Највећи укупан број бактерија (1×10^7/g) као и највећи број квасаца и плесни (148.000/g) констатовани су код једног узорка сунцокретове сачме.

Миколошки анализе протеинских хранива су установиле доминантност врста из родова *Aspergillus* (*A. flavus, A. fumigatus, A. niger* и *A. ochraceus*), *Fusarium* (*F. solani, F. subglutinans* и *F. verticillioides*) и *Mucor* (*Mucor hiemalis* f. *hiemalis* и *M. racemosuus* f. *racemosus*).

Испитивањем биохемијских карактеристика 10 гљивичних изолата из сунцокретове сачме, сојиног гриза и погаче утврђено је да: а) 2 културе *Aspergillus* spp. поседују антагонистичку активност према другим врстама гљива; б) 1 изолат *F. solani* биосинтетише Т-2 токсин; ц) 1 култура *F. subglutinans* производи зеараленон, док су д) 4 изолата *Mucor* spp. испољила способност разлагања било једног или оба трихотецена типа А (диацетоксисцирпенол – ДАС и Т-2 токсин).

Микотоксиколошким истраживањима 24 узорка протеинских хранива утврђено је одсуство микотоксина (афлатоксин Б1, охратоксина А, зеараленон, дезоксиниваленол, ДАС и Т-2 токсин) код свих 15 узорака сунцокретове сачме. У узорцима зрна соје и њених прерађевина (гриз и погача) детектован је само Т-2 токсин код 3 узорка у количини која није превазилазила 375 μg/kg.

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DIAGNOSIS OF MYCOTOXICOSES IN VETERINARY MEDICINE

ABSTRACT: The problem of mycotoxin presence in animal feed and the consequences that arise due to this, represent a great challenge for anyone encountering them. In the chain which includes studies from prevention to treatment, a very important place and a frequent source of confusion is the process of diagnosing diseases caused by mycotoxins. The aim of this paper is to present a long experience of the team of experts at the Institute of Veterinary Medicine of Serbia in Belgrade, who follows this issue in terms of clinical manifestations of mycotoxicoses in different animal species, pathomorphological and pathohistological changes that characterize them, and laboratory analysis of feed which is the source of those biological hazards and natural contaminants. Based on the findings it could be concluded that mycotoxin contamination is common. Although these levels usually do not exceed the limits laid by the legislation, considering the cumulative effects and possible chronic exposure of animals to their harmful influence, appropriate and competent approach is necessary. Namely, even when direct losses, such as animals' mortality, are not present, indirect losses, due to a drop of animal performances and production, as well as the occurrence of secondary infections, should not be neglected.

KEY WORDS: clinical signs, mycotoxicological feed analysis, histological changes

INTRODUCTION

Mycotoxicoses are nutritionally-medical (Ožegović and Pepeljnjak, 1995), but also a diagnostic problem (Hoerr and Thruston, 1986), because some mycotoxins cause changes in multiple organs and other diseases. Besides, mycotoxicoses can cause similar clinical signs or lesions. Scientists do not know yet how many mycotoxins really exist, even though more than 250 have been detected. They represent different kinds of chemicals, and as for their toxicological characteristics, they have not been fully determined for many of them.

Diseases caused by mycotoxins are not contagious. They are related to feed and/or specific feeding stuffs; they are similar to vitamin deficiencies, they cannot be treated with antibiotics or other drugs, and they do not cause

the immune response of the organism because of their low molecular weight. This leaves animals permanently unprotected from these mycotoxin effects. Low content of mycotoxins in feed and/or food in practical terms often causes the appearance of chronic mycotoxicoses (H u m p h r e y s, 1988). The effect of lower mycotoxin amounts over a long period of time has the same effect as the one caused by larger quantities during a short period.

Modern agricultural practices reduce the occurrence of acute mycotoxicoses with high mortality rate. Chronic mycotoxicoses are often suspected when clinical signs include poor performance, ill thrift, or increased incidence of infectious diseases. Diagnosing mycotoxicoses is not always easy. Exposure cannot be established by detection of mycotoxins in tissues from animals suspected of being poisoned by mycotoxins because analytical services for detection of mycotoxins in animal tissues are not commonly available. Therefore, we must rely on the detection of mycotoxins in grain or feed in order to help establish the diagnosis.

Investigation of suspected mycotoxicoses should begin by obtaining thorough histories. Clinical signs are very important because they can be used to select appropriate diagnostic tests to help confirm or refute mycotoxicoses. Investigation of suspected mycotoxicoses should include histopathological examination of tissues from affected animals whenever possible. Specific histopathological lesions may be evidence of mycotoxicoses. If there are no lesions or any kind of evidence of pathology in organs known to be affected by mycotoxins, then the possibility of mycotoxicosis presence is reduced.

Confirmation of suspected mycotoxicoses is assisted by either reproducing the clinical disease during a feeding trial, by using the suspected ration, or by detection of a known mycotoxin in feed or tissues of animals consuming the feed. Feeding trials are not routinely performed because they are difficult to conduct, expensive, and slow in providing the results. Detection of known mycotoxins in feeds is relatively easy with modern analytical chemical methods.

One of the most challenging aspects of diagnosing the cases of suspected mycotoxicoses is collecting the feed sample that would adequately represent the feed suspected of being contaminated. Mycotoxins are not evenly distributed in feeds, so the contaminated part of the feed may be consumed before disease is evident. The collection of meaningful feed specimens under such conditions is very difficult.

Determining the significance of mycotoxins present in the ration is difficult. If a mycotoxin is detected in feed, the disease it causes should match the clinical syndrome observed in the particular case, and its concentrations should be sufficient to cause mycotoxicosis. If not, it is unlikely that the presence of the mycotoxin in the feed is significant.

Treatment of animals suffering from mycotoxicoses is usually not very effective. Antidotes for mycotoxins are generally not available. Stopping and preventing further exposure by removing contaminated feed are important. It is not uncommon for animals to recover after they quit eating feed, or known, to be contaminated with mycotoxins. Such an occurrence supports the suspi-

cion of the feed as a causal factor, but does not prove that the animals suffered from mycotoxicosis (C a r l s o n and E n s l e y, 2003).

From the toxicological and economical point of view, five mycotoxin groups are currently significant worldwide. These are aflatoxins and ochratoxin A, produced mainly in storage conditions by *Aspergillus* and *Penicillium* fungi, and trichothecenes, zearalenone, and fumonisins produced by *Fusarium* species which contaminate cereals in the fields. Depending on dosage and time of exposure to mycotoxins, animals' intoxication may be acute, caused by high doses of mycotoxin intake, or chronic, resulting from long term low dose of mycotoxin intake (T r e n h o l m et al., 1985).

AFLATOXIN

Aflatoxins are products of secondary metabolism of molds *Aspergillus flavus* and *Aspergillus parasiticus*. However, there are opinions that other strains and species also have the ability to synthesize these toxins in the traces. On the basis of the test results, we determined that the grains originating from Serbia rarely contain aflatoxin in such a high concentration that it could cause acute aflatoxicosis (J a k i ć – D i m i ć et al., 2009b; 2010).

There are five important aflatoxins called aflatoxin B_1, B_2, G_1, G_2, and M_1. Aflatoxin M_1 is a metabolite of aflatoxin B_1 found in milk and urine. It is formed after aflatoxin B_1 enters the body. It is not found in feed. Aflatoxin B_1 is most frequently found in the highest concentrations in naturally contaminated feed.

Aflatoxin is a liver poison (hepatotoxin) that causes liver damage at higher doses and liver cancer at lower doses in all species that consume it. Aflatoxin exposure can depress the immune system. According to the relative toxicity (LD_{50}) the most susceptible animals are ducks and turkeys (0.3-0.6 mg/kg TM), then pigs (0.6 mg/kg TM) and ruminants (0.2 mg/kg TM), while hens are the most resistant (6.5-16.5 mg/kg TM) (L e e s o n et al., 1995).

Clinical signs of aflatoxicoses

Acute exposure, all species: Depression, anorexia, reduced gain or milk production, subnormal body temperature.

Chronic exposure, poultry: Decreased growth rate, reduced feed efficiency, steatorrhea (fat in feces), bruising.

Chronic exposure, swine: Anorexia, unthriftiness, slow growth, icterus, mild anemia, ascites, increased susceptibility to infection.

Chronic exposure, cattle: May slow rumen motility for 24 to 48 hours.

Lesions of aflatoxicosis

Acute exposure: Hemorrhage, ascites.

Chronic exposure: Pale, soft, clay-colored liver, mild anemia, icterus, ascites.

Histopathological: Hepatocyte degeneration and necrosis; centrilobular hemorrhagic hepatic necrosis, fatty changes and regeneration of hepatocytes; bile duct epithelial proliferation progressing to interlobular fibroplasia and extensive proliferation; Karyomegaly, atypical nuclei, hepatocytic vacuolization, bile retention.

Diagnostic aids for aflatoxicosis

Blood workup: Check for anemia, elevated liver enzymes, serum bile acids, albumin: globulin ratio; prothrombin activity. Tissue or fluid analysis: Aflatoxin M1 present in milk or urine; parent compound may be present in kidney or liver.

Grain or feed analysis: Among the grains originating from Serbia, aflatoxins are most likely to be present in corn. They are not likely to be present in forages or silage at significant concentrations. Dietary aflatoxin concentrations at which performance or clinical effects become noticeable depend upon species and effect. Decreased performance may occur at concentrations as low as 200 ppb in young, sensitive species. Immunity may become impaired at concentrations of about 200 ppb. Hepatic lesions may become noticeable at 200 to 400 ppb. Clinical illness may become obvious at about 400 ppb. Generally, ruminants are most resistant and swine and avian specimens least resistant to adverse effects. Violative residues in milk can occur at concentrations at or near 50 ppb aflatoxin (Carlson and Ensley, 2003). Pet food is also subject to regular control, having in mind the sensitivity and a few almost epidemic outbreaks of aflatoxicosis of these species (Garland and Reagor, 2007).

OCHRATOXIN

Ochratoxin A is the major mycotoxin of this group and it is produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum* (C A S T, 2003). Ochratoxin is mostly resorbed in the front parts of the digestive tract and, via bloodstream, it first reaches the kidneys and liver, and, to a lesser extent, the muscles where it also deposits. Excretion is mainly carried out through the urine and feces, while the elimination by milk is minimal. Excretion by eggs has also been noted (N e d e lj k o v i ć – T r a i l o v i ć, 2000).

Mice, rats, hamsters and chickens are sensitive, while pigs are resistant. Carcinogenic properties of ochratoxin exhibit high frequency of renal adenomas and cancer, especially in males, with frequent metastases in the liver and lymph nodes, while females suffer from multiplication of fibroadenoma in milk glands. Ochratoxin A exerts immunosuppressive activity (L e e s o n et al., 1995).

Toxic effects depend on the species and animal age, gender, health status, degree of contamination and the length of ingestion of contaminated feed. Toxicity is significantly affected by the form of toxins. LD50 for ducks is approximately 0.5 mg/kg of BW (to 3 mg/kg BW), for goats 3 mg/kg of BW,

pigs 6 mg/kg of BW, Beagle dogs 9 mg/kg of BW, cattle 13 mg/kg of BW, and female and male rats 21.4 and 30.3 mg/kg of BW, respectively.

Clinical signs of ochratoxicoses

Acute exposure: anorexia, fever, diarrhea and uremia. Typical symptoms, especially in pigs, are polidipsia and poliuria with glucozuria.

Chronic exposure: nonspecific clinical picture is characteristic with retard growth, decreased body weight, lower feed consumption and increased feed conversion.

Pathomorphological changes are primarily in the kidneys and liver. The kidneys are enlarged and pale (like boiled), and liver is fatty and degenerated.

Diagnostic aids for aflatoxicosis

Biochemical parameters: total protein level and albumin are the most sensitive indicator of acute ochratoxicosis. Also, by urine testing, besides the presence of OA and its metabolites, lower pH and the presence of protein may be determined (N e d e lj k o v i ć – T r a i l o v i ć, 2000).

A significant feature of ochratoxin is that it occurs in a wide variety of commodities such as different grains, soy products, coffee and raisins, in varying amounts but at relatively low levels (C A S T, 2003). Also, the results of feed mycotoxicology tests in our country showed low concentrations of ochratoxin (J a k i ć – D i m i ć et al., 2009a; J a k i ć – D i m i ć et al., 2010.) However, the levels may accumulate in body tissues and fluids, which together with slow elimination of ochratoxin from the body contribute to the development of harmful effects (R i c h a r d, 2007).

FUMONISIN

The most common are the B series fumonisins and molecular form of B1, a product of the molds of *Fusarium verticilloides* and *Fusarium proliferatum*. Corn is the major commodity affected by this group of toxins (C A S T , 2003).

Fumonisins are toxic to the central nervous system, liver, lungs, pancreas and kidneys of various animal species. Horses and pigs are the most sensitive species. Equine leukoencephalomalacia (ELE) is a fatal disease of horses caused by fumonisins. Porcine pulmonary syndrome is a form of the disease in swines. The mechanism is believed to be the inhibition of enzymes involved in the production of sphingosine from sphinganine. Sphingosine is an important component of cell membranes, especially for neurons.

Clinical signs of fumonisin toxicosis

Swine: Dyspnea, cyanosis and weakness, which develop 4 to 7 days after the consumption of fumonisin-contaminated feed. Death may occur within a few hours of onset of the syndrome.

Horses: Depression, blindness, ataxia, aimless wandering, facial paralysis, which may rapidly progress to coma and death. Death may occur 1 to 7 days after the sign onset.

Ruminants: May develop anorexia and suffer mild weight loss if fumonisin concentrations approach 200 ppm. There are few other significant or persistent signs.

Poultry: Appear to be more resistant than other species. Inappetence and skeletal abnormalities may develop at concentrations of 200 – 400 ppm.

Lesions of fumonisin toxicosis

Swine: Acute pulmonary edema characterized by marked to massive intralobular pulmonary edema and marked hydrothorax. Lungs are distended and turgid. Thoracic cavity is filled with straw-colored proteinaceous fluid. Microscopically, interstitial and interlobular edema can be observed. There is also a possibility of occurrence of multiple areas of focal pancreatic necrosis and hepatic lesions characterized by disorganized hepatocytes, increased mitotic figures, necrosis of single hepatocytes and mild bile retention. Icterus.

Horses: Leukoencephalomalacia – massive softening and liquefaction of cerebral white matter, ranging from discrete focal areas to large cavitations and inward collapse of the cortical gray matter. Hemorrhage is prominent. Microscopically, liquefaction and proliferation of macrophages in response to necrosis can be observed.

Diagnostic aids for fumonisin toxicosis

Blood workup: Evidence of hepatic dysfunction.

Tissue analysis: Sphinganine: sphingosine ratio is increased. Analysis of tissues for sphinganine and sphingosine is not easily available.

Analysis of corn or corn-containing feed for fumonisins: Concentrations in excess of 3 ppm fumonisin may be significant for horses. Concentrations above 5 ppm may be significant for pigs (C a r l s o n and E n s l e y, 2003).

DEOXYNIVALENOL

Deoxynivalenol (DON), also known as vomitoxin, belongs to the class of mycotoxins called trichothecenes and is mainly isolated from corn and other cereals, oil meals, hay and silage. It is produced by molds of the *Fusarium* genus*,* mainly by *Fusarium graminearum,* and in some regions by *Fusarium culmorum* (R i c h a r d, 2007).

Vomitoxin is not very toxic, but it is associated with feed refusal and decreased feed consumption, which can affect animal performance. Concentrations ranging from 5 to 10 ppm are associated with vomiting in pigs; hence its name. Thresholds for decreased feed intake are about 1 ppm in swine and 10 to 20 ppm in ruminants. The mechanism, by which vomitoxin acts, has not been elucidated. Other trichothecenes inhibit protein and nucleic acid synthesis. Cattle are very resistant to the effects of vomitoxin. Pigs are more sensitive. Cases of dogs contaminated with vomitoxin from pet foods containing grains have been reported.

Clinical signs of vomitoxin toxicosis

Swine, dogs, cats – feed refusal, vomiting; cattle – usually none, however some reports of feed refusal may be found in the scientific literature; poultry – usually none.

Lesions of vomitoxin toxicosis

No gross or histopathological lesions have been reported in animals consuming vomitoxin-contaminated feed.

Diagnostic aids for vomitoxin toxicosis: analysis of grains or feeds (C a r l s o n and E n s l e y, 2003).

ZEARALENONE

Zearalenone (F-2 toxin) is a secondary metabolite of fungi of the genus *Fusarium*, mainly *F. graminearum* and *F. culmorum*, as well as DON, so they are often found simultaneously (C A S T, 2003). It is a chemical that can act similarly to the female sex hormone estrogen. Excessive exposure does not cause death or abortions, but it can disrupt the estrus cycle in females, cause infertility and feminization in males, and precocious puberty in sexually immature females.

According to our laboratory findings, zearalenone content typically found in Serbian grains is usually not enough to adversely affect animals, but unusual environmental conditions during the growing season, or insufficiently dried grain put up for storage may increase zearalenone production.

Clinical signs of zearalenone toxicosis vary with species, sex and age of the animal.

Swine, sexually immature gilts: Behavioral estrus, swollen and edematous vulva, enlarged mammary glands, tenesmus (spasmodic contraction of anal or bladder sphincter), sometimes vaginal or rectal prolapse; clinical signs appear 2 to 7 days after exposure begins and subsides 4 to 10 days after exposure ends (N e š i ć, 2003).

Swine, mature sows: Early exposure in the estrus cycle – suppression of ovulation and signs of estrus that are severe and prolonged; exposure during the mid-cycle – pseudopregnancy, anestrus which may persist for 40 to 60 days after exposure stops.

Swine, castrated boars: Enlarged prepuce and nipples. Swine, immature boars: Reduced libido, retarded testicular development.

Swine, mature boars: Not inffected unless dietary concentrations reach 200 ppm or higher. Such concentrations are rarely encountered in U.S. grains.

Lesions of zearalenone toxicosis are present only in the reproductive system.

Prepubertal gilts: Swollen and edematous vulva; enlarged mammary glands; enlarged, hypertrophic and edematous uterus. Histopathologically, uterine and vaginal metaplasia, and follicular atresia can be observed.

Mature sows: Retained and functional corpora lutea with anestrus. Mammary alveolar development and ductular sqamous metaplasia.

Diagnostic aids for zearalenone toxicosis

Blood workup: Serum analysis for estrogen can help rule out an organic hormonal problem.

Feed or grain analysis: Since clinical effects are delayed several days after the ingestion, feed analysis may be of limited value. A sample collected after the problem is noticed may not contain detectable amounts of zearalenone (C a r l s o n and E n s l e y, 2003).

Differential considerations for zearalenone toxicosis are other estrogenic chemicals from plants (phytoestrogens) or other sources which could also produce some of the signs of zearalenone toxicosis.

T-2 TOXIN

T-2 toxin also belongs to the group of fusariotoxins and is produced by *F. sporotrichioides* (C A S T, 2003). It can be detected in various species of grains mainly to the level of 10 mg/g (R i c h a r d, 2007).

T-2 toxin poisoning is characterized by lower growth rate and increased feed conversion, while the level of detrimental effects is proportional to the amount of ingested mycotoxin and duration of exposure (Š e f e r, 1993). Decline in the production is increased if other mycotoxins are also present in the feed. Poultry is particularly sensitive.

Clinical symptoms are depression, lethargy and breathing problems. Local epithelonecrotic effects are manifested by stomatitis, oral necrosis and ulcerations. Early occurrence of vomiting may be accompanied by diarrhea, often bloody. Prolongation of prothrombin time and potential vascular damage cause widespread hemorrhages, up to hemorrhagic diathesis. Also, there is feverishness, anemia, growth inhibition and poor feed efficiency. Chronic effects of T-2 toxin poisoning are primarily reflected in the performance.

T-2 toxin causes patomorphological changes in the liver, gastrointestinal tract, kidney, bone marrow, skin and lungs, as well as in the heart, reproductive organs, spleen and nervous tissue (S i n o v e c, 2000).

Diagnosis can be confirmed by the presence of this toxin in the feed, with characteristic lesions appearing after a week of taking the feed containing 4 mg/kg of toxin, and after seven weeks when the consumed dose is 0.4 mg/kg (R i c h a r d, 2007).

PREVENTION OF MYCOTOXICOSES

Usage of feed that is free of mycotoxins is the best way to fight mycotoxins, but it may be difficult to find such feed because mycotoxins occur naturally. That is why we usually recommend the use of feed additives to bind mycotoxins.

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ДИЈАГНОСТИКА МИКОТОКСИКОЗА У ВЕТЕРИНАРСКОЈ МЕДИЦИНИ

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Резиме

Проблем присуства микотоксина у храни за животиње и последице које услед тога настају већ годинама представљају изазов за решавање за све који се са њима сусрећу. У том ланцу, који обухвата истраживања од превентиве до куративе, веома значајно место и чест извор недоумица чини процес дијагностиковања обољења изазваних микотоксинима.

Циљ рада је да се представи вишегодишње искуство тима стручњака Научног института за ветеринарство Србије из Београда који ову проблематику прати са аспекта клиничких манифестација микотоксикоза код различитих врста животиња, патоанатомских и патохистолошких промена које их карактеришу и лабораторијских анализа хране за животиње која је извор ових биолошких хазарда и природних контаминената.

На основу свих прикупљених налаза може се закључити да су микотоксини често присутни. Иако њихове количине углавном не прелазе лимите дозвољене важећом регулативом, с обзиром на кумулативни ефекат и могућу хроничну изложеност животиња штетном дејству микотоксина и у случају нижих концентрација, потребна је одговарајућа стручна реакција. Наиме, и онда када се не манифестују директни губици као последица угињавања животиња, не смеју се занемарити индиректне штете услед пада производње и појаве секундарних обољења.

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DERMATOMYCOSIS – CONDITIONS THAT CONTRIBUTE TO THE DISEASE DEVELOPMENT

ABSTRACT: Skin lesions caused by dermatophytes are classified depending on the infected skin structure: surface layer of cutis, fur layer, clutches or nails. Surface mycoses are caused by dermatophytes: *Epidermophyton, Microsporum* and *Trichophyton* species (also important are *Malassezia* spp., *Candida* spp., and *Trichosporon*). Skin is the target tissue for fungal infections if the epithelial layer is damaged and immune system cannot cope with the infection, or if the conditions are favorable for dermatophytes, which spread in the cutis due to the enzyme activities. Dermatophytes can be found on skin surface if they contaminate or colonize epidermis or hair follicles. However, clinical symptoms of lesion on the skin are sometimes absent. According to the literature data 6-9% of skin lesions are caused by dermatophyte in human medicine. Similar situation is in veterinary medicine. Fungus that cause dermatomycosis are widespread in the nature and could be divided into: zoophilic, geophilic and anthrophilic.

The goal of this paper is to present the latest knowledge in pathogenesis on dermatomycosis, predisposing factors important for the outcome of the disease, and immunological reaction of organism to the fungal infection. Our intention is to summarize the subject and present the facts related to specific problems in dermatomycosis.

KEY WORDS: dermatomycosis, dermatophyte, pathogenesis, immunity

INTRODUCTION

Among many microorganisms that are present in nature there are over 300 fungi that are actually pathogenic for animals and people (Outerbridge and Catherine, 2006). Mycoses are manifested differently and appear if the immune system of the host is weak, or under various conditions that support the growth of fungi. It is important to determine the factors that contribute to the mycoses development, such as: 1. Fungi are widespread in nature so eradication is difficult, 2. Clinical manifestation is variable (inflammation, allergic reaction), 3. Diagnosing is not easy since clinical appearance is different and depends on the host, 4. Therapy is difficult since number of available drugs is

restricted, 5. Prevention is available for some fungi and only for some animal species (B l a n c o and G a r c i a, 2008).

Dermatophytes present a part of the above mentioned group of fungi, among which zoonoses are some of the most important, since they are common for both people and animals. These fungi are widespread in nature and its classification depends on the habitat and their presence in various ecology niches. They are classified into zoophilic dermatophytes (which also include silvatic ones, those found in woods), geophilic and anthrophilic (C h a b a s s e a and P i h e t a, 2008).

Most of dermatophytes are located superficially and are localized on the surfaces of cutis, hair and nails. However, the mechanism between the host and fungus that actually contributes to the disease is not well understood. Lesions on skin induced by fungus depend on the location and structure of the skin, as well as on the skin product (superficial layer of the cutis, hair or nails). Dermatophytes cause superficial mycoses (most often *Microsporum, Trichophyton* and also *Malassezia* spp., *Candida* spp. and *Trichosporon*). If the protective barrier is damaged the skin presents main "door" for fungal infection. The skin infection may occur when fungus contaminates or colonizes epidermis or hair follicles, although it has been reported that clinical changes are not always present. According to the literature data, dermatomycosis in human medicine encompasses 6 to 9% of cases of all pathological changes in skin. This is similar in veterinary medicine (S t o j a n o v et al., 2009).

The most significant aspects of dermatomycosis are related to the broadening of knowledge on all the factors that participate in pathogenesis, such as: proteases, secretory enzymes, adhesion possibilities and ability to modulate defense mechanisms of the host (S a n d y et al., 2008). These data lead to the research of two problems: investigation of the pathogenicity mechanisms that transform ubiquitous fungi to pathogenic, and research on resistance mechanisms of the host related to the infection and disease.

The main goal of this paper is to present the latest knowledge on pathogenesis of dermatomycosis, predisposing factor important for the outcome of the disease, and immunological reaction of the organism to the fungal infection. Our intention is to summarize the subject and present the facts related to specific problems in dermatomycosis.

DERMATOPHYTES

Dermatophytes (gr. *derma* = skin + *phyton* = plant) (K l a j n and Š i p k a, 2006) includes three genera: *Epidermophyton, Microsporum* and *Trichophyton*. Fungi that belong to these genera can grow on keratinized tissue of animals and people (skin, hair, fur, nails, clutches) and induce dermatophytosis.

Infection appears on cutis and is restricted to dead cornified layers, since dermatophytes cannot penetrate deeply into the skin, and immune system of the host prevents the spread of this agent (S a n d y et al., 2008). These fungi are not part of the normal microflora of the skin in people and their presence on

the skin is a consequence of their ability to utilize keratin as a food source, that is opposite from other fungi (W e i t z m a n and S u m m e r b e l l, 1995).

Dermatophytes are classified in three anamorphic orders (asexual and imperfect): *Epidermophyton* spp., *Microsporidium* and *Trichophyton* from the class of anamorphic Hyphomycetes which belong to Fungi imperfecti. These genera are described in literature, depending on morphology and production of conidia.

Epidermophyton spp. Macroconidiae of this type of dermatophytes have enlarged wedge with typical smooth, thin and slightly bold wall. Macroconidiae have one to nine barriers that are 20 to 60 μm wide and 4–13 μm long. They appear in large number, single, or in a group. This genus has two species among which the pathogenic one is *E. floccosum*.

Microsporum spp. Macroconidiae have walls that are rough, uneven, wart and serrated. Macroconidiae of first isolated dermatophyte were described as fusiform, but later on new species were described with ovoid macroconidia (*Microsporum nanum*) (F u e n t e s, 1956), cylindrical form (*Microsporum vanbreuseghemii*) (G e o r g e t a l., 1962). Macroconidiae have thin, slightly large or large walls with 1–15 barriers, and are 6–160 μm x 6–25 μm in size. Macroconidiae can be stocky, with stalk or sphenoid appearance, usually individually situated along hyphae.

Trichophyton spp. Macroconidiae are smooth, with thin wall having 1 or 12 barriers. They appear alone or in the group and could have long appearance, as a pencil; they may become cylindrical, or resemble a long wedge. Macroconidia are 8–86 μm x 4–14 μm in size. In comparison to Macroconidiae, Microconidiae are present in larger number, have a shape of a ball or pear. They can be sphenoid, stocky or stalk "like", and can appear either individually, at one side of hyphae, or in a cluster.

DERMATOMYCOSIS

Dermatomycosis of dogs and cats

Most often, dermatomycosis is induced by *Microsporum canis* in both cats and dogs, while in dogs, the disease is most often caused by *Trichopyton mentagrophytes* and *T. mentagrophytes* var. *erinacci*. From the clinical point of view, characteristic lesions are: round hairless spots, with broken hair and inflamed skin, and milliary dermatitis present. Lesions are rarely generalized and appear if immunosuppression occurs, as well as in the case of hyperadrenocorticism. Folliculitis and onychomycosis (mycosis of clutches) can occur in dogs. Dogs' lesions can appear on muzzle and this is related to their activities (Q u i n n e t a l., 2002), such as digging and machination in ground with muzzle, hunting of rodents and attacking hedgehogs. As a consequence of such activities, specific type of dermatophytes that reside in the ground, rodents and wild animals, could be found on dogs. *M. gypseum* is present in ground, while *T. mentagrophytes* var. *erinacci* can be found in hedgehogs. It was confirmed

that arthospore of dermatophytes could be found on fur of dogs and cats, even if the clinical manifestation of the disease is absent.

Dermatomycosis of cattle

The main causative agent of dermatomycosis in cattle is *Trichophyton verrucosum*. Affected animals have lesions around their eyes and on head. In heifers and cows, lesions could appear on legs and neck. The characteristic lesions are: alopecia and spots with gray and white deposits similar to scabs (Q u i n n e t a l., 2002; G u d d i n g and Lu n d, 1995). Infection is most common in winter. In spring, when animals are outside on grasslands, the disease vanishes. If immune system is not able to cope with the disease, therapy or vaccine prevention is obligatory. Dermatomycosis are rare in goats and sheep, but if the infection does occur, it is caused by *M. canis*, *T. mentagrophytes* and *T. verrucosum*.

Dermatomycosis in horses

Trichophyton equinum is the main agent inducing dermatomycosis in horses. Still, there are two more types of dermatophytes that infect animals in various geographic regions, and those are *M. equinum* and *T. equinum* var *autotrophicum*. The agent is transferred by direct contact with infected animal or through contaminated equipment used for horse care. Changes are often present on parts of the skin that are in contact with belt and saddle, but could appear on all parts of the body if the brush, contaminated with this agent, is used for horse grooming. Infection with dermatophytes from the ground is possible with *M. gyseum*, if horses are rolling on the ground, with *M. canis* and *T. mentagrophytes* if they are in contact with dogs and cats, or with *T. verrucosum* if they are in contact with cows. Young animals are more susceptible to infection than the older ones.

Dermatomycosis in pigs

Dermatomycosis is significant in pig production. It could appear in all ages and is usually related to poor management. The main cause of this disease comes from the *M. nanum* that resides in the ground.

Dermatomycosis in poultry

Dermatomycosis in poultry is rare. It is caused by *M. gallinae* as a consequence of poor management. In hens and turkeys, it appears in the form of

white deposits on scabs and wattle, in severe cases it could attack the feather follicles and cause systemic disease.

Dermatomycosis in people

Infections caused by dermatophyte (ringworm) are named depending on the location of lesions. Word naming the lesion location is added after the Latin word *tinae*. *Tinae barbae* – stands for the infection of the chin which could be superficial or deep, with severe inflammatory pustular folliculitis. They are caused by zoophilic dermatophytes (Kwon–Chung and Bennett, 1992). *Tinae capitis* – represents head covered with hair. Changes could be subclinical with erythema or severe folliculitis, alopecia, sometimes with lymphadenopathia as well. It is caused by *Microsporum* and *Trichophyton* (Rippon, 1985). *Tinae corporis* – dermatomycosis that appears on body, shoulders and legs, and may also appear on face. Clinical signs could be severe with clearly limited erythematous vesicular spots. *Tinea cruris* – infection of crotch, perianal and perineal region. It appears mostly in older mail persons. Causative agents are *T. rubrum* and *E. floccosum*. The symptoms are flushing with dry dandruff. *Tinea favus* – causes lesion on head that appear as prominent yellow scabs and dry dandruff. It has been recorded mostly in Euro Asia and Africa. *Tinea imbricate* is a chronic infection that appears in places where skin folds, and causative agent is *T. concentricum*. It can be found in Asia, North and South America and Oceania. It is strictly anthrophilic dermatophyte (Rippon, 1988). *Tinea manuum* – its causative agent is *T. rubrum*. Lesions are found on the palms and interdigital areas of hands. Hyperkeratosis and cracking of the skin are present. *Tinea pedis* – is present on soles of feet and toes. It is also called athletes foot. It could be chronic with squamose epithelia, hyperkeratosis, redness and inflammation. Causative agents could be *Epidermaphyton floccosum* and a member of genera *Trychophyton*. *Tinea ungium* – attacks nails and appears under nails or superficially. The most frequent agents are *T. rubrum* and *T. mentagrophytes*.

Tab. 1 – Main type of fungi that could cause mycoses in people (Deacon J., 2005)

Primary site of pathogen entrance	Fungi	Pole stage	Disease	Type and the place of invasion
Skin	*Trichophyton* (22 species) *Microsporum* (19 spp.) but only 9 are involved in infections *Epidermophyton* (2 spp)	*Arthroderma* (Ascomycota)	Dermatomycoses: ringworm, tinea, athletes foot	Keratinized tissue people, wild and domestic animals

Table 2. Some of the major dermatophytes that could infect people (Deacon J., 2005)

Anthrophilic	Zoophilic	Geophilic
Epidermophyton floccosum	*Microsporum canis* (dogs, cats)	*Microsporum gypseum* (the most common infection in people)
Microsporum audouinii	*Microsporum equinum* (horses)	*Trichophyton terrestre*
Microsporum ferrugineum	*Microsporum nanum* (ground/pigs)	
Trichophyton mentagrophytes var interdigitale	*Microsporum persicolor* (rodents)	
Trichophyton rubrum	*Trichophyton equinum* (horses)	
Trichophyton tonsurans	*Trichophyton mentagrophytes* var *mentagrophytes* (mice, rodents)	
	Trichophyton verrucosum (cattle)	

PREDISPOSING FACTORS FOR SKIN FUNGAL DISEASE

It is very important to have the knowledge on predisposing factors that contribute to the development of fungal skin diseases. High humidity and hot climate, such as in tropical countries, contribute to the development of dermatomycosis (B l a n k et al., 1969). If the skin of laboratory animals or people is covered at the place of fungus inoculation it becomes softened and paired (G r e e n b e r g et al., 1976). The covered place enhances the humidity of the skin and keeps CO_2 produced by skin. This helps the growth of dermatophytes.

Many medical reasons contribute to predisposing factors for dermatomycosis. Dermatomycosis is usually found in chronically ill patients and animals suffering from vascular disease, corticosteroid therapy, Cushing disease, hemathological malignancy, chronic candidosis, diabetes mellitus or atopic dermatitis (allergy to many allergens present in the house and nature) (H a y , 1982). The age of a patient is also important for the development of dermatomycosis, and such infections usually occur without symptoms (G i l c h r e s t, 1979). Vascular disorders in peripheral blood stream that have not been diagnosed, and keratinization problems are related to chronic dermatomycosis. Nowadays, the predisposing factors for this disease are number of different allergies that are widespread in the world (W a g n e r and S o h n l e, 1995). Research has shown that sensitivity to dermatophytes could be connected to hereditary factors and that some recessive autosomal genes could transfer higher susceptibility to dermatomycosis (S e r j e a n t s o n and L a w r e n c e, 1977).

Effects of dermatophytes on host immune system

Results of the research indicate that dermatophytes are capable of "avoiding" the immune system and causing lesions in the host. Fungi may express several effects, including inhibition of lymphocyte by mannans – plant polysaccharides, impaired function of macrophages, disturbed activation of

keratinocytes and secretion of different protease (G i d d e y et al., 2007). The level of immune response and inflammation depend on how deep fungi have penetrated in the skin. Less invasive dermatophytes are sheltered from soluble components of the immune system (D a h l and G r a n d o, 1994). Also, secretion of subtilisin (Sub3) and metalloprotease (Mep3) produced by *M. canis* (B r o u t a et al., 2003) participate in the immunomodulation in host. Subtilisin and dypeptidyl protease V, secreted by *T. rubrum* and *T. tonsurans*, may induce immunity causing acute dermatomycosis and delayed type of hypersensitivity reaction (DTH) (W o o d f o l k and P l a t t s – M i l l s, 2001) with high *IgE* and *IgG4* level. Molecules of *Trichophyton rubrum* cell wall mannans (TRM) act immunosuppressively. They can inhibit the proliferation of mononuclear leukocytes against several antigens, including antigens of dermatophytes under laboratory conditions (M a c C a r t h y et al., 1994). Keratinocytes and monocytes/macrophages play an important role in the modulation of immune response. However, level of inteleukins (IL-1) secreted by these cells was lower when they were in contact with *T. Rubrum* than with *T. mentagrophytes* (O g a w a et al., 1998). The enzymes of dermatophytes, like dipeptidyl protease IV, may influence the immune response by dissolution of soluble immune components (L a n d i s et al., 2008).

PATHOGENESIS OF DERMATOMYCOSES

Adherence and invasion of skin surface

Dynamics of dermatophyte adherence to skin and keratinized skin tissues was studied in experimental models by using microscope techniques. It was determined that depending on time, the number of spores attached to skin and, consequently the number of germinated spores, increased. Also, the penetration through stratum corneum and spreading in different directions was observed (S a n d y et al., 2008). Zurita and Hay determined that maximal adherence of arthroconidia *Trichophyton* spp. to keratocytes occured in the first 3 to 4 hours. For some species of *Trichophyton* spp. (*T. mentagrophytes*) the adhesion takes place during the first 6 hours and the germination starts after only 4 hours (R a s h i d et al., 1995). In laboratory conditions, when skin sample of live tissue (explant) was used, maximal adherence was reached in the first 12 hours, and the spore germination started after one day (D u e k et al., 2004).

It is well known that there are factors that mediate the adhesion of dermatophytes. For example, at the surface of *T. rubrum* macroconidia, specific carbohydrate adhesives enable the adhesion of dermatophytes to the epithelial cells (E s q u e n a z i et al., 2004). Interestingly, one research showed that, on skin surface, long and free fibrils connect arthroconidia of fungi and keratocytes, while in deeper layers newly formed arthroconidia spread through the tissue creating a contact surface between skin and fungi (K a u f m a n et al., 2007).

Similar to the findings that confirmed aspartic protease in *Candida albicans*, which is necessary for the adherence to the host (D e B e r n a r d i s et

al., 2007), it was observed that protease enzymes, secreted by dermatophytes, facilitate the adherence, or are required for this process. The secretion of proteolytic enzyme subtilisin, metalloproteases and dipeptidyl peptidases by *M. canis* is regarded as important for the adhesion, or for the early phase of invasion of this microorganism (K u m a g a i et al., 2005).

Growth on hard keratinized skin products

Dermatophytes have several proteases essential for the transformation of keratine into useful oligopeptides or amino acids. Fungi secrete different forms of serine and metalloendoproteases (J o u s s o n et al., 2004) which are called keratinases. The level of importance of hydrolases, such as lipases or ceramidases, has not been precisely determined yet, but Viani et al. Have found that potent keratolytic hydrolase of *M. canis* is responsible for clinical infections. However, it remains unresolved whether the symptoms are caused by the activity of dermatophyte's keratinases, or the lesions develop because of inflammation and immune reaction. In any case, keratolytic effect of these enzymes is possible only after the reduction of disulphite bonds which maintain the protein structure of keratin tissue (K u n e r t, 1992). The excretion of sulphite depends on sulphite efflux pump that enables sulphitolysis of proteins and makes them available for proteases.

The secretion of proteases by fungi occurs under the circumstances of complex protein compounds being the only source of carbon and nitrogen, but not glucose and easily digestible peptides (J o u s s o n et al., 2004). This means that the keratolytic activity of dermatophytes is expressed under restricted nutrient conditions. Successful survival and growth of dermatophytes in some species actually depend on secretion of numerous proteases. The protein and protease structure differ depending on the species, in spite of extremely high similarity of orthologous genes. Specific features of dermatophytes that cause severe inflammation in host are probably related to different regulation of protein and protease secretion.

It should be mentioned that skin damage may occur without any activities of lytic enzymes of dermatophytes, but as a consequence of other biotic factors, such as bacteria or parasites. Moreover, host proteases may be activated and may contribute to the development of altered skin structures because of allergic reaction.

Skin immune response and inflammation

Superficial infections with dermatophytes cause different inflammatory reactions in organism depending on the pathogenicity of agent and chronicity of the process. Anthrophilic dermatophytes, such as *T. rubrum* and *E. Floccosum*, generally cause mild inflammation and small lesions on skin, but usually long term or persistent infections. On the other hand, geophilic and zoophilic

dermatophytes cause strong inflammatory reaction restricted to smaller surfaces because of higher immune response. The above mentioned points to the significant role of localized inflammatory process and the immune response of the host against dermatophytes (W a g n e r and S o h n l e, 1995).

Chemotactic mechanism

Superficial fungal infections are limited to the surface layers of skin, but sometimes the infection can be spread more deeply and cause strong inflammation. These deep changes include the occurrence of desquamation, vesicle and pustule formation, as well as considerable skin damage. Under microscope, aggregation of large number of neutrophils and formation of microabscesses can be observed in the acute phase, while in chronic cases monocytes dominate and hyper or para keratosis develop. Chemotactic mechanisms, complement activation, that participate in mobilization of neutrophils, are very important for inflammation (S w a n et al., 1983). The reason why some fungal infections like *T. rubrum* cause only mild inflammation is the fact that this fungus secretes substances which disable chemotactic mechanisms and hinder the activity of neutrophils (D a v i e s and Z a i n i, 1984). Chemotactic mechanisms that activate keratinocytes by secretion of cytokines, contribute to the inflammation and defense of the host are still unclear.

Role of phagocytic cells

The role of neutrophils in inflammatory reaction after the infection with dermatophytes is different. Their microbiocidic activity depends on oxidative activity of superoxide and hydrogen peroxide, hipochlorine acid and monochlor amines (T e s t et al., 1984). Nonoxidative substances, such as cathepsine, proteins that increase bactericidal effect or permeability, lactopherin, lysozime, elastase, azuricide and others, may act bactericidally (G a b a y et al., 1986). Antimicrobial features of macrophages/monocytes are expressed through the production of nitrogen oxide which inhibits fungal pathogens.

Superficial skin infections with severe clinical forms occur more often in immune compromised individuals, which leads to the conclusion that preserved functions of immune system are crucial for the protection against dermatomycoses. Proper functioning of defense system is necessary even in case of superficial infections reaching the stratum corneum (F i n d l i n g et al., 1981). Numerous researches indicate that epidermis is not just passive barrier against infectious agents, but also acts as immune surveillance which, by means of cell cooperation, successfully protects the organism from a wide palette of different noxae (W a g n e r and S o h n l e, 1995).

Cell wall of dermatophytes is primarily comprised of chitin and glucan that make glycopeptides, main antigens of these microorganisms. Like other fungi, dermatophytes possess complex antigens, such as glycopeptides, pep-

tides or carbohydrates (Moser and Pollack, 1978). Antigen features of these molecules are good and they sensibilize immune system even in case of superficial infection. Anitbodies against *T. rubrum* were determined in people not infected with this fungus, although cross reaction with antigen of some other microorganism remained possible (Sohnle et al., 1983). By using different serologic methods (ELISA, complement fixation, immunodiffusion and agglutination), investigations of humoral immune response in humans showed presence of antibodies against dermatophytes (Papini and Simonetti, 1985). Special immunologic problem is the occurrence of hypersensibilization mostly manifested as late allergic reaction of type DTH. It is not completely clear whether chronic dermatomycosis influences the development of *IgE* or the organism is predisposed due to atopa presence (Serjeantson and Lawrence, 1977). This allergic reaction is also related to dermatophyte species. In humans, DTH more frequently occurs in acute form of infection with *T. Mentagrophytes,* in comparison to the chronic form of infection with *T. rubrum* (Jones et al., 1973).

In conclusion, the presence of dermatophytes in humans and animals is often without clinical signs or with nonspecific skin changes, which delays prompt diagnostics. Dermatophytes are widely distributed and are well adjusted to specific ecologic niches. Therefore, immune system is not always prepared to respond in a completely satisfying manner. It can be stated that there are some differences between dermatomycoses in humans and animals, mainly because of the living conditions, that is the environment. The relationship between animals and humans, as well as the contemporary lifestyle, their living together or in close proximity, claims for better understanding of all the factors that can influence the infection, its spread and the reaction to dermatophytes in both humans and animals.

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ДЕРМАТОМИКОЗЕ – УСЛОВИ КОЈИ ДОПРИНОСЕ НАСТАНКУ БОЛЕСТИ

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Резиме

Промене на кожи изазване дерматофитима могу бити систематизоване у зависности од структуре или продукта коже који је захваћен на: површински слој кутиса, крзнено-длачни покривач или канџе – нокти. Површинске микозе изазивају дерматофите *Epidermophyton*, *Microsporum* и *Trichophiton* врсте (поред наведених врста значајне су још *Malassezia spp.* и *Candida spp*. и *Trichosporon*). Кожа представља улазна врата за гљивичне инфекције када је заштитна епителна баријера оштећена и имунолошки систем није у стању да се избори са инфекцијом или када су створени услови да дерматофите својом ензимском активношћу насеље кожу и прошире се ткивом кутиса. На површини коже се могу наћи дерматофете које контаминирају и/или колонизују површину епидерма или длачног фоликула, али се клинички знаци, промене на кожи, неће увек јавити. Дерматофите у хуманој медицини, према доступним подацима из литературе, представљају узрочнике 6% – 9% свих промена везаних за кожу и продукте коже. Сличан налаз је и код клиничких и лабораторијских испитивања узорака у ветеринарској пракси. Гљивице које узрокују дерматомикозу су група микроорганизама веома раширених у природи и њихова заступљеност, у односу на природно станиште и присуство појединих врста у појединим деловима животног станишта, дели их у зоофилне, геофилне и антропофилне.

Задатак нашег рада је да презентује досадашња истраживања везана за патогенезу дерматомикоза, предиспонирајуће факторе који имају важну улогу у настанку болести као и да прикаже имунолошку реакцију организама на гљивичну инфекцију. Намера нам је да се сумарним приказивањем наведене тематике на једном месту изнесу чињенице везане за специфичну проблематику дерматомикоза.

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THE EFFECT OF NEMATOPHAGOUS FUNGUS *DUDDINGTONIA FLAGRANS* ON THE GASTROINTESTINAL PARASITES IN SHEEP

ABSTRACT: Sheep production has serious problems due to the spread of intestinal parasites. These parasites cause loss of appetite, maldigestion, slow growth in body weight and wool, all of which results in economic losses as well. The control measures of infestation with strongyloid parasites in ruminants have until now been based mainly on the organization of grazing and the use of antihelmintics. However, due to the occurrence of resistance, alternative methods of control have been introduced. The use of nematophagous fungus *Duddingtonia flagrans*, which is capable of decreasing the number of infectious larvae and eggs in feces, has been successful. The aim of this study was to determine whether *Duddingtonia flagrans* decreases the number of eggs of *Trichostrongylus* spp *in* sheep feces. Fecal samples of thirty-four sheep were examined and the parasites were found in twelve sheep, six of which were fed with the fungus, and six of which were used as the control. According to χ^2 test, at the level of certainty of $p<0,005$, a statistically important difference in the number of eggs was observed between the sheep which were given the fungus and those which were not.

KEY WORDS: *Duddingtonia flagrans*, sheep, gastrointestinal parasite, biological control

INTRODUCTION

Sheep production has serious problems due to the spread of intestinal parasites. These parasites cause loss of appetite, maldigestion, slow growth in body weight and wool, all of which results in economic losses as well. The control measures of infestation with strongyloid parasites in ruminants have so far been mainly based on the organisation of grazing and the use of antihelmintics. However, due to the occurrence of resistance, alternative methods of control have been introduced. Grazing systems are often impractical and expensive, whereas frequent use of antihelmintics, which has been the main principle of therapy over the last fifty years, develops resistance of parasites to antiparasintics, especially significant in small ruminants, sheep and goats

(C o n d e r and J o h n s o n, 1995). Not less important aspect is the increase of consumers' demand for animal products without residua of drugs (D i e z – T a s c o n et al., 2005; L a l o š e v i ć et al., 2009). For these reasons, increasing number of alternative control methods is being introduced. The most promising one is the use of nematophagous fungus which is capable of reducing pasture contamination and the occurrence of infection (L a r s e n, 2000). In recent years, "nematode-destroying", or nematophagous fungi, has become focus of attention of many scientists around the world. They live in different cracks in land and rhizosphere where they feed on different free-living nematodes and dead organic matter. Among the most important and the most frequently investigated fungi are: *Duddingtonia flagrans, Monacrosporium* spp*., Arthrobotrys* spp*., Pochonia chlamydosporia* (C a r v a l h o et al., 2009). A kind of a predatory fungus which attracts the greatest attention is *Duddingtonia flagrans* (L a r s e n et al., 1997). Modern research shows that *D. flagrans* spores are capable of passing through the gastrointestinal tract of sheep and lessening the number of infectious larvae in feces, which was proved in the research of pasture contamination during spring grazing season (G o m e z – R i n c o n et al., 2006). *Duddingtonia flagrans* reduces the population of larvae on pastures which later results in a smaller number of parasites in animals up to the level where not only clinical signs but also subclinical effects caused by parasites will be prevented. At the same time, reducing the number of infective larvae on pastures should stimulate the development of naturally acquired immunity in young animals. In the fresh feces, *Duddingtonia flagrans* forms a three-dimensional net which leads either to egg destruction or larvae destruction (A r a u j o et al., 2009). Besides ruminants, the positive effect of *Duddingtonia flagrans* was observed in the control of intestinal parasites of pigs (*Ascaris suum*), and in nematodes of carnivores (*Ancylostoma caninum*) (L a r s e n et al., 1997, A r a u j o et al., 2009).

The aim of the study was to determine the effect of nematophagous fungus *Duddingtonia flagrans* on the gastrointestinal strongyloids, *Trichostrongylus* spp. in naturally infected sheep.

MATERIALS AND METHODS

This research was conducted in the Laboratory for Parasitology, Department of veterinary medicine, Faculty of Agriculture in Novi Sad. Feces samples were collected in the period from July to September 2010 from sheep in Zrenjanin municipality, raised freely on pastures.

Before the beginning of the experiment, stool samples were examined and natural infection by *Trichostrongylus* was found in all animals. Sheep were divided into two groups. The experimental group of six sheep was perorally given 17 ml of water solution of fungus (about $1x10^6$/ml chlamidospora) for five days. The control group of six animals did not receive the fungus suspension. Fecal samples were collected every week in the period of three weeks. The samples were examined by the method of helmint egg concentration with

a saturated solution of saccharose, and the number of eggs per gram of feces was calculated by the method of *Stoll*.

Duddingtonia flagrans (from the collection of Universite catholique de Louvian, Belgium) was multiplied on PDA base (potato dextrose agar) in the Laboratory for Microbiology at the Faculty of Agriculture in Novi Sad.

RESULTS AND DISCUSSION

With the aim of determining the presence of parasites from the genus *Trichostrongylus* spp., thirty-four feces samples were examined, and eggs of parasites were found in twelve sheep (35.9%), six of which were fed with the fungus (experimental group), and six of which were used as the control group. One sheep from the control group died during the research. Feces samples were taken from the sheep which had received the nematophagous fungi for three weeks in the amount of 20 grams. A statistically important difference in the number of eggs was observed between the sheep which received the fungus and those which did not.

According to a research conducted in Malaysia, already after the second day of the fungus intake, the decrease in larvae development in pastures was over 90%, and when the dose was doubled, the decrease was even 100% (C h a n d r a w a t h a n i et al., 2003.). A group of Spanish and Brazilian authors discovered that if sheep are fed with spores of fungus *Duddingtonia flagrans* at the right moment, from the point of view of epizootiology, this can have an important effect on infectious larvae on pastures, which was proved by finding 20% fewer parasites in lambs grazing for three weeks on the same pasture in spring (G o m e z – R i n c o n et al., 2006). Apart from the decreased spring contamination of the pasture, the lambs of the treated sheep had smaller total number of parasites and better growth than the lambs from the control group (Table 1).

Tab. 1 – The number of *Trichostrongylus* spp. eggs after one, two and three weeks after use of *D. flagrans*

Sheep number.	0 day	number eggs/g	1. week	number eggs/g	2. week	number eggs/g	3. week	number eggs/g
1. experimental	5700		11200		3000		400	
2. experimental	5200		3100		1700		600	
3. experimental	100		100		200		0	
7. experimental	4000		0		200		200	
8. experimental	4700		1200		900		100	
9. experimental	300		800		0		0	
4. control	13700		6300		8600		300	
5. control	9200		100		800		0	
6. control	1900		died					
10. control	500		1100		200		0	
11. control	1500		200		1300		800	
12. control	500		0		100		0	

A positive effect of *Duddingtonia flagrans* was also noticed in the control of intestinal parasites of pigs, which was confirmed by both *in vitro* experiments and on pastures (L a r s e n et al., 1997). High potential of the fungus to reduce the number of larvae of pulmonary strongyloid *Dictyocaulus viviparus* was noted in during a lab research, and the already mentioned results against gastrointestinal strongyloids show a wide scope of parasitic worms which can be controlled by this method (L a r s e n et al., 1997, S a r k ū n a s et al., 2000).

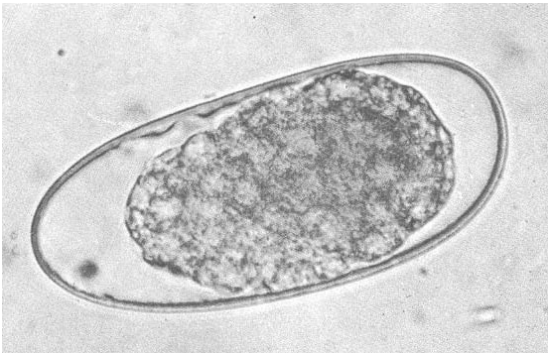

Fig. 1 – Trichostrongylus spp, egg, sheep feces

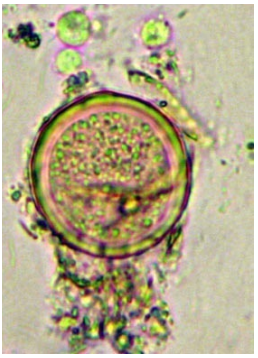

Fig. 2 – D. flagrans, chlamidospora

It appears that the passing of fungus spores through digestive tract depends on factors related to the nutrition and food flow in the animal, but a good sign is that spores of *Duddingtina flagrans* can survive in the digestive tract for a significant period of time under difficult conditions (C h a n d r a w a t h a n i et al., 2003). It is important to point out that during the implementation of this measure in the strongyloid control, it should be taken into account that some antihelmintics can have potentially negative or inhibiting influence on some nematophagous fungi. The time between the use and/or the possibility of combining the fungus treatment with medicines should be better explored (L a r s e n et al., 1997).

CONCLUSION

Our results show that *Duddingtonia flagrans* significantly reduces the number of eggs of *Trichostrongylus* spp. in the feces of naturally infected sheep. Since gastrointestinal parasites still represent a serious health problem, especially of small ruminants, antihelmintics are still necessary and prescribed in the control of these infections. Due to the resistance to these drugs, especially of *Trichostrongylus* spp. in sheep, the implementation of fungus *Duddingtonia flagrans* in cattle nutrition could be an important way of gastrointestinal and pulmonary strongyloids reduction. By simultaneous decrease

in the number of infected animals, the number of infectious larvae on pasture will decline, and the cycle of parasite development will be disturbed. From the aspect of greater need for safe food for human consumption, the occurrence of residua of drugs will be avoided in the food of animal origin.

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ЕФЕКАТ НЕМАТОФАГНЕ ГЉИВЕ *DUDDINGTONIA FLAGRANS* НА ГАСТРОИНТЕСТИНАЛНЕ ПАРАЗИТЕ КОД ОВАЦА

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Резиме

Производња оваца сусреће се са озбиљним проблемима услед раширености желудачно-цревних паразита. Њихов значај огледа се у економској штети због губитка апетита, малдигестије, слабог прираста телесне тежине и вуне. Контрола инфестације преживара стронгилидама до сада се заснивала углавном на организацији напасања и употреби антихелминтика, али, због појаве резистенције, уводе се алтернативне методе контроле. Употреба нематофагне гљиве *Duddingtonia flagrans*, која је способна да смањи број инфективних ларви и јаја у измету, показала се успешном. Циљ овог рада је да се утврди да ли *Duddingtonia flagrans* смањује присуство јаја *Trichostrongylus* spp. у измету оваца. Прегледана су 34 узорка фецеса а паразит је доказан код 12 оваца, од којих је 6 након тога добило гљиву, а 6 је коришћено као контрола. На основу χ^2 теста, на нивоу сигурности од $p<0,005$ доказана је статистички значајна разлика у броју јаја, у измету оваца које су добијале гљиву у односу на оне које нису.

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INTERACTION BETWEEN MYCOTOXINS AND CAUSATIVE AGENTS OF SWINE INFECTIVE DISEASES

ABSTRACT: Mycotoxins are secondary metabolites of fungi that can contaminate animal feeds at all stages of food production chain. Consumption of feed contaminated with mycotoxins may result in immunosuppression, which represents a predisposing factor for occurence of infectious diseases in livestock. The influence on immune system is of special interest in swine industry. The technology on swine farms demands frequent vaccinations, which may be a problem in the case of immunocompromised animals. The aim of this paper was evaluation of mycotoxin influence on swine farms, as secondary factors for destabilization of animals' immunological system.

Material for this research included the samples from five swine farms, where health disorders, i.e. clinical and patomorphological signs resembling the problem with infectious diseases in different swine categories, were detected. The applied research methods included: epidemiological and clinical evaluation, pathomorphological examination, laboratory testing of bacteriological and virusological tissue originating from dead animals, and microbiological feed testing in order to examine the presence of fungi and mycotoxins.

The obtained results indicated the existence of positive interaction between mycotoxins and causative agents of swine infective diseases. Despite continual pharmaco- and immunoprophylaxis in swine, the health problems of bacterial ethiology (colibacilosis, enteroxemia, dysentery, pneumonia, endometritis) were detected. From an epidemiological point of view, the presence of mycotoxins in animal feed may induce a breakdown of active immunity and occurrence of disease even in properly vaccinated animals.

KEY WORDS: infective diseases, imunoprophylaxis, swine mycotoxicoses

INTRODUCTION

Mycotoxins are secondary metabolites of fungi that can contaminate animal feeds at all stages of food production chain. At global level, it is considered that 25% of the world crop production is contaminated by mycotoxins, which may be a risk factor for human and animal health (Bouhet and Oswald, 2005). Consumption of feed contaminated with mycotoxins may result in immunosuppression, which represent a predisposing factor for infectious diseases

of livestock (M a r i n et al., 2002; O s w a l d et al., 2005). From a public health perspective, increased infections in animals may result in increased animal--to-human transmission of pathogens and/or increased antibiotic concentrations in meat, as a consequence of animal treatment (O s w a l d et al., 2005). The influence of mycotoxins on immune system is of special interest in swine industry. The technology on swine farms demands frequent vaccinations, which may be a problem in the case of immunocompromised animals (O s w e i l e r, 2006; P r o d a n o v et al., 2009). The aim of this paper was to evaluate the influence of mycotoxins in swine production, as secondary factors for destabilization of animals' immunological system.

MATERIALS AND METHODS

Material for this research included the samples from five swine farms, where health disorders, i.e. clinical and patomorphological signs resembling the problem with infectious diseases in different swine categories, were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: epidemiological and clinical evaluation, pathomorphological examination, standard laboratory testing for detection of the presence of aerobic and anaerobic bacteria, virological testing and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography.

RESULTS

Health disturbances in the youngest swine categories were recorded in the first examined farm (suckling piglets and weaners). Clinically, diarrhoea was detected in suckling piglets in the first 3 days of life after farrowing. After the analysis of the farm records several facts were discovered: diarrhoea occured in piglets of normal birth body weight, mortality rate was higher in animals that are in good body condition, and 30% of weaning piglets were small. Therapeutic treatment of piglets by oral and parenteral antibiotics application did not improve health problems. It should be emphasized that the dams were vaccinated during gestation with the aim to prevent disease in piglets (diarrhoea) in the first days of life. By clinical examination of certain number of suckling piglets, the clinical sign of vulvovaginitis (swelling and reddening of the vulva) was discovered. Diarrhoea and signs of pneumonia (cough, nasal secretion, fever) were detected in the weaned piglets. Pathomorphological examination of the dead suckling piglets revealed lesions present predominantly on the mucosal surface of the digestive tract (*Haemorrhagiae mucosae ventriculi*, *Enteritis catharralis acuta*). Besides the lesions in the digestive tract, prominent pathological changes in lungs were also discovered in dead weaners. (*Pneumonia fibrinosa in statu hepatisationis rubrae et griseae*, *Gastroenteritis*

haemorrhagica). Bacteriological testing of tissue samples derived from dead animals revealed the following bacteria: *Escherichia coli var. haemolytica*, *Streptococcus alfa haemolyticus*, *Pasteurella* sp. Despite the fact that all animals were medically treated after bearing, health status control of sows showed reduced appetite, clinical signs of endometritis and agalactia. By pathological control of the reproductive organs of excluded sows in the slaughterhouse, significant percent of endometritis was discovered (presence of liquid muddy content in the uterus with small pieces of destroyd tissue or content that looked like a sour cream). Bacteriological testing of tissue samples from the dams genital organs showed presence of *Staphylococcus haemolyticus*, *Escherichia coli*, *Streptococcus dysgalactiae subsp. equisimilis*. Considering the clinical and pathological symptoms observed, especially the signs of vulvovaginitis in farrowed piglets, a justified suspicion on the presence of mycotoxins in feed was made. Testing of first microbiological feed for piglets detected 3-fold increase in the number of fungi belonging to genera *Fusarium*, *Penicillium*, *Aspergillus*, *Rhizopus*, as compared to the level set by the regulation. By further laboratory testing, an increase of the total number of fungi in the large number of examinated feeds was discovered: corn ($887x10^3$ *Aspergillus*, *Rhizopus*), piglets second feed ($319x10^3$ *Aspergillus*, *Mucor*, *Rhizopus*), feed for pregnant sows ($123x10^3$ *Penicillium*, *Fusarium*) and feed for lactating sows ($526x10^3$ *Aspergillus*, *Penicillium*, *Mucor*). The presence of the following mycotoxins was detected: zearalenon ("ZEA") in the feed for pregnant sows (0.72 mg/kg), aflatoxin AB1 in the piglets first feed (0.018 mg/kg) and ochratoxin OTA in the piglets second feed (0.12 mg/kg).

Health problems on the second examined swine farm included increased incidence of clinical and pathomorphological signs of infective diseases and failed reaction to the applied therapy. By analyzing the existing data on the farm, high mortality in piglets 7 days before weaning was noticed, which did not decrease after medical treatment. With the aim to overcome the problem, the measure of medical treatment of piglets 3 days before weaning (parenteral application of antibiotics) was introduced, but with no results. Disease in the weaned piglets was clinically characterized by severe yellowish diarrhoea, dehidratation, huddling, roughly hair and sporadical coughing. Therapeutic treatment of the diseased animals was multiple: the antibiotics were given through feed, water and parenterally. Applying pathomorphological examination on the dead weaned piglets, the prominant changes of digestive and respiratory tract were detected (*Gastroenterotyphlitis haemorrhagica*, *Poliserositis fibrinosa massiva*, *Pneumonia complex*). By bacteriological testing of tissue samples from dead piglets the following bacteria were isolated: *Escherichia coli haemolytica, Pasteurella* sp**,** *Streptococcus uberis*. By laboratory feed testing of the available first feed for piglets the presence of OCT (0.5 mg/kg) and ZEA (4 mg/kg) was discovered.

Health problems in the third examined swine farm indicated a potential role of mycotoxins in fattening pigs. Clinically,the fatteners showed signs of broncopneumonia (cough), sporadic and intermitent bloody diarrhoea that was lately intensified, and fever (40–41 °C). Anamnestically, it was discovered

that in the last few days a total of 40 fatteners died. The pathomorphological examination on the dead fattenars was done. The existance of the pathological changes on the respiratory and digestive tract (*Pleuropneumonia actinobacillosa*, *Pericarditis fibrinosa diffusa*, *Gastritis catharrhalis*, *Typhlocolitis haemorrhagica – Dysenteria suum*) was discovered. Bacteriological testing of the tissue samples derived from the dead fatteners isolated the following bacteria: *Escherichia coli haemolytica*, *Pasteurella* sp., *Actinobacillus suis*. The presence of ZEA (0.8 mg/kg) in the samples of corn was microbiologically detected.

Complex health problems that, besides health disorders, included significant reproductive disturbances on the fourth swine farm were noticed. Anamnestic data showed that there were frequent periods of sows delivering mummified piglets, which decreased the litter size. Also, the increased number of rebreeding sows at irregular intervals was discovered. The conception rate has dramatically decreased and the problem with frequent abortions 2 months before was intensified. Besides this, farrowed piglets were nonviable and despite the medical treatment, they lived only 3-4 days after birth. In the pregnant dams, the immunoprophylaxis was carried out with the aim to prevent the outbreak of disease in suckling piglets. Also, the antibiotics were added in the sows feed 7 days before and 7 days after farrowing. Sporadically, the occurrence of severe yellowish diarrhoea in piglets and clinical signs of vulvovaginitis in just born piglets were evident. The clinical signs of diarrhoea and pneumonia were occasionally noticed in the weaners, while the fatteners faced the problem of outbreak of bronchopneumonia (over 80% of the fatteners were coughing and thumping, devloped dispnoa, had nasal secretion, individual vulvovaginitis and rectal prolapses). The pathomorphological examination of the dead fatteners revealed pathological lesions on the organs of respiratory (*Bronchitis catarrhalis*, *Pleuropneumonia actinobacillosa*, *Pneumonia interstitialis*) and digestive tract (*Typhlocolitis haemorrhagica acuta*). By applying bacteriological examination of the tissue samples derived from dead fatteners, the following bacteria were isolated: *Pasteurella* sp, *Actinobacillus suis*, *Mycoplasma* sp. After laboratory testing of swine feed samples, the simultaneous presence of several mycotoxins was established: ZEA (6.4 mg/kg), AB1 (0.0064 mg/kg), OTA (0.032 mg/kg) and trichothecenes: diacetoxyscirpenol ("DAS") 0.25 mg/kg and T-2 toxin (0.1 mg/kg).

The connection between the presence of mycotoxins in swine feed and an outbreak of viral infection of swine, *Morbus Aujeszky* ("MA") was established in the last examined farm. By microbiological testing of the feed for lactating sows the presence of fungi (*Fusarium* sp., *Mucor* sp.) and AB1 (0.02 mg/kg) were detected. Anamnestically, the health disorders in sows and their litters were observed. Epidemiological investigation revealed that 2 months before a total of 50 new sows had been brought on the swine farm. Serologically, the presence of specific antibodies against MA was detected in 6 sows. However, despite the fact that these animals were serologically positive, the origin of that immunological status from the aspect of MA remained unknown: vaccination or infection. On the other hand, the evaluated swine farm stopped with imunoprophylaxisis against MA 8 years before. By clinical examination of the sows, the signs of inapetence, mild apathy and agalactiae were observed.

In suckling piglets the sings of severe distrurbance of the central nervous system (wide open eyes, paddling, trembling, ataxia, paresis and paralysis) were clinically detected. In some cases the whole litter of piglets died within 48 hours. Despite the fact that the sows and piglets were therapeutically treated, there was no evident respond to the applied medication. Clinically, the fatteners also became anorectic, listless and apathic. The pathomorphological changes detected in dead sucklings indicated the lesions characteristic for MA infection (*Necroses miliares hepatis***,** *Haemorrhagiae corticis renis, Tonsillitis diphtheroides necroticans).* By applying virological testing (viral isolation on the susceptible cell culture) of the tissues derived from dead piglets, the *Morbus Aujeszky* virus was isolated.

DISCUSSION

The achieved results support the existence of positive interaction between the mycotoxins and causative agents of swine infective diseases. The continuos intake of small amounts of mycotoxins leads to chronic intoxication which is clinically characterized by the loss of weight, insufficient weight gain and increased susceptibility to infectious diseases (D i e k m a n and G r e e n , 1992; O s w e i l e r , 2006). The clinical toxicological syndroms caused by ingestion of moderate to high amounts of mycotoxins and their effects on immune system have been well characterized (M e i s s o n n i e r et al., 2008; P r o d a n o v et al., 2009). From an epidemiological point of view, mycotoxins may cause breakdown of active immunity and occurrence of disease even in properly vaccinated animals (M a r i n et al., 2002; T a r a n u et al., 2005).

The presence of mycotoxin in feed can be directly connected to the detected health disturbances on the examined swine farms. In our research we discovered persistant presence of various infections which had poor or no reaction at all to the applied antimicrobial therapy (gastroenteritis, pneumonia, dysenteria suum, endometritis). Also, the chronic disturbances, such as slow growth, malnutrition and persistant presence of infections of low intensity suggest the potential presence of mycotoxins. Normal immune function is expected to be restored after exposure to the toxin ends (O s w e i l e r , 2006). The consumption of mycotoxin-contaminated feed leads to the induction of teratogenic, cancerogenic, oestrogenic, neurotoxic and immunosuppressive effect in the organism (K a b a k et al., 2006). As a consequence of immunosuppressive action of mycotoxins, clinical and pathological lesions corresponding to infectious diseases of different ethiology occured (O s w a l d et al., 2005; O s w e i l e r , 2006). The obtained results offer an example of immunosuppressive effect, i.e. the occurence of enteroxemia in piglets, despite the fact that dams were vaccinated twice during the gestation. The enterotoxemia is caused by pathogenic bacterial strains and occurs frequently as a cause of mortality in the examined production phase. It can be provoked by feed quality, i. e. the presence of mycotoxins. On the other hand, young animals are much more

sensitive to the effects of mycotoxins in comparison to the adults. Also, dietary deficiencies of protein, selenium and vitamins have been sugested as predisposing factors for mycotoxicoses (K a b a k et al., 2006). Piglets of the nursing sows, exposed to AB1, may be immunocompromized because residues of AF-M1 occur in milk (O s w e i l e r, 2006). The biggest challenge with mycotoxicoses is the non-specific nature of symptoms in the affected animals. Consequently, the health disorders due to mycotoxins in the feed are difficult to diagnose (O s w e i l e r, 2006; P r o d a n o v et al, 2008). The acute symptoms are relatively more specific in pigs, for example zearalenone-induced reproductive disorders, fumonisins-induced pulmonary oedema and deoxynivalenol ("DON")--induced feed refusal (M e i s s o n n i e r et al., 2008). Mycotoxin mixtures, i.e. the combinations of several micotoxins are likely to occur naturally and may influence the immunity in an additional or synergistic manner. An example is combination of AB1 and T-2 toxin, AB1 and OTA. The latest combination was detected on the first examined swine farm. Economic losses that occur as a consequence of interaction of several mycotoxins are still unknown because in low concentrations several mycotoxins may interact in a way that is difficult to detect. Combinations of several and more moderate concentrations of different mycotoxins, which individually may appear in a level too low to be concerned with, can cause cumulative toxicoses, which affect the ability of the pigs organism to fight diseases (D i e k m a n and G r e e n, 1992; O s w e i l e r, 2006).

In the second evaluted case, the presence of OCT and ZEA in the feed was detected. Consequently, an evident decrease in the swine immunity against infective diseases (of the respiratory and digestive tract) was noticed on the farm, and there was no positive respond to the applied antibiotic therapy. Also, the occurrence of diarrhoea and an increased percent of waste piglets can be connected with the presence of mycotoxins in the feed, because the piglets display clinical feed refusal. Some effects caused by the consumption of mycotoxin contaminated feed were experimentally detected after long exposure (10-15 days). This long exposure is likely to occur in the field, as animals might eat the same batch of feed for an extended period of time (O s w e i l e r, 2006). The gastrointestinal tract represents the first barrier against ingested food contaminants and natural toxins. Upon the ingestion of mycotoxin contaminated feed, intestinal epithelial cells could be exposed to a high concentration of toxin (B o u h e t and O s w a l d, 2007). Stability of the intestinal flora appeared to be an important factor for animal health (O s w a l d et al., 2005). Finally, while systemic immunity is the focus of most investigations, it is highly probable that mycotoxins have their greatest effect on mucosal lymphoid tissue before they are absorbed and subsequently metabolized (B o u h e t and O s w a l d, 2005; O s w a l d et al., 2005). More recently, it has been experimentally demonstrated that the ingestion of OTA contaminated feed increases the susceptibility of pigs to natural infection by *Salmonella cholerasuis, Serpulina hyodysenteriae, Campylobacter coli.* Furthermore, it was discovered that, after the oral intake of purified fumonisin B1, susceptibility of animals to intestinal infections significantly increased. Although *Escherichia coli* is a normal inhabitant of intestinal microphlora, pathogenic bacterial strains poses

the virulence determinants which are involved in septicaemia development (O s w a l d et al., 2005). The presence of mycotoxin in feed for pregnant sows has an influence on the occurrence of embrional and fetal death, and decreased immunological defence in piglets. Reproduction disorders, fetal mummification and stillbirths may be connected with mycotoxin contaminated feed (P r o - d a n o v et al., 2009). Nutritional effects associated with feed refusal may also contribute to the observed decreased efficacy of therapeutic treatments and vaccination (O s w a l d et al., 2005).

Immune functions suppressed by mycotoxins may decrease resistance to infectious diseases, reactivate chronic infections and reduce vaccine and therapeutic efficacy (O s w a l d et al., 2005). In the last examined case, where the outbreak of MA on the farm was examined, mycotoxin (AF) in the feed can be connected with the possible reactivation of chronic (latent) infection in sows. It has been discovered that aflatoxin decrease resistance to bacterial, fungal, viral and parasitic diseases in swine. Subsequently, vaccinations against various infectous diseases may be less effective in animals exposed to mycotoxins (D i e k m a n and G r e e n, 1992). For example, AB1 interferes with the development of acquired immunity in swine after erysipelas vaccination (O s w a l d et al., 2005). Even when present in low doses, AF alters the immune response and this may predispose pigs to infectious diseases (M a r i n et al., 2002; T a r a n u et al., 2005). The titers of specific antibodies after vaccination are good indication of humoral immunoresponses. It has been demonstrated experimentally that DON and ZEA impaired the production of specific antibodies after vaccination against classical swine fever (C h e n et al., 2008). Therefore, the presence of mycotoxins in the feed may lead to a breakdown in vaccinal immunity and the occurrence of disease even in properly vaccinated animals (O s w a l d et al., 2005). It should be remembered that detected concentrations of mycotoxins in the feed are approximations, because sampling is never completely representative. Mycotoxin can be identifeid by chemical analyses, but sometimes causative cereal that initiated the problem is no longer available or representative sample (O s w e i l e r, 2006). On the other hand, when discussing the mycotoxin problem, feed dilution may reduce the exposure, but wet or contaminated grain can introduce new fungi, and develop the conditions that eventually may lead to the entire mixture being contaminated (O s w a l d et al., 2005). Mycotoxin contamination may not be suspected until most or all of the contaminated feed is consumed. A wise product practice is to save a representative sample of feed purchased, and keep it under stable conditions until swines are marketed. In case of any doubts regarding the feed quality, those samples may be valuable in documenting whether specific feeds were involved in the problem (O s w e i l e r, 2006).

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ИНТЕРАКЦИЈА МИКОТОКСИНА СА УЗРОЧНИЦИМА ИНФЕКТИВНИХ ОБОЉЕЊА СВИЊА

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Резиме

Микотоксини као секундарни метаболити плесни контаминирају анимална хранива у свим стадијумима њихове производње. Конзумација хранива контаминираних микотоксинима може имати за последицу имуносупресивно дејство у организму, што представља фактор предиспозиције за инфективна обољења. Дејство на имунолошки систем је од посебног значаја за свињарство, јер технологија на фармама свиња подразумева примену многобројних вакцинација, што представља проблем код имунолошки компромитованих јединки. Циљ рада је био испитивање утицаја микотоксина у свињарству, као секундарних фактора у дестабилизацији имунолошког система животиња.

Материјал за испитивање је обухватао пет фарми свиња, на којима су регистровани здравствени проблеми односно утврђено је присуство клиничких и патоморфолошких знакова инфективних обољења различитих категорија свиња. У оквиру примењених метода, обављена су епизоотиолошка и клиничка испитивања, патоморфолошки преглед угинулих јединки, вирусолошко и бактериолошко испитивање узорака органа угинулих јединки и микробиолошко испитивање узорака хране у циљу установљавања присуства плесни и микотоксина. Постигнути резултати испитивања указују на постојање позитивне интеракције између микотоксина и узрочника инфективних обољења свиња. Са епидемиолошког аспекта, значајно је да микотоксини у храни за животиње могу довести до пробоја активног имунитета и избијања обољења иако је имунопрофилакса обављена на прописан начин.

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THE IMPORTANCE OF ANTIOXIDANTS IN THE PROTECTION AGAINST MYCOTOXICOSES IN FARM ANIMALS

ABSTRACT: Mycotoxins are biologically active substances that are synthesized by saprophytic and parasitic fungi, and which, when taken into organism by ingestion, can provoke intoxications known as mycotoxicoses. Farm animals show different susceptibility to mycotoxins depending on various factors: genetic (species and breeds), physiological (age and obesity) and environmental (hygienic and climatic). One of the mechanisms of mycotoxin activities is peroxidation of lipids brought about directly by the production of free radicals or by increased sensitivity of tissue to peroxidation. Peroxidation of lipids provoked by mycotoxins is caused by low level of natural antioxidants, so they have a crucial role in the protection against mycotoxins. Nutritive stress can influence negatively the relationship between antioxidants/pro-oxidants, and mycotoxins are nowadays regarded as leading factors of stress induced by nutrition. This optimal relationship can be regulated by the use of antioxidants in food (selenium, vitamin E, carotenoids, etc.) known to prevent tissue damages caused by free radicals. Selenium and vitamin E are essential nutrients which contribute to the preservation of animal health by realizing mutual biological activities in the organism. This paper presents the findings on mechanisms of the action of different species of mycotoxins and the importance of antioxidative protection in farm animals, as well as the results of our investigations of influence of mycotoxins on the occurrence of some reproductive disorders in pigs.

KEY WORDS: antioxidants, farm animals, mycotoxins

INTRODUCTION

Mycotoxins are secondary products in the metabolism of molds and they represent a global problem in supplying people and animals with food. For a number of years it has been thought that mycotoxins appear only in a small number of feedstuffs. However, today it is known that they are present in almost all feedstuffs. More than 30 mold metabolites are considered toxic for both people and animals whilst 25% of cereals in the world are contaminated by mycotoxins. *Fusarium* species can inhabit the cereals during the period of

their growth and can create "field mycotoxins". *Aspergillus* and *Penicillium* mostly develop after the harvest and their toxins are called "storage mycotoxins". The trade of cereals results in the spread of mycotoxins, so they may also appear in feedstuffs in the regions where it is rather unusual (S u r a i and D v o r s k a , 2005).

A growing presence of mycotoxins is a consequence of significant climatic changes. Extremely high, but also low temperatures, heavy precipitations and drought favor the development of molds and production of their toxic metabolites in animal and human food. Due to their harmful effect on the health of animals and people, mycotoxins are called "silent killers", "invisible thieves" and "natural toxins" (W o o d, 1992). There are more than 300 mycotoxins present in nature, but so far the toxic effect has been identified for only about 30 mycotoxins. Therefore, negative laboratory findings do not indicate the absence of mycotoxins, but the absence of 30 mycotoxins identified so far. As for the rest of mycotoxins, no precise answer can be given. Sampling of material for the analysis of mycotoxins is rather complicated since synergistic action in low concentrations can cause bigger problems than one mycotoxin in greater quantity (S u r a i et al., 2008).

MECHANISMS OF TOXICITY OF MYCOTOXINS

The main mechanisms of toxicity of mycotoxins are: inhibition of the protein, DNA and RNA synthesis, damages of DNA, lipid peroxidation, the change in structure and function of membrane and starting off the programed death of cell. The consequences are: immunosuppression, hepatotoxicity, nephrotoxicity, neurotoxicity and gonadotoxicity (S u r a i and D v o r s k a , 2004). Mycotoxins may inhibit the activity of gluconeogenetic enzymes, the function of mitochondria (inhibition of the enzymes of tricarbonate cycle), they may disturb the functions of nucleic acids and the synthesis of proteins. They demonstrate hepatotoxic and hepatocarcinogenic action through the peroxidation of lipids. They can also have immunomodulatory effect even when they are present in food in concentrations below the limit of detection. They suppress humoral and cellular immunity, change the activity of T and B lymphocytes, decrease the production of antibodies and disturb the function of macrophage.

Not all domestic animals are susceptible to mycotoxins to a same degree. The most susceptible ones are pigs, cattle and poultry, while the least susceptible are sheep and goats. Also, young and quite old animals are the most susceptible. Poor feeding, bad hygiene and the way of keeping may increase the susceptability of animals to mycotoxins. High concentration of mycotoxins in food can even lead to death of a great number of heads in a short time period. However, low contents of mycotoxins are also problematic, due to persistent depression of animals (J o k i ć et al., 2004).

Research by J o k i ć et al. (2003) pointed to the negative effects of some mycotoxins on sows' reproductive characteristics in two different periods. In the first period of examination the animals were fed with silage, wet maize

grain, soya and sunflower grits all having high levels of mycotoxins. In the second period, the animals were fed with diets which did not contain prohibited quantities of mycotoxins (artificially dried maize grain, soya and sunflower grits) (Table 1).

Tab. 1 – Average content of mycotoxins in some fodder

Fodder	Mycotoxins, mg · kg^{-1}			
	Zearalenon	Aflatoxin B_1	Aflatoxin G_1	Ochratoxin
First Period				
Silaged wet corn kernels	3.4	0.08	0.01	3.0
Soybean meal	1.6	-	-	0.1
Sunflower meal	5.32	0.052	-	0.66
Second Period				
Artificially dried corn kernels	0.1	-	-	0.1
Soybean meal	0.07	-	-	-
Sunflower meal	0.2	-	-	0.02

The mean values for the level of mycotoxins in some feeds in the second period were lower than those prescribed by the Book of Regulations on maximum quantities of harmful matters and ingredients in animal feeds. The level of F-2 toxin ranged from 0.07 mg/kg in soya grits to 0.2 mg/kg in sunflower grits. Ochratoxin was present at the level of 0.02 mg/kg in sunflower grits, and 0.1 mg/kg in artificially dried maize grain, but the presence of aflatoxin B_1 and aflatoxin G_1 was not confirmed.

The results of the insemination of sows in the first and second period of trial are shown in Table 2.

Tab. 2 – Results of insemination of sows

Parameter		Period		Difference
		I	II	
Number of attempts		6180	7052	
Delivery Parity		3.95	3.83	
Drying-estrus interval (days)		21.01	15.87	
Success of insemination (conception)	n	4415	5932	12.68***
	%	71.44	84.12	
Failure to impregnate	n	1765	1120	12.68***
	%	28.56	15.88	
Miscarriage	n	86	95	0.04 NS
	%	1.39	1.35	
Deliveries	n	4237	5690	12.13***
	%	68.56	80.69	

The occurrence of oestrus in sows was rather long in both periods of trial. After the weaning, the oestrus was manifested after 15.87 days in sows fed with diets containing lower content of mycotoxins, while in animals fed with mixture of high level of mycotoxins it appeared after 21.01 days. The success

in sows insemination was better in the second period than in the first (84.12% in comparison to 71.44%). Obtained differences were statistically significant ($P<0.001$). The number of services was greater in the first period than in the second, with 28.56% and 15.88%, respectively, expressed in relative values. The percent of farrowed sows was greater in the second period (80.69%) than in the first (68.56%) and these differences were statistically highly significant.

MYCOTOXINS AND APOPTOSIS

Mycotoxins incorporated into the cell membranes may lead to lethal changes. They may provoke the change in the composition of fatty acids and peroxidation of polyunsaturated fatty acids in membrane, leading to a damage of membrane receptors and loss of membrane function due to the change in elasticity and porousness. The maintenance of tissue homoestasis involves removing of spent and damaged cells. This process is a programed apoptosis of cells. It is developed by initiating the death signal in the plasma membrane, creating pro-apoptotic oncoproteins, by activation of proteases and endonucleases. Final result is irreversible process that leads to cell's death. In apoptosis, cells shrink, the nucleus gets smaller, chromatin is condensed, DNA splits into fragments and proteins of kinase are activated.

Reactive oxygen species have an important role in the process of apoptosis, including the onset of the reinforcement of apoptosis. The decrease of GSH level within the cell, that is, its consumption by the cell, may intensify apoptosis. On the contrary, the increase of GSH level decreases this process. Many studies have confirmed that mycotoxins decrease the level of GSH, thus initiating apoptosis. T_2 toxin is a potent apoptosis agent. Trichotecenes induce nucleosomal fragmentation of DNA (N a g a s e et al., 2001). Today the apoptosis is being considered as a mutual mechanism of toxicity of various mycotoxins.

MYCOTOXINS AND ANTIOXIDANTS

Mycotoxins can lead to lipid peroxidation directly, or they can increase the tissue sensitivity to peroxidation. Lipid peroxidation caused by mycotoxins is a consequence of decreased level of natural antioxidants (D v o r s k a and S u r a i, 2001). Antioxidants are of great importance in the protection against mycotoxins (G a l v a n o et al., 2001). Selenium and vitamin E are key ingredients of food and they play the role in antioxidative protection. Vitamin E, as an integral component of lipid membrane, neutralizes free radicals. It represents the first line of cell`s defence against free radicals and it is the keeper of cell's integrity. Selenium plays its role through the enzyme GSH-Px. The level of this enzyme increases in plasma along with the increase of the concentration of selenium in food or water, what is a good indicator of biological adoption of selenium (S a n k a r i, 1985; H a s s a n, 1987; T o d o r o v i ć, 1990; M i h a i l o v i ć et al., 1991). With na increase of selenium level above the

necessary, the activity of GSH-Px shows the effect of plateau so that higher concentrations of selenium do not lead to further increase of the activity of this selenoenzyme (Meyer et al., 1981; Mihailović et al., 1997; Joksimović-Todorović et al. 2005a, b, 2006a, b). Organic selenium demonstrates better antioxidative protection than non-organic, due to better resorption (Jokić et al., 2005), and in combination with vitamine E it demonstrates protection in chicks under stress and/or infected by aflatoxin (Stanley et al., 1998).

Sometimes selenium and vitamin E show synergistic activities and sometimes not. The lack of synergism shows that selenium and vitamin E have different forms of protection against oxidative stress, or that some mechanisms in which peroxidation is not present are also involved in the defence. These mechanisms can function together, but they can also occupy different places depending on situations created (Allison and Laven, 2000).

Protective action of selenium against aflatoxin has been confirmed in mammals as well. Pigs were protected against the action of AFB1 when they received 2.5 mg Se/kg through diet (Davila et al., 1983). Selenium also displays protective effect in toxicosis induced by T-2 and DON mycotoxins. Furthermore, acute lethal toxicity of T-2 toxin decreases with the use of selenium (Yazdanpanah et al., 1997). Not only selenium but also some other antioxidants show protective effect in lipid peroxidation caused by mycotoxins: vitamin A and E, ascorbic acid, coenzyme Q 10, synthetic antioxidants and plant extracts. The use of Mycosorb and organic selenium is highly efficient in preventing peroxidation in the liver of chicks infected by T-2 toxin (Surai, 2002). A simultaneous use of antioxidants and absorbents of mycotoxins (of wide range) today represents the best protection of poultry against mycotoxicoses (Weber et al., 2006).

CONCLUSION

Mycotoxins are today regarded as leading factors of nutritive stress which affect unfavorably the relationship of antioxidants/pro-oxidants. Considering the fact that the antioxidants/pro-oxidants balance in the cell (redox status) is responsible for regulating apoptosis, it is likely that natural antioxidants and selenoproteins (GSH-Px, tireodoxin reductase and methionine sulphoxid reductase B) may be involved in the prevention of apoptosis caused by mycotoxins.

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ЗНАЧАЈ АНТИОКСИДАНАТА У ЗАШТИТИ ОД МИКОТОКСИКОЗА КОД ДОМАЋИХ ЖИВОТИЊА

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Резиме

Микотоксини су биолошки активне материје које синтетишу сапрофитне и паразитске гљивице, а унети ингестијом у организам изазивају тровања која се називају микотоксикозе. Домаће животиње су различито осетљиве на микотоксине зависно од различитих фактора: генетских (врсте и расе), физиолошких (старости и ухрањености) и услова средине (хигијенских и климатских). Један од механизама деловања микотоксина је пероксидација липида директно производњом слободних радикала или повећањем осетљивости ткива на пероксидацију. Пероксидација липида изазвана микотоксинима је проузрокована ниским нивоом природних антиоксиданата, тако да они имају кључну улогу у заштити од микотоксина. Нутритивни стрес неповољно утиче на однос антиоксиданта/про-оксиданта, а микотоксини се данас сматрају водећим факторима стреса изазваних исхраном. Овај оптималан однос може се регулисати употребом антиоксиданата у храни (селен, витамин Е, каротиноиди, и др.) који спречавају ткивна оштећења узрокована слободним радикалима. Селен и витамин Е су есенцијални нутрицијенти који доприносе очувању здравља животиња остваривањем заједничких биолошких активности у организму. У овом раду биће приказана сазнања о механизмима деловања различитих врста микотоксина и значаја антиоксидативне заштите код домаћих животиња, као и резултати наших испитивања утицаја микотоксина на појаву појединих репродуктивних поремећаја код свиња.

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INVESTIGATION OF DISSEMINATION OF ASPERGILLOSIS IN POULTRY AND POSSIBLE CONTROL MEASURES

ABSTRACT: Fungi belonging to genus *Aspegillus* are ubiquitous saprophytic microorganisms which are, in certain circumstances, responsible for clinical infections of respiratory tract in all poultry, particularly in young birds. In case of a lung form, *Aspergillus fumigatus*, *A. niger* and *A. glaucus* are the most frequently isolated fungi. In general, poultry is constantly exposed to these fungi in its environment. Predisposing factors, such as long exposition and highly contaminated environment and litter, high humidity in poultry houses, poor ventilation, malnutrition and stress, all contribute to clinical aspergillosis. Some geographic and seasonal regularities are observed in relation to the distribution of disease outbreaks. In this sense, cases of aspergillosis in our country were more frequently noted in wild areas located northern from the rivers Sava and Danube. Influence of some factors on the outbreak and spreading, as well as predominant clinical features of aspergillosis in poultry were investigated in this paper. Possible prophylactic and intervention measures were discussed. The occurrence of *Aspergillus sp.* in poultry was analyzed according to the clinical and laboratory investigations performed during the two selected years, 2000 and 2010. Widespread aspergillosis was noted in poultry flocks of different age, both in young and adult birds. During the years 2000 and 2010, acute aspergillosis was found in 12 and 16 commercial flocks of chickens and turkeys, respectively. Ocular infection with *Aspergillus* was determined in 10 day old broilers from two flocks. *Aspergillus sp.* was isolated from unhatched eggs (6.86%), litter (23.07%), environmental (36.17%) and hatchery swabs (3.85%). Besides the appropriate antifungal therapy, enforcement of proper sanitary-hygiene measures on poultry farms and hatcheries, as well as microbiological control of feed are considered essential for an efficient control of infection and its spreading.

KEY WORDS: avian aspergillosis, humidity, environment, temperature

INTRODUCTION

Aspergillosis is a fungal disease of all poultry species, particularly of young birds. Most frequently it occurs in turkey poults, chicks, ducklings and goslings (K u n k l e, 2003; B e y t u t et al., 2004). *Aspergillus fumigatus* is considered to be the most pathogenic and is the most frequent isolate from pathologic lesions, while others like *A. niger*, *A. flavus*, *A. terreus* and *A. glaucus*

can induce the disease, too. The fungal spores are ubiquitous in nature. Exposure of poultry to fungi or spores occurs after the introduction of contaminated litter and feed. Early infection is possible in hatcheries if fungal contamination occurs.

Certain infectious diseases may contribute to aspergillosis, e.g. infectious bronchitis, coryza, chronic respiratory disease, laryngotracheitis, Newcastle disease and fowlpox. It has been speculated that extremly dry air and dust can cause the infection with aspergillus because they dry out the respiratory mucoses and protective effect of mucus is absent (K r i s t e n s e n and W a t h e s , 2000).

Some geographic and seasonal regularities are observed in relation to the distribution of aspergillosis outbreaks. Incidence of the disease decreases on poultry farms with stringent hygiene and good nutrition menagement.

Influence of certain factors on the outbreak and spreading, as well as clinical features of aspergillosis, were investigated in this paper. The possibility to control the disease by introduction of prophylactic and intervention measures was discussed.

MATERIAL AND METHODS

The presence of *Aspergillus* spp**.** on poultry farms was analyzed from data collected after the clinical and laboratory investigations performed during the two selected years, 2000 and 2010.

Poultry flocks were clinically observed and postmortem examination of chickens and turkeys of different age was carried out. If aspergillosis was detected in the findings, the attacked tissues were taken for microbiological investigations. Having been processed first, tissue samples were cultured on solid media using standard methods. Mycological investigations were done in accredited laboratory at the Veterinary Institute Novi Sad.

Litter taken directly from different surfaces in poultry houses and swabs was cultured on solid media. Unhatched eggs, hatchery waste and environmental swabs taken from different surfaces in hatcheries were sampled in order to determine the presence of fungi and its frequency, as well as for the comparison of mycological contamination with the environmental conditions.

The influence of different factors on the occurrence, spreading and source of aspergillosis was investigated using data from the official report of the Republic Hydrometeorological Service of Serbia, regarding the outer temperature and rainfalls in 2010, for the purpose of comparison with the control period from 1961 to 1990.

RESULTS AND DISCUSSION

The spreadout of aspergillosis was noted in poultry of different age, from very young birds to adult ones. It is well known that the disease can occur in

other young and adult poultry species, including poults, gooslings, ducklings, swans, wild and pet birds, particularly if kept in intensive manner. While resistant and healthy poultry can overcome the infection with high number of *Aspergillus*, week and young birds generally become ill easily. It is experimentally demonstrated that chickens up to three days of age are the most susceptible to the infection, while older are more resistant. I s l a m et al. (2009) found significant differences in morbidity (up to 70%) and mortality rate depending on age and category; aspergillosis was most frequently detected in cockereles (9.03%), then in broiler chickens (5.48%) and in laying hens (1.92%). The disease was clinically present in broilers at the earliest age (13 days) and in layers and cockereles at the oldest age (76 weeks). In epizootiological investigation S a j i d et al. (2006) found the majority of sick flocks at the age of 14 days.

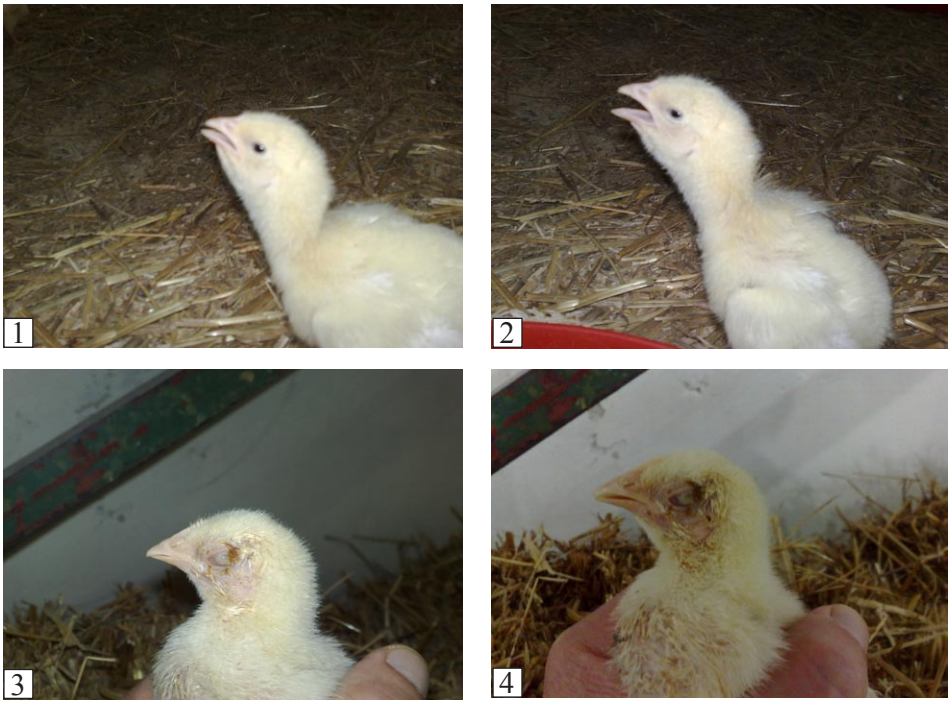
Aspergillosis apears in two clinical forms: 1) acute form with high morbidity and mortality rates, the disease persists from only few days to two weeks, and 2) subacute and chronic form in adult poultry, disease being persistant several weeks or rarely several months.

During the years 2000 and 2010, acute aspergillosis was detected in 12 and 16 commercial flocks of chickens and turkeys, respectively. In 2010, the disease was acute with high morbidity and mortality within only few days. Numerous flocks suferred detrimental losses in a short period of time and were destroyed because their further raise was economically unjustified. Clinical symptoms during the acute source of infection are described in literature, including depression, inapetence, thirst and hyperventilation with dyspnoe (A k a n et al., 2001; I s l a m et al., 2009; S t o u t e et al., 2009). Chickens have ruffled feathers; they drowse, get weaker and, in complicated cases of dyspnoe, pathologic wheese is observed, which differs from other respiratory diseases. Besides the symptoms previously mentioned, the investigation of mass pneumomycoses discovered one distinctive finding which was grupping of the chickens toward the source of fresh air ("hunger for oxygen"; Figures 1 and 2).

Besides respiratory form, infection of eyes can also occur (A k a n et al., 2001). In that case protruding eye lids are observed because of formation of yellowish-cheesy small pellets around the membrane nicticans with central ulceration (Figures 3 and 4). In our investigations, ocular infection with *Aspergillus* was determined in two broiler flocks, at the age of 10 days.

In subacute and chronic infection, only one or several individuals in flock became ill. Clinical signs of depression, inapetence, ruffled feathers and emaciation are common, sometimes chronic bronchopneumonia, too (M a r t i n et al., 2007; I s l a m et al., 2009). during the year 2010, subacute and chronic aspergillosis was not detected.

In the investigated period, acute aspergillosis was clinically noted. Postmortem findings included nodules – aspergillus granuloma, oval or round shaped, single or in conglomerate, size of a pin head to pea, located on air sacs, lungs and on visceral serosae of abdominal cavity, liver and intestines (Figures 5, 6, 7 and 8). Granulomas, oval or round, single or in group of conglomerate, are typical section finding in *Aspergillus* infected birds. They are located in tho-

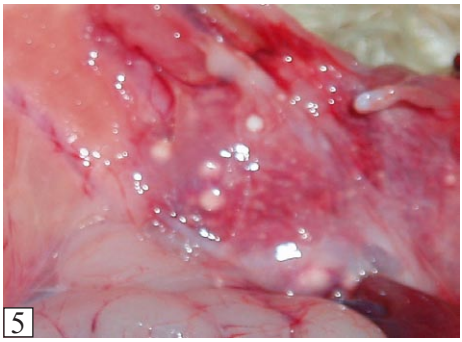


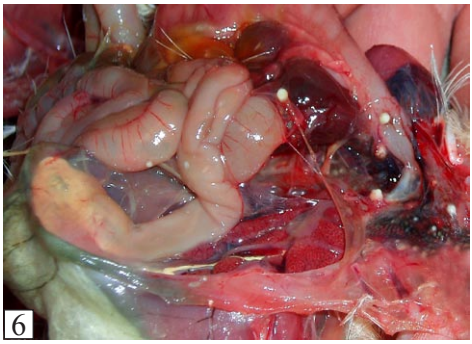
Slika 1 i 2. Oboleli pilići od aspergiloze – izražena dispnoja.
Slika 3 i 4. Očni oblik aspergiloze – žućkaste siraste kuglice oko membrane niktikans.

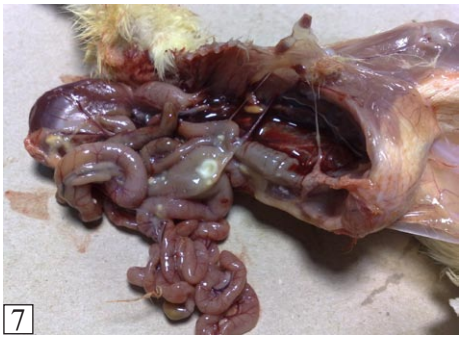
racic and abdominal cavity and on the liver surface. Their appearance resembles the one found in avian tubercullosis, but with radial hyphae nets surrounded by reactive zone like granulation tissue. Generally, nodules are present in almost all tissues, even in eyes and brain (Throne Steinlage et al., 2002; Mukaratirwa, 2006; Martin et al., 2007; Cacciuttolo et al., 2009; Islam et al., 2009; Stoute et al., 2009).

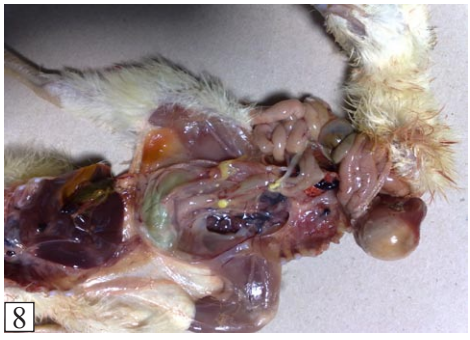
Clinical signs and pathologic findings are not sufficient for a diagnose, so laboratory confirmation by isolation of *Aspergillus sp.* is necessary. The spores germinate well in laboratory conditions, on standard media and at room temperature, producing green to green-blue colored colonies that become darker, even black over time (Figures 9 and 10). In order for the disease to be diagnosed other respiratory agents need to be excluded, such as infectious bronchitis, coryza, chronic respiratory disease and Newcastle disease, infectious laryngitis, tuberculosis and fowlpox.

In intensive poultry keeping, there are numerous sources of this important fungal infection. Feed, particularly mashed, can be contaminated with high number of fungi and molds (Akan et al., 2001; Nešić et al., 2005; Škrinjar et al., 2009). According to the Regulation of microbiological quality of feed, complete diets for young and adult poultry may contain not more then 50000 and 300000 colonies of molds, respectively. Based on our knowledge, findings

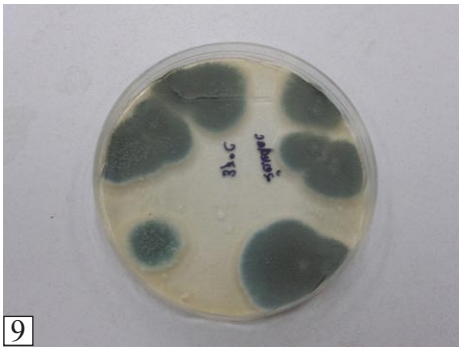


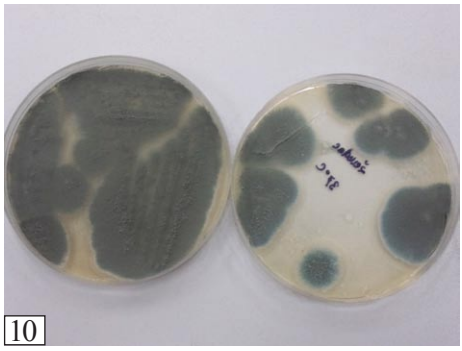






Slika 5-8. Oboleli ćurići od aspergiloze; patoanatomski nalaz: diseminirani čvorići-aspergilusni granulomi na plućima i zamućenim vazdušnim kesama (slika 5), i po serozama organa u abdominalnoj duplji (slika 6,7,8)





Slika 9 i 10. Rast kolonija Aspergillus flavus na Sabour-o agaru

of microbiologically inadequate complete diets with *Aspergillus* contamination are not rare (data not presented here).

The infection in poultry may occur if litter, environment or hatcheries are contaminated. Fertile eggs and embryos can be contaminated before or during the incubation, and *Aspergillus sp.* is found in tissues of unhatched eggs and hatchery waste (J a c o b s e n et al., 2010). Warm and humid air in hatchery provides ideal conditions for the survival of *Aspergillus*. Infected embryos die

between 15th and 18th incubation day, which may cause a dicrease in hatchability of up to 30%. Dust containing more than 800 colonies per gram leads to prospective embryo infection (K o z i ć, 1967). Eggshell can be contaminated in case of inadequate collection and storage of eggs. The spores are most frequently isolated from alanto-chorion liquid, producing green colonies up to 2 cm in size. During the years 2000 and 2010, *Aspergillus sp.* colonies were often detected in unhatched eggs and swabs taken from the hatchery (Table 1). In order to prevent the contamination of embryos and chickens at hatch, different sanitation programs are applied (I v a n o v, 2008). The sanitation programs are designed in such manner that the number of fungi and other potential pathogens is maintained at an acceptable level. However, their total elimination is not realistic. Lately, some reports have brought the attention to possible infection of reproductive tissues of hens and concomitant disease (*Egg Borne Aspergillosis* or congenital aspergillosis). It is demonstrated that intravenous and inhalation infection is possible (F e m e n i a et al., 2007). Still, under natural conditions, infection is predominantly induced aerogenically and orally.

Using inadequate litter increases the possibility of infection at early age. Higher number of fungi, longer exposition, particularly in combination with stressing procedures, like vaccination, beek trimming etc., all contribute to aspergillosis. Different types of litter may be contaminated unequally. S a j i d et al. (2006) found significantly higher rate of infected eggs in flocks kept on sawdust (67.74%) in compairison to rice hulls (32.26%). On the other hand, I s l a m et al. (2009) could not clearly relate the incidence of aspergillosis to the type of litter used. In our investigations, mycological control of litter and swabs taken from surfaces in poultry houses points to oversights during sanitation and disinfection (Table 1). With respect to year 2000, the total number of samples and the number of mycologically inadequate samples was nominally higher in 2010. Raising poultry under higher fungal contamination increases the risk of aspergillosis, especially in immune compromised or sick birds. The influence of particular outer and ambiental factors to fungal survival are numerous (D e B e y et al., 1995; G i g l i et al., 2005; K a r w o w s k a, 2005; N i c h i t a and T i r z i u, 2008). The spores of *Aspergillus* germinate better when the air is dry. *Aspergillus sp.* is often isolated from dust, and since the largest proportion of dust particles is inhalable, dust is considered to be the predisposing factor for aspergillosis outbreak (D e B e y et al., 1995). The type of ventilation and air humidity are also relevant (D e B e y et al., 1995; G i g l i et al., 2005; S a j i d et al., 2006) as they can impact the concentration of *Aspergillus* in the poultry house (N i c h i t a and T i r z i u, 2008).

By using available data on meteorological conditions in the region, this paper aims at establishing the connection between clinical aspergillosis and overall laboratory findings, as well as the climatic factors. The official report of Hydrometeorological Service of the Republic Serbia for the year 2010 show extremely warm percentile distribution. During the summer of 2010 maximal daily temperatures were often above 30^0C, and the total number of tropical days was higher then the usual in most parts of Serbia, except in mountain regions.

Tab. 1 – Contamination of unhatched eggs, litter, swabs taken from the poultry houses and hatcheries by *Aspergillus sp.*

Sample type	Year 2000		Year 2010	
	Total number of samples	Number and % of positive samples	Total number of samples	Number and % of positive samples
Unhatched eggs	481	33 – 6.86	617	62 – 10.05
Litter	39	9 – 23.07	42	15 – 35.71
Swabs from houses	94	34 – 36.17	111	39 – 36.14
Hatchery swabs	182	7 – 3.85	268	15 – 5.60

The Hydrometeorological reports also show rainfalls of totally 110 to 150% of the average values (taken from the period between 1961 and 1990) in most parts of Serbia, and the percentile distribution was rainy, very rainy and extremely rainy. The rainfalls were extremely rainy in the northern regions. During the selected years (2000 and 2010) average outer temperatures were high throughout the whole year. The rainfalls, watery alluvium and relative humidity of air, were significantly higher than usual. In order to survive and reproduce, fungi need certain humidity and temperature level which is around and higher than 37°C (De Bey et al., 1995; Karwowska, 2005; Gigli et al., 2005; Sajid et al., 2006; Chate and Bhivgade, 2010).

The distribution of the aspergillosis outbreaks is influenced by some geographic and seasonal differences, so it is more frequent in areas with high outer temperature and rainfalls (De Bey et al., 1995; Karwowska, 2005; Madsen, 2006; Sajid et al., 2006). On the other hand, during winter, due to inadequate ambiental conditions and the fact that sometimes it is impossible to provide optimal ambient for poultry, aspergillosis incidence increases. Some daily aberrance in the number of *Aspergillus* in poultry houses was determined by Nayak et al. (1998). In our country, aspegillosis outbreaks are more frequent in areas northern from the rivers Sava and Danube.

Microflora in poultry houses and farms in general, in our case the "population" of *Aspergillus sp.,* contributes to the emission of biosol into atmosphere, especially during the warmer months (Karwowska, 2005). The concentration of unwanted and potentially harmful gasses, dust, bacteria and fungi in poultry house changes depending on the age of poultry, particularly in broiler chickens (Vučemilo et al., 2007). The airpollution impacts the health and productivity of poultry (Nešić et al., 2005). Also, certain species of fungi have toxinogenic and allergenic features so they can disturb the health of people, either directly, in case of farmers, or in case of the rest of the population, indirectly, after emission into atmosphere (Škrinjar et al., 2009; Chate and Bhivgade, 2010). It seems to be necessary to create and implement standards for adequate hygiene, epizootiological and epidemiological conditions on farms and their environment.

CONCLUSIONS

1. The global warming and high relative humidity induced increase of incidence of some diseases, including aspergillosis.

2. Poultry is constantly exposed to *Aspergillus sp.* in its environment, so high contamination and long exposal contribute to clinical aspergillosis.

3. The success of therapy basically depends on the source of disease and degree of dissemination of process. In acute cases, particularly in very young chickens, it would be wrong to rely on antifungal therapy solely. Implementation of sanitary-hygiene measures in poultry houses and hatcheries, as well as microbiological control of feed, is essential for prevention of significant losses.

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ИСТРАЖИВАЊА РАШИРЕНОСТИ АСПЕРГИЛОЗЕ КОД ЖИВИНЕ И МЕРЕ КОНТРОЛЕ

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Резиме

Гљивице из рода *Aspegillus* су убиквитарни сапрофити који у одређеним околностима могу да изазову респираторне инфекције свих врста живине, а нарочито младих категорија. *Aspergillus fumigatus*, *A. niger* и *A. glaucus* су најчешће изоловане врсте у форми плућне аспергилозе живине. Живина је у мањој или већој мери континуирано изложена овој врсти плесни у животном окружењу, па развоју клинички манифестне аспергилозе доприносе висока експозиција, тј. јака контаминираност плеснима, висока влажност у објектима, слаба вентилација, неухрањеност и стрес. У раду је испитиван утицај појединих фактора на појаву, раширеност и клиничку форму аспергилозе код живине. Разматрана је могућност контроле болести увођењем профилактичких мера. Присуство *Aspergillus* sp. код живине је анализирано на основу резултата клиничких и лабораторијских испитивања извршених у току 2000. и 2010. године. Раширеност аспергилозе је констатована код живине у различитом узрасту, од младих категорија па до узраста постигнуте технолошке старости. У току 2010. године аспергилоза живине, код пилића и ћурића регистрована је у акутном току у укупно 16 јата, нешто више него у току 2000. године, када је утврђено 12 клинички оболелих комерцијалних јата пилића и ћурића. Регистрован је случај инфекције на очима у два јата бројлерских пилића у узрасту од 10 дана. Миколошким контролама узорака неизлежених пилића, простирке, брисева са површина из објеката и инкубатора, *Aspergillus* sp. је изолован у распону од 3,85% до 36,14 %.

За контролу ширења инфекције од пресудног је значаја примена санитарно-хигијенских мера у објектима за смештај живине и инкубаторским станицама, као и контрола микробиолошког квалитета хране.

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ANTIOXIDANT PROPERTIES OF HOT WATER EXTRACTS FROM CARPOPHORE AND SPORES OF MUSHROOM *GANODERMA LUCIDUM*

ABSTRACT: *Ganoderma lucidum* (Leyss.:Fr.) Karst is one of the medicinal mushrooms, which possesses enviable antioxidant properties. Objective of this investigation was to evaluate antioxidant activity, reducing power, scavenging abilities on 1.1-diphenyl-2-picryl-hydrazyl (DPPH) radicals and chelating effects on ferrous ions of hot water extracts obtained from carpophore and spores of this mushroom. Hot water extract from carpophore (Gl-I) showed high antioxidant activity of 85.7 ± 0.7%, at 10 mg/ml, while antioxidant activity of hot water extract from spores (Gl-Is) was 9.2 ± 0.3% at 10 mg/ml. Reducing power of Gl-I reached a plateau of 3.4 ± 0.1 at 20 mg/ml, and 0.3 ± 0.0 at 20 mg/ml for Gl-Is. At 10 mg/ml, scavenging ability on DPPH radicals of Gl-I increased to 96.8 ± 2.5%, whereas Gl-Is scavenged DPPH radicals by 69.6 ± 2.5% at 10 mg/ml. Gl-I chelated 81.6 ± 3.6 % of ferrous ions at 20 mg/ml, while the chelating effect of Gl-Is was 73.8 ± 1.7%. The antioxidative activities of hot water extracts from carpophore and spores of the mushroom *G. lucidum* were concentration dependent and increased with an increase in the concentration.

KEY WORDS: *Ganoderma lucidum*, antioxidant activity, scavenging effect, reducing power, chelating effect, polysaccharide, mushroom extract

INTRODUCTION

Free radicals are abundantly present in food components and have an effect on many reactions in food systems. Also, they are produced during normal and pathological cell metabolism as a consequence of exogenous chemical and endogenous metabolic processes. Free radicals, especially oxygen derived ones, are highly reactive and capable of oxidizing biomolecules which results in cell death and tissue damage. Degenerative diseases including brain dysfunction, cancer, cardiovascular disease, cataract, diabetes, and immune-system decline are supposed to be associated with these processes (A m e s et al., 1993). Oxidative damages of DNA, proteins and other macromolecules accumulate with

age and are considered to be decisive factors of endogenous damage (F r a g a et al., 1990). All organisms possess natural defense systems against free radical damage, such as superoxide dismutase, which converts superoxide radicals into hydrogen peroxide, and catalase, which converts hydrogen peroxide into water and oxygen gas. In addition, they contain anti-oxidants, such as α-tocopherol, ascorbic acid, carotenoids, glutathione and polyphenols (N i k i et al.,1994), that trap free radicals, thus inhibiting the oxidative mechanisms that lead to degenerative diseases. If the mechanism of antioxidant protection becomes unbalanced by aging, a constant supply of natural products with antioxidant activity would be suitable to help the human body to reduce oxidative damage. Naturally occurring antioxidants found in whole grains, fruits, vegetables, teas, herbs may have a role in natural protection.

Mushrooms have also been reported as organisms with antioxidant activity. This is correlated with their phenolic and polysaccharide compounds (M a u et al., 2005; D u b o s t , O u and B e e l m a n , 2007; S o n g and V a n G r i e n s v e n , 2008). They are not only appreciated for their taste and high nutritional value, but also as a significant source of biologically active compounds of medicinal value. *Ganoderma lucidum* (Leyss.:Fr.) Karst, commonly known as lacquered mushroom, is not edible due to its coarse and hard texture and bitter taste, but it is the most often used one in traditional medicine of Far Eastern people. For thousands of years it has been well known for its treatment of various diseases, including cancers. Recent investigations also point to high antioxidative properties of this mushroom.

MATERIALS AND METHODS

Preparation of polysaccharides

Hundred grams of fine mushroom powder was washed with 96% ethanol at room temperature for 24 h under stirring, filtered and dried in vacuum (60 min at 42°C). Dried filtercake was extracted with 2 l Milli-Q water (MQ) by autoclaving (45 min. at 121°C), the extract was chilled and centrifuged for 20 min at 9000 g. Supernatant was concentrated to 10% of its initial volume, and polysaccharides were precipitated by addition of 2 volumes of cold 96% ethanol and left at 4°C overnight. After centrifugation, the pellets were washed with 70% ethanol, dried in vacuum and dialyzed using a ZelluTrans/Roth® 6.0 regenerated cellulose tubular membrane (MWCO: 8.000-10.000) against MQ for 24 h at room temperature to remove residual small molecules as polyphenols, peptides and polysaccharides < 8-10 kD. After centrifugation, the high molecular weight polysaccharides were ethanol precipitated and vacuum dried for later use.

Evaluation of the Antioxidant Properties

This examination was performed by measuring DPPH free radical scavenging activity, reducing power, antioxidative effect and chelating ability on ferrous ions. Each extract was analyzed in three replicates for each antioxidant test, and the results were reported as mean values ± standard deviation.

Antioxidant Activity

The antioxidant activity was determined by the conjugated diene method (L i n g n e r t et al., 1979) with slight modification. Each polysaccharide powder (0.1 to 10 mg/ml, 100 µl) in MQ was mixed with 2 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer. Then, 6.5 mM Tween 20 was added to provide a stable emulsion and the mixture was incubated for 15 h in the dark at 37°C, while shaken to accelerate oxidation. Afterwards, 0.2 ml of the antioxidant mixture was added to 6 ml of absolute methanol. The absorbance of the supernatant mixture was measured at 234 nm against a blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. The antioxidant activity was calculated as follows: antioxidant activity (%) = $[(A_0 – A_1)/ A_0] \times 100$, where A_0 was the absorbance of the control reaction and A1 the absorbance in the presence of the sample. Ascorbic acid and α-tocopherol were used as the positive control. Value of 100% indicated the strongest inhibitory ability.

DPPH Free Radical Scavenging Activity Assay

The assay was done according to the modified method of Bilos (1958). In the first series, each polysaccharide powder (0.1–10 mg/ml, 2 ml) in MQ was mixed with 1 ml of freshly prepared DMSO solution of 0.2 mM DPPH. In the second series, each sample was mixed with 1 ml DMSO solution. The reaction mixture was vortexed vigorously for 1 min and kept in the dark at 20°C for 40 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm against the blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). DPPH free radical scavenging activity was calculated according to the following equation: % scavenging = [1-(Ai-Aj)/Ac] x100, where Ai was the absorbance of 2 ml extract mixed with 1 ml DPPH solution, Aj was the absorbance of 2 ml extract mixed with 1 ml DMSO solution and Ac was the absorbance of blank-2 ml of DMSO mixed with 1 ml of DPPH solution. Ascorbic acid, BHT and α-tocopherol dissolved in DMSO were used as the positive control.

Reducing Power

The reducing power was determined according to O y a i z u (1986). Each polysaccharide powder (0.1 to 20 mg/ml, 2.5 ml) in MQ was mixed with 2.5 ml 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was vortexed and incubated at 50°C for 20 minutes. Then, 2.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 2000 g for 10 min. The upper layer (5 ml) was mixed with 5 ml of MQ and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. Higher absorbance indicated higher reducing power. Ascorbic acid was used as the positive control.

Chelating Ability on Ferrous Ions

Chelating ability was determined according to D i n i s et al. (1994). Each polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in MQ was mixed with 3.7 ml of MQ and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. Citric acid and EDTA were used for comparison. The control did not contain ferrous chloride or ferrozine, complex formation molecules. Lower absorbance indicated higher chelating power.

Statistical Analysis

All measurements were done in triplicate and data were expressed as mean ± standard deviation. Free statistical regression calculations online (http://easycalculation.com/statistics/regression.php) was used to calculate EC_{50} values by linear regression analyses.

RESULTS AND DISCUSSION

After hot water extraction and alcohol precipitation, the obtained polysaccharides from the carpophores and the spores of the mushroom *G. lucidum* were refined by dialyses and dry extracts were used for the investigation of antioxidant properties.

Antioxidant Activity

Conjugated diene is formed from a moiety with two double bonds separated by a single methylene group which occurs in polyunsaturated fatty acids. Formed conjugated diene can be monitored spectrophotometrically using its characteristic absorption at 234 nm. This method is suitable for the investigation of an early stage of lipid peroxidation (M o o n and S h i b a m o t o , 2009).

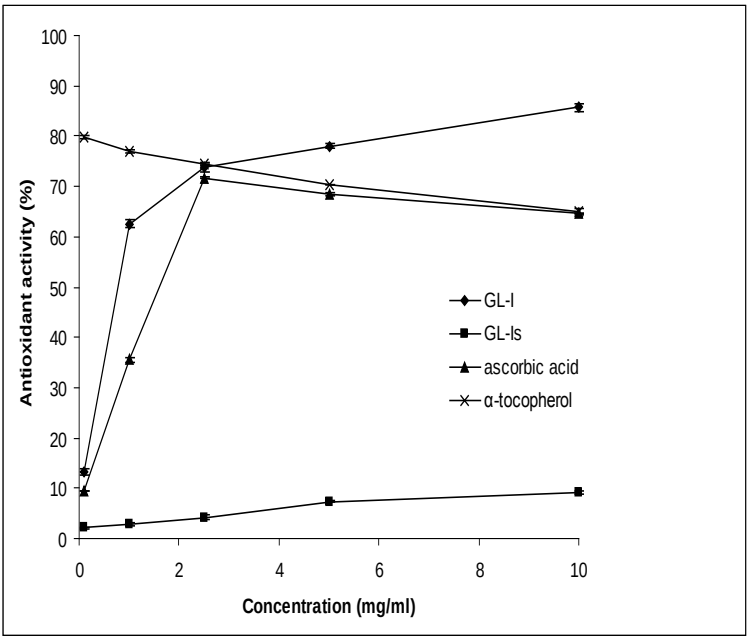

Fig. 1 – Antioxidant activity of Gl-I and Gl-Is from *Ganoderma lucidum*. Each value is expressed as mean ± standard deviation (n = 3).

Gl-I showed high antioxidant activity of 85.7 ± 0.7%, at 10 mg/ml, while antioxidant activity of Gl-Is was 9.2 ± 0.3% at 10 mg/ml (Figure 1.). Gl-I had higher antioxidant activity than Gl-Is at all other concentrations as well. Antioxidant activities of ascorbic acid and α-tocopherol were 64.7 ± 0.1 and 65.0 ± 0.5% at 10 mg/ml. Gl-I was even more effective than ascorbic acid and α-tocopherol at 5 and 10 mg/ml.

DPPH Free Radical Scavenging Activity Assay

The proton radical scavenger reacts with the stable free radical DPPH (deep violet color) and converts it to 1.1-diphenyl-2-picrylhydrazine with discoloration, suggesting that antioxidant activity of *G. lucidum* is due to its proton

donating ability. Medicinal mushrooms are free radical inhibitors or scavengers, acting as primary antioxidants which possess capability of terminating the chain reaction by reacting with free radicals which are major propagators of the autoxidation chain of fat (G o r d o n, 1990).

At 10 mg/ml, scavenging ability on DPPH radicals of Gl-I was very high and increased to 96.8 ± 2.5%, whereas Gl-Is scavenged DPPH radicals by 69.6 ± 2.5% at 10 mg/ml (Figure 2). Obviously, Gl-I appeared more effective than Gl-Is at all concentrations tested. However, ascorbic acid and BHT, the positive controls used in this test, scavenged DPPH radicals by 87.6 ± 0.3 and 55.2 ± 0.2% at 10 mg/ml, whereas α-tocopherol scavenged DPPH radicals by 96.1 ± 0.1% at 5 mg/ml.

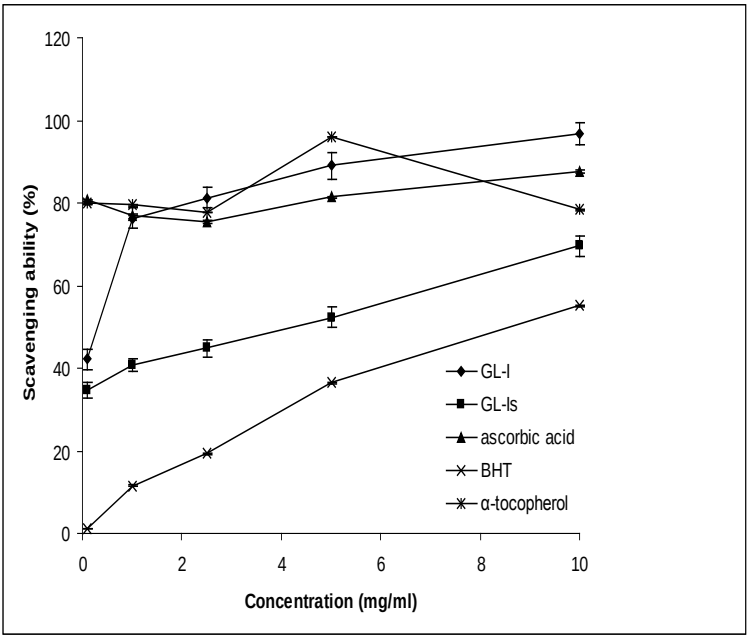

Fig. 2 – Scavenging ability of Gl-I and Gl-Is from *Ganoderma lucidum*. Each value is expressed as mean ± standard deviation (n = 3).

Reducing Power

Test solutions changed color from yellow to different shades of green and blue, depending on the reducing power. Conversion of the Fe^{3+}/ferricyanide complex to the ferrous form was caused by the presence of reducers. It seems that *G. lucidum* possesses hydrogen-donating ability.

Reducing power of Gl-I reached a plateau of 3.4 ± 0.1 at 20 mg/ml and only 0.3 ± 0.0 at 20 mg/ml for Gl-Is (Figure 3.). Compared with Gl-Is, Gl-I was

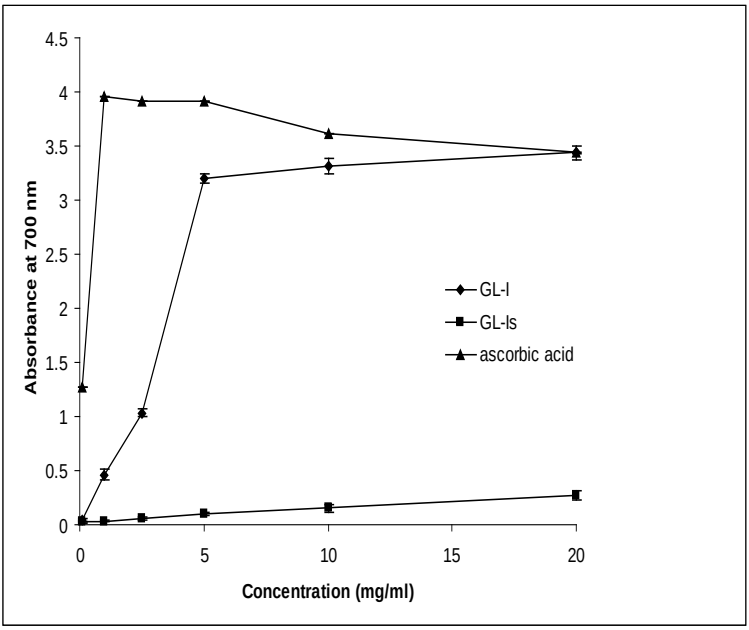

Fig. 3 – Reducing power of Gl-I and Gl-Is from *Ganoderma lucidum*. Each value is expressed as mean ± standard deviation (n = 3).

more effective (i.e. the difference between Gl-I and Gl-Is was approximately thirty three-fold at 5 mg/ml). Ascorbic acid, used as a positive control, had a reducing power of 3.4 ± 0.0 at 20 mg/ml, the same as Gl-I.

Chelating Ability on Ferrous Ions

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, such as mushroom extracts, the complex formation is disrupted and red color of the complex is decreased. Change of color points to chelating activity of extracts and captures the ferrous ion before ferrozine does it (W o n g a n d C h y e, 2009).

Gl-I chelated 81.6 ± 3.6 % of ferrous ions at 20 mg/ml, while the chelating effect of Gl-Is was 73.8 ± 1.7% (Figure 4). Lower absorbance indicated higher chelating ability. However, the chelating effect of the synthetic metal chelator EDTA was 100% at 0.1-20 mg/ml, while citric acid was not a strong chelator in this assay (it chelated only 10.3 ± 0.1% at 20 mg/ml). With regard to ferrous ion chelation, Gl-Is was less active than Gl-I.

The antioxidative properties of hot water extracts from carpophore and spores of the mushroom *G. lucidum* in all applied tests were concentration dependent and increased with increasing concentration.

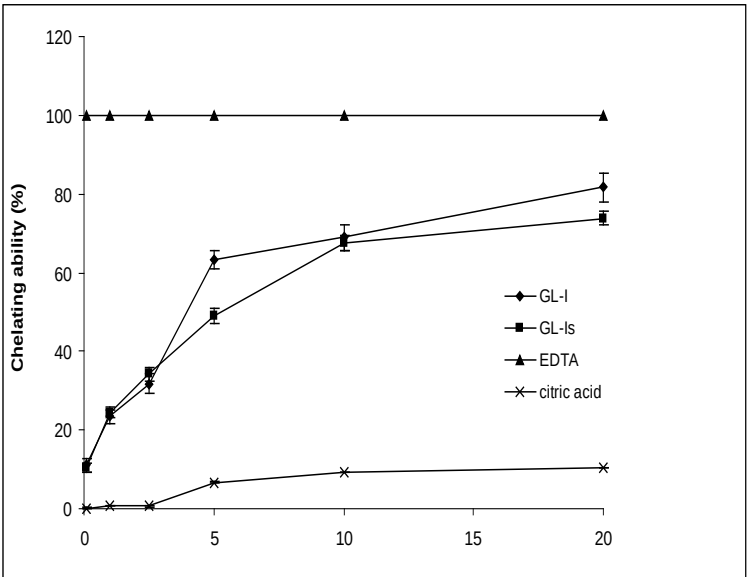

Fig. 4 – Chelating ability of Gl-I and Gl-Is from *Ganoderma lucidum*. Each value is expressed as mean ± standard deviation (n = 3).

EC_{50} values in antioxidant properties

The results of antioxidant activity, DPPH free radical scavenging activity, reducing power and chelating effect on ferrous ion were normalized and expressed as EC_{50} (mg/ml) values which represented the effective concentration of each mushroom extract required to show 50% of antioxidant properties. Lower EC_{50} value corresponds to higher antioxidant activity of the mushroom extract.

Gl-I showed very good antioxidant activity, which was confirmed by low EC_{50} value (1.2 ± 0.0 mg/ml). On the other hand, EC_{50} for Gl-Is was high (44.8 ± 0.9 mg/ml) which indicated weak antioxidant activity. However, α-tocopherol showed excellent antioxidant activity (EC_{50} < 0.1 mg/ml), while ascorbic acid was also quite active, as it was shown by its low EC_{50} value (1.6 ± 0.0 mg/ml).

The investigated extracts appeared to be very good DPPH radical scavengers, especially Gl-I having the EC_{50} value 0.1 ± 0.1 mg/ml, while EC_{50} value of Gl-Is was 3.6 ± 0.5 mg/ml. Ascorbic acid and α-tocopherol were both excellently scavenging DPPH radicals (EC_{50}<0.1 mg/ml), while BHT was a good DPPH radical scavenger (EC_{50} = 8.5 ± 0.0 mg/ml).

EC_{50} value of the reducing power for Gl-I was very good (0.5 ± 0.1 mg/ml), whereas Gl-Is showed weak reducing power (38.69 ± 6.6 mg/ml). Ascorbic acid showed excellent reducing activity (EC_{50}<0.1 mg/ml).

EC_{50} values of the chelating abilities on ferrous ions were good for both Gl-I (3.86 ± 0.0 mg/ml) and Gl-Is (4.9 ± 0.3 mg/ml). EDTA showed excellent chelating activity (EC_{50} < 0.1 mg/ml), while citric acid was not a good chelator (EC_{50} > 20 mg/ml).

CONCLUSION

Our results suggest that mushroom *Ganoderma lucidum* and its extracts could be very good sources of naturally-derived antioxidants. The applied tests showed that hot water extract obtained from fruiting body of this mushroom had better antioxidant effects than that of the spores, probably due to the fact that spore cell walls were not broken. Use of mushrooms in daily diet might be beneficial for human health in preventing or reducing oxidative damage. Although EDTA is an excellent metal chelator, ascorbic acid, α-tocopherol and BHT also appear to have good antioxidant activity, reducing power and scavenging ability on DPPH radicals. However, these compounds are synthetic antioxidants.Since some of them, such as BHT, may possess mutagenic activity (N a m i k i, 1990), it would be very useful to replace them with natural antioxidants in food and pharmaceuticals. Mushroom and its extracts could be used for that purpose.

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АНТИОКСИДАТИВНЕ АКТИВНОСТИ ВРЕЛИХ ВОДЕНИХ ЕКСТРАКАТА ИЗ КАРПОФОРА И СПОРА ГЉИВЕ *GANODERMA LUCIDUM*

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Резиме

Ganoderma lucidum (Leyss.: Fr.) Karst је једна од медицински важних гљива, која поседује изузетна антиоксидативна својства. Циљ овог истраживања био је потврда антиоксидативне активности, редукционе способности, могућности хватања 1,1-дифенил-2-пикрилхидразил (DPPH) радикала и способности хелирања јона гвожђа врелих водених екстраката добијених из карпофора и спора ове гљиве. Врели водени екстракт из карпофора (Gl-I) показао је високу антиоксидативну активност 85.7 ± 0.7%, при 10 mg/ml, док је антиоксидативна активност врелог воденог екстракта спора (Gl-Is) била 9.2 ± 0.3% при 10 mg/ml. Редукциона способност екстракта Gl-I достигла је ниво 3.4 ± 0.1 при 20 mg/ml и 0.3 ± 0.0 при 20 mg/ml за екстракт Gl-Is. При концентрацији 10 mg/ml способност хватања DPPH радикала екстракта Gl-I достигла је 96.8 ± 2.5%, док је екстракт Gl-Is везао 69.6 ± 2.5% DPPH радикала при 10 mg/ml. Екстракт Gl-I хелирао је 81.6 ± 3.6 % феро јона при концентрацији 20 mg/ml, а хелирајући ефекат екстракта Gl-Is био је 73.8 ± 1.7%. Антиоксидативне активности врелих водених екстраката из карпофора и спора гљиве *G. lucidum* зависиле су од концентрације и повећавале су се са повећањем концентрације.

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EXTRACT FROM WILD STRAIN OF MUSHROOM *GANODERMA LUCIDUM* AS NATURAL ANTIOXIDANT

ABSTRACT: Recently, much attention has been paid to revealing natural biomaterials for clinical purposes since use of synthetic antioxidants is restricted due to their carcinogenicity. Among various natural antioxidants, polysaccharides, in general, have strong antioxidant activities and can be explored as novel potential antioxidants. The aim of this work was to examine the antioxidant properties of hot water extracted polysaccharides from *Ganoderma lucidum* in the form of mature fruit bodies, collected from the Bojčinska forest near Belgrade, the Republic of Serbia. Antioxidant properties were assayed *in vitro*, by the conjugated diene method, reducing power, scavenging abilities against 1.1-diphenyl-2-picrylhydrazyl (DPPH) radicals and chelating ability on ferrous ions. At concentrations of 1 mg/ml, the scavenging ability of *G. lucidum* polysaccharide extract on DPPH radicals was 74.7 %. At 1 mg/ml, the radical scavenging ability of the positive controls BHT, ascorbic acid and α-tocopherol were 11.5, 77.1 and 79.4 %, respectively. The antioxidant activity of the polysaccharide extract increased as the concentration increased to 78.0% at 20 mg/ml. Antioxidant activities of ascorbic acid and α-tocopherol were 63.8 % and 65.4% at 20 mg/ml. Polysaccharide extract from *G. lucidum* showed steady increase in the reducing activity as concentrations increased to 2.9 at 20 mg/ml. Ascorbic acid, used as a positive control, had a reducing power of 3.9 at 5 mg/ml. Chelating effects of the polysaccharide extract on ferrous ion increased with the increased concentrations. At 0.1-20 mg/ml, the chelating ability of *G. lucidum* polysaccharide extract was between 10.3-87.8%. The chelating effect of the synthetic metal chelator EDTA was 100% at 0.1-20 mg/ml, while citric acid did not prove to be good chelating agent for ferrous ions in this assay since its chelating ability was 10.3% at 20 mg/ml.

KEY WORDS: antioxidant properties, *Ganoderma lucidum*, polysaccharide extract

INTRODUCTION

Free radical species produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging (C r o s s , 1987; B e c m a n and A m e s , 1998). Almost all organisms are well protected against free radical damage by enzymes,

such as superoxide dismutase and catalase, or compounds, such as ascorbic acid, tocopherols and glutathione (N i k i et al., 1994). In order to reduce the damage of free radicals, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and *tert*-butylhydroquinone (TBHQ) are used. However, according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing, such as BHT and BHA, have already been documented. For example, higher levels of BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis (D e O l i v e i r a et al., 2009; P r a s a d et al., 2009). Recently, much attention has been paid to screening natural biomaterials.

Among various natural antioxidants, polysaccharides, in general, have strong antioxidant activities and can be explored as novel potential antioxidants (J i a n g et al., 2005; N g et al., 2006). Also, polysaccharides are potentially useful for pharmaceutical purposes due to a variety of their biological activities, such as immunological, anti-radiation, anti-blood coagulation, anticancer, anti-HIV and hypoglycemic activities (Y a n g et al., 2005; Y o o n et al., 2003).

Therefore, the aim of the present work is to evaluate the antioxidant properties of hot water extracted polysaccharides from wild strain of the mushroom *Ganoderma lucidum* in the form of mature fruit bodies, collected from the Bojčinska forest near Belgrade, the Republic of Serbia. In recent years, wild edible mushrooms have become increasingly important in our diet for their nutritional and pharmacological characteristics. Their nutritional values and taste components have been studied. However, there is little information available about antioxidant properties of wild edible mushrooms.

Antioxidant properties were assayed *in vitro* in terms of antioxidant activity, by the conjugated diene method, reducing power, scavenging abilities on 1.1-diphenyl-2-picrylhydrazyl (DPPH) radicals and chelating ability on ferrous ions.

MATERIALS AND METHODS

Sample preparation

Fresh wild-growing fruiting bodies of mushroom *Ganoderma lucidum* were collected from the Bojčinska forest near Belgrade, the Republic of Serbia. After collecting, the fruiting bodies were brush-cleaned, air-dried to constant mass at 40 ^{o}C and ground into fine particles which were stored in the dark prior to the analysis.

Extraction of water soluble polysaccharide fraction

Up to 10% of dried powdered tissue was suspended in Milli-Q water. Polysaccharide extraction was done by autoclaving at 121^{o}C for 45 minutes.

The extract was cooled down and centrifuged at 9000 x g for 20 minutes. Supernatant was concentrated to 10% of the starting volume. Two volumes of 96% ethanol were added and left at 4 ºC overnight. After centrifugation, the pellets were washed with 70% ethanol, and dried in vacuum at 42 ºC. Purification was done by dialysis using ZelluTrans/Roth® 6.0 regenerated cellulose tubular membrane (MWCO: 8.000-10.000) against Milli-Q for 24 h at room temperature in order to remove residual small molecules as polyphenols, peptides and polysaccharides < 8-10 kDa. After centrifugation high MW polysaccharides were ethanol precipitated and vacuum dried for later use.

DPPH (2.2-diphenyl-1-picryhydrazyl) radical scavenging activity

The assay was done according to the modified method of Bilos (1958). In the first series, each polysaccharide powder (0.1–10 mg/ml, 2ml) in Milli-Q water was mixed with 1ml of freshly prepared DMSO solution containing 0.2 mM DPPH. In the second series, each sample was mixed with 1 ml DMSO solution. The reaction mixture was vortexed vigorously for 1 min and kept in the dark at 20 ºC for 40 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm against the blank using UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). DPPH free radical scavenging activity was calculated by the equation $[1-(A_i-A_j)/Ac]$ x100, where Ai was the absorbance of 2 ml extract mixed with 1 ml DPPH solution, Aj was the absorbance of 2 ml extract mixed with 1 ml DMSO solution, and Ac was the absorbance of blank-2 ml of DMSO mixed with 1 ml of DPPH solution. Ascorbic acid, BHT and α-tocopherol dissolved in DMSO were used as the positive control. The EC_{50} value (milligrams of extract per milliliter) was the effective concentration at which DPPH radicals were scavenged by 50%, and it was obtained by interpolation from linear regression analysis.

Antioxidant activity

The antioxidant activity was determined by the conjugated diene method with slight modifications (Lingnert et al., 1979). Each polysaccharide powder (0.1 to 20 mg/ml, 100µL) in Milli-Q water was mixed with 2 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer (pH 6.5). Then, 6.5 mM Tween 20 was added to provide a stable emulsion which was shaken in the dark at 37 °C to accelerate the oxidation. After incubation for 15 h in 0.2 ml of antioxidant mixture, 6 ml of absolute methanol was added. The absorbance of the supernatant mixture was measured at 234 nm against a blank using a UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without extract. The proportional antioxidant activity was calculated from the equation $[(A_0 – A_1)/ A_0]$ x 100, where A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence

of the sample. Ascorbic acid and α-tocopherol were used as positive controls. A value of 100% indicated the strongest inhibitory ability.

Ferric-reducing antioxidant power assay

Reducing power was determined according to O y a i z u (1986). Each polysaccharide powder (0.1 to 20 mg/ml, 2.5 ml) in Milli-Q water was mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was vortexed and incubated at 50 °C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 2000*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of Milli-Q water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank using a UV/VIS spectrophotometer (Shimadzu UV-1650 PC, Japan). The blank was the solution with all reagents but without the extract. Higher absorbance indicated higher reducing power. Ascorbic acid was used as the positive control.

Chelating ability on ferrous ions

Chelating ability was determined according to the method of D i n i s et al. (1994). Each polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in Milli-Q water was mixed with 3.7 ml of Milli-Q water and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min. at room temperature, the absorbance of the mixture was determined at 562 nm against the blank. Blank was the solution with all reagents but without the extract. Lower absorbance indicated higher chelating ability. The EC_{50} value (mg extract/ml) was the effective concentration at which ferrous ions were chelated by 50%. Citric acid and ethylenediaminetetraacetic acid (EDTA) were used for comparison.

RESULTS AND DISCUSSION

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples (S u et al., 2008). At concentrations of 1 mg/ml, the scavenging ability of *G. lucidum* polysaccharide extract on DPPH radicals was 74.7 % (Figure 1). At 1 mg/ml, the radical scavenging ability of the positive controls BHT, ascorbic acid and α-tocopherol were 11.5, 77.1 and 79.4 %, respectively (Figure 1). The radical scavenging ability of the extract and positive controls at 1 mg/ml decreased in the following order: α-tocopherol ≈ ascorbic acid > *G. lucidum* > BHT. At 10 mg/ml, the radical scavenging ability decreased in the following order: ascorbic acid > α-tocopherol ≈ *G. lucidum* > BHT.

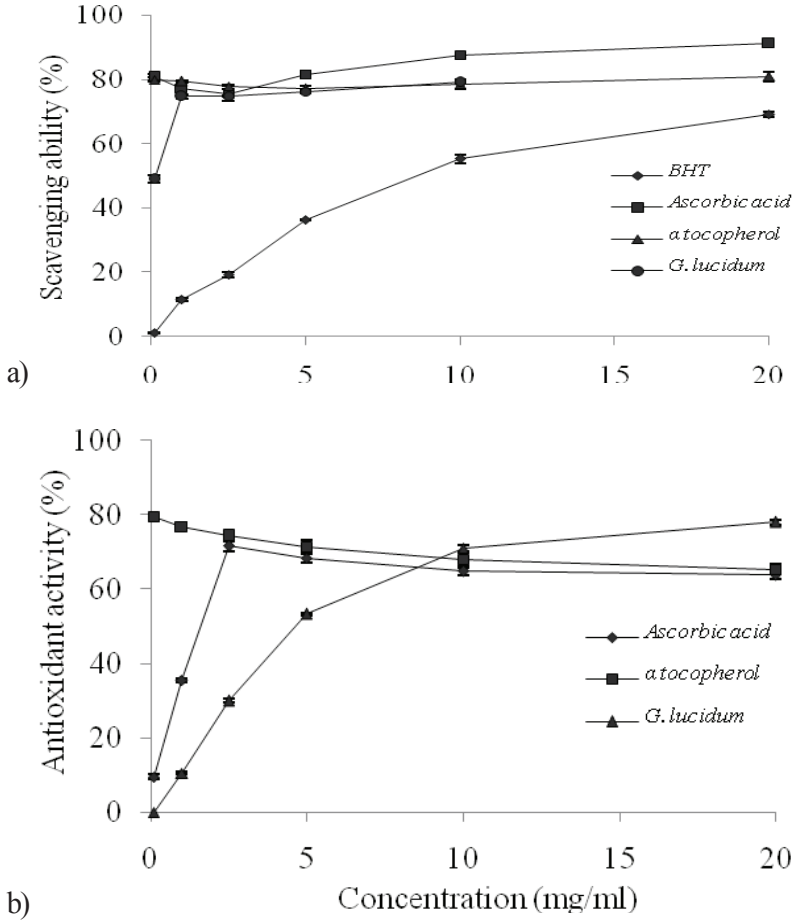

Fig. 1 – Scavenging ability on 1.1-diphenyl-2-picrylhydrazyl radicals (a); antioxidant activity evaluated in the linoleic acid model system (b) of hot water polysaccharide extract from *G. lucidum*. Each value is expressed as mean ± SEM (n = 3).

Using the conjugated diene method, the antioxidant activity of the polysaccharide extract from *G. lucidum* increased as the concentration increased to 78.0% at 20 mg/ml (Figure 1). Antioxidant activities of ascorbic acid and α-tocopherol were 63.8 % and 65.4% at 20 mg/ml, respectively.

Polysaccharide extract from *G. lucidum* showed steadily increasing reducing activity as concentrations increased to 2.9 at 20 mg/ml (Figure 2). Ascorbic acid, used as a positive control, had a reducing power of 3.5 at 20 mg/ml. High reducing power of *G. lucidum* polysaccharide extracts at 20 mg/ml suggested high potential in hydrogen-donating ability (A d e s u n g et al., 2007).

Chelating effects of the polysaccharide extract on ferrous ion increased with the increased concentrations (Figure 2). At 0.1-20 mg/ml, the chelating

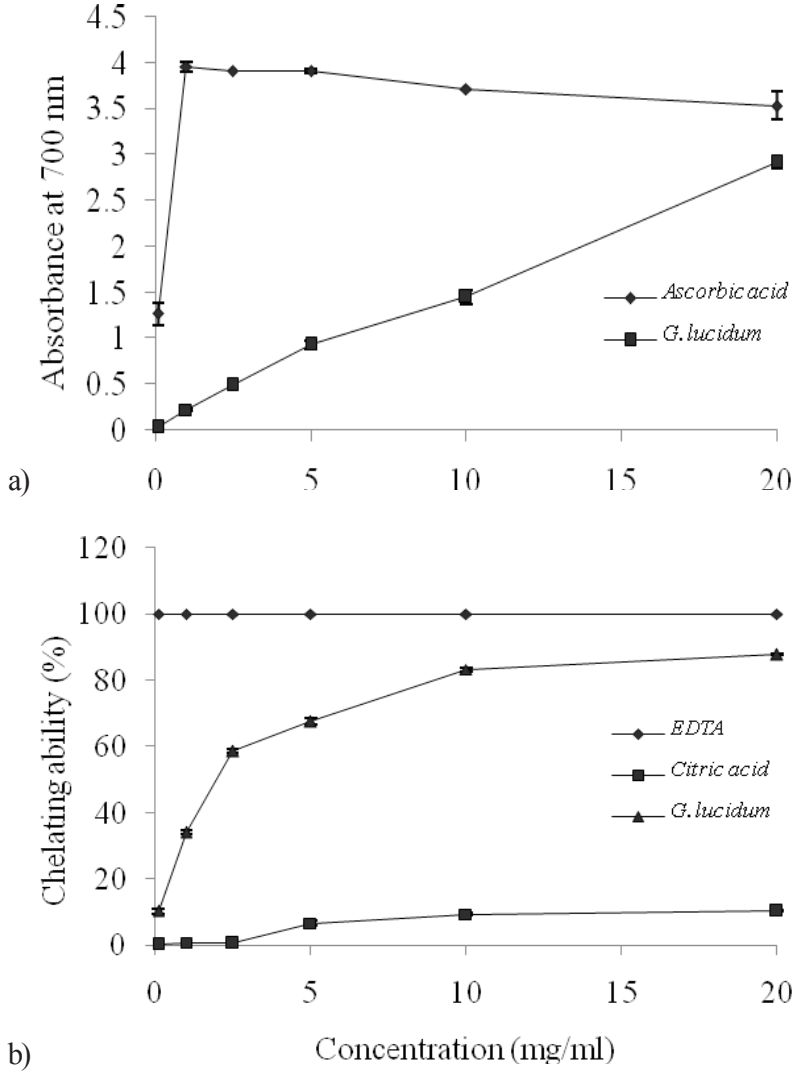

Fig. 2 – Reducing power (a), and chelating ability on ferrous ions (b) of hot water polysaccharide extract from *G. lucidum*. Each value is expressed as mean ± SEM (n = 3).

ability of *G. lucidum* polysaccharide extract was between 10.3-87.8%. The chelating effect of the synthetic metal chelator EDTA was 100% at 0.1-20 mg/ml, while citric acid was not a good chelating agent for ferrous ions in this assay, and its chelating ability was 10.3% at 20 mg/ml.

EC_{50} values in antioxidant properties

The effectiveness of antioxidant properties was expressed as EC_{50} (mg/ml) value which represented the effective concentration of mushroom extract required to show 50% antioxidant property (Table 1). Lower EC_{50} value corresponded to higher antioxidant activity of the mushroom extract.

Tab. 1 – EC_{50} values of polysaccharide extract from *G. lucidum* in antioxidant properties. Each value is expressed as mean ± SEM (n = 3).

	EC_{50}[a] (mg extract/ml)
Scavenging ability on DPPH radicals	0.16 ± 0.03
Antioxidant activity	8.89 ± 0.12
Reducing power	2.69 ± 0.17
Chelating ability on ferrous ions	3.48 ± 0.10

[a] EC_{50} value: The effective concentration at which 1.1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50%; the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; and ferrous ions were chelated by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.

With regard to scavenging ability on DPPH radicals, the polysaccharides from wild strain of *G. lucidum* showed very good scavenging ability which could be observed in their low EC_{50} values (0.16 ± 0.03 mg/ml). Ascorbic acid and α-tocopherol were both confirmed as excellent scavengers of DPPH radicals (EC_{50} < 0.1 mg/ml). BHT also proved to be a good DPPH radical scavenger (EC_{50} = 11.86 mg/ml). An EC_{50} value of the antioxidant activity was 8.89 ± 0.12 mg/ml. α-tocopherol showed excellent antioxidant activity (EC_{50} < 0.1 mg/ml), whereas ascorbic acid also had a good activity as shown by its low EC_{50} value (1.64 mg/ml).

For reducing power EC_{50} value was 2.69 ± 0.17 mg/ml. Ascorbic acid showed excellent reducing activity (EC_{50} < 0.1 mg/ml). EC_{50} value of the chelating ability of *G. lucidum* extract was 3.48 ± 0.10 mg/ml. The chelator EDTA showed higher activity (EC_{50} < 0.1 mg/ml). An EC_{50} value of the citric acid was < 20 mg/ml.

CONCLUSION

The results of the present study suggest that polysaccharide extract from wild strain of the *Ganoderma lucidum* mushroom acts as natural antioxidant. The extract exhibited good antioxidant activities, and some even showed higher potency than the standard synthetic antioxidants in some instances; for example, *G. lucidum* polysaccharide extract had higher activity in the DPPH assay when compared to BHT. Polysaccharide extract may be a good source for the development of antioxidant food additives. Further investigations are necessary to verify these activities *in vivo*.

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ЕКСТРАКТ ШУМСКОГ СОЈА ГЉИВЕ *GANODERMA LUCIDUM* КАО ПРИРОДНИ АНТИОКСИДАНТ

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Резиме

У данашње време све веће пажња се поклања проналажењу природних биолошких материјала који имају антиоксидативна својстава. Нежељени ефекти, оштећење јетре и карциногенеза, од стране синтетичких антиоксиданата који се користе као адитиви исхрани су увелико потврђени. Међу различитим природним антиоксидантима полисахариди привлаче све већу пажњу као једињења са израженом антиоксидативном активношћу. Циљ рада је био да се испитају антиоксидативна својства врелог воденог екстракта полисахарида гљиве *Ganoderma lucidum* добијеног из зрелих плодоносних тела, прикупљених у Бојчинској шуми у близини Београда, Република Србија. Антиоксидативна својства су испитивана помоћу антиоксидативне методе у модел систему линолеинске киселине, мерењем снаге редукције, способности хватања 1,1-дифенил-2-пикрилхидразил (DPPH) радикала и способности хелирања јона гвожђа. При концентрацији од 1 mg/ml способност хватања DPPH радикала полисахаридног екстракта шумског соја *Ganoderma lucidum* достигла је ниво од 74.7%, док су измерене вредности, при истој концентрацији, за позитивне контроле BHT, аскорбинску киселину и α-токоферол износиле 11.5, 77.1 и 79.4%. Антиоксидативна активност измерена у модел систему линолеинске киселине зависила је од концентрације и повећавала се са повећањем концентрације. Измерена је вредност од 78.0% при концентрацији од 20 mg/ml. Антиоксидативне активности аскорбинске киселине и α-токоферола при концентрацији од 20 mg/ml биле су 63.8 и 65.4%. Редукциона способност полисахаридног екстракта достигла је ниво од 2.9 при 20 mg/ml и 3.9 при 5 mg/ml код аскорбинске киселине која је коришћена као позитивна контрола. Способност хелирања јона гвожђа се повећавала са повећањем концентрације. При концентрацији од 0.1-20 mg/ml измерена способност хелирања била је између 10.3-87.8%. Хелатни ефекат EDTA био је 100% у опсегу од 0.1-20 mg/ml. Лимунска киселина се није показала као добар хелирајући агенс (10.3% при 20 mg/ml).

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ANTIMICROBIAL ACTIVITY OF AQUEOUS EXTRACT OF *LAETIPORUS SULPHUREUS* (BULL.: FR.) MURILL

ABSTRACT: Wood-rotting basidiomycete, *Laetiporus sulphureus* (Bull.: Fr.) Murill., also known as chicken of the woods, is known for its nutritional value. In this study, aqueous extract obtained from *L. sulphureus* was investigated for its antimicrobial properties using microdilution *in vitro* assay. Plant, animal and human pathogens, as well as food spoilage agents, were tested. Aqueous extract obtained from *L. sulphureus* showed strong activity against the tested microorganisms in a dose dependent manner. Considering that there is a constant emerging of pathogen resistance to the known synthetic agents, there is an undeniable need for new therapeutical drugs and preservatives in food industry. Thus, these results that indicate activity of natural products may be of practical use.

KEY WORDS: antimicrobial activity, aqueous extract, *Laetiporus sulphureus*

INTRODUCTION

Researches in the past decade suggest that significant number of people is involved in various forms of alternative medicine. Estimates for using complementary and alternative medicine in adults worldwide have increased in the last twenty years (B a r n e s et al., 2004.). The main reason for this lies in the fact that many people are to a certain extent dissatisfied with the conventional treatment (A s t i n, 1998). Also, constant emerging resistance of bacterial and fungal species, known for the ailments they cause in humans, to commercial synthetic medicines is probably the most important reason why people turn to alternative medicine. While the use of herbs is rather common in the Western hemisphere, medicinal use of mushrooms, which has a long tradition in the Asian countries, has been slightly increased in Europe since the last decades (L i n d e q u i s t et al., 2005). Although there have been extensive researches on the use of medicinal mushrooms, their true potential is yet to be revealed.

Excessive use of antibiotics and antimycotics in the treatment of infectious diseases caused by human pathogenic microorganisms led to multiple

drug resistance. This motivated scientists to search for new substances with antimicrobial activity in natural products. Plants and mushrooms turned out to be an excellent source of novel chemotherapeutic agents (K a r a m a n et al., 2003). A number of compounds that have been proved to posses significant antimicrobial activities were isolated from polypore fungi. They provide a rich variety of active secondary metabolites and polysaccharides. Polysaccharides emerged as an important class of bioactive substances, and many medicinal and therapeutic properties are attributed to them (A l q u i n i et al., 2004). *Trametes versicolor*, *Laetiporus sulphureus* and *Ganoderma lucidum* are just some of the known mushrooms with this potential. This fact alone made them good candidates for critically needed new antibiotics and antimycotics (Z j a w i o n y, 2004).

Laetiporus sulphureus (Bull.: Fr.) Murill is a wood-rotting basidiomycete, growing on several tree species and producing shelf-shaped fruit bodies with a bright yellow fleshy margin. This recognizable pigmentation along with the fruit body form is responsible for the trivial name under which this fungus is known, and that is sulfur shelf (W e b e r et al., 2003).

Even though it is known as a source of active compounds, and is widely used as food among Anatolian people, literature reports on antimicrobial activity of *L. sulphureus* extracts are scarce (Z j a w i o n y, 2004; T u r k o g l u et al., 2007).

The potential barrier to everyday use of medicinal mushrooms as therapy is the manner in which the mushroom is consumed. Most of the researches on fungi as potential antimicrobial agents are based on ethanolic and methanolic extracts of the fungal fruit body (T u r k o g l u et al., 2007; B a r r o s et al., 2007). Consumption of products on this basis is of no practical use.

Therefore, the aim of the present study is to evaluate the antimicrobial potentials of aqueous extracts of *L. sulphureus* fruit bodies on several microorganisms of medicinal importance. This would perhaps allow daily use of mushrooms that are available in various forms.

MATERIALS AND METHODS

Mushroom

Fruiting bodies of *L. sulphureus* were collected in the woods near Belgrade. Identification and classification were carried out and all specimens were deposited at the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. Mature basidiocarps of *L. sulphureus*, were collected in *Salix alba* in 2009. Fresh mushrooms were randomly divided, dried in an oven at 40 °C before analysis. Dried mushroom material was grounded to a fine powder with blender. The sample (30 mg) was dissolved in 1ml of distilled water and left for 24 h at room temperature. The aqueous solution was then centrifuged at 5000 rpm for 10 minutes; the supernatant was poured off and used in the *in vitro* assay.

Antimicrobial activity

Antibacterial activity

The following Gram negative bacteria were used: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *and Salmonella typhimurium* (ATCC 13311), *and Enterobacter cloacae* (human isolate), as well as the following Gram positive bacteria: *Listeria monocytogenes* (NCTC 7973), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The antibacterial assay was carried out by modified microdilution method (D a o u k et al., 1995; H a n e l and R a e t h e r 1988; E s p i n e l – I n g r o f f, 2001) in order to determine the antibacterial activity of compounds tested against the human pathogenic bacteria.

The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/ml. The inocula were prepared daily and stored at +4°C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and check the validity of the inoculum.

Antifungal activity

For the antifungal bioassays, seven fungi were used: *Aspergillus ochraceus* (ATCC 12066), *Aspergillus fumigatus* (plant isolate), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Trichoderma viride* (IAM 5061). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

The micromycetes were maintained on malt agar and the cultures were stored at + 4 °C and sub-cultured once a month (B o o t h, 1971). In order to investigate the antifungal activity of the tested aqueous extract, a modified microdilution technique was used (D a o u k et al., 1995; H a n e l and R a e t h e r, 1988; E s p i n e l – I n g r o f f, 2001.). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 10^5 CFU in a final volume of 100 µl per well. The inocula were stored at +4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and check the validity of the inoculum.

Microdilution test

The minimum inhibitory, bactericidal and fungicidal concentrations (MICs, MBCs and MFCs, respectively) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/ml. The microplates were incubated for 24 h at

37°C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial sub-cultivation of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 72 h. The lowest concentration with no visible growth was defined as the MBC value, indicating 99.5% killing of the original inoculum. Streptomycin was used as a positive control (1 mg/ml DMSO).

Minimum inhibitory concentration (MIC) determinations for fungi were performed using 96-well microtiter plates. The microplates were incubated for 7 days at 25 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

The fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µl into microtiter plates containing 100 µl of broth per well and further incubation for 5 days at 25 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole was used as positive control (1mg/1ml DMSO).

RESULTS

The antimicrobial effect of aqueous extract of *L. sulphureus* was tested against four species of Gram negative bacteria, four species of Gram positive bacteria, and seven species of fungi. The extract generally exhibited better antifungal than antibacterial activity (Figure 1). Among the tested bacteria, *L. sulphureus* extract strongly inhibited *M. flavus* and *L. monocytogenes*, with MIC values of only 1.50 mg/ml. As for the remaining bacterial strains, in the tested concentrations (0.15-1.50 mg/ml) aqueous extract showed activity, but insufficient to be considered as inhibitory. Antibiotic streptomycine (1mg/1ml DMSO) was used as the positive control. In the tested concentrations, streptomycine showed inhibitory activity in the range of 0.63-12.50 x 10^{-3} mg/ml, and bactericidal activity on the tested bacteria in the range of 1.25-25.00 x 10^{-3} mg/ml. The bacteria most resistant to the effect of streptomycine was proved to be *L. monocytogenes*, which could be of practical use, because it was also proved to be the most susceptible to the effects of extract of *L. sulphureus*.

DISCUSSION

Presented results of antibacterial activity are consistent with the results for *M. flavus* published by T u r k o g l u et al. It proved to be the most susceptible species, though it should be noted that in the mentioned study agar-well diffusion method was used, and the extract was methanolic (T u r k o g l u et al., 2007).

It should be mentioned that there are no previous reports on the antifungal activity of extract of *L. sulphureus*. In the present study, aqueous extract

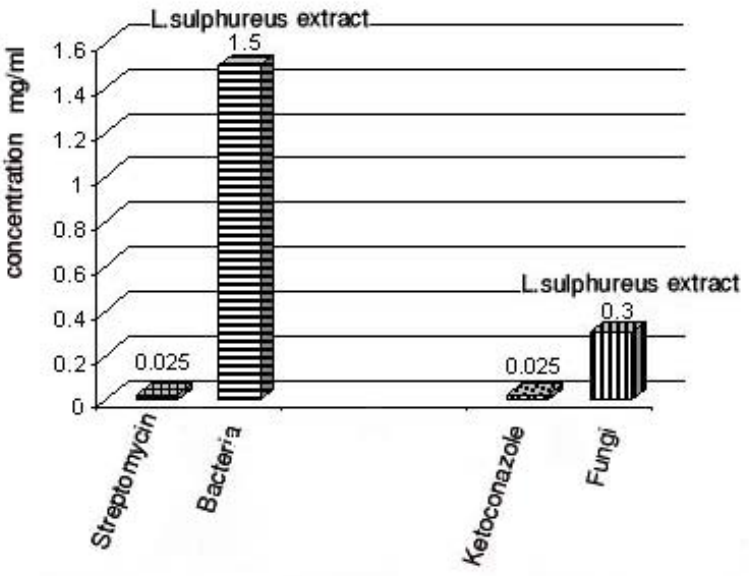

Fig. 1 – Antibacterial and antifungal effect of *L. sulphureus* extract in comparison to streptomycin and ketoconazole

showed excellent fungicidal activity against all the tested fungi, with 0.30 mg/ml as MFC value. In comparison with antifungal activity of antimycotic ketoconazole (1mg/ml DMSO), the tested extract showed lower activity, but still good enough to be considered as a promising antifungal agent. Ketoconazole exhibited inhibitory activity against all the tested fungi in the range of 0.63-25.00 x 10^{-3} mg/ml, and fungicidal activity in range of 1.25-25.00 x 10^{-3} mg/ml.

Resistance to antibiotics and antifungal agents is emerging in a wide variety of organisms, and multiple drug resistant organisms pose a serious threat to the treatment of infectious diseases. Hence, medicinal mushrooms, or mushroom derived antimicrobial substances must receive proper attention, especially when they include a harmless basis, which is water in this case.

Terpenes, lectins, polysaccharides etc. are known to have an effect on bacterial cytoplasmatic membrane, making it vulnerable (L i n and C h o u, 1998; Y a n g et al., 2002). *L. sulphureus* is rich source of these compounds, which are potentially responsible for its antimicrobial activity.

C o w a n (1999) reported that the most active components are generally water-insoluble, but O l e n n i k o v et al. (2009) determined the existence of water-soluble polysaccharides in *L. sulphureus* that could be bioactive principles playing an important role in the activity of mushroom (C o w a n, 1999; O l e n n i k o v et al., 2009). It is expected that low polarity organic solvents would yield more active extracts, but at the same time organic solvent is what makes these extracts unsafe and impractical for everyday use (C o w a n , 1999).

Reports on antimicrobial activity of *L. sulphureus* are very scarce, and this is the first report on the antibacterial and antifungal activity of aqueous extract of this mushroom.

Results of this study confirm once more the true potential of natural products as antimicrobial agents. *L. sulphureus* presents a valuable source in the fight against the tested pathogens, as a sole therapy, which requires further clinical trials, or in combination with other chemotherapeutical agents.

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АНТИМИКРОБНА АКТИВНОСТ ВОДЕНОГ ЕКСТРАКТА *L. SULPHUREUS* (BULL.: FR.) MURILL

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Резиме

Гљива мрке трулежи, *Laetiporus sulphureus* (Bull.: Fr) Murill., позната и као шумско пиле, већ је позната по својој нутритивној вредности. У овој студији испитивана је антимикробна способност воденог екстракта *L. sulphureus*, микродилуционом *in vitro* методом. Биљни, животињски и хумани патогени, као и проузроковачи кварења хране су тестирани. Водени екстракт добијен од *L. sulphureus* показао је снажну активност на тестиране микроорганизме уз дозну зависност. С обзиром да постоји константни пораст резистентности патогена на познате синтетичке агенсе, потреба за новим терапеутским лековима и конзервансима у прехрамбеној индустрији је неопходност. Стога, резултати који указују на активност природних продуката могу бити од великог практичног значаја.

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ANTIOXIDANT ACTIVITY OF WATER EXTRACTS FROM FRUIT BODY OF *LENTINUS EDODES* ENRICHED WITH SELENIUM

ABSTRACT: Shiitake *(Lentinus edodes)* belongs to medically important and delicious fungi. It is recognizable for its healing properties, excellent taste and rich aroma. According to the traditional Japanese and Chinese medicine, shiitake mushroom significantly increases the strength and vitality of the body. Shiitake contains immunostimulants, compounds that lower cholesterol, prevents clogging of blood vessels, regulates the pressure, balances blood sugar levels, regulates digestion, and improves the performance of respiratory organs by its antirheumatic and antiallergic activities. Shiitake is recommended to use as food, prevention and cure, usually in a form of a spice (dried and ground) or tea. It can be consumed fresh, too.

The objective of this study was to test the effect of enrichment in selenium on antioxidant, reducing and free radical scavenging activity of water extracts from fruit body of *Lentinus edodes*. The fungus was enhanced by adding organic selenium, zinc (II) complex with the ligand 2.6-bis diacetylpyridine (selenosemicarbazon) and inorganic compounds (Na_2SeO_3) of selenium in nutritional substrate where the fungus was grown. The total selenium content in fruit body was around 50 ppm for the sample enriched with selenium originating from organic sources, and 80 ppm for the sample enriched with selenium from inorganic sources. Samples were prepared by extraction of fruiting bodies in heated water. The results indicated that water extracts of whole fruit bodies, from both control and mushrooms supplemented with selenium, had quite good antioxidant activity. However, there was no significant difference between the samples supplemented with selenium content and those that were not.

KEY WORDS: *Antioxidant activity, Extract, Selenium, Shiitake*

INTRODUCTION

Selenium is essential micronutrient for mammals and birds. It is essential antioxidant, necessary for the proper functioning of hormones and immune system. The content of selenium in plants that are known as the source of this compound has been reduced due to the poverty of the land on which they grow (J i a n ' a n et. al., 2002; K l a p e c et. al., 1998; S a n j i v et. al., 2005). Sele-

nium deficiency can cause many disorders in the body (G r o m a d z i n s k a et. al., 2008). Based on previous studies, it is known that the fungi are good accumulators of selenium (B o r o v i č k a and R a n d, 2007; S a v i ć et. al., 2009). Selenium content in dry mass of fungi is between 0.57 and 19.46 mg/kg, depending on the type, age and location of fungi (F a l a n d y s z , 2008). The aim of the study was to compare the possibility of adoption of selenium in fruit body of industrial mushroom *Lentinus edodes* from organic and inorganic selenium sources. *L.edodes* is medicinal mushroom originating from Asian countries. Fruit body of the fungus is used as food, but also as medicine. It builds up the immune system, lowers cholesterol, helps blood coagulation and relieves symptoms in the cancer treatment. Mentioned fungi can be consumed as freshly prepared, concentrated extracts, or dietary supplements (DS). Several types of DS are derived from mushrooms *L.edodes*: dried and pulverized fruiting bodies, extracts in hot water and alcohol extracts, biomass or extract of mycelium. Commercial products are available on the market in the form of tablets, capsules and teas.

The total selenium content in enriched mushrooms is determined by the optical emission spectrometer with inductively coupled plasma, ICP-OES. Antioxidant potential, scavenging effect, as well as the reduction potential of fungi with and without addition of selenium in the form of extract of fruit bodies in hot water was determined in the experiment.

MATERIAL

Possibility of accumulation of selenium from nutrient rich substrate in mushroom fruit bodies of *Lentinus edodes* (commercial designation L-31) was examined. This strain was grown at Department of Microbiology, Faculty of Agriculture, University of Belgrade. Sodium selenite, Na_2SO_3 was used as inorganic source of selenium, while the organic compound used in the work was newly synthesized organic complex of Zn (II) with the ligand 2,6-bis-diacetylpyridine (selenosemicarbazon) (H2dapsesc) – [Zn (dapsesc)] (T o d o r o v i ć et. al., 2007). The compounds were added in the nutrient substrate where the mushrooms were grown in the concentrations of 50 mg/kg selenium and 15 mg/kg selenium, in the form of inorganic salts and organic complexes. The total selenium content was determined in dry mass of the sample by ICP-OES. The average content of selenium in the substrate without the addition of supplements was about 0.2 mg/kg, while this value in the fruit bodies of the control fungi was 0.4-0.6 mg/kg. This confirms the initial statement that the total selenium content in the substrate, and fungi that grow on it, was low. The average content of selenium in the fruit body of fungi that grew on media supplemented with selenium from organic sources (15 mg/kg Se) was around 50.4 mg/kg. The content of selenium in fruit body of the fungi that grew on the substrate with the addition of inorganic salt (52.3 mg/kg Se) was about 81.0 mg/kg.

METHODS

The antioxidant activity was determined by the conjugated diene method with slight modification (Turley et al., 2010; Yu – Hsiu et. al., 2008). The negative control was the solution with all reagents but without extract. The antioxidant activity was calculated as follows: antioxidant activity (%) = [(A_0 – A_1)/ A_0] x 100, where A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence of sample. Ascorbic acid and α-tocopherol were used as the positive control. Value of 100% indicated the strongest inhibitory ability.

Test for determination of the potential neutralization of 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical was prepared in accordance with the modified method by Bilos (Prashani et. al., 2005). Binding capacity of DPPH free radical method was calculated based on the following equation: % scavenging = [1-(A_i-A_j)/Ac] x100, where Ai was the absorbance of 2 mL extract mixed with 1 mL DPPH solution; Aj was the absorbance of 2 mL extract mixed with 1 mL DMSO solution, and Ac was the absorbance of blank-2 mL of DMSO mixed with 1 mL of DPPH solution. Ascorbic acid, BHT and α-tocopherol dissolved in DMSO were used as the positive control.

The reducing power was determined according to the method of Oyaizu (Turley et al., 2010). The blank was the solution with all reagents but without extract. Higher absorbance indicated higher reducing power. Ascorbic acid was used as the positive control.

Results were expressed as mean ± standard deviation of three parallel measurements. Tests were performed using computer program Microsoft Excel 2007. The data were analyzed using one-way analysis of variance (ANOVA) and Student's *t* test at significance level 0.05. The lowest effective concentration (EC_{50}) was obtained by interpolation from linear regression analysis.

RESULTS AND DISCUSSION

Antioxidant activity

Using a modified method of conjugated diene, water extracts of whole mushrooms showed strong antioxidant activity at concentrations of 10 mg/ml (Figure 1). The potential values of fungi *L. edodes* at the concentration of 10 mg/ml for control samples and of mushrooms enriched with selenium from selenite and selenium from organic sources were 16.41%, 26.33% and 49.27%, respectively. The antioxidant activity of ascorbic acid was most pronounced at concentrations of 2.5 mg/ml and amounted to 71.7%, while for α-tocopherol this activity was at concentrations of 0.1 mg/ml, and it was 79.7%.

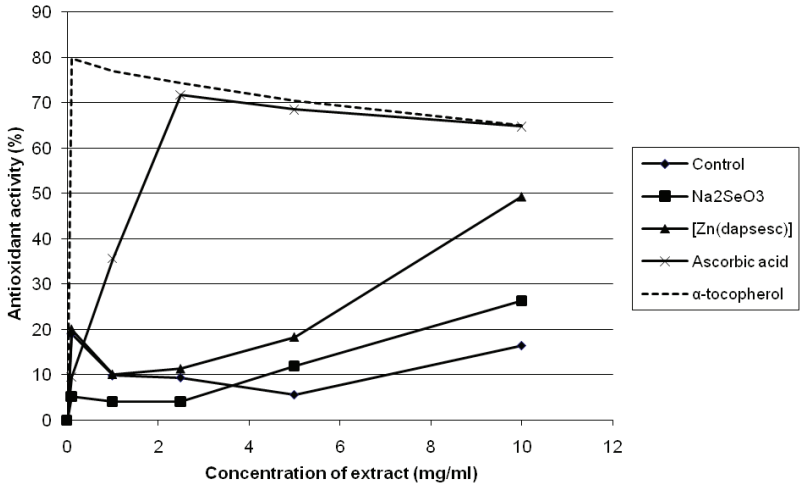

Fig. 1 – The antioxidant activity of hot water extracts of *Lentinus edodes* L31. Values are expressed as mean ± standard deviation (n = 3).

Reducing power

Reducing power of hot water extracts from fruit bodies of *L. edodes* increases with higher concentration. At concentrations of 20 mg/ml, the reducing power of control mushrooms *L.edodes* and samples with selenium from inorganic and organic compounds was 1566, 1645, 1156, respectively (Figure 2). Reducing power of ascorbic acid was significantly higher in comparison to the samples and it amounted to 3.956 at the concentration of 1 mg/ml.

Scavenging activity

Absorbance of DPPH radical binding is shown in Figure 3. Hot water extract of fruit body showed a strong ability to bind DPPH radicals. The ability of radical binding of ascorbic acid, BHT and α-tocopherol at concentrations of 0.1-10 mg/ml, was 80.6-87.7%, 1.13-55.23% and 79.9-78.4%. Scavenging activity of *L. edodes* at concentrations 0.1-10 mg/ml without addition of selenium, with the addition of inorganic and organic selenium was 83.21-108.39%, 89.11-108.31% and 79.01-106.8%. EC_{50}

The antioxidant activity of hot water extract from whole mushroom is summerized in Table 1. Results are expressed as EC_{50} values for easier comparison of the results. EC_{50} is the lowest effective concentration related with antioxidant activity.

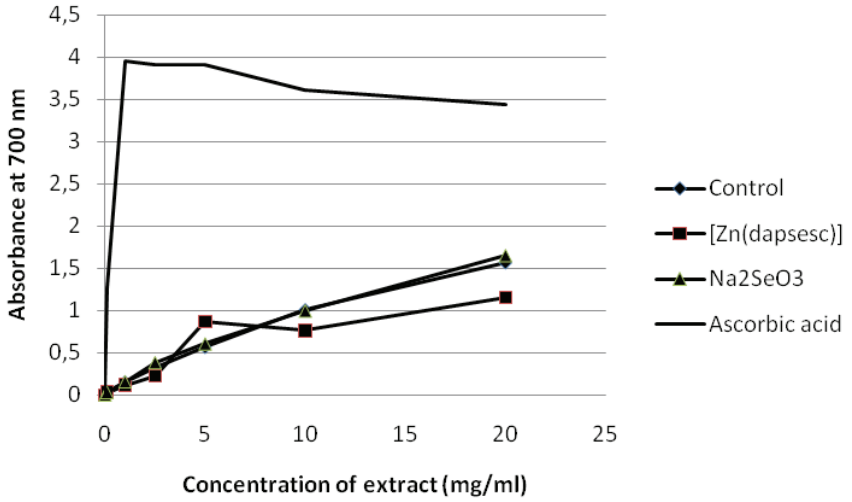

Fig. 2 – Reducing power of hot water extracts of whole mushrooms *L.edodes* L31. Each value is expressed as the mean ± standard deviation (n = 3).

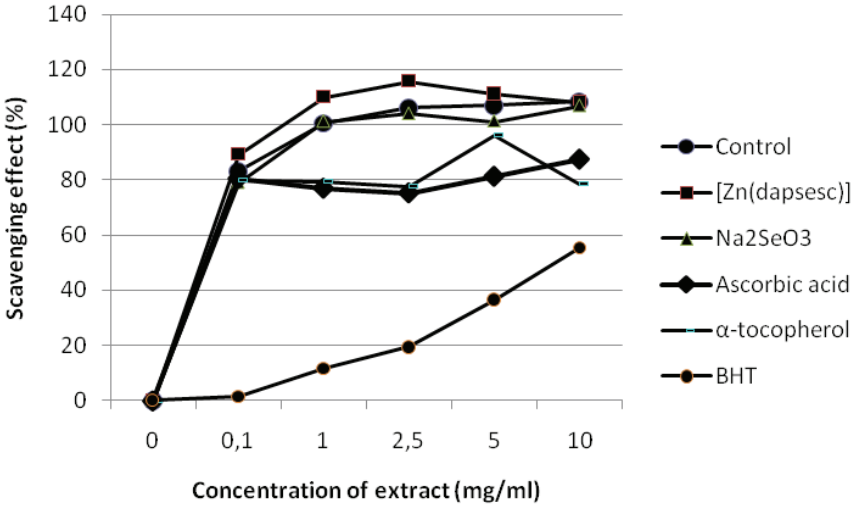

Fig. 3 – The possibility of binding DPPH radicals of hot water extract from whole mushrooms *L.edodes*. Each value is expressed as mean ± standard deviation (n = 3)

Table 1- EC_{50} values for the antioxidant activity of hot water extract from whole mushroom enriched with selenium

	EC_{50}[a] (mg/ml)		
	Antioxidant activity	Reducing power	Scavenging effect
Ascorbic acid	1.63±0.25[b]	<0.1	<0.1
α-tocopherol	< 0.1	NA	<0.1
BHT	NA[c]	NA	8.49±0.03
Lentinus edodes L31, control	12.02±0.09	>20	<0.1
Lentinus edodes L31 with inorganic selenium (50mg/kg Se)	5.1±0.06	>20	<0.1
Lentinus edodes L31 with organic selenium (15mg/kg Se)	3.79±0.04	>20	<0.1

a EC_{50} value: The effective concentration at which the antioxidant potential was 50%, the absorbance was 0.5 for reduction power, the power of neutralizing the DPPH radical was 50%. EC_{50} value was obtained by interpolation from linear regression analysis.
b Each value is expressed as the mean ± standard deviation (n = 3).
c NA: not analyzed

CONCLUSION

Mushrooms enriched with selenium are potential dietary supplements. Samples enriched with selenium from the organic sources showed significantly higher antioxidant activity than samples enriched with selenium from the inorganic sources. The results indicated that hot water extracts of whole mushrooms enriched with selenium showed good antioxidant activity at higher concentrations (10 mg/ml), regardless of the presence of molecules in the aqueous extract. The results of other studies indicate that the samples that have undergone dialysis showed higher antioxidant potential than extracts of whole mushrooms (Yu – Hsiu, 2008). From the results presented in the previous chapter, it is obvious that the reducing power of the control sample was 20-50% higher than the power of the enriched samples. Values of the samples enriched with inorganic selenium were slightly higher than those obtained from the samples enriched with organic selenium. Results of the previous studies show significant reducing capability of the aqueous extract from whole mushroom compared to the polysaccharide extracts (Yu – Hsiu, 2008). It is assumed that this is due to the presence of small molecules in the extract of whole mushrooms. Scavenging ability of both control and enriched fungus compared with positive tests was higher. Ascorbic acid and α-tocopherol showed the scavenging activity similar to that of fungi samples, while the BHT showed significantly lower activity, which differs from the results of the previous studies (Turley et al., 2010). It can be concluded that the hot water extracts have very good ability to bond DPPH radicals, similar to samples that passed the dialysis (Yu – Hsiu, 2008). Further research should include dialysis of the samples in order to remove small molecules from the extract. It is assumed that the hot

water extraction degrades polysaccharide molecules to smaller molecules that can later cause problems during sample analyses.

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АНТИОКСИДАТИВНА АКТИВНОСТ ВОДЕНОГ ЕКСТРАКТА ГЉИВЕ *LENTINUS EDODES* ОБОГАЋЕНЕ СЕЛЕНОМ

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Резиме

Shiitake (*Lentinus edodes*) припада групи медицински значајних и деликатесних гљива. Препознатљива је по својој лековитости, изванредном укусу и богатој ароми. Према традиционалној јапанској и кинеској медицини, гљива shiitake значајно повећава снагу и виталност организма. Shiitake садрже имуностимулансе, састојке који снижавају холестерол, спречавају зачепљење крвних судова, регулишу притисак, уравнотежују ниво шећера у крви, регулишу пробаву, побољшавају рад дисајних органа, делују антиреуматски и антиалергијски. Препорука је да се shiitake користе као укусна храна, превентива и лек, најчешће као зачин (сушене и млевене) или чај. Могу се конзумирати и као свеже припремљене.

Циљ рада био је да се разјасни да ли селен додат у супстрат за гајење гљиве *Lentinus edodes* утиче на редукциона својства екстракта, антиоксидативну активност екстракта, као и процена реактивности екстраката према радикалским врстама. Гљива је обогаћена селеном додавањем органских, Zn (II) комплекс са лигандом 2,6-диацетилпиридин бис (селеносемицарбазон), и неорганских једињења (Na_2SeO_3) селена у хранљиви супстрат на којем је гљива узгајана. Укупан садржај селена у плодоносном телу кретао се око 50 ppm за узорак обогаћен селеном из органског извора и 80 ppm за узорак обогаћен селеном из неорганског извора. Узорци су припремљени екстракцијом плодоносних тела у загрејаној води. Добијени резултати указују на то да водени екстракти целих гљива, како контролних тако и са додатком селена, имају добру антиоксидативну активност. Међутим, није примећена значајна разлика између узорака са и без садржаја селена.

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COMPARATIVE STUDY OF CELLULOLYTIC ACTIVITY OF THREE RUMEN FUNGI ON DIFFERENT SUBSTRATES

ABSTRACT: Anaerobic chytridiomycete fungi are found in the gastrointestinal tracts of many domesticated ruminant and nonruminant herbivores and of a wide variety of wild herbivorous mammals. They produce high levels of cellulases and hemicellulases; these enzymes are regulated by substrate (especially soluble sugars) available to the organisms.

The aim of this paper was to do a comparative study of cellulolytic activity of three rumen fungi on carboxymethyl cellulose and Avicel. The capacity of enzymes was determined by monitoring the growth on carboxymethyl cellulose (CMC) and Avicel. Enzyme activity was detected extracellularly in culture supernatants after vegetative growth. All of the isolates degraded CMC and avicel, and exhibited cellulolytic activities (carboxymethyl cellulose-(CMC-ase) and avicelase).

KEY WORDS: anaerobic fungi, cellulases, gas production, *Neocallimastix*, *Piromyces*

INTRODUCTION

All animals, including humans, need to consume food on a fairly regular basis in an effort to assimilate specific essential nutrients necessary to support body structure and functions. Fortunately, not all animals attempt to consume the same foods in order to obtain these essential nutrients. Otherwise, tremendous competition would occur for available food reserves resulting in minimization of species diversity. The greatest amounts of stored nutrients in the world are in the form of plant cell wall material, which is indigestible by all mammalian digestive enzymes. Only bacteria and fungi possess the capacity to degrade plant cell wall materials.

The rumen is a highly complex ecosystem that contains different microbial species. Ruminant's performance depends on the activities of their microorganisms to utilize dietary feeds. The rumen microbial ecosystem is comprised of at least 30 bacterial (10^{10} to 10^{11}/ml rumen fluid) (Stewart et al., 1997), 40 protozoa (10^5 to 10^7 (Williams and Coleman, 1997), and 6 fungal

species (<10^5) (O z k o s e et al., 2001; N a g p a l et al., 2009b). Bacteria, fungi, and protozoa are responsible for 50 to 82% of cell-wall degradation (L e e et al., 2000).

Anaerobic fungi inhabit the gastrointestinal tract of herbivores, especially ruminants, and make a significant contribution to rumen metabolism, particularly to the digestion of plant structural biomass. Rumen fungi can even colonize highly recalcitrant material, including wheat and rice straw, maize stems, soybean hulls, temperate and tropical grasses or palm press fiber (H o et al., 1991; R o g e r et al., 1992; L e e et al., 2000). These properties make anaerobic fungi interesting for the scientific community.

Besides digestive tract and faeces (D a v i e s et al., 1993), anaerobic fungi have also been isolated from saliva of a sheep (L o w e et al., 1987a).

Rumen fungi produce a wide range of polysaccharide degrading enzymes (such as cellulase and xylanase) that degrade lignin-containing plant cell walls and have the ability to degrade up to 65% of dry weight of plant tissues in pure cultures (O r p i n and J o b l i n , 1988).

MATERIALS AND METHODS

Isolates

The anaerobic fungi used were isolates OEM1, C1 and G1, isolated from faeces of *Cervus dama* (from the Skopje ZOO), domestic cow and domestic goat, respectively. The methods used for isolation from the faeces, as well as the maintenance of pure fungal cultures, have been already described (A t a n a s o v a – P a n c e v s k a, 2006). Strain OEM1 resembled *N. frontalis* (H e a t h et al., 1983; O r p i n, 1975), whereas strains C1 and G1, resembled *P. communis* (O r p i n, 1977) and *P. mae* (L i et al., 1990), respectively.

Culture purity

Fungal isolates were routinely checked for purity by examination of wet mounts, Gram staining and transfer of isolates from liquid culture to agar plates containing medium with 0.2% cellobiose to check the bacterial colony formation.

Medium

Complex medium for growth and maintenance of fungi was medium 10 of C a l d w e l l and B r y a n t (1966), except that glucose (4 g/l) was the only sugar present, and 10% (v/v) of clarified rumen fluid was added. The pH was adjusted to 7.0-7.2. Medium was prepared anaerobically using cysteine – HCl (0.05%) as reducing agent.

Culture conditions

Incubations were carried out in 20 ml flasks closed by butyl rubber stoppers, under O_2-free CO_2 atmosphere. The medium (15 ml) was inoculated by 1 ml of 3 days old fungus culture. The cultures were examined in triplicate. Inoculated cultures were grown at 39°C for 4 days.

Assay of cellulolytic enzymes in culture supernatants

Avicel and carboxymethyl-cellulose (CMC) were used as growth substrates for the production of cellulolytic enzymes. The inoculated serum bottles were incubated at 39ºC for 12, 24, 36, 48, 72 and 96 hours. Enzyme activities were measured at the end of each incubation period. The utilization of the substrates was assayed at each time point using three biological replicates per fungal isolate. Five uninoculated serum bottles were used as negative controls.

After incubation, the medium was centrifuged at 1500 g for 15 minutes, and the supernatant was tested for the presence of active enzymes.

With CMC as the substrate, 0.2 ml of supernatant was incubated with 1.8 ml of 50 mM citrate-phosphate buffer (pH 6.8) containing 10 mg of CMC for 30 minutes at 50°C. The reaction was terminated and reducing sugars were detected by the addition of 3 ml of dinitrosalicylic acid reagent (DNS). The A550 values were read with glucose representing the standard.

With Avicel, 0.25 ml of culture supernatant was incubated with 50 mg of substrate in 1.75 ml of 50 mM citrate-phosphate buffer (pH 6.6) at 40°C for 4 hours. The reaction was terminated by placing the reaction tubes in boiling water for 5 minutes. The samples were centrifuged to pellet the residual Avicel, and the reducing sugars that were liberated were analyzed with DNS.

In vitro gas production

The total gas production during fermentation was measured with a 25-ml glass syringe connected to a needle, which pierced through the butyl stopper into the head-space of the flask.

RESULTS

This is relatively new area of rumen microbiology in Macedonia because of lack of information about cellulolytic activity of rumen fungi (Atanasova-Pancevska and Kungulovski, 2003/2004; Atanasova-Pancevska, 2006; Atanasova-Pancevska and Kungulovski, 2008). In the present study, three monocentric type ruminal fungi were isolated from ruminant herbivores. These isolates were characterized by their morphologies, gas production, and production of cellulolytic enzymes.

Anaerobic fungi are obligate anaerobes and gain energy from the fermentation of carbohydrates (O r p i n, 1994).

The changes of pH in cultures of the three fungi are shown in Table 1 and Figure 1. The pH of OEM1 cultures grown on Avicel was the lowest, while C1 cultures grown on CMC had the highest peak after 24 h of incubation, followed by gradual reduction to the final pH value of 7. The pH of all other cultures was virtually identical.

Tab. 1 – pH of cultures of isolates OEM1, C1 and G1, grown on CMC and Avicel

pH	CMC			Avicel		
	OEM1	C1	G1	OEM1	C1	G1
0	7.1	7.1	7.1	7.1	7.1	7.1
12	7.15	7.19	7.12	7.13	7.15	7.14
24	7.2	7.47	7.27	7.15	7.21	7.23
36	7.23	7.39	7.2	7.15	7.18	7.18
48	7.2	7.4	7.16	7.12	7.16	7.16
72	7.1	7	7	7.1	6.98	6.99

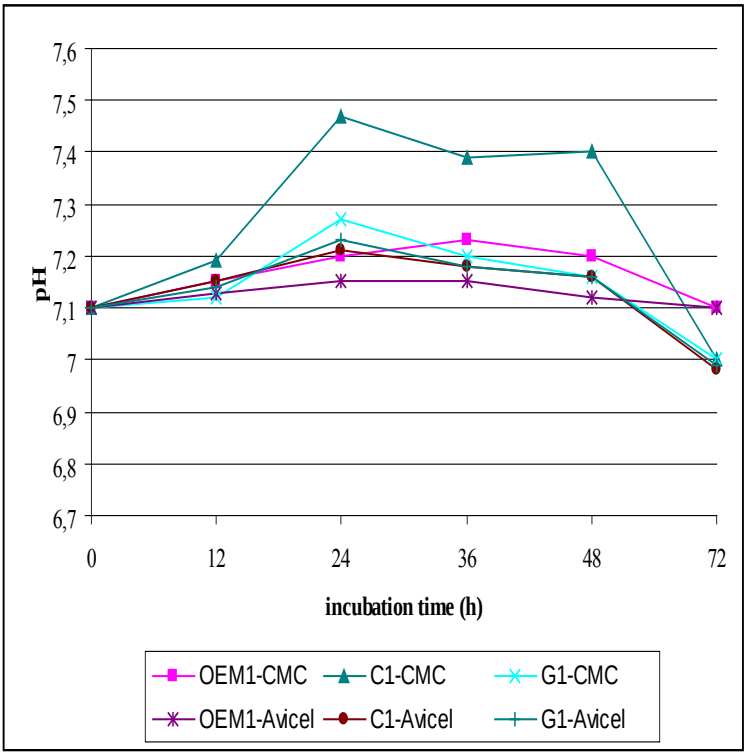

Fig. 1 – pH of cultures of isolates OEM1, C1 and G1, grown on CMC and Avicel

Fungal growth in cellulose cultures was measured by gas production during fermentation of CMC and Avicel. The amount of gas produced was determined after 12, 24, 36, 48, and 72 hours. The results are shown in Table 2 and Figure 2.

Tab. 2 – Gas production by OEM1, C1 and G1, grown on CMC and Avicel

gas (mL)	CMC			Avicel		
hours	OEM1	C1	G1	OEM1	C1	G1
0	0	0	0	0	0	0
12	5	4	3	6	5	5
24	32	28	25	29	27	26
36	30	27	24	27	28	26
48	15	13	13	15	12	13
72	9	7	6	8	7	8

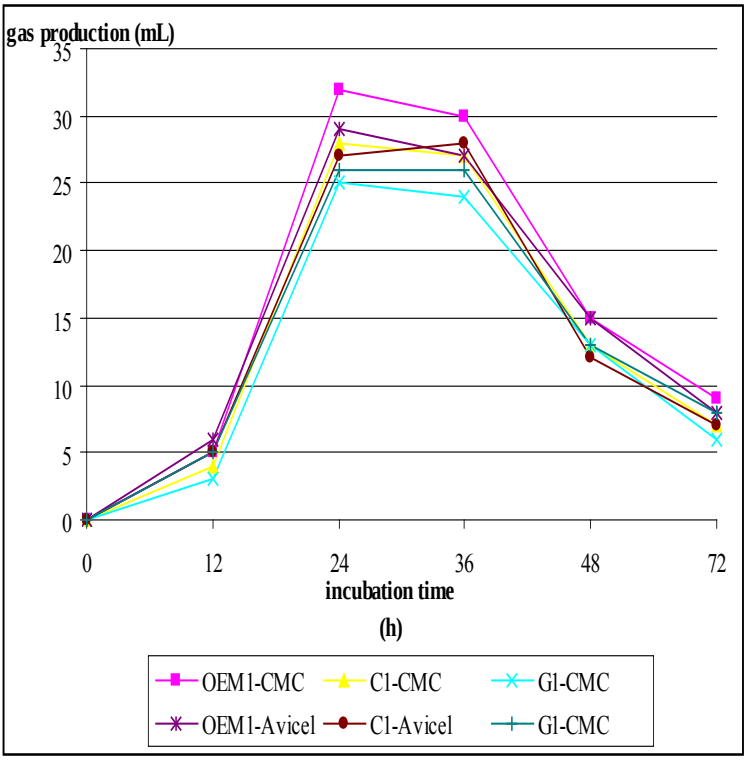

Fig. 2 – Gas production by OEM1, C1 and G1, grown on CMC and Avicel

The isolates were initially isolated on medium M10 with glucose as a sole carbohydrate source, and were then grown on Avicel (crystalline cellulose) and CMC, as a growth substrate. All isolates produced an array of enzymes that allowed them to hydrolyze plant cell walls. The enzymatic activity was simultaneous with the growth of the isolate (Table 3), as it was the case with other ruminal fungi (Lowe et al., 1987b; Mountfort and Asher, 1985).

The activities of CMC-ase and Avicelase of OEM1, C1 and G1 grown on both media proceeded in a similar manner (Table 3). After an initial lag phase of about 12-24 h, the activity of enzymes increased rapidly, reaching its maximum in 96th hour. The increase in the production of total gas for all strains was accompanied by an increase of enzyme activity.

Tab. 3 – Activities for carboxymethylcellulose (CMC-ase) and avicelase of OEM1, C1 and G1 grown on M10 medium with CMC and Avicel

Fungal isolate	Incubation period (h)	Enzyme (μmol/min/mL)			
		CMC-ase on CMC	Avicelase on CMC	CMC-ase on Avicel	Avicelase on Avicel
OEM1	12	0.0821	0	0	0.1910
	24	0.2356	0.0461	0.1966	0.2865
	36	0.4775	0.5730	0.2865	0.3820
	48	0.6685	0.6685	0.2865	0.3820
	72	0.7640	0.8596	0.3820	0.6685
	96	0.8893	0.8596	0.8596	0.6685
C1	12	0	0.2865	0	0
	24	0.0562	0.5730	0	0.0225
	36	0.2022	0.6685	0.2865	0.1461
	48	0.4775	0.6685	0.3427	0.3820
	72	0.6236	0.7640	0.3483	0.6685
	96	0.8202	0.7697	0.4775	0.7640
G1	12	0	0.3820	0	0
	24	0	0.5730	0	0.0225
	36	0.0506	0.5730	0.1517	0.2865
	48	0.4382	0.5730	0.2865	0.4775
	72	0.4775	0.6685	0.4382	0.7640
	96	0.5281	0.7191	0.6685	0.8596

pH	CMC			Avicel		
	OEM1	C1	G1	OEM1	C1	G1
0	7.1	7.1	7.1	7.1	7.1	7.1
12	7.15	7.19	7.12	7.13	7.15	7.14
24	7.2	7.47	7.27	7.15	7.21	7.23
36	7.23	7.39	7.2	7.15	7.18	7.18
48	7.2	7.4	7.16	7.12	7.16	7.16
72	7.1	7	7	7.1	6.98	6.99

DISCUSSION

The rumen provides an environment rich in nutrients and cofactors, including aminoacids, peptides, vitamins, and minerals, required by microorganisms for fermentation and growth (H u n g a t e, 1966).

The three fungal isolates from faeces used in this study belong to the groups of morphologically similar fungi.

The in vitro growth of the ruminal fungi followed a pattern typical of a wide variety of other fungi. When introduced into fresh media, these fungi proceed through a succession of phases beginning with a lag phase, continuing with a growth phase and then a stationary phase, and ending with a death phase (G r i f f e n, 1981). Often, the initial growth can be exponential, and it is usually followed by a longer period with a declining growth rate, which can appear as linear growth (Figures 1 and 2).

During the last 3 decades, measurement of *in vitro* microbial gas production (MGP) has received great impetus and become increasingly popular for determining the rate of fermentation (M e n k e et al., 1979; T h e o d o r o u et al., 1994; D a v i e s et al., 2000).

The syringe system is used widely to record the gas values at different times of incubation (D u a n et al., 2006).

This method for measuring gas production as an index of activity *in vitro* was first described by M e n k e et al. (M e n k e et al., 1979).

Changes in pH and total gas production of the culture were closely related to the extent of CMC and Avicel digestion. After an initial lag period of 12 hours, the fungal gas increased rapidly between 12 and 24 h and reached its maximum. After that, the stationary period of about 12 hours occurred, followed by a decrease to 6 mL gas at 72nd hour. Subsequently, the pH decreased rapidly between 48 and 72 h before stabilizing. The production of gas was identical, with slight differences (Figure 2).

The fermentation rate of various carbohydrates was present during the total gas production. As it was expected, gas production increased simultaneously with enzyme production in all the tested isolates (Figure 2). Isolates OEM1, C1 and G1 produced a maximum of 32, 28 and 25 ml gas, respectively, when incubated on M10 with CMC as the sole energy source, and 29, 27and 26 ml gas, respectively, when incubated on M10 with Avicel as the sole energy source.

The effectiveness of anaerobic fungi in ruminal cellulolysis depends on their ability to degrade complex polysaccharides which occur in plant cell walls. In addition to utilizing cellulose, *N. patriciarum* utilizes xylan and other grass hemicelluloses (O r p i n and L e t c h e r, 1979). Cultures of *N. patriciarum*, *Piromyces communis*, and *Sphaeromonas communis* (O r p i n, 1984), as well as several unnamed isolates of ruminal fungi which morphologically resemble *Neocallimastix* spp. or *S. communis* (G o r d o n, 1985; G o r d o n and A s h e s, 1984), all degrade various polysaccharide components of wheat straw cell walls. About half of the total cell walls, including about half of the cellulose and hemicellulose components, were lost from 4 to 5 days old cultures of *Neocallimastix* and *Piromonas* spp., whereas only smaller proportions

of these cell wall components disappeared from *Sphaeromonas* cultures grown for the same period of time (G o r d o n, 1985; O r p i n, 1984).

The extent to which ruminal fungi digest substrate depends on both the strain of fungi and the type of substrate. Cellulolytic enzymes were produced by our isolates after growth on CMC and Avicel. The effect of growth substrate on enzyme production by isolates OEM1, C1 and G1 was examined (Table 3). Although, cellulose was expected to be better inducer of cellulolytic enzymes, avicelase and CMC-ase activities were almost identical. Enzyme production was substrate dependent but differences were less obvious than in the case of ruminal *Piromyces* species (W i l l i a m s and O r p i n, 1987a, b).

The anaerobic fungi produce a wide range of polysaccharide degrading enzymes. Enzymes have been found associated with the rhizomycelium and many were also secreted into the surrounding environment (W i l l i a m s and O r p i n, 1987; L o w e et al., 1987d; B r e t o n et al., 1995; G e r b i et al., 1996a). The presence and activity of some surface associated enzymes fluctuated according to the stage of the life cycle (B r e t o n et al. 1995; G e r b i et al. 1996a). Also, the growth conditions greatly influence enzyme production, with three times the level of fibrolytic enzymes being produced in a stirred fermenter compared with static batch cultures in bottles (D i j k e r m a n et al., 1996a), whereas other continuous flow cultures produced up to twenty times the level of enzymes of batch cultures (Z h u et al., 1996). Fibrolytic enzymes were generally repressed by the presence of the sugar monomers resulting from degradation of polysaccharide: glucose for cellulases and xylose, and arabinose for xylanases (M o u n t f o r t, 1994).

CONCLUSION

Increased interest and research activities in the anaerobic gut fungi in the last decade or so have provided much information on their biology, taxonomy, physiology and enzymology. However, at present, there is still very little information on the range and diversity of fungal species inhabiting different host species, different substrates and different parts of the alimentary tract. It is not known whether the gut fungi are host or substrate specific. Further work is needed to elucidate this and some other aspects of their life, but in order to achieve these more specific techniques, involving molecular biology and molecular biotechnology, will be required.

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ЦЕЛУЛИТИЧКА АКТИВНОСТ ТРИ ВРСТЕ РУМЕНСКИХ ПЛЕСНИ КУЛТИВИРАНИХ НА РАЗЛИЧИТИМ СУПСТРАТИМА – КОМПАРАТИВНА СТУДИЈА

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Резиме

Румен представља изразито комплексан екосистем који садржи различите врсте микроба. Перформансе румена зависе од активности и способности његових микроорганизама у утилизацији нутритијената. Руменски микробни екосистем садржи бар 30 врста бактерија (10^{10} до 10^{11}/ml руменске течности) (Stewart et al., 1997), 40 врста протозоа (10^5 до 10^7 (Williams and Coleman, 1997), и 6 врста плесни (<10^5) (Ozkose et al., 2001; Nagpal et al., 2009б). Бактерије, плесни и протозоиски организми су одговорни за деградацију од 50 до 82% ћелијског зида (Lee et al., 2000).

Анаеробне хитридиомицетне плесни су пронађене у гастро-интестиналном тракту великог броја домаћих руминентних и неруминентних хербиворних животиња како и код великог броју дивљих хербиворних сисара. Ове плесни производе велике количине ензима целулазе и хемицелулазе, регулисаних од стране супстрата (посебно од стране растворљивих шећера) доступних организму.

Циљ овог рада је да се одреди целулолитичка активност код три врсте руменских плесни култивираних на карбоксиметилцелулазној (CMC) и Avicel-скоj подлози. Ензимска активност је детектована екстрацелуларно у супернатанту култура након вегетативног раста. Сви изолати деградирају CMC и Avicel и показују целулолитичку активност (карбоксиметил целулаза и авицелаза).

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DYNAMICS OF LIGNINOLYTIC ENZYME PRODUCTION IN *GANODERMA APPLANATUM* DEPENDING ON CULTIVATION TYPE

ABSTRACT: *Ganoderma applanatum* belongs to the group of white-rot fungi, due to a well-developed ligninolytic enzyme system. White-rot fungi have attracted great scientific attention in recent years, especially with respect to their enzymatic potential for the bioremediation of persistent pollutants. Contrary to *G. lucidum*, which medicinal properties, as well as ligninolytic enzyme system have been extensively studied, enzymatic system of *G. applanatum* has not been studied yet. Thus, the aim of this study was to analyze the dynamics of laccase, Mn-dependent peroxidase, and versatile peroxidase activity during submerged and solid state cultivation on two selected plant raw materials. Enzyme activity was determined spectrophotometrically after 7, 10 and 14 days of cultivation. The peak of laccase activity (220.14 Ul^{-1}) was noted after 14 days of submerged wheat straw fermentation. Maximum level of Mn-dependent peroxidase (110.91 Ul^{-1}) and versatile peroxidase (116.20 Ul^{-1}) activity was obtained in the medium with oak sawdust after 14 days of submerged cultivation.

KEY WORDS: laccase, *Ganoderma applanatum*, Mn-dependent peroxidase, versatile peroxidase, plant raw materials

INTRODUCTION

Ganoderma applanatum is a mushroom which fruiting bodies have been used as traditional medicine in China and reported to have diverse medicinal activities, such as antitumor, antiviral, immunomodulated etc. (L e e et al., 2007). Contrary to its medicinal properties, which were studied extensively, its ligninolytic enzyme system has not been studied yet. This species belongs to the group of white-rot fungi capable of degrading all basic wood polymers due to their ability to synthesize relevant hydrolytic and oxidative extracellular enzymes. These enzymes are responsible for degradation of cellulose, hemicellulose and lignin into low-molecular-weight compounds that can be assimilated for fungal nutrition (S o n g u l a s h v i l i 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). *G. lucidum* synthesizes three extracellular ligninolytic enzymes: lacasse (EC 1.10.3.2), Mn-dependant peroxidase (EC 1.11.1.13), and lignin peroxidase (EC 1.11.1.14) (D'Souza et al., 1996, 1999; Varela et al., 2000; Silva et al. 2005). In regard to the fact that enzymatic system of *G. applanatum* has not been studied yet, the aim of this study was to research the dynamics of selected ligninolytic enzyme production during submerged and solid state fermentation of plant residues.

MATERIAL AND METHODS

Organism and growth conditions

Ganoderma applanatum BEOFB 411, collected in Bojčin forest (near Belgrade), was used in this study. The culture was preserved on malt agar medium, in culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

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) of 7 day old *G. applanatum* culture. Incubation was performed at room temperature (22 ± 2°C) on a rotary shaker (160 rpm) for 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.

Analyzed plant raw materials were wheat straw and oak sawdust. Solid-state fermentation (SSF) was carried out at 25°C in 100 ml flasks containing 2g of wheat straw or 5g of oak sawdust soaked with 10 ml of the modified synthetic medium (without glucose, with nitrogen in a concentration of 25 mM, pH 5.0). Submerged fermentation (SF) was carried out in 250 ml flasks containing the same weight of ground plant materials soaked with 50 ml of the modified synthetic medium. Flasks prepared in this way were inoculated with 3 ml of homogenized inoculum. The samples from flasks were harvested after 7, 10 and 14 days of cultivation. Extraction of ligninolytic enzymes obtained under SSF conditions was performed by stirring samples with 50 ml of dH_2O on magnetic stirrer for 10 min at the temperature of 4ºC and centrifugation (4 ºC, 3000 rpm, 10 min). The obtained biomasses after SF fermentation were separated by centrifugation (4 ºC, 3000 rpm, 10 min) and supernatants were used to estimate enzyme activity. Three replications for each analyzed plant residue were prepared in order to decrease statistical error.

Enzyme activity assays

Laccase (Lac) and Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and versatile peroxidase (VP)] activities were determined spectropho-

tometrically. Lac activity was assayed by using 50 mM ABTS (ε_{436} = 29300 $M^{-1}cm^{-1}$) as a substrate, in a phosphate buffer (pH 6.0) and at the temperature of 35ºC. The reaction mixture contained: buffer, ABTS, and sample (V_{tot} = 1 ml). Mn-oxidizing peroxidases activities were determined with 3 mM phenol red (ε_{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 2 M NaOH. Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μmol of substrate/min. The Spectrophotometer –BioQuest (CECIL CE2500) was used for these assays.

Determination of total protein content

The amount of total proteins was performed 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; $mg \cdot ml^{-1}$), 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 at room temperature for 5 min. Total protein content is shown in $mg \cdot ml^{-1}$.

RESULTS AND DISCUSSION

The maximal level of MnP activity (110.9 ± 5.4 Ul^{-1}) was obtained after 14 days of oak sawdust SF, while the lowest value (8.4 ± 0.6 Ul^{-1}) was measured after 7 days of wheat straw SSF (Figure 1). The obtained results showed that oak sawdust was better carbon source and submerged cultivation was better cultivation type for MnP production.

The VP activity profile was similar to MnP profile. VP activity reached the peak after 14 days of submerged cultivation on oak sawdust (116.2 ± 3.9 Ul^{-1}), which was at least three-fold higher than after wheat straw fermentation. The lowest VP activity was obtained after 7 days of SSF of the both plant residues (10.8 ± 1.5 Ul^{-1}) (Figure 1).

The peak of Lac activity (220.1 ± 22.3 Ul^{-1}) was obtained after 10 days of oak sawdust SF, while minimum value (18.2 ± 0.2 Ul^{-1}) was noted in the wheat straw medium after 7 days of solid state cultivation (Figure 1).

Maximum of total protein content (11.5 ± 1.0 $mgml^{-1}$) was detected after 7 days of oak sawdust SF, and the minimum one (1.5 ± 0.1 $mgml^{-1}$) after 10 days of SSF of the same substrate, which was reflected on the specific enzyme activities.

The obtained results showed that oak sawdust is better substrate for the production of all tested enzymes, which was expected considering the fact that it is a natural substrate for *G. applanatum*, but the obtained values of the

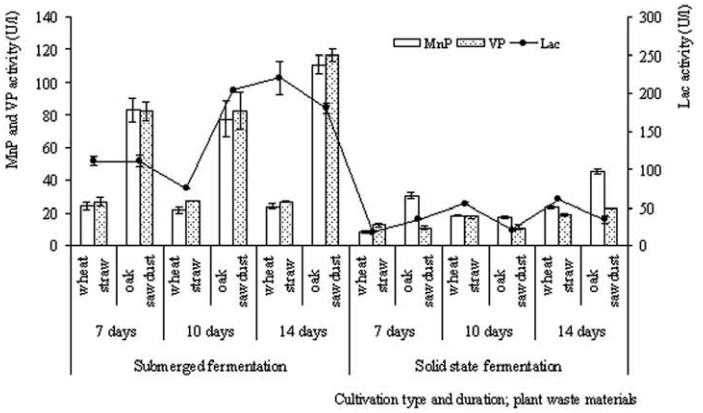

Fig. 1 – Effect of cultivation type, carbon source, and duration of cultivation on Mn-oxidizing peroxidases and laccase activity in *Ganoderma applanatum*

enzyme activity in wheat straw medium were not negligible, though it is unspecific substrate for this species. Annual worldwide production of agricultural plant residues is considerable, 123 x 10^6 t, and approximately half of that amount is used neither for food and feed, nor for the production of textile and paper (Villas-Bôas et al., 2002). Therefore, these abundant and available biowastes could present serious environmental pollutants, but also good substrates for obtaining large amounts of low-cost enzymes and bioconversion into fungal biomass (Moldes et al., 2004; Songulashvili et al., 2007). The utilization of raw plant materials by various mushroom species could contribute to more environmentally friendly solutions for the pollution.

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ДИНАМИКА ПРОДУКЦИЈЕ ЛИГНИНОЛИТИЧКИХ ЕНЗИМА *GANODERMA APPLANATUM* У ЗАВИСНОСТИ ОД ТИПА КУЛТИВАЦИЈЕ

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Резиме

Ganoderma applanatum припада групи гљива изазивача беле трулежи захваљујући добро развијеном лигнинолитичком ензимском систему. Ова група организама привлачи велику научну пажњу последњих година с обзиром да поседују велики ензимски потенцијал за разградњу тешко разградивих полутаната. За разлику од врсте *G. lucidum*, чија су лековита својства и лигнинолитички ензимски систем детаљно проучавани, ензимски систем *G. applanatum* је још увек недовољно познат. Циљ ових истраживања је био да се анализира динамика продукције Mn-зависне пероксидазе (MnP), верзатил пероксидазе (VP) и лаказе (Lac) у медијуму са пшеничном сламом или пиљевином храста као изворима угљеника, током течне и чврсте културе. Мерења су вршена након 7, 10 и 14 дана културе. Активност ензима је мерена спектрофотометријски, коришћењем ABTS-а као супстрата за лаказе и фенол црвеног за Mn-оксидујуће пероксидазе. Максимални ниво активности MnP (110.9 Ul^{-1}) и VP (116.2 Ul^{-1}) је добијен у медијуму са пиљевином храста, након 14 дана течне културе. Највећа активност Lac (220.1 Ul^{-1}) је измерена у медијуму са пшеничном сламом као изворoм угљеника, након 14 дана течне културе.

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LIGNINOLYTIC ENZYME PRODUCTION BY *LENZITES BETULINUS* ON SELECTED PLANT RAW MATERIALS

ABSTRACT: To get a better insight into the ligninolytic system of *Lenzites betulinus*, the effect of wheat straw and oak sawdust, as carbon sources, on production of Mn-oxidizing peroxidases and laccase, under solid-state and submerged fermentation, was studied. Obtained results revealed considerable differences related to the both factors affecting enzyme activities. Wheat straw was more favorable carbon source for Mn-oxidizing peroxidases and oak sawdust for laccase activity. Solid-state fermentation of wheat straw was optimal for Mn-dependent peroxidase activity (72.1 Ul^{-1}). In contrary to this, submerged fermentation of the same residue gave the highest level of versatile peroxidase activity (25.4 Ul^{-1}). The peak of laccase activity was noted during solid-state fermentation of oak sawdust (32.3 Ul^{-1}), while this enzyme was not detected under submerged fermentation of any plant residues.

KEY WORDS: laccase, *Lenzites betulinus*, oak sawdust, Mn-dependent peroxidase, versatile peroxidase, wheat straw

INTRODUCTION

Lenzites betulinus (L.) Fr. is a plant pathogen and common white-rot species grows scattered or clustered on deciduous wood such as birch, beech and oak, and less frequently on coniferous woods. This species has been used in traditional Chinese medicine for haunch and femoral pain, acropathy, apoplexy, and cold (R e n et al., 2006), and a wide range of medicinal effects were confirmed. A water extract of *L. betulinus* demonstrated mild antitumor activity against Sarcoma 180 (I k e k a w a et al., 1968), and the methanol extract showed free-radical scavenging (L e e et al., 1996), immunomodulating (F u j i m o t o et al., 1994) and antimicrobial activity (Y a m a c and B i l g i l i , 2006).

Nowadays, the ligninolytic enzyme systems of different mushrooms present an important area of interest which aim is to introduce the types and characteristics of produced enzymes, as well as the possibilities of their application in various biotechnological processes. Considering the literature data where ligninolytic enzyme system of *L. betulinus* has been only rarely reported, the aim of this study was to discover whether cultivation conditions

affect the production and activity of the selected enzymes during solid-state and submerged fermentation of different types of agricultural wastes by this species.

MATERIAL AND METHODS

Organism and growth conditions

Lenzites betulinus BEOFB 500 was collected from *Populus* sp. in Permskiy Kray, Russia, and maintained on malt agar medium (MA) in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

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 7 H_2O, 0.5 gl^{-1}; yeast extract, 2.0 gl^{-1}; pH 6.5) with 25 mycelial agar plugs (0.5cm in diameter) of 7-day-old *L. betulinus* culture on MA. The Erlenmayer flasks (250 ml) were incubated at room temperature (22 ± 2°C) on a rotary shaker (100 rpm) for 7 days. The obtained biomass was washed (3 times) by sterile distilled water (dH_2O) and homogenized with 100 ml of dH_2O in laboratory blender.

The ligninolytic enzyme activity was determined after solid-state (SSF) and submerged (SF) fermentation of wheat straw and oak sawdust. SSF was carried out at 25ºC in 100 ml flasks containing 2 g of wheat straw or 5 g of oak sawdust soaked with 10 ml of modified synthetic medium (without glucose, with nitrogen in a concentration of 25 mM, and pH 6.5). Homogenized suspensions (of 5 ml) were used for inoculation of one flask. On the other hand, SF was performed in 250 ml flasks containing 2 g of wheat straw or 5 g of oak sawdust, grounded to pass at least 40-mash screen, and 50 ml of modified synthetic medium, at room temperature (22 ± 2°C) on a rotary shaker (100 rpm). Prepared flasks were inoculated with 3 ml of homogenized inoculum. Samples from flasks were harvested successively after 7 and 10 days of cultivation. Extraction of ligninolytic enzymes obtained under SSF conditions was performed by stirring samples with 50 ml of dH_2O on magnetic stirrer for 10 minutes at the temperature of 4ºC and centrifugation (4°C, 3000 rpm, 10 min). Obtained biomasses after SF fermentation were separated by centrifugation (4 °C, 3000 rpm, 10 min) and supernatants were used to estimate the enzyme activity. Five replicates for each plant residue were analyzed.

Enzyme activity assays

Laccase (Lac) and Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and versatile peroxidase (VP)] were determined spectrophotometrically using CECIL CE 2501 spectrophotometer. Lac activity was estimated by monitoring the A_{436} change related to the rate of oxidation of 50 mM 2.2'-azino--bis-[3-ethyltiazoline-6-sulfonate] (ABTS) (ε_{436} = 29300 M^{-1} cm^{-1}) in 0.1 M

phosphate buffer (pH 6.0), at 35ºC. The reaction mixture contained: buffer, ABTS, and sample (V_{tot} = 1 ml). Mn-oxidizing peroxidases activities were determined with 3 mM phenol red (ε_{610} = 22000 $M^{-1}cm^{-1}$) as a substrate in a buffer with the following content: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt (pH 4.5). The reaction mixture (V_{tot} = 1 mL) contained: buffer, sample, 2 mM H_2O_2 and phenol red, with or without 2 mM $MnSO_4$ (for MnP and VP, respectively). Reaction was stopped by 2M NaOH.

Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μmol of substrate/min.

RESULTS AND DISCUSSION

Studied species was able to grow at both substrates and produce all three assayed enzymes. The calculated enzyme activity at different time points shows significant differences. Lower MnP activity was associated with initial phases of experiment while Lac showed the opposite effect. Production of ligninolytic enzymes was dependent on the carbon source and cultivation type. Wheat straw appeared to be a better carbon source for activity of Mn-oxidizing peroxidases, while oak sawdust was more favorable for Lac production. SSF of wheat straw was optimal for MnP activity (72.12 Ul^{-1}), while VP activity reached peak after 10 days of wheat straw SF (29.09 Ul^{-1}) (Figures 1, 2). *L. betulinus* was able to produce Lac by solid state cultivation on both wheat straw and oak sawdust substrates, but its activity was not noted in liquid medium (Figures 1,2). The obtained results demonstrated that initial stages of SSF are associated with considerable Lac production, and the highest level was noted after oak sawdust SSF (32.25 Ul^{-1}) (Fig. 1).

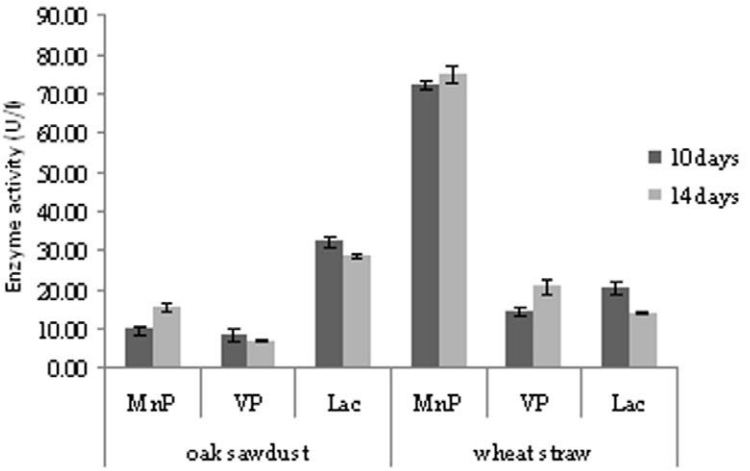

Fig. 1 – Effect of carbon source and duration of cultivation on Mn-oxidizing peroxidases and laccase activity in solid state cultivation of *Lenzites betulinus*

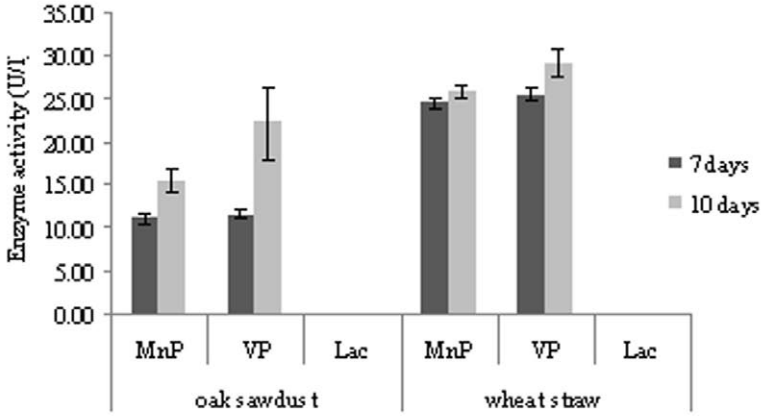

Fig. 2 – Effect of carbon source and duration of cultivation on Mn-oxidizing peroxidases and laccase activity in submerged cultivation of *Lenzites betulinus*

L e k o u n o u g o u et al. (2008) came to the same conclusion researching *Trametes versicolor*. Namely, the authors suggested that under conditions of beech SSF Mn-oxidizing peroxidases are not involved in initial phases of wood material colonization. The lack of Lac production could be strongly related with aeration level, i.e. the oxygen deficit under SF conditions, as oxygen is required for the enzyme catalytical proccesses (R e i n h a m m a r, 1984; T h u r s t o n, 1994; E g g e r t et al., 1996; S o l o m o n et al., 1996). In contrary to this, solid-state cultivation has proved to be particularly suitable for the Lac production since it reproduces the natural living conditions of fungi (P a n d e y et al., 1999; M o o – Y o u n g et al., 1983). Based on this fact, higher enzyme activities during SSF in comparison to SF could also be explained.

Although numerous studies of different medicinal effects of *L. betulinus* were done, data on its ligninolytic enzyme system have not been reported until now. This type of study is significant because, recently, special attention in biotechnology has been given to obtaining large amounts of low-cost enzymes by usage of various agricultural and food industry residues, which can often be serious environmental pollutants. Residues could be mineralized to low-molecular weight compounds by various ligninolytic enzymes, which are better digested by animals and could be used in further processing, such as in producing feeds and basic commodities for different industrial purposes.

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ПРОДУКЦИЈА ЛИГНИНОЛИТИЧКИХ ЕНЗИМА *LENZITES BETULINUS* НА ОДАБРАНОМ БИЉНОМ ОТПАДНОМ МАТЕРИЈАЛУ

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Резиме

Многобројна истраживања су показала различите медицинске ефекте екстраката *Lenzytes betulinus*. Међутим, проучавање лигнинолитичког ензимског система ове врсте до сада није рађено. Да ли услови култивације утичу на продукцију и активност Mn-оксидујућих пероксидаза и лаказе у току чврсте и течне ферментације пшеничне сламе и пиљевине храста овом врстом било је питање на које смо желели да добијемо одговор. Добијени резултати су показали да и тип култивације и извор угљеника утичу на ензимску активност. Пшенична слама је била знатно бољи извор угљеника за активност Mn-оксидујућих пероксидаза, а пиљевина храста за активност лаказе. Чврста ферментација пшеничне сламе је била оптимална за активност Mn-зависне пероксидазе (72.1 UI^{-1}), док је при течној ферментацији истог материјала добијен највиши ниво активности верзатил пероксидазе (25.4 UI^{-1}). Пик лаказне активности је забележен 10. дана чврсте ферментације пиљевине храста (32.3 UI^{-1}). Међутим лаказна активност није била детектована након течне ферментације тестираног биљног материјала.

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MOLD ATTACK ON FRESCOES AND STONE WALLS OF GRADAC MONASTERY

ABSTRACT**:** Microfungi can colonize stone surfaces and form sub-aerial biofilms which can lead to biodeterioration of historic monuments. In this investigation samples for mycological analyses were collected from stone material with visible alteration on stone walls of Gradac monastery exterior. The prevailing fungi found on stone walls were dematiaceous hyphomycetes with melanized hyphae and reproductive structures (*Alternaria, Aureobasidium*, *Cladosporium* and *Epicoccum* species). The frescoes inside the monastery building were also analyzed for the presence of mycobiota. The predominant fungi found on frescoes were osmophilic species from genera *Aspergillus* and *Penicillium*. The significant result is identification of human pathogen species *Aspergillus fumigatus* on frescoes.

KEY WORDS: *Aspergillus fumigatus*, biodeterioration, biofilm, cultural heritage, mycobiota

INTRODUCTION

During the recent decades there has been a general concern regarding the deterioration of historic and art buildings. The deterioration problem of works of art is significantly relevant in countries that are rich in cultural heritage, such as Serbia. Therefore, it is important to protect the cultural heritage objects from damages caused by air pollution, physical and chemical agents and biodegrading activity of microorganisms. Microorganisms can colonize monuments' surface and form microbial sub-aerial community, biofilm. All biofilm-forming microorganisms may cause biodeterioration and degrade monuments mechanically, chemically and aesthetically through the metabolic activities and biomineralization process in these biofilms (S u i h k o et al., 2007). Filamentous fungi are also part of sub-aerial biofilms and they can cause biodeterioration of historic monuments resulting in weathering of monuments. There are many publications focused on fungal deterioration of stone monuments, historical

buildings, frescoes and art objects (G a y l a r d e and M o r t o n, 2005). The two main mechanisms of fungal biodeterioration activity is hyphal penetration through the substratum and biocorossive activity as a result of production of organic acids and pigments (W a r s c h e i d and B r a m s, 2000).

Object of this research was the Gradac monastery, which was founded in 13th century by Helen of Anjou, and it was declared as Monument of Culture of Exceptional Importance in 1979, due to its architecture, wall paintings, and role in the history. As a part of the conservation process of cultural heritage of the Republic of Serbia, aim of this study was to isolate and identify biodegrading filamentous fungi from stone walls and frescoes.

MATERIAL AND METHOD

Samples for mycological analyses were collected from stone walls and frescoes with visible alterations of Gradac monastery. Thirty six samples from stone walls and 20 samples from frescoes were taken by swabbing with sterile cotton swabs. In the laboratory, swab samples were shaken mechanically for 10 minutes in 10 ml sterile distilled water and 1 ml aliquots of the resulting suspension were used for inoculation of spread plates on Malt agar medium containing 500 mg streptomycin per litre (MSA) in three replications. The inoculated plates were incubated at 25°C. Fungal growth was observed daily, for the period of 10 days, and they were submitted to the routine laboratory procedure to obtain pure fungal culture. Reisolations were done successively to the selective nutrient media: Potato dextrose agar (PDA), Czapek's agar (CzA) and Malt extract agar (MA) using standard mycological methods (B o o t h, 1971). Reisolated cultures were also incubated at 25°C. Identification of the obtained isolates to species level was done by macroscopic and microscopic examination. Microscopic preparates were dyed with lactophenol or fuchsine acid, observed by light microscopy (Zeiss AxioImager M.1, with software AxioVision Release 4.6) and determined by appropriate keys (A i n s w o r t h et al., 1973; E l l i s and E l l i s, 1997; P i t t, 1979; R a p p e r and F e n n e l, 1965).

RESULTS AND DISCUSSION

Out of a total of 56 samples, 17 fungal taxa were identified (Table 1). All isolates were obtained in pure culture by single conidial transfer. Mycological analyses showed that mycobiota was specific to different substrata (stone walls and frescoes).

The results of identification by morpho-physiological methods showed the abundance of dematiaceous hyphomycetes with melanized hyphae and reproductive structures on exterior stone walls of the monastery, while the prevailing taxa isolated from frescoes were osmophilic *Aspergillus* and *Penicillium* species. Non-sporulating isolates that could not be assigned to any

Tab. 1 – Molds isolated from stone walls and frescoes of Gradac monastery

Micromicetes	stone walls	frescoes
Alternaria alternata	+	
Alternaria sp	+	
Aspergillus fumigatus		+
Aspergillus nidulans		+
Aspergillus versicolor		+
Aureobasiduum pullulans	+	
Cladosporium cladosporoides	+	
Epicoccum purpurascens	+	
Fusarium sp.	+	
Mycelia sterilia	+	
Mycelia sterilia (melanized)	+	
Nigrospora nigra	+	
Paecylomyces sp.		+
Penicillium sp.	+	
Penicillium sp. 2		+
Sporobolomyces roseus	+	
Ulocladium sp.		+

taxonomic group were referred to as *Mycelia sterilia*. The production of dark conidia and pigments was recorded in culture media during the cultivation of melanized fungi isolated from stone walls. According to M i l a n e s i et al. (2005), species from genera *Alternaria*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, *Nigrospora*, and *Ulocladium*, which were found during our study, cause discoloration, as well as the mechanical exfoliation of building stone material that was analyzed through mechanical hyphae penetration and the production of different pigments and organic acids. *Aureobasidium pullulans* is a stain fungus that usually causes staining of different stone surfaces which decreases the aesthetic qualities of historic monuments. *Penicillium* and *Aspergillus* species are ubiquitous taxa that can produce numerous conidia and are widespread in the environment and easily dispersed by air. These fungi are usually found as contaminants or biodeterioration agents in many different habitats and materials, including those considered as representative of historical and cultural heritage. The significant result is the presence of *A. fumigatus* on frescoes inside the monastery. This mold can cause opportunistic infections in humans including aspergillosis which can sometimes be fatal. Indoor mold presence, including the interior of monasteries and other culture heritage buildings, can lead to sick building syndrome. This is why the fungal presence in indoor environment should not be neglected.

In conclusion, deterioration and alteration of stone walls and frescoes can be related to growth of filamentous fungi. The most frequent fungal contaminants of the historical and cultural monuments are air-borne and soil-borne micromycetes with cosmopolitan distribution that can colonize many kinds of surfaces.

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ПЛЕСНИ НА ФРЕСКАМА И КАМЕНИМ ЗИДОВИМА МАНАСТИРА „ГРАДАЦ”

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Резиме

Плесни могу да колонизују камени супстрат и да са другим микроорганизмима формирају специфичну микробијалну заједницу, биофилм. Присуство биофилма на каменим површинама доводи до процеса биодетериоризације који нарушава естетски изглед објеката од културно историјског значаја. Објекат истраживања, манастир Градац, који је основала Јелена Анжујска у 13 веку, је због посебног архитектонског значаја, зидног сликарства и његове улоге у историји 1979. године проглашен за културни споменик од изузетног значаја за Републику Србију. Као део конзервационог процеса, у оквиру програма очувања културне баштине, постављен је циљ – изолације и идентификације филаментозних гљива са камених зидова и фресака. Узорковање је извршено са 56 места са видљивим променама и оштећењима и стандардним миколошким методама изоловане чисте културе. Највећи број идентификованих плесни са спољашњих зидина манастира су из групе Dematiaceae са карактеристичних меланизованим хифама и конидијама (врсте родова *Alternaria, Aureobasidium*, *Cladosporium* и *Epucoccum*). Међутим, у узорцима са фресака доминирале су врсте родова *Aspergillus* и *Penicillium*. Значајно је истаћи да је на фрескама идентификована врста *A. fumigatus*, опортунистички хумани патоген, изазивач респираторне аспергилозе, и њено пренамножавање у затвореном простору је опасно по здравље људи.

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CHARM SCIENCES INC

Jednostavna analiza mikotoksina u žitaricama

Odobrenje GIPSA :
ROSA Aflatoksin
Kukuruz
Kukuruzno brašno
Kukuruzna krupica
Mešavina kukuruza i soje
Oštećen kukuruz
Kukuruzni griz
Suve žitarice za destilaciju
Mleven pirinač
Ovas
Kukuruz kokičar
Pirinač
Raž
Sirak
Soja
Pšenica
ROSA DON
Ječam
Kukuruz
Ječam za slad
Mleven pirinač
Ovas
Pirinač
Sirak
Pšenica
Pšenično brašno
ROSA Fumonizin
Kukuruz
ROSA Ohratoksin
Ječam
Ječam za slad
Pšenica
ROSA Zearalenon
Ječam
Kukuruz
Kukuruzni griz
Suve žitarice za destilaciju
Mleven pirinač
Ovas
Pirinač
Sirak
Pšenica
Pšenično brašno

CHARM-ovi testovi za mikotoksine su na bazi inovativne **ROSA** (*Rapid One Step Assays* - brza analiza u jednom koraku) tehnologije, lake za korišćenje, brze i tačne. Princip izvođenja testa se zasniva na pipetiranju ekstrakta uzorka na **ROSA** tračicu, inkubiranju i očitavanju u **ROSA-M** čitaču.

CHARM-ov kvantitativan aflatoksin test je prvi dobio GIPSA (Grain Inspection, Packers & Stockyards Administration) odobrenje za kvantitativno merenje nivoa aflatoksina u žitaricama. Od tada, **CHARM** je proširio detekciju mikotoksina od A do Z, uključujući:

- Trominutni kvantitativan test za aflatoksine u kukuruzu
- Trominutni kvalitativan test za aflatoksine u kukuruzu i za DON u pšenici i ječmu
- Desetominutni kvantitativni testovi za aflatoksine, DON, fumonizin, ohratoksine, T-2/HT-2 i zearalenon u različitim uzorcima

JEDNOSTAVNA PRIPREMA UZORKA (EKSTRAKCIJA)

CHARM obezbeđuje etanol i metanol u razblaženoj koncentraciji koji su spremni za upotrebu. Dodatne prednosti su sledeće:

- Jednostavna priprema uzorka i ušteda vremena
- Eliminisanje pripreme rastvora određene koncentracije

Zašto je CHARM-ova tehnologija bolja ?

- ✓ Analiza u jednom koraku
- ✓ Tračica za kvantitativno očitavanje
- ✓ Prikaz rezultata na Rosa-M čitaču u ppb i/ili ppm
- ✓ Tračice su zaštićene plastificiranim koricama koje ih štite od spoljašnjih uticaja
- ✓ Nije potrebna kalibracija instrumenta
- ✓ Minimalno mesta i potrošnog materijala potrebno za rad
- ✓ 2 u 1 test na aflatoksine: ekstrakt uzorka može da se koristi za kvalitativan i kvantitativan test
- ✓ Štampanje ili čuvanje rezultata uz pomoć **MYCOsoft** softvera

CHARM MYCOsoft™

VICAM FLUOROMETRIJSKA ANALIZA MIKOTOKSINA

➲ Jedna od najsavremenijih metoda analize mikotoksina obuhvata ekstrakciju uzorka i prečišćavanje sa Vicam-ovim imunoafinitetnim kolonicama (SAD) koje dalje idu na tečnu hromatografiju HPLC ili se mogu koristiti za kvantitativno određivanje prisutnih mikotoksina upotrebom fluorometra.

➲ Metoda je jednostavna i brza (analiza traje manje od 30 minuta). Jednostavnost testa je jedna od prednosti – pritiskom na taster fluorometra podešava se koji će mikotoksin da se određuje. Posle izolovanja mikotoksina primenom imunoafinitetne kolonice, uzorak se stavi u fluorometar. Fluorometar očitava fluoroscenciju direktno proporcionalnu količini prisutnog mikotoksina i daje vrednost izraženu u ppb ili ppm. Rezultat se štampa na ugrađenom štampaču. Instrument može da određuje Aflatoksin, DON, Zearalenon, Fumonizin, Ohratoksin, T-2 toksin.

➲ Vicam-ov sistem je odobren od Američkog ministarstva za poljoprivredu (USDA) i Udruženje analitičkih hemičara (AOAC).

Колектив Научног института за ветеринарство Србије драгим колегама и сарадницима жели срећан и успешан рад !

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