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# *ASPERGILLUS* SPECIES AS MYCOTOXIN PRODUCERS IN AGRICULTURAL PRODUCTS IN CENTRAL EUROPE

ABSTRACT: *Aspergillus* species are able to produce a range of mycotoxins, including e.g. aflatoxins, ochratoxins, fumonisins and patulin. Aflatoxins are mainly produced by members of *Aspergillus* section *Flavi*, and they contaminate various agricultural products in several parts of the world. Several recent reports have indicated that aflatoxin-producing fungi and consequently aflatoxin contamination occur in agricultural commodities in a number of European countries which have not been faced with this problem before. Indeed, recent surveys have clarified that concentrations of aflatoxins in maize products and milk has been exceeding the EU limit in several regions of Central Europe including Serbia, Slovenia, Croatia, Northern Italy and Romania. However, aflatoxin contamination and aflatoxin-producing *Aspergillus* species have not been identified yet in maize in Hungary. We examined the presence of potential aflatoxin-producing Aspergilli in maize samples collected in southern parts of Hungary. Several *A. flavus* isolates were identified, and preliminary results indicated that some of the isolates were able to produce aflatoxins. Contamination of other agricultural products with aflatoxins can also pose problems in Central Europe due to global warming. Ochratoxin contamination of grapes and grape-derived products is usually caused by black Aspergilli, especially by *A. carbonarius* and *A. niger*, although these species have been rare in Central European vineyards due to climatic factors. Ochratoxin contamination of other agricultural products including spices and cereals was also observed in the region. Besides, ochratoxin producing Aspergilli are frequently isolated from imported products including coffee beans, dried fruits and spices, and ochratoxin contamination of these samples was also observed. Fumonisins are produced mainly by *Fusarium* species, and by the recently identified producers *Aspergillus niger* and *A. awamori*. We examined fumonisin producing abilities of *A. niger / A. awamori* isolates collected from the variety of substrates including raisins, figs, dates, maize and onions. The isolates, which came from dried vine fruits, produced several fumonisin isomers also present in the raisin samples, indicating that fumonisin contamination of these products was probably caused by black Aspergilli. Besides, strains collected from figs, dates and onions were also able to produce fumonisins, and preliminary data indicated that figs and onions were also contaminated with low but significant amount of fumonisins. Potential fumonisin producing *A. awamori* isolates were also identified on maize samples. Further studies on

the examination of the occurrence of fumonisins and their potential producers in other agricultural products are in progress. Regarding patulin, contamination of apple based products is a serious problem in the region, mainly caused by *Penicillium* species. Although patulin producing Aspergilli have also been identified in cereals, patulin contamination of cereals and cereal based products is usually low in Central Europe.

KEY WORDS: *Aspergillus*, aflatoxins, fumonisins, ochratoxins, patulin

## INTRODUCTION

*Aspergillus* is among the most economically important of the fungal genera. *Aspergillus* isolates are used in Oriental food fermentation processes for the production of soy sauce, and in the biotechnological and pharmaceutical industry for the production of several organic acids and enzymes, and biologically active metabolites such as lovastatin (V a r g a et al., 2008). Although it is not considered to be major causes of plant disease, *Aspergillus* species may be responsible for several disorders in various plants (Table 1). The most notorious plant pathogens are *Aspergillus niger* and *A. flavus*, which may cause various plant diseases in susceptible plant varieties. In contrast with the specialized plant pathogens such as powdery mildews, rusts or most *Fusarium* species, these species are opportunistic pathogens without host specialization as proved in *A. flavus* (S t. L e g e r et al., 2000).

Tab. 1 – *Aspergillus* species involved in plant pathogenesis (modified after V a r g a et al., 2008)

| Plant | Disease | Species involved |
|---|---|---|
| Almond | Kernel decay | *A. niger, A. flavus, A. parasiticus* |
|  | Chlorosis | *A. niger* |
| Apples | Fruit rot | *A. sclerotiorum, A. terreus* |
| Apricot, peach | Ripe fruit rot | *A. niger* |
| Carrot | Sooty rot | *A. niger* |
| Citrus (*Citrus* spp.) | Albinism | *A. flavus* |
|  | Black mold rot | *A. niger* |
|  | Minor ear rots | *A. niger, Aspergillus* sp. |
|  | Fruit and root rot | *A. flavus* |
| Date Palm | Fruit rots | *Aspergillus* sp. |
| Fig | Fig smut | *A. niger* |
| Grape | Vine canker | *A. niger* |
|  | Bunch rot (sour rot) | *A. niger* |
|  | Berry rots | *A. aculeatus, Aspergillus* sp. |
| Maize | Ear rot | *A. flavus* |
| Mango | Black mold rot | *A. niger* |
| Onion, garlic | Black rot | *A. niger, A. alliaceus* |
| Peanut | Crown rot | *A. niger* |
| Pineapple | *Aspergillus* rot | *A. flavus* |
| Pistachio | *Aspergillus* fruit rot | *A. niger* |
| Strawberry | Fruit rots | *A. niger* |

While only a limited number of *Aspergillus* species can invade living plant tissues, they are frequently seen as post-harvest contaminants of agricultural products. Aspergilli can contaminate agricultural products at different stages, including harvest, processing and handling. The most important aspect of food spoilage caused by these organisms is the formation of mycotoxins, which may have harmful effects on human and animal health. Several mycotoxins, which may contaminate various agricultural products, have been identified and the economically most important are aflatoxins, ochratoxins, fumonisins and patulin (Table 2, Figure 1). In this review we will provide details on the occurrence of these mycotoxins and their producers in Central Europe.

Tab. 2 – Some economically important mycotoxins produced by *Aspergillus* species in various agricultural products

| Mycotoxins | Agricultural product | Species |
|---|---|---|
| Aflatoxins | Peanut, maize, cotton, spices | *A. flavus, A. parasiticus* |
| | Brazil nuts | *A. nomius* |
| Ochratoxins | Cereals | *P. verrucosum* |
| | Meat, cheese | *P. nordicum* |
| | Grape, wine | *A. niger, A. carbonarius* |
| | Coffee, spices | *A. ochraceus, A. steynii, A. westerdijkiae, A. niger, A. carbonarius* |
| | Figs | *A. alliaceus, A. niger* |
| | Pistachio nuts | *A. ochraceus* |
| Fumonisins | Grape, raisins, figs, onion | *A. niger, A. awamori* |
| Patulin | Cereals, malt | *P. expansum, A. clavatus* |
| | Apple, pear, cherry | *P. expansum, P. roquefortii, P. brevicompactum* |



Ochratoxin A          Aflatoxin B₁          Patulin

Fumonisin B₂
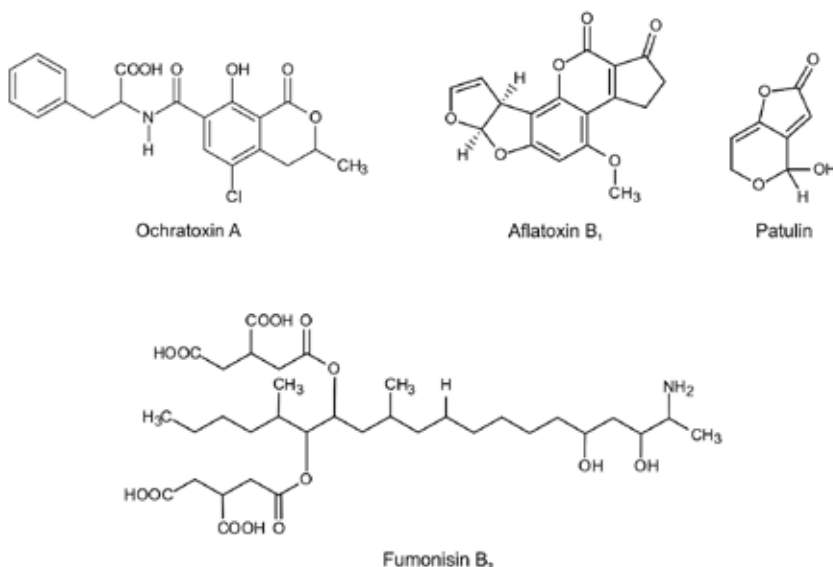
Fig. 1 – Chemical structures of the most important mycotoxins produced by *Aspergillus* species

# AFLATOXINS

Aflatoxins are decaketide-derived secondary metabolites which are produced by a complex biosynthetic pathway. Aflatoxins are among the economically most important mycotoxins. Aflatoxin $B_1$ exhibits hepatocarcinogenic and hepatotoxic properties, and is frequently referred to as the most potent naturally occurring carcinogen. Acute aflatoxicosis epidemics have occurred recently in several parts of Asia and Africa leading to death of several hundred people. These toxins are produced by at least 14 species assigned to three sections of the genus *Aspergillus*: section *Flavi* (*A. flavus, A. pseudotamarii, A. parasiticus, A. nomius, A. bombycis, A. parvisclerotigenus, A. minisclerotigenes, A. arachidicola, A. togoensis*), section *Nidulantes* (*Emericella astellata, E. venezuelensis, E. olivicola*) and section *Ochraceorosei* (*A. ochraceoroseus, A. rambellii*) (V a r g a et al., 2009; R a n k et al., 2011). Due to climatic factors, aflatoxins are mainly detected in products imported (e.g. Brazil nuts, pistachio nuts, peanuts and figs) into Central Europe. The famous "paprika scandal", which occurred in Hungary in 2004, was also caused by mixing the imported aflatoxin contaminated red pepper powder with Hungarian red pepper (F a z e k a s et al., 2005). However, aflatoxin contamination of some agricultural products does occur in Central European countries including Hungary (average aflatoxin contamination of Hungarian red pepper in 2000-2003: 0.76 μg/kg, (S z e i t z n é S z a b ó et al., 2007).

Although aflatoxins have been rare in Central European foods and feeds, global warming could alter the climatic conditions considerably. Recently, several papers have assessed the effects of climate change on food safety, including the occurrence of mycotoxin producing fungi and mycotoxins in foods (P a t e r s o n and L i m a, 2010; M i r a g l i a et al., 2009; C o t t y and J a i m e - G a r c i a, 2007). All these studies emphasize that aflatoxin producing fungi and consequently aflatoxins are expected to become more prevalent with climate change. Indeed, several recent reports have indicated that aflatoxin producing fungi and consequently aflatoxin contamination occurs in agricultural commodities in several European countries which have not been faced with this problem before (G i o r n i et al., 2007). In Romania, Tabuc et al. (2009) found that about 30% of maize samples collected between 2002 and 2004 in southeastern Romania were contaminated with $AFB_1$. In Serbia, J a k i c - D i m i c et al. (2009) could isolate *A. flavus* from 18.7% of the maize samples analyzed, and aflatoxins were also detected in 18.3% of the samples, while P o l o v i n s k i - H o r v a t o v i c et al. (2009) detected aflatoxin $M_1$ in 30.4% of milk samples collected from small farms in amounts exceeding the allowable limits of the European Union. Similarly, J a n k o v i c et al. (2009) also detected aflatoxin $M_1$ in 39% of the examined milk samples. T o r k a r and V e n g u s t (2007) detected aflatoxin $M_1$ above the EU limit in 10% of the examined milk samples in Slovenia. Furthermore, Halt (1994) detected aflatoxins in 9.4% of Croatian flour samples. During the research on potential aflatoxin producing fungi in southern Hungarian maize fields, we could isolate and identify a large number of *A. flavus* strains from maize. About 25%

of these isolates were found to be able to produce aflatoxins in varying quantities based on TLC and ELISA analyses. These preliminary data indicated that aflatoxin producing Aspergilli were also present in Hungarian maize fields. Consequently, climate change with elevated temperatures could lead to aflatoxin contamination of Hungarian agricultural products too, similarly to that observed in neighboring countries recently (T ó t h  et al., in preparation; D o b o l y i  et al., submitted). Investigation on the resistance levels of maize hybrids most frequently cultivated in the region is also in progress both in Hungary and in Serbia.

Potential aflatoxin producing *A. flavus* isolates were also identified in other agricultural products including stored wheat, onions, grapes and rice (V a r g a  et al., unpublished) and in cattle feed (K r n j a j a  et al., 2008). Aflatoxins were also detected in sunflower flour samples (M a s i c  et al., 2003) and in spices in Serbia (S a r i c  and S k r i n j a r, 2008).


## OCHRATOXINS


Ochratoxin A is a pentaketide mycotoxin that contaminates different plant products, including cereals, coffee beans, nuts, cocoa, pulses, beer, wine, spices and dried vine fruits (V a r g a  et al., 2001). Ochratoxins exhibit nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties. Several nephropathies affecting animals as well as humans have been attributed to ochratoxin A. This mycotoxin is the etiological agent of Danish porcine nephropathy, and renal disorders observed in other animals. In humans, ochratoxin is frequently cited as the possible causative agent of Balkan endemic nephropathy, a syndrome characterized by contracted kidneys with tubular degeneration, interstitial fibrosis and hyalinization of glomeruli. In 1993, the International Agency for Research on Cancer (IARC) classified ochratoxin A as a possible human carcinogen (Group 2B). Besides *Penicillium verrucosum* and *P. nordicum*, ochratoxins are produced by a range of *Aspergillus* species assigned to sections *Circumdati*, *Nigri* and *Flavi* (V a r g a  et al., 2008). In section *Circumdati*, ten species produce large amount of ochratoxin A and those are: *A. cretensis, Neopetromyces muricatus, A. pseudoelegans, A. roseoglobulosus, A. westerdijkiae, A. flocculosus, A. sulphureus, A. steynii, A. ochraceus* and *A. sclerotiorum*. In section *Flavi*, *A. alliaceus* and *A. albertensis* produce ochratoxins, while in section *Nigri* ochratoxin A is produced by *A. carbonarius*, *A. niger*, *A. lacticoffeatus* (a possible synonym of *A. niger*) and *A. sclerotioniger*. Regarding the imported products, recent surveys have clarified that ochratoxin contamination of coffee is caused mainly by *A. westerdijkiae* and black Aspergilli (N o o n i m  et al., 2008), while in pistachio, *A. ochraceus* is primarily responsible for ochratoxin contamination (S e d a g h a t i  et al., 2011).

Ochratoxin A has been frequently identified in cereal samples in several Central European countries including Hungary, Serbia and Croatia (V a r g a  et al., 2004; L e v i c  et al., 2004; P u n t a r i c  et al., 2001). According to a recent survey on mycotoxins worldwide, aflatoxins have been detected in

only 6% of cereal samples collected in Central Europe, while fumonisins and ochratoxins have been found in 29% and 41% of the samples, respectively (http://www.biomin.net/mycoreport/mtsurvey.html). Ochratoxins are produced by *P. verrucosum* in cereals under colder climate conditions (e.g. in northern parts of Europe: Lund and Frisvad, 2003), while Aspergilli dominate in the tropical region (M a g n o l i et al., 2007). There is an important question regarding which species is responsible for OTA contamination of cereals in countries with warmer climate, including southern Hungary. Due to the importance of cereal kernel mycotoxin contamination, it is highly necessary to assess mycotoxin content throughout the region immediately after harvest and during storage. Such studies are in progress in our laboratories.

Ochratoxin contamination of other agricultural commodities in Central Europe including red pepper (F a z e k a s et al., 2005) and grape derived products is mainly caused by *Aspergillus* species including black Aspergilli and members of *Aspergillus* section *Circumdati* (V a r g a and K o z a k i e w i c z, 2006). Ochratoxin A is a frequent contaminant of wine, with red wines being more contaminated than white wines of the same wine growing region. Besides this, ochratoxins are more frequently observed in wines originating from southern areas of Europe with their warmer climates (V a r g a and K o z a k i e w i c z, 2006). The amount of ochratoxins was also found to be dependent upon the latitude of the vineyard: OTA contamination is usually higher at lower latitudes. Ochratoxins were detected in Hungarian (V a r g a et al., 2005b), Croatian (D o m i j a n and P e r a i c a, 2005) and Slovenian wines (C i g i c et al., 2006). Furthermore, ochratoxin producing black Aspergilli were also identified in Hungarian grapes, but no potential ochratoxin producers were detected in Czech vineyards (V a r g a et al., 2007b).


FUMONISINS


Fumonisins are carcinogenic mycotoxins which were originally identified in *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*). Fumonisins are mycotoxins produced by several species of the genus *Fusarium*, including e.g. *Fusarium proliferatum, F. subglutinans, F. oxysporum* and *F. globosum*. Regarding the toxicity of fumonisins, high levels of fumonisin contamination in home-grown maize were found to be associated with high prevalence of human esophageal cancer in several parts of the world, including Transkei region in South Africa, Linxian province in China, Northern Italy, Mazandaran and Isfahan provinces in Iran, southeastern USA, India, Kenya, Zimbabwe and Brazil, and they were also involved in leukoencephalomalacia in horses, pulmonary edema in pigs, and liver cancer and neural tube defects in experimental rodents (S t o c k m a n - J u v a l a and S a v o l a i n e n, 2008). In a recent study, P e l et al. (2007) have identified a putative gene cluster for fumonisin biosynthesis in the phylogenetically very distantly related fungus *Aspergillus niger*, and fumonisin production has also been proved for several *A. niger* isolates coming from culture collections, coffee beans and grapes (F r i s v a d

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et al., 2007; M o g e n s e n  et al., 2010). Most of these reports claimed that *A. niger* produced only fumonisins $B_2$ and $B_4$, although other isomers were also detected in smaller quantities (V a r g a  et al., 2010). Whereas *F. verticillioides* produces fumonisins on agar media based on plant extracts such as barley malt, oat, rice, potatoes, and carrots, *A. niger* is able to produce fumonisins in high quantities on agar media with low water activity (F r i s v a d  et al., 2007). Recently, we have examined the mycobiota and fumonisin content in several agricultural products. The range of fumonisin isomers present in the various substrates, and produced by *A. niger* isolates collected from dried vine fruits (raisins, sultanas) was examined. Among the *A. niger* isolates identified, about 65% were found to be able to produce fumonisins. The average fumonisin content in the dried vine fruit samples contaminated with potential fumonisin producing black Aspergilli was 7 mg kg$^{-1}$. The isolates produced several fumonisin isomers also present in the dried vine fruit samples, including fumonisins $B_{1-4}$, 3-epi-$FB_3$, 3-epi-$FB_4$, iso-$FB_1$, and two iso-$FB_{2,3}$ forms (Figure 2, V a r g a  et al., 2010). Besides this, several strains collected from figs, dates and onions were also able to produce fumonisins, and preliminary data indicated that figs and onions were also contaminated with low but significant amount of fumonisins (data not shown). Black Aspergilli were suspected to be responsible for fumonisin contamination of grape-derived products, figs and onions (V a r g a  et al., 2010; V a r g a  et al., submitted).
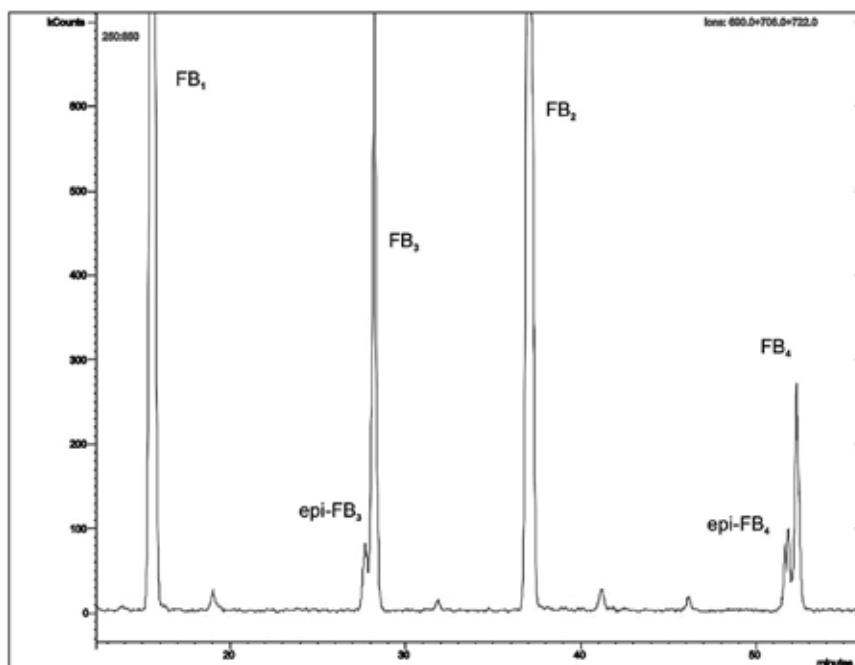


Fig. 2 – Reversed-phase, high-performance liquid chromatography/electrospray ionization – ion trap mass spectrometry (RP-HPLC/ESI-ITMS) chromatogram of the extract of a raisin sample. The fumonisin isomers detected in the sample are indicated.

# PATULIN

Patulin is produced by a number of fungi belonging to the *Aspergillus*, *Penicillium*, *Byssochlamys* and *Paecilomyces* genera. *Penicillium expansum*, causing blue mold (soft rot) of apples, pears, cherries and other fruits, is recognized as one of the most common causes of patulin contamination (T r u c k - s e s s and T a n g, 2001). Several *Aspergillus* species are also able to produce patulin, including species assigned to *Aspergillus* sections *Clavati* (V a r g a et al., 2007a) and *Terrei* (V a r g a et al., 2005a). These species frequently occur in cereals and cereal products (L o p e z - D i a z and F l a n n i g a n, 1997; A b r a m s o n et al., 1987). The most well-known species *A. clavatus* can be isolated mainly from soil and dung, but it also occurs in stored products (mainly cereals) with high moisture content, e.g. inadequately stored rice, corn and millet (F l a n n i g a n and P e a r c e, 1994). *A. clavatus* isolates appeared to be particularly well adapted for growth during malting (F l a n n i g a n and P e a r c e, 1994). Although patulin producing species (mainly Penicillia) are frequently found in cereal products in Central Europe, usually low patulin contamination of these products does not pose a serious health hazard to humans.

Patulin contamination of apple products caused by *P. expansum* is the main risk factor in the region. In the nineties, about 0-25% of the apple juice concentrates were found to contain patulin above the allowed limit in Hungary (V a r g a et al., 2004). These findings were in accordance with those found in other European countries (data not shown). The average content of patulin in Hungarian apple juice concentrates was above 100 μg kg$^{-1}$ in 2000. In that case, one of the examined samples contained 440 μg kg$^{-1}$ of patulin which was nine times higher than the maximum allowable limit (V a r g a et al., 2004).

In conclusion, although *Aspergillus* species are usually considered to prefer warmer climates, several important mycotoxin producing Aspergilli do occur in agricultural products in Central Europe, leading to mycotoxin accumulation in products such as cereals, grapes, dried fruits, onions and spices. Mycotoxin producing fungi and consequently mycotoxin contamination are expected to become more prevalent in this region due to climate change. A continuous thorough investigation of the occurrence, and the potential danger posed by these fungi and their mycotoxins are needed for the agricultural products in this region.

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# *ASPERGILLUS* ВРСТЕ КАО ПРОДУЦЕНТИ МИКОТОКСИНА У ПОЉОПРИВРЕДНИМ ПРОИЗВОДИМА У ЦЕНТРАЛНОЈ ЕВРОПИ

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## Резиме

*Aspergillus* врсте су способне да произведу велик број микотоксина, укључујући афлатоксине, охратоксине, фумонизине и патулин. Афлатоксине претежно производе припадници врсте *Aspergillus,* секције *Flavi,* и контаминирају различите пољопривредне производе у неколико светских региона. Неколико последњих истраживања указују да контаминације пољопривредних производа плеснима, продуцентима афлатоксина а самим тим и афлатоксинима почињу да се појављују и у земљама Европске уније које се раније нису суочавале са овим проблемом. Према недавно објављеним подацима, потврђено је присуство афлатоксина у кукурузу и млеку у количинама које прелазе максимално дозвољене границе према ЕУ регулативи, у неколико региона централне Европе укључујући Србију, Словенију, Хрватску, северну Италију и Румунију. Међутим, контаминација кукуруза афлатоксинима и токсигеним плеснима још није примећена у Мађарској. Ми смо испитивали присуство плесни, потенцијалних продуцената токсина из рода *Aspergillus,* у узорцима кукуруза прикупљених у јужним областима Мађарске. Идентификовано је присуство неколико изолата *A. flavus* и прелиминарни подаци указују на њихову способност да продукују афлатоксине. Очекује се да ће контаминација афлатоксинима и других пољопривредних производа осим кукуруза постати све озбиљнији проблем за централноевропске земље као последица глобалног отопљавања. Контаминацију грожђа и сродних производа охратоксином изазивају црни Aspergilli, нарочито *A. carbonarius* и *A. niger,* иако се ове врсте ретко срећу у виноградима централне Европе услед климатских фактора. У овом региону је такође запажена појава контаминације охратоксинима и других производа као што су жита и зачини. Такође, плесни овог рода, продуценти охратоксина, често могу да се изолују из увозних производа као што су зрна кафе, сушено воће и зачини а регистрована је и контаминација ових производа охратоксином. Фумонизине производе врсте рода *Fusarium* као и недавно идентификовани *Aspergillus niger* и *A. awamori.* У нашим истраживањима смо испитивали способност продукције фумонизина од стране *A. niger / A. awamori* у различитим супстратима, укључујући суво грожђе, смокве, урме, кукуруз и црни лук. Изолати из сувог грожђа су били способни да продукују неколико изомера фумонизина који су били присутни у узорцима сувог грожђа, указујући да је конатминација сувог грожђа фумонизима највероватније узрокована црним *Aspergillus* врстама. Поред тога, сојеви изоловани из смокве, урме и црног лука су такође показали способност производње фумонизина а прелиминарни подаци показују да су смоква и црни лук били контаминирани ниским али значајним количинама фумонизина. Изолати потенцијалног продуцента фумонизина *A. awamori* су такође изоловани у узорцима кукуруза. У току су истраживања о присуству фумонизина и њихових потенцијалних продуцената и у другим пољопривредним производима. Што се тиче патулина, контаминација производа од јабука је веома озбиљан проблем у овој регији а углавном га изазивају врсте рода *Penicillium.* Иако је у

житу идентификовано присуство продуцената патулина из рода *Aspergillus*, кон-
таминација жита и производа од жита патулином је углавном незнатна у централ-
ној Европи.

КЉУЧНЕ РЕЧИ: *Aspergillus*, афлатоксини, фумонизини, охратоксини, па-
тулин

*B i l j a n a   S t o j a n o v s k a – D i m z o s k a,*
*Z e h r a   H a j r u l a i – M u s l i u,*
*E l i z a b e t a   D i m i t r i e s k a – S t o j k o v i ć,*
*R i s t o   U z u n o v,   P a v l e   S e k u l o v s k i*

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# OCCURRENCE OF AFLATOXINS IN PEANUTS AND PEANUT PRODUCTS DETERMINED BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

ABSTRACT: Liquid chromatography with fluorescence detection using immunoaffinity column clean-up was a method described for determination of aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$) in peanuts and peanut based products. The validation of the procedure was performed. Good coefficient of correlation was found for all aflatoxins in the range of 0.9993–0.9999. Limit of detection (LOD) and limit of quantification (LOQ) ranged from 0.003–0.005 μg/kg and 0.009–0.023 μg/kg, respectively, which was acceptable. The mean recovery for total aflatoxins was 88.21%. The method also showed acceptable precision values in the range of 0.171–2.626% at proposed concentration levels for all four aflatoxins. $RSD_R$ values (within laboratory reproducibility) calculated from the results showed good correlation between two analysts for all aflatoxins and they ranged from 4.93-11.87%. The developed method was applied for the determination of aflatoxins in 27 samples of peanuts and peanut based products. The results showed that 21 peanut samples (77.7%) were below LOD of the method. Three samples had positive results over the MRL. There was one extreme value recorded for the total aflatoxins in peanut (289.2 μg/kg) and two peanut based products, peanut snack and peanut, with total content of aflatoxins being 16.3 μg/kg and 8.0 μg/kg, respectively. The obtained results demonstrated that the procedure was suitable for the determination of aflatoxins in peanuts and peanut based products and it could be implemented for the routine analysis.

KEY WORDS: aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$), imunoaffinity column clean-up, HPLC-FLD, peanut samples, peanut based products, validation

## INTRODUCTION

Aflatoxins are toxic secondary metabolites produced mostly by *Aspergillus flavus* and *A. parasiticus*. Among them, aflatoxin $B_1$ exhibits the highest toxicity and carcinogenicity and it can be found in many commodities (groundnuts, nuts, cereals and their products, dried fruits, herbs) (S o l e i m a n y F. et al., 2012). The International Agency for Research on Cancer (IARC) classified naturally occurring aflatoxin $B_1$ as carcinogenic to humans (Group 1) (IARC, 1993).

Peanuts and peanut based products are considered as popular food among all age groups, especially peanut snacks and peanut flips (D i s s a n a y a n e D M M P et al., 2009). Peanut has proved to be a good substrate for the growth of *Aspergillus* sp. and for the production of aflatoxins (B a k h i e t  et M u s a, 2010). Factors responsible for the high incidence of aflatoxin contamination of peanuts include poor agricultural practices during planting, harvesting, drying, transportation and storage of the product (O l i v e i r a et al., 2009). There are several reports from all over the world concerning the aflatoxin contamination in peanuts and peanut products. Y e n t ü r et al., (2009) analyzed 20 samples of peanut butter in Turkey and found that all samples contained aflatoxins ranging from 8.16-75.74 µg/kg. O l i v e i r a et al. (2009) reported that 52% of the peanut samples analyzed in Brazil were positive for aflatoxins, ranging from 51-420 µg/kg. P a r k (2006) analyzed 40 peanut and 30 peanut butter samples. $AFB_1$ was found in 5 peanut butter samples with mean $AFB_1$ concentration of 12 µg/kg, and 10 peanut samples had $AFB_1$ concentration ranging from 19-32 µg/kg. In another study, M u t e g i et al., (2009) carried out a survey on a total of 1260 peanut product samples. Thirty eight per cent of all samples tested were noted safe according to the EU regulatory limits. The most contaminated product was peanut flour with 88% of all its products, having aflatoxin levels of more than 10 µg/kg.

Total aflatoxin content in food is regulated by legislation worldwide (Commission Regulation 466/2001, 2174/2003 and 1881/2006). Our country has adopted the EU regulations since December 2005 (118/2005). Different analytical methods are used for aflatoxins analysis (R e n et al., 2007; I b á ñ e z – V e a et al., 2011). The HPLC methods for mycotoxin analysis have gained more attention due to their efficiency and high sensitivity, especially when fluorescence detection is used because it provides high selectivity, low LOQ and accurate analysis (B a o et al., 2010). Very important aspect concerning mycotoxin analysis is sample preparation and clean-up. Application of immunoaffinity column (IAC) provides clean extracts due to the specificity of the antibody, applicability to complex matrices, good precision, accuracy and sensitivity of analytical methods (S c o t t and T r u c k s e s s, 1997; C i c h n a – M a r k l, 2011).

The aim of this research was to evaluate and validate reliable analytical method for determination of total aflatoxins in peanuts and peanut based products, using immunoaffinity column clean-up and liquid chromatography with postcolumn bromination in Kobra cell and fluorescence detection.

## 2. MATERIALS AND METHODS

### *2.1. Apparatus*

HPLC analysis was performed with Perkin Elmer (PE) chromatographic system equipped with binary pump (PE LC-250), manual injector (PE Rheodyne 7125) and fluorescence detector (PE LC-240). Mycotoxins were separated on

Supelco column (250 mm x 4.6 mm, 5μm) at room temperature. The mobile phase was a mixture of water: acetonitrile: methanol (600:50:350, *V/V/V*) with addition of 119 mg KBr and 350 μl 4N $HNO_3$. The mobile phase was degasified in the ultrasonic bath before use. The flow rate was 1 ml/min and the injection volume was 100 μl. The detection was carried out at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 440$ nm.

## 2.2. Reagents and standard solutions

HPLC reagents (methanol, acetonitrile, water) and chemicals (benzene, KBr, NaCl, $HNO_3$) were purchased from Merck (Darmstadt, Germany). For clean-up purification immunoaffinity columns Aflaprep (R-Biopharm Rhône, Glasgow, Scotland) were used. As a standard aflatoxin mix from Supelco, with concentrations of $AFB_1$ 982 ng/ml, $AFB_2$ 284 ng/ml, $AFG_1$ 1034 ng/ml, $AFG_2$ 333 ng/ml dissolved in benzene:acetonitrile (98:2), was used. Aflatoxin mix stock solution with concentrations of $AFB_1$ 100 ng/ml, $AFB_2$ 28.4 ng/ml, $AFG_1$ 103.4 ng/ml, $AFG_2$ 33.3 ng/ml was prepared from the aflatoxin mix standard, dissolving aliquot (1.01 ml) in a volumetric amber flask of 10 ml with solvent mixture benzene:acetonitrile (98:2). Seven working standard solutions were prepared from the stock solution in volumetric amber flasks of 5 ml. The aliquot of solution needed for preparation of working standards was evaporated under stream of nitrogen and the dry residue was dissolved in methanol:water (1:1). All working standards were kept in a refrigerator at 2-8°C.

## 2.3. Samples

Total of 27 peanuts and peanut based products samples were collected during 2011-2012. All the samples were brought to our laboratory by border health inspectors or food operators. The samples were kept in their original packages in dark, dry and cool place until analysis. For the recovery experiment, aflatoxin-free peanut samples were spiked with known amount of aflatoxin $B_1$ at three concentration levels (1.0, 2.0 and 5.0 μg/kg) and all of them were around maximum residual level (MRL) for peanuts. Those portions (500 μl) of aflatoxin $B_1$ standards were applied to the tested samples and they were kept for app. 15 min before the addition of extraction solvent.

## 2.4. Analytical procedure

The extraction and purification of aflatoxins from peanut samples was done according to AOAC method (AOAC, 2005). HPLC-FLD procedure was performed according to ISO standard (ISO 16050:2003). Twenty five grams of tested sample with addition of 5g NaCl and 125 ml 70% methanol was mixed in a blender jar for 2 min at high speed. The mixture was filtered through a

fluted filter paper. Thirty ml of water was added to a 15 ml of filtrate and filtered again through microfiber filter paper. Fifteen ml of the second filtrate was quantitatively passed through the immunoaffinity column at flow rate of 1-2 drop/sec. The column was washed with 10 ml of water. Aflatoxins were eluted with 1 ml of methanol in an amber vial at flow rate of 1-2 drop/sec. The elution step was repeated one more time with 1 ml of water. Then, 100 μl of methanol--water solution was applied to HPLC-FLD system, followed by derivatization with bromine in Kobra cell (R-Biopharm Rhône).

## 2.5. Validation procedure

The validation procedure was performed according to Decision 2002/657/EC and Regulation 401/2006/EC. Seven working standard solutions were used for the linearity testing in the following range: for $AFB_1$ 0.25-15.0 ng/ml, for $AFB_2$ 0.071-4.260 ng/ml, for $AFG_1$ 0.258-15.51 ng/ml and for $AFG_2$ 0.083-4.990 ng/ml. The LOD was calculated as 3.3xSD/slope (according to the ICH regulation – Validation of analytical procedures:Q2), where the slope was estimated from the calibration curve in the lower concentration range and SD (standard deviation) was based on the measurement of analytical background response of ten (10) blank samples. The LOQ was calculated as 10xSD/slope in the same manner as LOD. Recovery, as a part of method validation, was determined according to the method of standard addition. The following fortified concentration levels were applied: for $AFB_1$ (1.0; 2.0 and 5.0 μg/kg), for $AFB_2$ (0.310; 0.568 and 1.550 μg/kg), for $AFG_1$ (1.028; 2.068 and 5.128) and for $AFG_2$ (0.293; 0.666 and 1.464 μg/kg), by using an aflatoxin-free peanut sample (previously determined with HPLC-FLD). At each fortified level the analysis was performed with six replicates. Repeteability was estimated based on standard deviation and relative standard deviation (coefficient of variation – CV), using the data from the recovery experiment. Within-laboratory reproducibility of the method was performed in the same way using fortified analyte concentration: for $AFB_1$ 2.000 μg/kg, for $AFB_2$ 0.568 μg/kg, for $AFG_1$ 2.068 μg/kg and for $AFG_2$ 0.666 μg/kg. The analysis was also performed with six replicates. Those steps were repeated by different analysts in different days, using the same method, same samples and the same apparatus. The precision of the HPLC-FLD system was achieved with six injections in series of seven working standards.

## 3. RESULTS AND DISCUSSION

Seven-point calibration curves were linear in the proposed concentration range for all four aflatoxins and they had satisfactory coefficient of correlation ($R^2$) in the range of 0.9993-0.9999. The method was appropriate for the tested concentration range, having in mind the European legislation regarding the maximum permitted level of aflatoxins in peanuts (2.0 μg/kg for $AFB_1$

and 4.0 μg/kg for sum of AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ for nuts and processed products thereof, intended for human consumption or use as ingredients in foodstuff).

Limits of detection were in the range of 0.003-0.008 μg/kg for all four aflatoxins and limits of quantification were in the range of 0.009-0.023 μg/kg.

The results obtained from repeatability, which was estimated by relative standard deviation (RSD$_r$), were satisfactory and they are presented in Table 1. The obtained values are in the acceptable range (0.171 – 2.626%) at proposed concentration level for all four aflatoxins.

Tab. 1. – Repeatability, estimated by relative standard deviation (RSDr) and recovery of the method

|  | spiked concentration level (mg/kg) | determined concentration level (mg/kg) | SD$_r$* | RSD$_r$ (%) | recovery (%) |
|---|---|---|---|---|---|
| AFB$_1$ | 8.940 | 7.608 | 0.174 | 2.290 | 92.33 |
| AFB$_2$ | 2.530 | 3.263 | 0.006 | 0.171 | 127.86 |
| AFG$_1$ | 9.240 | 7.344 | 0.154 | 2.098 | 84.37 |
| AFG$_2$ | 2.970 | 1.447 | 0.038 | 2.626 | 48.31 |

n* = 6

The results for the recovery are also given in Table 1. The values for AFB$_1$ and AFG$_1$ were in compliance with the European legislation, and performance criteria for aflatoxins were in line with Commission Regulation 401/2006. For AFB$_1$ the recovery was 92.33% and the recovery for AFG$_1$ was 84.37%. There were some minor deviations concerning the values of AFB$_2$ and AFG$_2$. However, having in mind that AFB$_1$ usually contributes to the total content of aflatoxins and that all EU Directives (Commission Regulation 466/2001, 2174/2003 and 1881/2006) offer a possibility to express the results as AFB$_1$ content or sum of aflatoxins, those deviations are not likely to affect the result significantly.

Table 2 gives results for the within-laboratory reproducibility expressed with standard deviation (SD$_R$) and relative standard deviation (RSD$_R$). RSD$_R$ values calculated from the results showed acceptable correlation between two analysts in all four aflatoxins. For AFB$_1$, RSD$_R$ values were 6.602% for the first analyst and 4.934% for the second analyst, for AFB$_2$ values are 10.041% and 6.865%, for AFG$_1$ values are 8.356% and 6.380% and for AFG$_2$ values are 5.967% and 11.870%.

The stability of the method was tested following the AFB$_1$ concentration by applying the method of 20 toxin-free peanut samples fortified with AFB$_1$ 2.0 μg/kg. The obtained mean concentration was 1.624 μg/kg. Each series of analyzed samples was controlled. The obtained values showed that the method was stable over the tested period, having in mind the Shewhart control chart (Fig. 1).

Tab. 2. – Within-laboratory reproducibility estimated by standard deviation ($SD_R$) and relative standard deviation ($RSD_R$)

| | | I analyst | II analyst |
|---|---|---|---|
| spiked concentration level (mg/kg) | $AFB_1$ | 2.000 | 2.000 |
| | $AFB_2$ | 0.568 | 0.558 |
| | $AFG_1$ | 2.068 | 2.068 |
| | $AFG_2$ | 0.666 | 0.666 |
| determined concentration level (mg/kg) | $AFB_1$ | 1.644 | 1.617 |
| | $AFB_2$ | 0.498 | 0.493 |
| | $AFG_1$ | 1.158 | 1.145 |
| | $AFG_2$ | 0.260 | 0.252 |
| $SD_R$* n* = 6 | $AFB_1$ | 0.109 | 0.080 |
| | $AFB_2$ | 0.050 | 0.034 |
| | $AFG_1$ | 0.097 | 0.070 |
| | $AFG_2$ | 0.015 | 0.030 |
| $RSD_R$ (%) | $AFB_1$ | 6.602 | 4.934 |
| | $AFB_2$ | 10.041 | 6.865 |
| | $AFG_1$ | 8.356 | 6.380 |
| | $AFG_2$ | 5.967 | 11.87 |
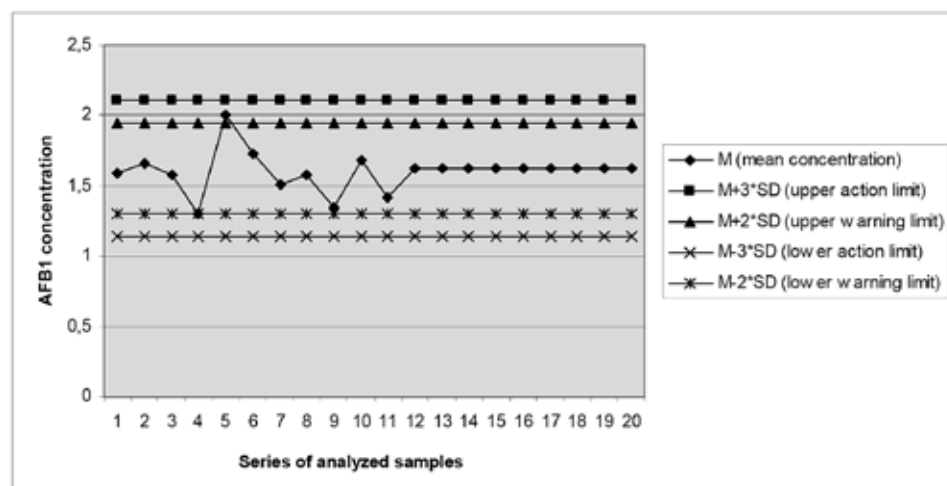


Fig. 1. – Shewhart control chart

The results from 27 analyzed samples are presented in Table 3. As it can be seen from the table, most of the peanut samples are below LOD with exception of one extreme (289.2 μg/kg for total content of aflatoxins). One sample of peanut snack had maximum content of total aflatoxins 16.3 μg/kg, which was over the permitted MRL of 4.0 μg/kg for total aflatoxins. One sample of peanut flips also had content of total aflatoxins over the MRL (8.0 μg/kg).

Tab. 3. – Results from the analysis of total aflatoxins in peanuts and peanut based products

| Sample type | No. of samples | No. of samples below LOD | No. of samples over the MRL | Concentration range for sum of AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ (mg/kg) |
|---|---|---|---|---|
| Peanuts | 13 | 9 | 4 | 1.5-289.2 mg/kg |
| Peanut snacks | 10 | 9 | 1 | 16.3 mg/kg |
| Peanut flips | 2 | 1 | 1 | 8.0 mg/kg |
| Peanut butter | 2 | 2 | – | – |

Chromatograms for three positive samples (a-peanut snack sample, b-peanut flips sample and c-peanut samples) are presented in Figure 2. They are well defined, without matrix effects and practically without impurities and unidentified peaks. This is due to the IAC selectivity and selectivity of the procedure.
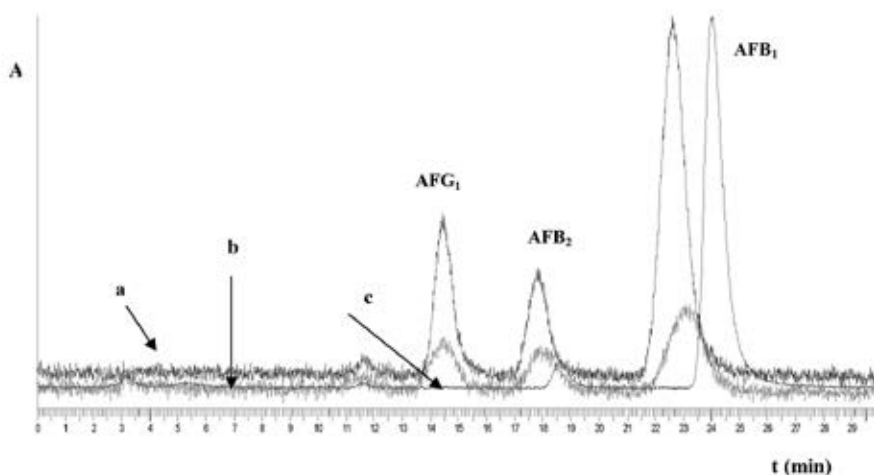


Fig. 2. – Chromatograms of positive (a) peanut snack sample (total concentration of aflatoxins 16.3 µg/kg), (b) peanut flips sample (total concentration of aflatoxins 8.0 µg/kg) and (c) peanut sample (total concentration of aflatoxins 289.2 µg/kg)

# 4. CONCLUSION

This paper presented the results of application of reliable HPLC-FLD method for the analysis of total aflatoxin. The validation procedure confirmed that the proposed method provides satisfactory aflatoxin recoveries (mean value for total aflatoxins was 88.21%), with acceptable precision values in the range of 0.171–2.626% at proposed concentration levels for all four aflatoxins. The method also showed high level of peak selectivity and low values for limit of detection and limit of quantification. The results from 27 analyzed samples showed that 21 samples (77.7%) were below the LOD. Three samples had

positive results over the MRL. One extreme result was obtained for the presence of total aflatoxins in peanut (289.2 µg/kg) and two peanut based products, peanut snack and peanut flips, with total content of aflatoxins being 16.3 µg/kg and 8.0 µg/kg, respectively.

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ОДРЕЂИВАЊЕ ПРИСУСТВА АФЛАТОКСИНА У КИКИРИКИЈУ
И ПРОИЗВОДИМА ОД КИКИРИКИЈА ПРИМЕНОМ ТЕЧНЕ
ХРОМАТОГРАФИЈЕ СА ФЛУОРЕСЦЕНТНИМ ДЕТЕКТОРОМ

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Резиме

У раду је описан метод за одређивање афлатоксина (AFB$_1$, AFB$_2$, AFG$_1$ и AFG$_2$) у кикирикију и производима од кикирикија применом метода течне хроматографије са флуоресцентним детектором уз примену имуноафинитетне колоне за пречишћавање. Такође је приказан и поступак валидације метода. За све испитиване афлатоксине, показан је добар коефицијент корелације који се кретао у опсегу 0.9993–0.9999. Лимит детекције (LOD) и лимит квантификације (LOQ) се кретао у прихватљивим границама од 0.003–0.005 µg/kg и 0.009–0.023 µg/kg, респективно. Средња вредност за рикавери за суму афлатоксина је био 88.21%. Метод је такође показао задовољавајућу прецизност за испитивање концентрација токсина и кретао се у опсегу 0.171–2.626%. Интралабораторијска репродуктивност (RSDr) је показала добру корелацију између два аналитичара за све испитиване афлатоксине и кретала се у границама 4.93–11.87%. Испитиван метод је примењен у одређивању садржаја афлатоксина у 27 узорака кикирикија и производа од кикирикија. Резултати испитивања су показали да је садржај афлатоксина у 21 узорку (77.7%) био испод лимита детекције (LOD) метода. Три узорка су били позитивни на садржај токсина и садржали су их у дози вишој од максимално дозвољених вредности. Постојао је један узорак кикирикија са екстремно високим садржајем афлатоксина (289.2 µg/kg) и два производа на бази кикирикија (кикирики снек) са укупним садржајем афлатоксина од 16.3 µg/kg и 8.0 µg/kg. Резултати потврђују да је приказан метод одговарајући за одређивање афлатоксина у кикирикију и сродним производима и препоручује се његова примена у рутинским анализама.

КЉУЧНЕ РЕЧИ: афлатоксини (AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$), пречишћавање имуноафинитетним колонама, HPLC-FLD, кикирики, производи од кикирикија, валидација

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# VALIDATION OF IMMUNOENZYMATIC TESTS FOR THE DETECTION OF AFLATOXIN PRESENT IN FOOD

ABSTRACT: The significance of this study is reflected in proving the reliability of the ELISA test for the detection of aflatoxin in food and feed. Verification tests were based on the use of reference materials with known concentrations of aflatoxins, contamination of samples by adding known concentrations of analyte and participation in inter-laboratory studies, by different perpetrators and different laboratories. By measuring the known concentrations of analytes it was proved that aflatoxins, in the selected samples, were determined in accordance with the requirements defined for a given method, and it was demonstrated that it is possible to determine the level / concentration of mycotoxins in relation to the MRL defined by the Regulation on amendments to the Ordinance on the maximum allowable residue levels of pesticides in food and feed and feed and feed for which maximum allowed quantities of residues of plant protection products (Službeni Glasnik RS broj 28/2011 član 2. prilog 5. tačka 2).

KEY WORDS: aflatoxins, ELISA test, validation, verification

## INTRODUCTION

Globalization and international trade, as well as the growing nutritional needs of the population in the world, point to the constant need of improving food production in order to ensure adequate protection of public health. Influenced by consumer demands, food safety is still a major focus of the public due to the foodborne diseases. According to the World Health Organization, chemical hazards, such as mycotoxins, fitotoxins (algal toxins) and plant toxins in food are important causes of diseases in humans and animals (WHO, 2002).

In relation to this group of toxins, mycotoxins are the biggest problem in the process of providing safe food chain, especially in the developing countries. Therefore, their presence in food is a subject of constant evaluation of numerous national and international agencies. Mycotoxins are toxic secondary metabolites of a number of saprophytic molds which humans and animals mostly intake through contaminated food infested with spores, conidia and /

or mycelium fragments. Alimentary intake of mycotoxins by humans and animals can cause intoxication.

It is supposed that mycotoxins have always been present in food and the first data on the harmful effects of mycotoxin consumption was recorded in China 5000 years ago. Today, it is well known that, in the past millennium, mycotoxins were the cause of several large-scale poisoning and death of hundreds of thousands of both animals and people in Europe and other continents. The occurrence of unknown disease ("turkey X disease"), which in 1960 led to the death of more than 100,000 turkeys and 20,000 other poultry with signs of acute hepatic necrosis, ppointed to the necessity of determining the causal factors. A year later, a culture of fungi *Aspergillus flavus* was isolated from the imported peanut meal, the component material used for livestock feed. Discovery of aflatoxins which are fluorescent under UV light represents a milestone in the research. As a result of ingestion of aflatoxins different health disorders appear and they are manifested differently with respect to the level, character and intensity, depending on the amount and type of ingested aflatoxin, length of input, the general state of an organism, as well as the age groups of people. The most severe disorder of aflamycotoxicosis is hepatocellular carcinoma.

Changes caused by mycotoxins depend on the type and quantity of mycotoxins in food, as well as the length of input. Diseases caused by mycotoxins are not contagious; they are similar to vitamin deficiencies and they are not treated with antibiotics or other drugs. Poisoning is manifested in the form of primary acute or chronic toxicosis, as well as in the form of secondary toxicosis. The operating mechanism of mycotoxins is very complex, causing functional and structural changes in various tissues, organs and organ systems. Detrimental effects on human and animal health are often cumulative and occur after a long period of introduction and accumulation in the target tissue. Considering a range of differences in their chemical structure, certain mycotoxins exhibit different biological effects that can be manifested as carcinogenic, mutagenic, teratogenic, embryotoxic and immunomodulatory (S i n o v e c et al., 2006).

Detection of mycotoxins is invaluable when it comes to people's health because their detection and elimination of contaminated food can prevent fatal consequences for human health. Therefore, the production conditions must practice a multistage continuous monitoring of hygienic quality of food for a rapid and effective response, as for now this is the only way for a successful prevention of harmful effects of mycotoxins.

National legislation in Serbia prescribes the maximum residual levels (MRLs) of aflatoxin in food and feed. The prescribed values are concentrations ranging from 2-15μg/kg (Službeni Glasnik RS, br.28/2011a). These values are harmonized with the regulations and directives in the European Union (Off. J. EU Communities Commision Regulation, 2002, 2003), which is necessary to ensure a single market with a permanent principle of proportionality. Generally, aflatoxin MRLs are based on a compromise between the protection of consumers and mitigation of limited trade.

This study was based on a validation study and application of immunological methods for the detection of mycotoxins, ELISA test, which is based on a selective antigen-antibody reaction.

# MATERIAL AND METHODS

## *Principle*

Enzyme linked immunosorbent assay-ELISA is a method for detecting and measuring antigens or antibodies. The use of enzymes related to one of the reagents in immunoassays, in which the enzyme acts as a bookmark, can be quantified. Enzymes are usually associated with the antibody and the antibodies that are either specific to the antigen or antibody to Ig. The amount of enzyme in the Ag-Ab complex is determined indirectly by measuring the amount of substrate degradation products appropriate to be included in the final stage of the test. The use of enzymes which substrates give colored product decomposition and the amount of light which can be measured colorimetrically (A r s i c et al., 2000).

## *ELISA type*

ELISA tests may be qualitative or quantitative and they provide highly sensitive and specific antibodies. The choice of ELISA test is not simple. It depends on the required sensitivity and the detection limits of the analyte or the antibody response to the antigen. ELISA tests were used in the validation and verification of detection of aflatoxins in food. Sample preparation is the organic-methanol extraction (Table 9).

Tab. 1. – Materials and ELISA tests

| ELISA kit, chemicals and supplies | | |
|---|---|---|
| Product | Manufacturer/quality | Lot |
| Celer Afla total | Tecna | 01049 |
| Celer Afla B1 | Tecna | 03110 |
| Metanol | Metanol LGC, HPLC pure | SO-9260-BO25 |
| NaCl | Merck | K40124804919 |
| Filter paper | Macherey-Nagel/MN 615 $^1/_4$ • ø240 mm | 1005708 |
| Analitical standard Mycotoxin Mix 1 (Aflatoxin) | Biopure | LO9272B |

*Competitive ELISA*

The essence of competitive ELISA is that it is a combination of an unknown amount of analyte in the sample and the reference analyte compete for binding to a limited number of binding sites on the antibody. This ELISA can be performed either in the form of solid phase absorbed analyte or antibody. In the case of a competitive ELISA added analyte in the sample competes with the reference analyte absorbed by a solid phase for binding to a limited number of labeled antibodies.

**Solutions of reference substances (analytical standard) for verification Concentrations of calibration standards:**
Total aflatoxins: 0 ppb, 2 ppb, 8 ppb, 30 ppb, 80 ppb;
Aflatoxin $B_1$: 0 ppb, 1 ppb, 5 ppb, 20 ppb, 40 ppb.

**Working solutions obtained by adding the standard analytical matrix-**spiking:
Concentrations of total aflatoxin in flour: LOD-2 ppb, $N_1$-5 ppb, $N_2$-10 ppb, $N_3$-20 ppb.

## RESULTS AND DISSCUSION

Validation of the method is the process used to confirm that the applied analytical procedure for a specific test is suitable for its intended use. The terms validation and verification are often used together when evaluating the requirements for validation of the methods. Verification process is less rigorous than validation and it is used in situations when full validation has already been achieved. The results of validation and verification methods are used to assess the quality, reliability and consistency of analytical results, and they are integral parts of good laboratory practice (H e s s, 2010).

The aim of this study was to confirm the required performance characteristics, technical competence and independence by ELISA test (confirmation of validation methods of manufacturer's data) in the detection of aflatoxin and aflatoxin B1. Verification tests are based on the use of reference materials with known concentrations of mycotoxins – CRM (Table 10).

The samples contaminated by adding the specified concentration of analyte participated in inter-laboratory tests on various matrices, by different perpetrators and different laboratories. By measuring the known concentrations of analyte (verification methods) objective evidence of the determination of mycotoxins in the samples selected according to defined requirements for a given method was provided, and it was possible to determine the level / concentration of mycotoxins in relation to the MRL defined in the relevant legislation (Službeni Glasnik, br. 28/2011b).

Tab. 2. – Certified reference materials – CRM

| Parametar | Product | Manufacturer | Concentration | Unit number |
|---|---|---|---|---|
| Total aflatoxins | Peanut butter (kikiriki buter) BCR – 385R | Institute for Reference Materials and Measurements | 3.6 µg/kg (ppb) | 0063 |
| Total aflatoxins | Protein mixture | Trilogy | 9.9 µg/kg (ppb) | TR-A100 A-SF-10 |
| Aflatoksin $B_1$, | MA1140B-1, maize starch | Progetto Trieste, Test Veritas, 2011 Mycotoxin round | 9.67 µg/kg (ppb) | MA1140B-1 |
| Aflatoksin $B_1$, | MA1140B-2, maize starch | Progetto Trieste, Test Veritas, 2011 Mycotoxin round | 2.71 µg/kg | MA1140B-2 |
| Blank corn | Corn | VICAM, USA | 0 ppb (blank) | A-215 |
| Aflatoksin B1 | Maize test material-maize | FAPAS | 9.32 µg/kg | Test material 04192 |

Test parameters are based on the decision of the European Commission EC/657/2002 (Official Journal of the European Communities, 2002 and 2003), AOAC manual (AOAC Offical Methods of Analysis 2012) and the requirements for the performance characteristics given by the manufacturer – Tecna (F r a n c e s c a  D i a n a et al. 2008a).

Requirements for performance characteristics are given by the manufacturer, resulting in the validation study.

Tab. 3. – ELISA Requests for verification

| Parametar | Range | LOD, LOQ | Recovery (%) | Precision (CV%) |
|---|---|---|---|---|
| Total aflatoxins | 2-20 µg/kg | 2 µg/kg | 134±19 | 11.6 ± 3.5 |
| Afla B1 | 1- 40 µg/kg | 1 µg/kg | 124 ± 22 | 20 |

Limit of detection of total aflatoxins is 2 mg / kg for aflatoxin B1 1 mg / kg. Accuracy percentage is 134 ± 19% for total aflatoxins and 124 ± 22 % for aflatoxin B1. In the laboratory it should range from 10-20% (F r a n c e s c a D i a n a et al., 2008b).

**Confirmation of the limit of detection (LoD), limit of quantification (LOQ)**
**Limit of detection (LOD):**
The lowest concentration of analyte which can be detected by a specific analytical method with a given probability (typically 95%). It is the concentration at which the risk of false negative results is low. It is defined as the lowest level of analyte that can be determined by using specific methods required under the circumstances. In other words, the concentration for which the risk of false negative results is low.

**Limit of quantitation (LOQ):**
The lowest concentration of analyte in a sample that can be quantitatively determined with acceptable precision and accuracy. If the analytical result is

below this limit it is reported as less than (<). Another term used is limits and reporting.

### Description of procedure

LOD is determined by simultaneous calibration standards which are integral part of the test kit, and by testing of contaminated samples at level corresponding to LOD in 6 series. Statistical analysis of the results (K o s t i c et al. 2011), provided the data for the LOD.

Tab. 4. – a), b), c) – Results for the LOD for total aflatoxins

| 1.a) | Total aflatoxins µg/kg |
|------|------------------------|
| **n** | **Xi** |
| 1) | 2.11 |
| 2) | 2.21 |
| 3) | 2.25 |
| 4) | 2.05 |
| 5) | 2.11 |
| 6) | 2.29 |

| 1.b) | |
|------|---|
| n | 6 |
| $t_{0.95}$ | 2.015 |

| 1. c) | | | | | | | |
|-------|-----|-----|------|-------|-----|-----|------|
| **Parameter** | **Xsr** | **SD** | **3 SD** | **10 SD** | **Sr** | **CD** | **L$_O$D** |
| **Total aflatoxins** | 2.16 | 0.11 | 0.33 | 1.11 | 0.13 | 0.17 | 0.49 |

### Accuracy (recovery, bias)

The accuracy of the analytical method is the degree of agreement of the results obtained by the tested method and the true value. It is usually expressed as a value recovery (%). Accuracy can be described as a close agreement between the values adopted as law or reference value and the measured value. The real value for the assessment of accuracy can be defined in several ways (CRM, contaminated control sample). Certified reference materials (CRM) samples contain analyte in a given matrix (Table 10). It is calculated as the ratio of individual measurements and the exact value multiplied by 100. The expected recovery depends on the sample matrix, the sample processing procedure and concentration. AOAC manual provides a table of values in the recovery position of analytical concentration (AOAC Offical Methods of Analysis, 2012, Table 7).

Tab. 5. – Relation of units analytes and expected accuracy (recovery)

| **Active content** | **Analytical relationship** | **Unit** | **Mid recovery (%)** |
|--------------------|------------------------------|----------|----------------------|
| 0.001 | $10^{-5}$ | 10 ppm | 80-110 |
| 0.0001 | $10^{-6}$ | 1 ppm | 80-110 |
| 0.00001 | $10^{-7}$ | 100 ppb | 80-110 |
| 0.000001 | $10^{-8}$ | 10 ppb | 60-115 |
| 0.0000001 | $10^{-9}$ | 1 ppb | 40-120 |

**The precision (intra-reproducibility)**

The precision of the method is the degree of agreement between mutually independent test results expressed as standard deviation. Standard deviation is a measure for the value of random variable dispersion around the mean and it is expressed in the units used to express the accident variable. Standardna deviation can be expressed relative to the mean and then called a relative standard deviation RSD, or coefficient of variation CV (M a g n u s s o n et al., 2007).

Accuracy depends on the concentration of the analyte and the dependence should be determined and documented. Eligibility criteria for precision depend on the type of analysis or testing purposes. Accuracy can vary between 2% and more than 20% RSD (AOAC Offical Methods of Analysis, 2012, Table 8).

Tab. 6. – Relation of units analytes and expected precision (CV, RSD)

| Active content | Analytical relationship | Unit | CV (RSD%) |
|---|---|---|---|
| 0.001 | $10^{-5}$ | 10 ppm | 7.3 |
| 0.0001 | $10^{-6}$ | 1 ppm | 11 |
| 0.00001 | $10^{-7}$ | 100 ppb | 15 |
| 0.000001 | $10^{-8}$ | 10 ppb | 21 |
| 0.0000001 | $10^{-9}$ | 1 ppb | 30 |

**Interlaboratory reproducibility**

It expresses the results achieved by participating in inter-laboratory comparison (PT-schemes). Interlaboratory comparative study was defined as an organization run for and evaluation of tests on the same or similar samples, materials, or by two or more different testing laboratories in accordance with predetermined conditions.

Interlaboratory reproducibility precision is required between different laboratories. The aim is to verify that different methods give the same results in different laboratories, by different analysts, different environmental conditions, different equipment, but in terms of methodology specified parameters. The results of PT-scheme are given by z-score. Z-score represents the score of truth for laboratories participating in the measured concentration of the analyte.

Regardless of the number of laboratory tests and the number of repetitions, the critical values are as follows:

Results with Z <| 2 | – fair value

Results from | 2 | <Z <| 3 | – difficult, occurring as a random error

Results with Z> | 3 | – unsatisfactory

The significance of this work is reflected in the formal confirmation of ELISA test competence for the specific test which is necessary for accreditation and recognition of test results by the European Association of accredited laboratories.

### Description of the procedure and results

The experimental work was based on the analysis of spiked samples by adding known amounts of analyte to the sample. By statistical processing of experimental results in ValHemMet (K o s t i c et al. 2011b) the values for:

Sb – standard deviation between series
Sw – standard deviation within a series
Sx – standard deviation of the mean values of a group
S tot -total standard deviation
CV – coefficient of variation

**Truthfulness method** (or system error bias) is defined as a ratio of the value of all Xsr determining the actual value (contaminated samples, CRM); the accuracy is defined as the ratio between the minimum and maximum obtained values and the actual value (contaminated samples, CRM).

### Total aflatoxins

Celer AFLA(Tecna) is immunoenzyme method for the quantitative determination of total aflatoxins in cereals, nuts, dried fruit and feed.

The significance of this test was investigated experimentally in the laboratory for biological and biochemical testing, at the Department of Microbiology of the Enological London Station. The results of six series of measurements in duplicate were compared within the laboratory at different times, by different perpetrators at different matrices.

Tab. 7. – Verification plan for total aflatoxins

| Tested sample/matrix | Concentration | Parameters |
|---|---|---|
| Corn flour – $N_1$ | 5 ppb | precision and accuracy |
| Corn flour – $N_2$ | 10 ppb | precision and accuracy |
| Corn flour – $N_3$ | 20 ppb | precision and accuracy |
| Peanut paste | 3.6 ppb | precision and accuracy |
| Protein mixtures | 9.9 ppb | precision and accuracy |

Statistical processing of the results (K o s t i c et al., 2011c) of contaminated samples at three different levels-5ppb N1, N2-10ppb, 20ppb-N3, gave the data on the accuracy and CV.

Tab. 8. – a), b), c), d) – Results for accuracy, truthfulness and CV, Level N1

| 2. a) | | | |
|---|---|---|---|
| **n** | **I** | **II** | **Xsr** |
| **1)** | 5.7 | 5.5 | 5.6 |
| **2)** | 5.0 | 5.1 | 5.1 |
| **3)** | 5.2 | 5.2 | 5.2 |
| **4)** | 5.4 | 5.5 | 5.4 |
| **5)** | 5.5 | 5.3 | 5.4 |
| **6)** | 5.4 | 5.5 | 5.5 |

| 2. b) | |
|---|---|
| **Sw** | **0.115** |
| Xsr | 5.363 |
| Sx | 0.199 |
| $Sx^2$ | 0.040 |
| $Sb^2$ | 0.033 |
| **Sb** | **0.182** |
| **Stot/Sr** | **0.210** |

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| 2. c) | |
|---|---|
| **n** | 6 |
| **min** | 4.98 |
| **max** | 5.7 |
| **Ref. value** | 5 |

| 2. d) | | | |
|---|---|---|---|
| **Accuracy** | | **Truthf.** | **CV** |
| from % | **100** | **107%** | **4.018%** |
| to % | **114** | | |

Tab. 9. – a), b), c), d) – Results for accuracy, truthfulness and CV, Level N2

| 3. a) | | | |
|---|---|---|---|
| **n** | **I** | **II** | **Xsr** |
| **1)** | 11.4 | 9.8 | 10.57 |
| **2)** | 10.2 | 10.3 | 10.23 |
| **3)** | 10.7 | 12.8 | 11.78 |
| **4)** | 11.4 | 11.8 | 11.58 |
| **5)** | 9.9 | 9.24 | 9.55 |
| **6)** | 11.4 | 11.03 | 11.21 |

| 3. b) | |
|---|---|
| **Sw** | **0.79** |
| Xsr | 10.82 |
| $Sx$ | 0.86 |
| $Sx^2$ | 0.74 |
| $Sb^2$ | 0.42 |
| **Sb** | **0.65** |
| **Stot/Sr** | **1.026** |

| 3. c) | |
|---|---|
| **n** | 6 |
| **min** | 9.24 |
| **max** | 12.82 |
| **Ref. value** | 10 |

| 3. d) | | | |
|---|---|---|---|
| **Accuracy** | | **Truthf.** | **CV** |
| from % | **92** | **108%** | **9.5%** |
| to % | **128** | | |

Tab. 10. – a), b), c), d) – Results for accuracy, truthfulness and CV, Level N3

| 4. a) | | | |
|---|---|---|---|
| **n** | **I** | **II** | **Xsr** |
| **1)** | 21.5 | 20.7 | 21.12 |
| **2)** | 19.8 | 20.04 | 19.9 |
| **3)** | 23.3 | 21.5 | 22.4 |
| **4)** | 21.12 | 20.1 | 20.6 |
| **5)** | 22.7 | 20.56 | 21.65 |
| **6)** | 20.55 | 20.65 | 20.6 |

| 4. b) | |
|---|---|
| **Sw** | **0.86** |
| Xsr | 21.12 |
| $Sx$ | 0.85 |
| $Sx^2$ | 0.73 |
| $Sb^2$ | 0.36 |
| **Sb** | **0.60** |
| **Stot/Sr** | **1.05** |

| 4. c) | |
|---|---|
| **n** | 9 |
| **min** | 19.8 |
| **max** | 21.5 |
| **Ref. value** | 20 |

| 4. d) | | | |
|---|---|---|---|
| **Accuracy** | | **Truthf.** | **CV** |
| from % | **99** | **104%** | **5.2%** |
| to % | **108** | | |

Statistical processing of the results (Kostic et al. 2011d, Sr) samples CRM peanut paste obtained data for accuracy, truthfulness, and CV.

Tab. 11. – a), b), c), d) – Results for accuracy, truthfulness and CV, CRM peanut paste

5. a)

| n | I | II | Xsr |
|---|---|---|---|
| 1) | 3.4 | 3.2 | 3.39 |
| 2) | 3.7 | 3.8 | 3.75 |
| 3) | 3.3 | 3.5 | 3.38 |
| 4) | 3.5 | 4.2 | 3.87 |
| 5) | 3.6 | 4.4 | 4.00 |
| 6) | 3.5 | 3.8 | 3.67 |

5. b)

| Sw | 0.335 |
|---|---|
| Xsr | 3.674 |
| Sx | 0.250 |
| $Sx^2$ | 0.062 |
| $Sb^2$ | 0.006 |
| Sb | 0.080 |
| Stot/Sr | 0.344 |

5. c)

| n | 6 |
|---|---|
| min | 3.20 |
| max | 4.37 |
| Ref. value | 3.6 |

5. d)

| Accuracy | | Truthf. | CV |
|---|---|---|---|
| from % | 87 | | 9.4% |
| to % | 119 | 100% | |

Statistical processing of the results (Kostic et al. 2011e, Sr) samples CRM-protein mixtures obtained the data for accuracy, truthfulness, and CV.

Tab. 12. – a), b), c), d) – The results of the accuracy, truthfulness and CV, CRM-protein mixture

6. a)

| n | I | II | Xsr |
|---|---|---|---|
| 1) | 11.1 | 11.04 | 11.085 |
| 2) | 11.2 | 11.75 | 11.46 |
| 3) | 10.4 | 9.05 | 9.72 |
| 4) | 12.5 | 12.45 | 12.48 |
| 5) | 14.9 | 14.9 | 14.93 |
| 6) | 13.3 | 12.6 | 12.97 |

6. b)

| Sw | 0.48 |
|---|---|
| Xsr | 12.11 |
| Sx | 1.79 |
| $Sx^2$ | 3.21 |
| $Sb^2$ | 3.098 |
| Sb | 1.76 |
| Stot/Sr | 1.82 |

6. c)

| n | 6 |
|---|---|
| min | 9.05 |
| max | 14.99 |
| Ref. value | 9.9 |

6. d)

| Accuracy | | Truthf. | CV |
|---|---|---|---|
| from % | 91 | | 15.1% |
| to % | 151 | 122% | |

Based on the results of testing for accuracy, truthfulness and CV verified by a high level of agreement of mutually independent test results for different

samples with different concentrations of aflatoxins by different perpetrators. The values for accuracy, authenticity, and CV were found within the eligibility criteria set by the manufacturer (D i a n a et al., 2008b) and the AOAC manual (AOAC Offical Methods of Analysis, 2012b). The minimum and maximum contents of aflatoxins obtained by measuring chart are shown graphically (Graphic 1 – Recovery method for Celer Afla). At least deviation contaminated samples, recovery was noted in the third level of concentration, and the highest accuracy in the first level of concentration. In terms of testing the aflatoxin content of CRM, better precision and recovery were recorded for peanut paste.
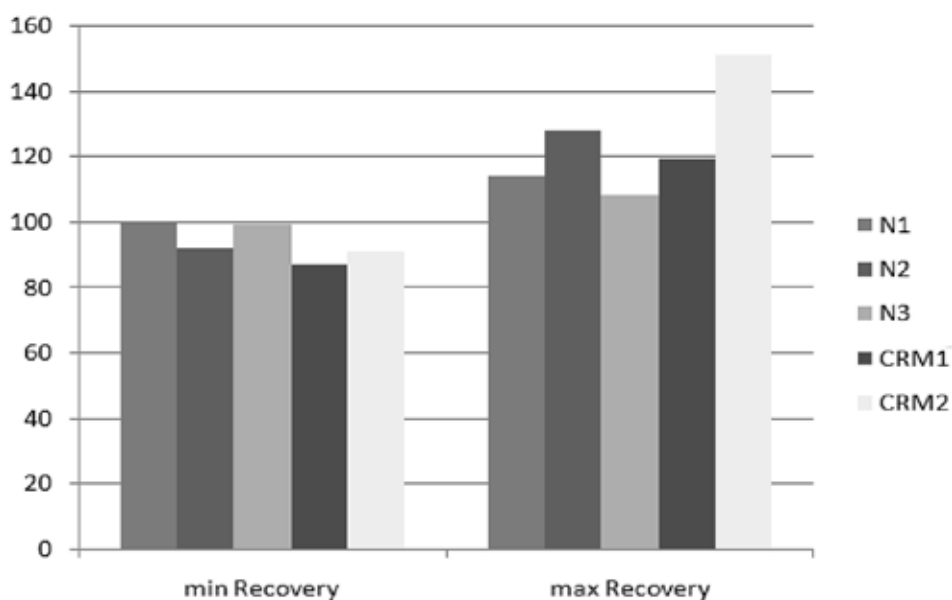


Graph. 1. – Recovery method for Celery Aflalo, contaminated corn samples levels N1, N2, N3, CRM peanut paste (CRM1) and CRM-protein mixture (CRM2)

**Aflatoxin B$_1$**

Quantitative determination of the content of aflatoxin B1, with test Celer AFLA B1 confirmed the results of the proficiency test.

The significance of this test in detecting the presence of mycotoxins was examined by participating in the interlaboratory study (MLI) in June 2011, organized by Progetto Trieste-Test Veritas. Fourteen laboratories were compared, in two concentration levels: 9.67 and 2.71 mg/kg. Z values were found within the limits Z<|2| – fair value (Test Veritas, Progetto Trieste 2011). MLI results are summarized in Table 13.

Tab. 13. – Results and Z-scores for the MLI Progetto Trieste – Test Veritas, June 2011

| MLI Progetto Trieste | Aflatoksin B1 (Celer Afla B$_1$, Tecna) | |
|---|---|---|
| sample | MA-1140-1 | MA-1140-2 |
| unit | **µg/kg** | |
| X-Ref. value | 9.67 | 2.71 |
| x-laboratory | 7.6 | 2 |
| z-score | -0.86 | -1.05 |
| %RSD- confir | 37.66 | 43.79 |
| N-screening | 14 | 13 |
| N-confir. | 27 | |
| %RSD- screening | 33.17 | 45.89 |

The results of an international interlaboratory study confirmed the importance of applying immunoenzymatic tests in the detection of mycotoxins. The content of mycotoxins, in the MLI in a sample of corn 04192, organized by FAPAS, was determined by ELISA in 110 laboratories. Measuring the content of aflatoxin B1 in corn is meant for laboratories that use this type of test methods. Laboratory Enological station is London screening of mycotoxins examined by ELISA assay Celery Afla B1. The results of the examination and treatment involved four people. Test method Celery Afla B1 showed excellent results of concentration levels, z score $<| 2 |$ – fair values. This result confirmed that the ELISA test can safely be used in this area. Results are shown for clarity in Table 14.

Tab. 14. – Results for MLI FAPAS

| MLI FAPAS | Aflatoksin B1 (Celer Afla B$_1$, Tecna) |
|---|---|
| sample | Test material 04192 |
| unit | µg/kg |
| X-Ref. value | 9.32 |
| x-laboratory | 11.35 |
| z-score | 1 |

## CONCLUSIONS

Test results (verification methods, MLI) provided objective evidence that the determination of aflatoxins in selected samples was in accordance with the requirements defined for a given method (Table 15) and that it was possible to determine the level / concentration of aflatoxin in relation to MRL defined in the Regulations (Službeni Glasnik RS, br. 28/2011).

Results of verification and the results of inter-laboratory comparisons showed the significance of immunoenzymatic tests in the detection of mycotoxins. Specific, precise and accurate quantitative detection of aflatoxin, aflatoxin B1 in food was provided. The agreement between the reference values and the values indicated high accuracy of the results.

Tab. 15. – Results for all test parameters and comparison with default values

| Parametar | Results | Range (µg/kg) | LOD /LOQ (µg/kg) | Recovery (%) | Precision (CV%) |
|---|---|---|---|---|---|
| Total aflatoxins | Set value: | 2–20 | 2 | 134 ± 19 | 11.6 ± 3.5 |
| | Obtained value: | Confirmed | 1,1 | 100–114<br>92–128<br>99–108<br>87–119<br>91–151 | 4<br>9.5<br>5.2<br>9.4<br>15 |
| Afla B1 | Set value: | 1–40 | 1 | 124 ± 22 | 20 |
| | Obtained value: | Confirmed | Z = \|0.86–1.05\| | | |

Results of inter-laboratory comparisons showed that the ELISA test is reliable for testing of low and high levels of contamination that are consistent with the laws in our country and in the countries of the European Union. An important characteristic of immunoenzymatic tests is reflected in the speed of sample preparation, simultaneous analysis of numerous samples, simple implementation and a wide measurement range.

However, routine analysis requires periodic use of certified reference materials, checking of each manufacturing lot number of the test, the use of immunoaffinity column clean complex matrix (spice, tea, cocoa) in order to avoid false positive results. If the ELISA test establishes the content of the analyte above the maximum allowable concentration, before issuing a confirmatory method, such result should to be checked for possible cross-reactivity with components of the matrix.

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## ВАЛИДАЦИЈА ИМУНОЕНЗИМСКИХ ТЕСТОВА ЗА ДЕТЕКЦИЈУ АФЛАТОКСИНА ПРИСУТНИХ У ХРАНИ

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Резиме

Значај овог рада се огледа у доказивању да је ELISA тест компетентан за детекцију афлатоксина у храни и храни за животиње. Верификациона испитивања се базирају на употреби референтних материјала познатих концентрација афлатоксина, узорака контаминираних додавањем аналита у познатој концентрацији и учешћем у међулабораторијским испитивањима, од стране различитих извршилаца и различитих лабораторија. Мерењем познатих концентрација аналита доказано је да је одређивање афлатоксина у одабраним узорцима у складу са дефинисаним захтевима за дату методу као и да је могуће одредити ниво/концентрацију микотоксина у односу на МДК дефинисане Правилником о допуни Правилника о максимално дозвољеним количинама остатака средстава за заштиту биља у храни и храни за животиње и о храни и храни за животиње за коју се утврђују максимално дозвољене количине остатака средстава за заштиту биља („Службени Гласник РС" број 28/2011 члан 2. прилог 5. тачка 2.).

КЉУЧНЕ РЕЧИ: афлатоксини, ELISA тест, валидација, верификација

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# ANALYSIS OF AFLATOXINS B1 AND G1 IN MAIZE BY QUECHERS

ABSTRACT: A reliable and easy method has been developed for the determination of aflatoxins B1 and G2 in maize samples. High performance liquid chromatography coupled with FLD (HPLC-FLD) with photochemical derivatization was used. Mycotoxins were extracted from maize using a QuEChERS-based extraction procedure. The optimized analytical conditions were evaluated in terms of recoveries, reproducibility, LOD, LOQ and linearity for aflatoxin B1 and aflatoxin G1 in maize. Extraction, chromatographic and detection conditions were optimized in order to increase sample sensitivity. The linearity was analyzed in the range of 0.4-20 μg/kg and the correlation coefficients ($R^2$) were higher than 0.99 for aflatoxins B1 and G1. Blank samples were spiked at 1.0, 2.0 and 4.0 μg/kg, and the average recovery for aflatoxin G1 was 96.96±1.72% and for aflatoxin B1 it was 86.80±1.24%. RSDs were lower than 25% for both mycotoxins. LOD for both aflatoxins was 0.5 μg/kg and LOQ was 1.0 μg/kg, respectively.
KEY WORDS: aflatoxin B1, aflatoxin G1, QuEChERS, HPLC

## INTRODUCTION

Aflatoxins, a group of chemicals produced by *Aspergillus flavus, Aspergillus parasiticus* and the less common *Aspergillus nomius* (S i r h a n et al., 2011), can be recognized by olive green or grey green color on maize kernels in the field or in storage. Although aflatoxins are not automatically produced whenever grains become moldy, the risk of aflatoxin contamination is greater in damaged, moldy maize than in maize with little mold. Aflatoxins are harmful or fatal to livestock and are considered carcinogenic to humans and animals.

The current mycotoxin extractions have comprised a liquid-liquid extraction (LLE) which requires great amounts of organic solvents. The extraction takes long and depends on the matrix as well as on the studied mycotoxins

while the main disadvantage of LLE is the possibility of mycotoxins loss due to their absorption on the walls of glass containers (T u r n e r et al., 2009); supercritical fluid extraction (SFE) (H u o p a l a h t i et al., 1997; H u o p a l a h t i and J ä r v e n p ä ä, 2000) which greatest advantage is high extraction selectivity from the obtained relatively pure extracts and carbon-dioxide is used as an extraction reagent; solid phase extraction (SPE) which has proved to be an excellent extraction for mycotoxins from various matrices based on the columns filled with silica gel and joint phases in a stationery phase (B u r s i ć et al., 2012); pressure liquid extraction (PLE), matrix solid phase dispersion (MSPD), ultrasound and homogenizing extraction with various mixtures of organic solvents (P a s t o r i n i, 2006) are only some of the techniques in the stream of those which can be applied to the extraction of mycotoxins from various matrices.

After extraction, it was necessary to remove the substances which, by their presence, could hinder the detection and determination of mycotoxins such as lipids, carbo-hydrates or proteins from raw extracts. For the purification of raw extracts, the columns used were those filled with active charcoal and aluminium-oxide, with or without ion exchanger and the columns filled with silica-gel or florisil. In recent years, immuno-affinity columns (MycoSep columns, AlfaOta clean etc.) have been used and QuEChERS has been recognized as the most modern procedure for extraction and extract purification.

In recent years, the trends have been directed towards the decrease in the sample amount for analyses with the approach which is safer and less damaging to the environment, such as QuEChERS, and which, at the same time, implies a quicker and simpler way of sample preparation, ensuring high yields and good precision. A n a s t a i d e s e t al. (2003) developed quick, easy, cheap, efficient, rugged and safe method (QuEChERS) in order to overcome the limitations of the existing preparation methods, which was successfully used in the analyses of pesticides from various matrices. With certain modifications, QuEChERS has become a method of interest for the extraction and purification of samples and for the mycotoxin determination. Only 5-10 g of a sample was taken during the analysis, and the extraction in PTT tubes was performed with water and acidified acetonitril. On addition of anhydrous magnesium sulfate and sodium acetate (or sodium chloride), a sample is centrifuged. Supernatant can immediately be injected into a liquid chromatograph (R o m e r o-G o n z a l e z et al., 2011; G a r r i d o F r e n i c h et al., 2011) or its purification can be done by adding primary-secondary amines (PSA) and anhydrous magnesium sulfate and after the repeated centrifugation, supernatant is analyzed by liquid chromatography (W u et al., 2010).

The aim of this experimental work was the validation of QuEChERS method used for the detection of the content of aflatoxin B1 and aflatoxin G1 in maize.

# MATERIAL AND METHOD

**Chemicals and materials:** Aflatoxin standards were purchased from Sigma Aldrich (Germany). Standard stock solutions were prepared in methanol. Working solutions were prepared by diluting the stock standards with mobile phase to the final mass concentration of 0.45 µg of aflatoxin B1/ml and 1.0 µg of aflatoxin G1/ml. The QuEChERS extraction comprised sodium chloride (J.T. Baker, Holland), trisodium citrate 5.5-hydrate (Zorka pharma, Šabac, Srbija), anhydrous magnesium sulfate (Hemrad, Belgrade, Serbia), anhydrous sodium acetate (Sigma–Aldrich, Germany), methanol, acetonitrile and cc acetic acid – 99.5% (J.T. Baker, Holland).

**HPLC analysis:** The HPLC analyses were carried out with Agilent 1100 system, consisting of a degasser, binary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with the Zorbax EclipsePlus C18 (3.5 µm, 3.6 mm x 150 mm) chromatographic column. The mobile phase was (A) water and (B) MeCN. The gradient conditions were 0-15 min 35% B, 15-18 min 35% B, 18-20 min 60% B and 20-25 min 70% B. The column temperature was 30 °C at the flow rate of 1.5 ml/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 50 µl for both standard and sample solutions.

**Validation parameters:** The linearity was checked by preparing the mycotoxins mixture standard in mass concentrations of 0.4, 1.0, 5.0, 10.0, 15.0 and 20 ng/ml.

The recovery: Untreated samples of maize were enriched with 23.45 and 89 µl working solution of aflatoxin B1 and with 10, 20 and 40 µl of working solution of aflatoxin G1 in three replicates. The final mass concentrations of aflatoxins B1 and G1 in the spiked samples were 1, 2 and 4 µg/kg. After thirty minutes, the QuEChERS extraction was carried out and presented in Scheme 1.

Scheme 1 – Sample preparation (QuEChERS method)

10 g sample + 10 ml 1% $CH_3COOH$ in MeCN + 5 ml $H_2O$
↓
Shake vigorously for 1 min

↓

Add 1.67 g $CH_3COONa$
↓
Shake tube directly after the salt addition for 2 min
↓
Add 4 g $MgSO_4$
↓
Centrifuge for 20 min at 3000 g
↓
Upper layer analyzed by HPLC
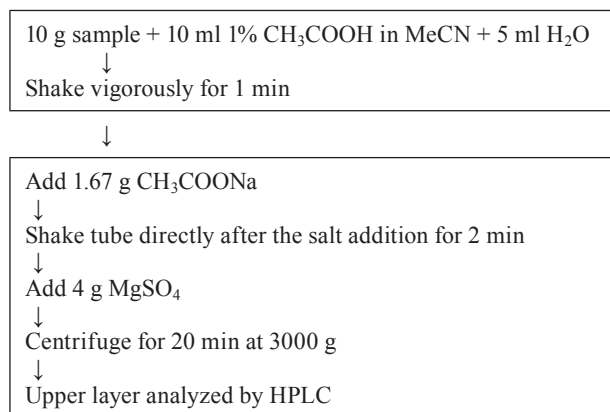
Reproducibility of the method was determined by analyzing the sample of the same concentration level (5.0 μg/kg) in six replications and it was expressed as relative standard deviation – RSD (%).

The limits of detection (LOD) were calculated by means of "Calculate Signal-to-Noise" calculator within the Qualitative MussHunter B.03.01 program (Agilent Technologies, 2010) based on the relation of standard deviation of the peak height and noise height in the chromatograms for the mycotoxin mixture standard at concentration of 0.05 μg/kg.

The limits of quantification (LOQ) were determined by adding 100 μl of mycotoxin mixture standard in the concentration of 1.0 μg/ml.

## RESULTS AND DISCUSSION

Validation parameters: LOD for aflatoxin B1 was 0.16 μg/kg and for aflatoxin G1 it was 0.08 μg/kg. LOQ was 0.4 μg/kg for both mycotoxins. The linearity range for both aflatoxins was from 0.4 to 20.0 μg/kg. Reproducibility of the method expressed as a RSD (%) was 9% for aflatoxin B1 and 27% for aflatoxin G1 and they were in accordance with the Commission Regulation (EC) No 401/2006, which permitted RSD to be up to 30%.

Table 1 shows the recovery values obtained by QuEChERS method.

Tab. 1. – Obtained recovery by QuEChERS

| Aflatoxin concentration in samples (μg/kg) | | Replicates | | | Average of recovery (%) | RSD (%) |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | |
| G1 | 1 | 78.42 | 79.50 | 80.42 | 79.45 | 1.26 |
| | 2 | 80.50 | 81.40 | 82.70 | 81.53 | 1.32 |
| | 4 | 83.40 | 85.20 | 84.70 | 84.43 | 1.10 |
| B1 | 1 | 98.42 | 95.17 | 96.24 | 96.61 | 1.71 |
| | 2 | 95.14 | 92.17 | 96.34 | 94.55 | 2.27 |
| | 4 | 100.66 | 100.10 | 98.42 | 99.73 | 1.17 |

By employing QuEChERS techniques, high extraction recoveries were obtained with average recoveries for aflatoxin G1 being 96.96±1.72% and for aflatoxin B1 being 86.80±1.24%.

Figures 1, 2 and 3 show chromatograms of analyzed samples.
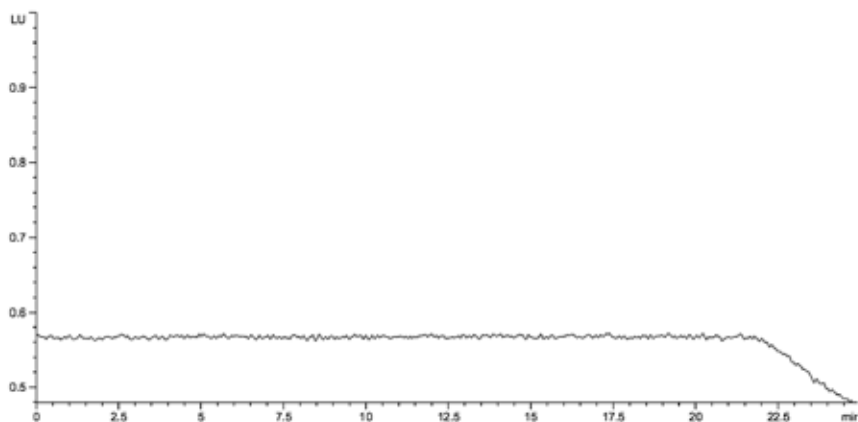
Fig. 1 – Control maize sample
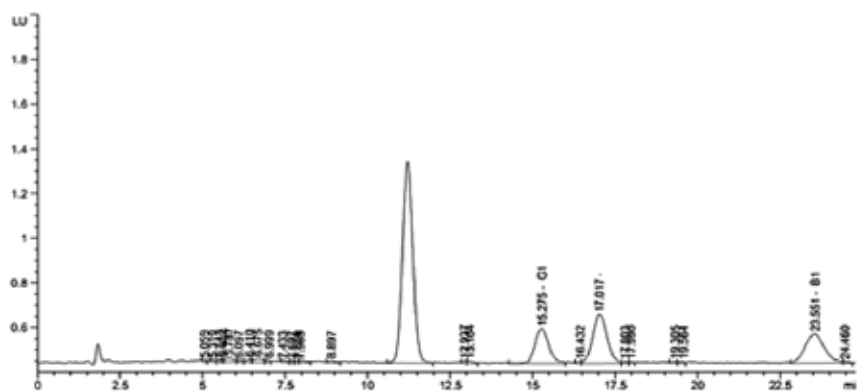


Fig. 2 – Standard of aflatoxins G1 and B1
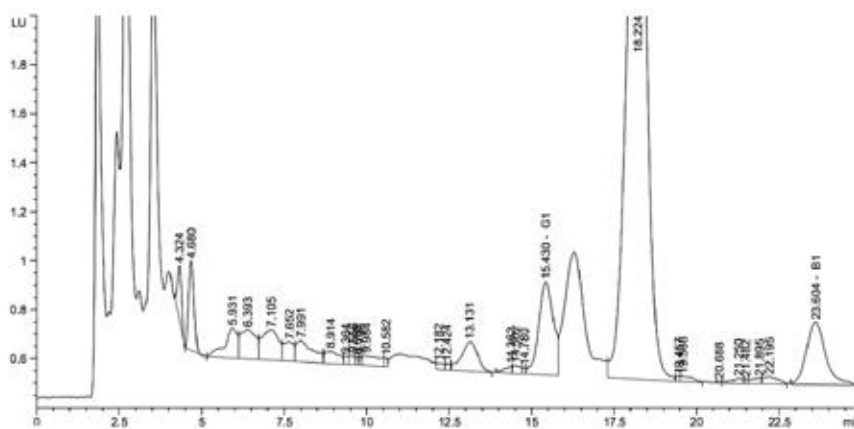


Fig. 3 – Chromagram of spiked samples with aflatoxins B1 and G1

## CONCLUSION

A new method based on QuEChERS extraction procedure and HPLC-FLD was developed for the simultaneous determination of aflatoxin B1 and aflatoxin G1. The extraction procedure, using water/acetonitril acidified with acetic acid, was simple and it required no further clean-up steps. Therefore, it can be recommended as an alternative to the time-consuming extraction as well to the more expensive imunoaffinity columns and multifunction column for aflatoxins determination in maize.

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QUECHERS АНАЛИЗА АФЛАТОКСИНА B1 И G1 У КУКУРУЗУ

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Резиме

Развијен је поуздан и лак метод одређивања афлатоксина B1 и G1 у узорцима кукуруза. Коришћене су високо перформантна течна хроматографија уз FLD (HPLC-FLD) и фотохемијска дериватизација. Микотоксини су екстраховани из кукуруза употребом QuEChERS екстракционе процедуре. Оптимизација аналитичких услова је процењена на нивоу приноса екстракције, репродуктивности, LOD, LOQ и линеарности афлатоксина B1 и афлатоксина G1 у кукурузу. Екстракција, хроматографија и услови одређивања су оптимизовани у циљу повећања осетљивости. Линеарност је одређена за опсег концентрација од 0.4-20 µg/kg са коефицијентом корелације ($R^2$) вишим од 0.99 за афлатоксине B1 и G1. Контролни узорци су обогаћени на нивоу 1.0, 2.0 и 4.0 µg/kg, са просечним вредностима проноса екстракције од 96.96±1.72% за афлатоксин G1 и 86.80±1.24% за афлатоксин B1. RSD вредности су биле ниже од 25% за оба микотоксина. LOD оба афлатоксина је износио 0.5 µg/kgг са LOQ од 1.0 µg/kg.
КЉУЧНЕ РЕЧИ: афлатоксин B1, афлатоксин G1, QuEChERS, HPLC

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# THE INFLUENCE OF DROUGHT ON THE OCCURENCE OF AFLATOXINS IN MAIZE

ABSTRACT: In this study, a total of 78 maize samples harvested during September and October 2012 in Vojvodina were analyzed. Presence of aflatoxins (AFs) was determined by enzyme-linked immunosorbent assay (ELISA) method. Among the 78 analyzed maize samples, even 44 (56.4%) samples were contaminated with AFs. Concentration interval between 1-10 µg/kg, 10-50 µg/kg and 50-80 µg/kg were found in 23.1%, 17.9% and 15.4% of analyzed maize samples, respectively. It was supposed that prolonged drought during spring and summer of 2012 had a great influence on high contamination frequency and concentration of AFs.

KEY WORDS: Aflatoxins, maize, drought, ELISA

## INTRODUCTION

Aflatoxins (AFs) are secondary metabolites produced mainly by three species of *Aspergillus* including *A. flavus*, *A. parasiticus* and *A. nomius*. The most known AFs which can be found as contaminants of food and feed are $B_1$, $B_2$, $G_1$ and $G_2$ and their two metabolic products M1 and M2 (D e c a s t e l l i et al., 2007). AFs are most common in granular food, including cereals, grains and groundnuts. Milk, eggs, meat and their products can be contaminated if animal consume contaminated feed. However, the commodities with the highest risk of AFs contamination are corn, peanuts, and cottonseed (E l l i s et al., 1991).

The occurrence of AFs is influenced by certain environmental factors and they are especially found in tropical and subtropical regions where temperature and humidity are optimum for growth of molds and for the production of toxins (R u s t o m, 1997). The incidence of AFs and their amount in contaminated products especially depend on the conditions of temperature and humidity during crop growth and storage. Furthermore, the extent of AFs contamination will vary with geographic location, agricultural and agronomic

practices, and the susceptibility of commodities to fungal invasion during pre harvest, storage, and/or processing periods (G a r r i d o et al., 2012).

AFs have been associated with various diseases, such as aflatoxicosis, in livestock and humans throughout the world and they have received great attention because of their potential high toxic, mutagenic, teratogenic and carcinogenic effect on humans and animals. The International Agency for Research on Cancer classified AFs as primary carcinogenic compounds (IARC, 2012).

According to the literature data, *Aspergillus* species and their metabolites AFs are one of the most known contaminants of maize and maize products (B a n k o l e et al., 2003). Maize is one of the most important crops cultivated in Serbia with approximate annual production of six million tons. The weather conditions and drought in spring and summer of 2012 significantly contributed to the reduction in maize production (M a s l a c, 2012). However, it should be noted that there is still a lack of data regarding the occurrence of *Aspegillus* toxins in maize from Serbia.

Many countries, including Serbia, have introduced regulations which stipulate maximum allowed limits of AFs in maize intended for food and feed production. The Serbian regulations (Sl. Glasnik RS 4/2010; Sl. Glasnik RS, 28/2011;) on the control of mycotoxins in food and feed were adopted and complied with the European Union regulation (2002/32/EC, 2010/165/EC) in 2010 and 2011, respectively.

Due to the significant health risks associated with the presence of AFs in food and feed, it is important to establish a data collection on the occurrence of these toxins in Serbian commodities. There are no enough data regarding the occurrence of AFs in maize from Serbia. Hence, the purpose of this work was to screen the presence of AFs in maize harvested in Vojvodina, Northern Province of Serbia, during 2012.


MATERIALS AND METHOD

*Samples*

A total of 78 maize samples were collected in Vojvodina. Samples were collected after harvest, during September and October 2012. All samples were stored at the temperature of 4 °C in refrigerator before analysis.


*Determination of AFs*

Content of AFs ($B_1$, $B_2$, $G_1$ i $G_2$) was determined by the enzyme immu-nosorbent assay method (ELISA). All samples were analyzed in duplicate with Quantitative Aflatoxin High Sensitivity (HS) test kit (Neogen Veratox®, Lansing, USA). Range of quantitation for AFs HS test kit was between 1 and 8 μg/kg and the analyses were done according to the manufacturer´s description. The analyzed samples which contained AFs more than 8 μg/kg were analyzed

with Aflatoxins Quantitative test kits (standards: 0, 5, 10, 50 μg/kg). Samples with content of AFs more than 50 μg/kg were analyzed again after dilution.


RESULTS AND DISCUSION

In this study, 78 maize samples were analyzed in order to evaluate the AFs contamination. The obtained results (Table 1) showed that 44 (56.4%) of analyzed maize samples were positive. AFs concentration in the positive samples ranged from 1.20 to 80.0 μg/kg. Positive results were classified into three groups. Concentration interval from 1 to 10 μg/kg of AFs were found in 18 (23.1%) of analyzed maize samples, while concentration interval 10-50 μg/kg and 50-80 μg/kg were found in 14 (17.9%) and 12 (15.4%) samples, respectively. Mean level of aflatoxin concentration for all positive samples was 27.0 μg/kg. The obtained results show that negative samples (34) and samples with AFs concentration less than 10 μg/kg (18) can be used for human consumption. Maximum allowed limit for AFs in maize intended for human consumption was stipulated by Serbian (Sl. List 28/2011) and the European Union (2010/165/EC) regulation. Both regulations prescribe 10 μg/kg as the maximum allowed level for sum of AFs ($B_1$, $B_2$, $G_1$ and $G_2$). Fourteen samples of maize were found to be contaminated with AFs in range of concentration from 10 to 50 μg/kg, and those samples can be used as feed by Serbian (Sl. List 4/2010) regulation. This regulation set maximum level of 50 μg/kg for AFs in maize intended for animal feed. The regulation of the European Union (2002/32/EC) prescribed 20 μg/kg as the maximum allowed limit only for $AFB_1$ in maize intended for animal feed.

Tab. 1. – Contamination frequency (CF), interval (CI) and mean level (CM±SD) of AFs

|  | 1–10 μg/kg | 10–50 μg/kg | 50–80 μg/kg |
|---|---|---|---|
| CF | 23.1 | 17.9 | 15.4 |
| CI | 1.20–9.97 | 10.2–43.2 | 51.2–80.0 |
| CM | 4.87 ± 2.99 | 18.9 ± 12.0 | 68.1 ± 12.4 |

CF(%), CI (μg/kg), CM (μg/kg) and SD (standard deviation)


The results from Table 1 show that AFs were found in 12 (15.4%) samples with concentration higher than 50 μg/kg. Those samples with high AFs contamination cannot be used for human and animal consumption. After mixing with non-contaminated maize, those samples can be used in animal diet, with the use of an appropriate adsorbent.

The obtained results also show that weather conditions in spring and summer of 2012 had a very strong influence on the production of AFs in the examined maize samples. According to the few previously published literature data, *Aspergillus* species and AFs are rarely found in Serbia (B o č a r o v -

- S t a n č i ć, et al., 2001). Furthermore, our previous results showed that none of the analyzed maize sample from Serbia was contaminated with AFs (M a t i ć et al, 2009; M a t i ć et al., 2010).

Maize planting in Serbia usually starts in the middle of April and lasts until middle of May, and farmers harvest the crops during October and November. Due to unusually high temperatures in August and September 2012, harvest was finished earlier, in the second part of September. Considering the fact that high contamination frequency of AFs was found (56.4%) in maize harvested during 2012, weather conditions for that year were analyzed (Figure 1, Figure 2).

Figure 1 shows the average amount of precipitation in Vojvodina for the period from April to September 2012 compared to the average values for the long-term period of 1971–2000 (Republic Hydrometeorogical Service of Serbia, 2012). The figure shows that lack of rainfall characterized the period from April to September. Extremely low amount of rainfall was recorded in the period of four months (June-September), and during that period, the amount of rainfall was lower by 66, 78, 93 and 60% than average rainfall for multiannual period (1971-2000), respectively. For the period of four months, precipitation covered more than 50% of Vojvodina for only 11 days, while during August, there was only 4 mm of rain in the entire region.
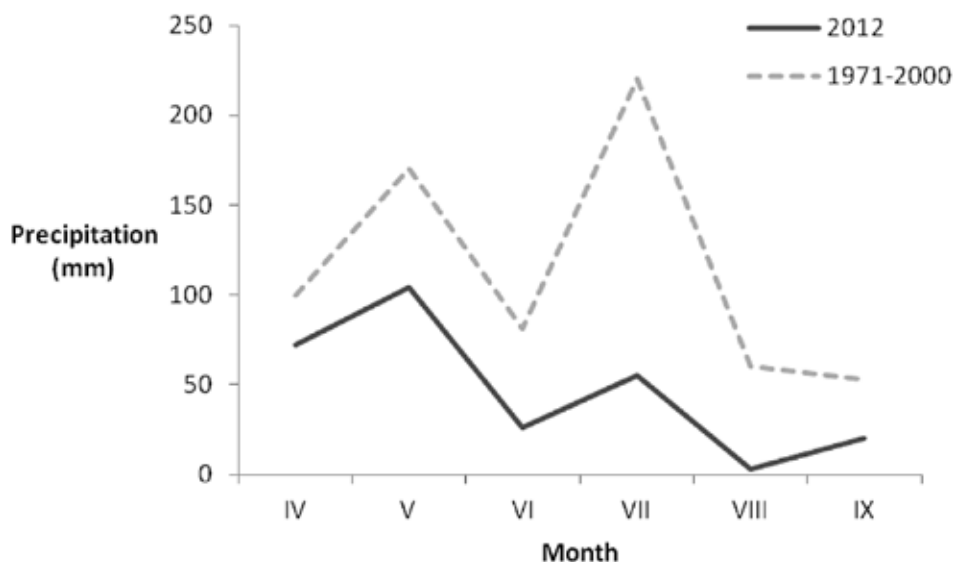


Fig. 1. – Average amount of precipitation in Vojvodina for the period April-September 2012 compared to the average values for the long-term period of 1971–2000

Despite the lack of precipitation, period from June to September was characterized with high temperatures. Monthly average temperature was higher

than the average value, from 1.2 °C (May) to 4.1 °C (July), (Figure 2). The period of four months from June to September was very warm with average monthly temperature in the interval from 20.2 to 25.2 °C and very often daily temperature was near 40 °C. This phenomenon resulted in the favorable conditions for mold growth and emergence of various pests and damage to corn. The lack of moisture and high temperature in these months caused considerable damage to corn.
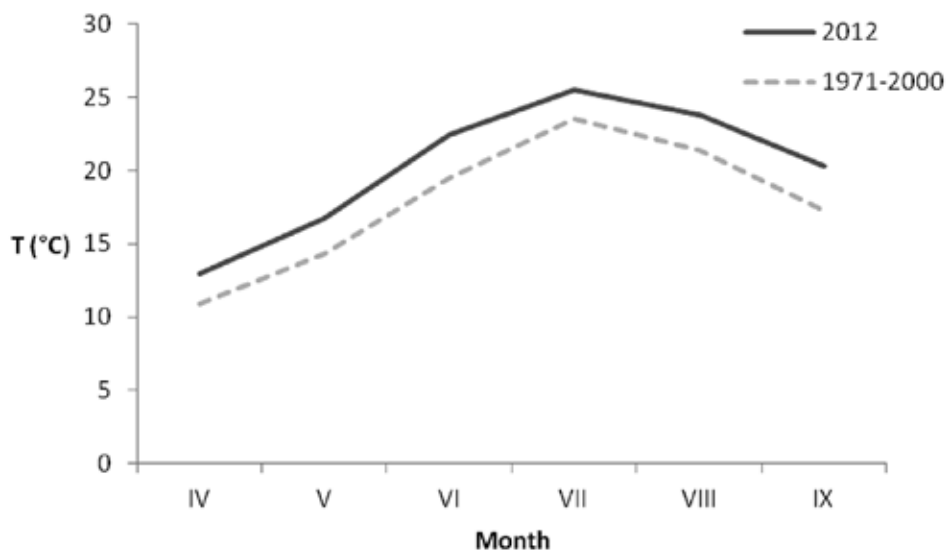


Fig. 2. – Average temperature in Vojvodina for the period April-September 2012 compared to the average values for the long-term period of 1971–2000

According to the figures, it can be concluded that hot and dry weather with prolonged drought characterized spring and summer of 2012 and it was assumed that these weather conditions caused higher presence of AFs. Previous reports have confirmed that if the maize growth and development is followed by hot and dry weather conditions, it can be contaminated with *Aspergillus flavus* with increased probability for AFs synthesis (S t a c k et al., 2003). These authors reported that prolonged drought and high temperatures during the growing season favored the development of *A. flavus*, restricted the development of competitors of *A. flavus*, and inhibited normal pollination in the corn plant. This often leads to an increase in the extent and amount of AFs contamination. Furthermore, K a r a m i-O s b o o et al. (2012) analyzed the presence of $AFB_1$ in maize harvested for three years and concluded that weather conditions, primarily low rainfall and drought, had a great influence on the presence of $AFB_1$.

The obtained results for maize harvested in 2012 confirmed that this crop should be continuously controlled in order to protect the population against the unacceptable risk of AFs contamination.

Prevention and control of AFs in food and feed is required, considering the fact that AFs have toxic effect on humans and animals and for the economic reasons as well.

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# УТИЦАЈ СУШЕ НА ПОЈАВУ АФЛАТОКСИНА У КУКУРУЗУ

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Резиме

У току истраживања анализирани су узорци кукуруза који су сакупљани на територији Војводине непосредно након бербе 2012. године. Имуноафинитетном методом (ELISA) анализирано је присуство афлатоксина ($B_1$, $B_2$, $G_1$ и $G_2$) у 78 узорака кукуруза. Од укупног броја анализираних узорака кукуруза у 34 (43,6%) није детектовано присуство афлатоксина, док је чак 44 (56,4%) узорка било позитивно на присуство овог токсина. У 18 испитаних (23,1%) узорака концентрација афлатоксина је била између 1 и 10 µg/kg, у 14 (17,9%) између 10 и 50 µg/kg, а чак у 12 (15,4%) узорака концентрација афлатоксина је била изнад 50 µг/кг. Овако висока учесталост и концентрација афлатоксина у узорцима кукуруза највероватније је последица екстремних климатских услова током вегетационог периода 2012. године. Сезону развоја и сазревања кукуруза у периоду од априла до октобра 2012. године обележиле су изузетно високе просечне температуре ваздуха уз ретку појаву кише. Топло време и суша на територији Војводине допринели су и развоју *Aspergillus* врста и синтези афлатоксина. С обзиром на добијене резултате и изузетну токсичност афлатоксина на организам људи и животиња, неопходно је спроводити појачану контролу рода кукуруза из 2012. године.
КЉУЧНЕ РЕЧИ: афлатоксин, кукуруз, суша, ELISA

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# REDUCING THE LEVEL OF MYCOTOXINS IN CORN BY REMOVAL OF FINES

ABSTRACT: Mycotoxins are highly toxic compounds produced by molds that commonly occur in cereal grains. These toxins can have adverse effects on human and animal health. Mycotoxin decontamination procedures can be divided into three different groups: chemical, biological and physical. Procedures from the first two groups are often regarded as unacceptable for lowering the mycotoxin level. A laboratory brush was developed for the purpose of physical cleaning of corn kernel. Three samples of commercially available corn kernels were subjected to the brushing procedure in order for the experimental study to be conducted. The mass of 100g of corn kernels was placed on motionless screen of the brush. A rotating part of the experimental device, the polypropylene bristle brush was set to higher speed (higher than 800 rpm). During the corn brushing, dust and broken kernels were brushed out through the motionless screen. Corn samples were taken before and after the brushing procedure and they were analyzed for aflatoxin concentration by HPLC-UV RED. By comparing the control and brushed samples, it can be noticed that removal of fines caused the reduction in the level of mycotoxins in all three brushed samples.

KEY WORDS: mycotoxins, corn, brushing

## INTRODUCTION

Mycotoxins are highly toxic compounds produced by molds that commonly occur in cereal grains. They are produced by molds (fungi) under field conditions and harvest, or during storage (M a r g u a r d t , 1996; S t o j a n o v i ć et al., 2005). Mycotoxins can have adverse effects on human and animal health by causing toxic response when ingested. Intake of contaminated material potentially induces acute and chronic diseases, which may result in teratogenic, carcinogenic, and immune-suppressive effects (W u , 2004; P e s t k a , 2007, J a j i ć et. al., 2008).

Mycotoxins are thermostable molecules which can be totally destroyed only at very high temperatures, which have deteriorating effects on nutritive value of contaminated material. Therefore, value parameters (such as temperature and pressure) in standard procedures applied to food and feed processing are not normally as high as it is required for total decontamination of material. However, many of those processes affect the reduction in mycotoxins (Scot, 1984; Erdington et al., 1997, Bullerman and Bianchini, 2007).

With respect to the applied processes, mycotoxin decontamination procedures can be divided into three main groups: chemical, biological, and physical. Chemical substances used for decontamination of material are acids, alkali, oxidizing reagents, reducing agents, and chlorinating agents. These substances can cause mycotoxin content to be reduced by up to 99%. Although they are very effective, chemical treatments are not widely used due to practical problems: they are expensive and time consuming; they can change palatability and nutritive value of material; they decrease material quality, and can induce the formation of toxic by-products (Hameed, 1993, Avantaggiato, 2012).

Biological detoxification is also used for decreasing of mycotoxin content in material. The main action of these agents is biotransformation and/or biodegradation of mycotoxins by microorganisms or enzymes, which results in production of non-toxic metabolites. After mixing with contaminated material, these bio-adsorbents must be activated in gastrointestinal tract of the animal. Therefore, the effects of their addition could not be noticed before the intake of material (Dänicke, 2004, Avantaggiato, 2012).

Physical procedures include screening, sorting, removal of infested grains, γ irradiation, thermal treatments, adsorbing, etc. Most of the food and feed production processes can be considered as physical. By milling and fractionation of milled material, mycotoxin contamination may be redistributed and concentrated in certain milling fractions. However, this procedure could not totally remove mycotoxins (Abbas et al., 1985, Scudamore et al., 2003). Thermal processing can also be used for decontamination. However, there is a difference in effects on mycotoxin content depending on the specific process. For example, cooking, baking or roasting have inappreciable effects on toxin content. On the other hand, extrusion cooking process, apart from high temperature, includes high pressures and shear forces. Under these conditions, mycotoxin content can be reduced. Although extrusion process can be effective, there are high investment and operational costs for the implementation of this process (Ryu et al., 2003; Scudamore et al., 2004).

The aim of this experimental work was to investigate the influence that removal of fines by brushing process can have on the reduction of aflatoxin level in corn.

## MATERIAL AND METHOD

Three samples of commercially available corn kernels were subjected to a brushing procedure for the purposes of this experimental study. A laboratory brush was developed for the purpose of physical cleaning of the kernels. The mass of approximately 100g of corn kernels was placed on motionless screen of the brush. A rotating part of the experimental device, the polypropylene bristle brush was set to higher speed (higher than 800 rpm). During the corn brushing, dust and broken kernels were brushed out through the motionless screen.

Corn samples were taken before and after the brushing procedure and they were analyzed for aflatoxin level by HPLC-UV RED. The basic solution containing AFLATOXINS LC Tech P/N 10837 (aflatoxin B1 2.02 µg/ml and aflatoxin B2 0.5 µg/ml) of acetonitrile was used for the determination of aflatoxin content. The basic standard (1 ml) was diluted to 50 ml with mobile phase. Samples were homogenized and part of it was extracted with methanol/water mixture. Afterwards, the extract was filtrated and an aliquot was diluted in PBS. Immuno-affinity column (AlfaCLEAN, 3ml widebore) was used for the purification of prepared samples. Column was washed out with distilled water and air stream dried. Elution was done in methanol; eluate was dried in the nitrogen stream and reconstructed with mobile phase. The prepared samples were analyzed with HPLC/FLD. The HPLC analyses ware carried out on 1100 Agilent system with Zorbax EclipsePlus C18 (150x4.6 mm, 3.5 µm) column. The mobile phase used was: (A) water and (B) acetonitrile in gradient mode, with the flow rate of 1.54 ml/min. The injection volume was 50 µl. The detection limit for aflatoxin B2 was 0.05 ng/ml, while for aflatoxin B1 it was 0.2 ng/ml. The quantification limit for aflatoxin B2 was 0.1 ng/ml, while for aflatoxin B1 it was 0.4 ng/ml. The average recovery rate (n=3) for aflatoxin B1 was 86.3% and for aflatoxin B2 it was 83.4%. The repeatability was in accordance with the European Commission (EC) regulation No. 401/2006, with the RSD less than 30%. The triness for aflatoxin B1 was -19% and for aflatoxin B2 it was -8%, which was also in accordance with the EC decision 2002/657/EC.

STATISTICA software version 9 (Statsoft, Tulsa, OK, USA) was used for Tukey's HSD comparison of means of samples. Single-factor ANOVA calculations were used for comparing samples before and after the brushing procedure. Differences among means with probability value of $p \leq 0.05$ were accepted as statistically significant differences, and differences among means with the value of $0.05 \leq p \leq 0.10$ were accepted as tendencies to differences.

## RESULTS AND DISCUSSION

Figure 1 shows the concentration of aflatoxin B1 in unbrushed and brushed samples. Logarithmic distribution is used for expressing the values of aflatoxin B1 concentration (µg/kg). Concentration of aflatoxin B1 in all samples was very high before physical cleaning process, and it ranged from 199.7
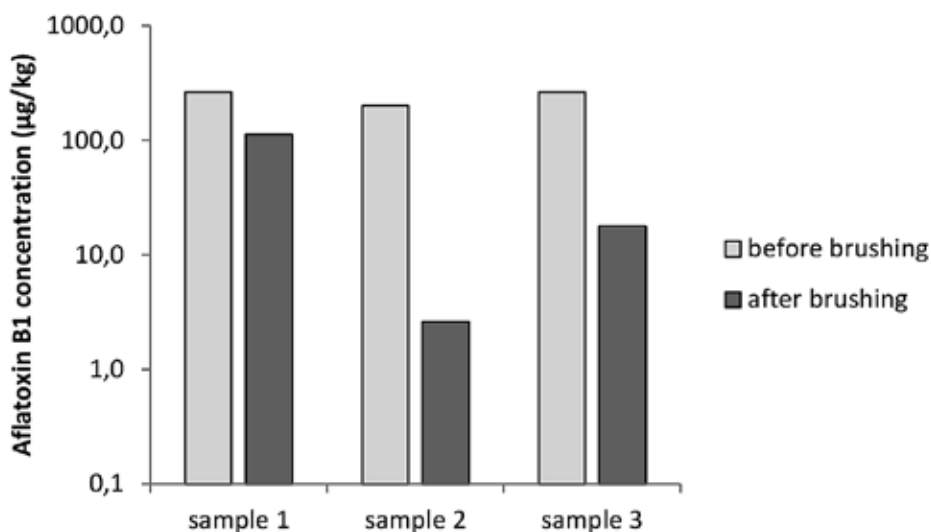
Fig. 1 – Concentration of aflatoxin B1 in unbrushed and brushed samples.
The values are presented as mean values, n = 3. All treated samples have significantly ($p \leq 0.05$) lower aflatoxin content.
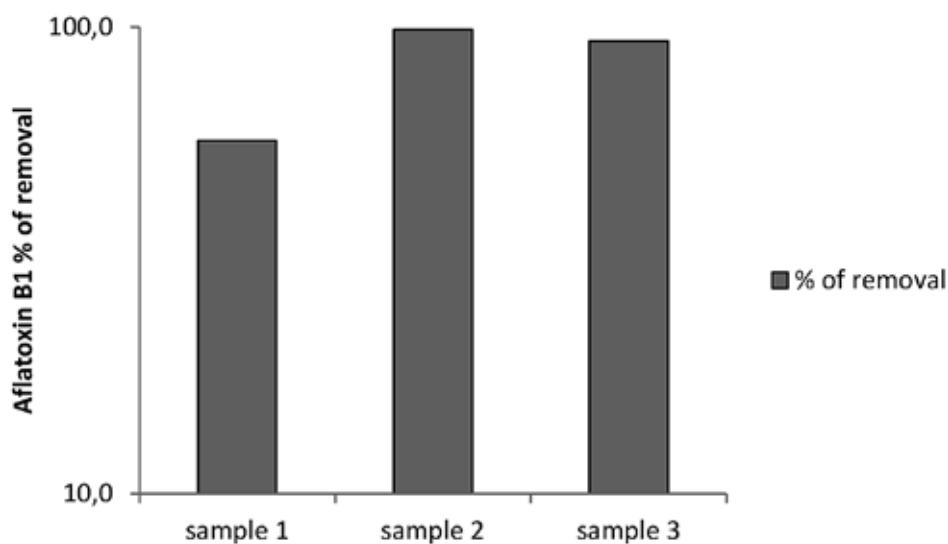


Fig. 2 – Percentage of removal of aflatoxin B1 in brushing process.

to 263.5 μg/kg. The application of brushing process had significant influence ($p < 0.05$) on reducing the content of aflatoxin B1 in all three samples. The use of brushes for corn kernel surface cleaning induced the reduction of the specified mycotoxin content from 57.0 to 98.7% (Fig. 2).

70
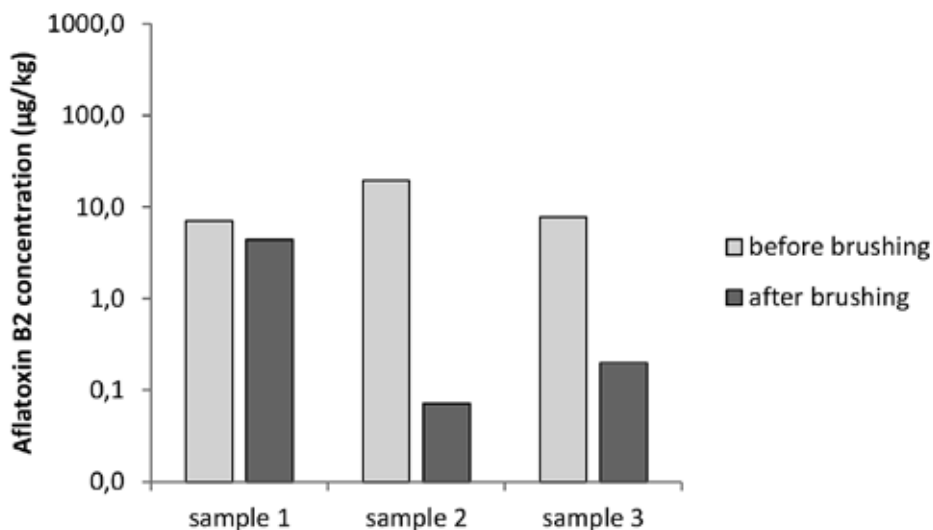
Fig. 3 – Concentration of aflatoxin B2 in unbrushed and brushed samples.
The values are presented as mean, n = 3. All treated samples have significantly (p≤0.05) lower aflatoxin content.

Figure 3 shows the concentration of aflatoxin B2 in unbrushed and brushed samples. It can be seen that for the sample 1 concentration of aflatoxin B2 content was reduced from 7.0 to 4.4 μg/kg. By expressing the results in percentages, aflatoxin B2 was removed from the sample 1 by 36 % (Fig. 2). Aflatoxin B2 content in samples 2 and 3 was reduced from 19.4 μg/kg (sample 2) and 7.8 μg/kg (sample 3) to 0.1 and 0.2 μg/kg, respectively, which was lower by 99.6 (sample 2) and 97.4% (sample 3).

P a r k and L i a n g, 1993, reported the results obtained from the removal of aflatoxin from peanut by applying physical procedures, such as cleaning and segregation. They applied six different procedures for decontamination. Cumulative reduction, achieved by all procedures, was 99.3 %. Removal of aflatoxin per procedure is presented in Table 1.

Tab. 1 – Physical cleaning of infected peanut (P a r k  and  L i a n g , 1993)

| Technology | Aflatoxin concentration (μg/kg) | Reduction (%) | Cumulative reduction (%) |
|---|---|---|---|
| Farmers' stock | 217 | – | – |
| Belt separator | 140 | 35 | 35 |
| Shelling plans | 100 | 29 | 54 |
| Color sorting | 30 | 70 | 86 |
| Gravity table | 25 | 16 | 88 |
| Blanching/color sorting | 2.2 | 91 | 99.0 |
| Re-color sorting | 1.6 | 27 | 99.3 |
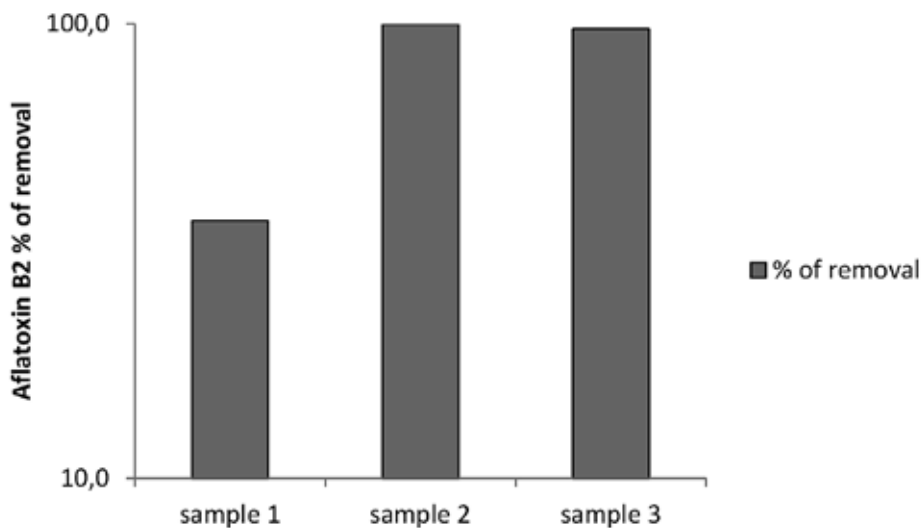
Fig. 4 – Percentage of removal of aflatoxin B1 in brushing process.

Although high reduction results were obtained, several unit operations were combined in this process and they required high investment costs for equipment purchasing.

P a r k, 2002, used physical cleaning for the reduction of aflatoxin content in corn. By removing kernels with extensive mold growth and cleaning of corn kernels, 40% to 80% of reduction was achieved. This author also used dry milling process for fractionation of alatoxin B1 content. The highest levels of mycotoxin were found in germ and hull fractions. Grits, low-fat meal and low fat flour contained only 6-10% of aflatoxin. However, this type of operation can be used only for the concentration of toxin in separate fractions, and not for removal from overall mass.

C o n w a y et al., 1978, reduced aflatoxin content in corn by microwave roasting. In comparison with physical procedures, microwave roasting requires high energy consumption.

A v a n t a g g i a t o, 2012, reported that screening/cleaning process could achieve significant reduction of aflatoxin content (by 30-40%). The same author reported that corn dehulling could cause the reduction of aflatoxin content by up to 93%, and with density segregation in floating water and/or saturated NaCl solution, aflatoxin could be reduced by up to 70%.

When comparing the experimental results with the data from literature, it can be noticed that brushing process used in this study appeared to be very effective in aflatoxin reduction. When it is applied to remove mycotoxin, this procedure does not alter physical or chemical status of the kernel, as it is the case with most procedures applied to lowering of mycotoxins. Additionally, comparing with other processes used for decontamination of material, investment and operational costs for utilization of brushing process are considerably low.

## CONCLUSION

Results obtained from this study showed that application of brushing process to mycotoxin removal from corn kernel caused significant (p < 0.05) reduction of aflatoxin content. The brushing process reduced aflatoxin B1 content in the range from 57.0 to 98.7%, while aflatoxin B2 was reduced from 36% to 99.6%, depending on the sample. This is above the values reported in the literature for most of the other decontamination procedures. By applying brushing process, physical and chemical characteristics of corn kernel remained unchanged, in contrast to many other processes used for mycotoxin removal.

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# СНИЖАВАЊЕ НИВОА МИКОТОКСИНА У КУКУРУЗУ УКЛАЊАЊЕМ ЧЕСТИЦА ПРАШИНЕ

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Резиме

Микотоксини су веома токсична једињења која продукују плесни, а најчешће се појављују у житарицама. Ови токсини могу негативно утицати на људско и животињско здравље. Поступци за деконтаминацију материјала заражених микотоксинима могу се поделити у три групе: хемијски, биолошки и физички. Хемијски и биолошки поступци често нису прихватљиви за снижавање нивоа микотоксина у сировинама и храни. За потребе физичког чишћења кукурузног зрна развијена је лабораторијска четкалица. У експерименталном раду узета су три комерцијално доступна узорка кукуруза у зрну, која су подвргнута поступку четкања. Маса од око 100 грама зрна кукуруза је стављена на непокретну перфорирану површину. Ротирајући део експерименталног уређаја, полипропиленска четка је постављена на велик број обртаја (већи од 800 обртаја у минути). Током четкања зрна кукуруза прашина и сломљена зрна су одстрањени кроз отворе на перфорираној површини. Пре и после четкања узети су узорци зрна кукуруза, који су анализирани на садржај афлатоксина поступком високопритисне течне хроматографије (HPLC-UV RED). Упоређивањем контролних и четканих узорака може се приметити да је уклањање прашине изазвало снижавање микотоксина код сва три третирана узорка.

КЉУЧНЕ РЕЧИ: микотоксини, кукуруз, чишћење

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# INTERLABORATORY COMPARISON FOR DETERMINATION OF OCHRATOXIN A BY ELISA IN MAIZE
## (Running title: DETERMINATION OF OCHRATOXIN A IN MAIZE)

ABSTRACT: Participation in interlaboratory comparison and proficiency testing schemes is important for laboratories to control the work quality. In this study, a sample of naturally contaminated maize was analyzed for the content of ochratoxin A (OTA) in three laboratories in Serbia. Participating laboratories used enzymatic immunoaffinity method (ELISA) for the determination of OTA and selection of the ELISA kit was free. Between-laboratory precision was acceptable as evidenced by Cochran's C test. Moreover, z-scores for all three laboratories were $z < \pm 2$, which is considered acceptable. Used OTA confirmation methods were thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC), with fluorescence detector. The results of different methods were comparable.

KEY WORDS: confirmation methods, ELISA, interlaboratory comparison, maize, ochratoxin A

## INTRODUCTION

The primary aim of proficiency testing (PT) was to provide quality assurance for laboratories and demonstration of competence to an accreditation body by comparing their results with similar laboratories (I S O / I E C   1 7 0 4 3, 2010; S a n t o v a c et al., 2010). An improvement and maintenance of quality in the laboratory can also be achieved by regular participation in interlabora-

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tory comparisons (ILC) (E A - 4 / 1 8   T A, 2010). They are also useful tools for demonstrating the competence of laboratories, similar to PT in accreditation procedures. Both PT and ILC should be carefully and competently planned, prepared, carried out, interpreted and documented (I S O / I E C   1 7 0 4 3, 2010).

It is also important that the cost-effective aspects and fitness-for-purpose of the use of PT were taken into account. Thus, in some cases it is useful to participate in ILC. ILC definition**:** "Organization, performance and evaluation of test on the same or similar test items by two or more laboratories in accordance with pre-determined conditions. Note – In some circumstances, one of the laboratories involved in the intercomparison may be the laboratory, which provided the assigned value for the test item" (I S O / I E C   1 7 0 4 3, 2010). ILC can be designed for purposes other than PT: a) the validation of methods (for determining performance characteristics such as reproducibility, comparability, confidence intervals under comparable conditions, limiting values or robustness, measurement uncertainty etc.; b) the characterization of reference materials (to assign the certified value and estimate uncertainty of this value); c) self-assessment of a laboratory's performance in a test. ILC is useful and cost-effective external quality control in the following cases: due to changes of personnel; for the test methods to another matrix; for the extension of the scope of accreditation; for documented in-house methods; if laboratory use some procedural steps deviating from the standard methods; if the results of the PTs are unsatisfactory and corrective actions are necessary; if assistance in detecting systematic errors in the laboratory is required; and if the laboratory has no other means to provide evidence of its technical competence and quality of measurement (I L A C - G 2 2, 2004).

Test materials used in ILC should be of appropriate quality. Sample must be carefully selected and prepared. It is very important that all laboratories get a homogeneous and stable test sample (I S O   G u i d e   3 5, 2006).

If the laboratory did not have satisfactory results in the PT or in case of critical results, it should check and improve its work and implement any necessary corrective actions. The accreditation procedures defined for such cases should be followed (I L A C - G 2 2 , 2004; I S O / I E C   1 7 0 4 3, 2010).

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* spp. and *Penicillium* spp.; it can be found in cereal grains and other food. OTA is possibly carcinogenic to humans (Group 2B) (W H O, 1997), and therefore, its confident and accurate determination and detection is important. This paper shows an example of organization of an ILC for mycotoxin determination and the obtained results were discussed.

## MATERIAL AND METHODS

In this study, organizer-lab used maize sample, which was previously proved to contain a significant amount of *Penicillium* molds and ochratoxin A. The 1 kg sample was roughly grinded and homogenized and divided into parts.

An interlaboratory comparison involved three participating laboratories, codes labeled as Lh0, Lh1 and Lh2. The organizer-lab delivered 150 g of maize

sample to the participating laboratories. Participating laboratories applied enzymatic immunoaffinity method (ELISA) for the determination of OTA and ELISA kits from different producers were used: R-Biopharm AG, Romer Labs® and Tecna S.r.l.

For a thin-layer chromatographic method of maize analysis, extraction was done with acetonitrile–water. Sodium bicarbonate was added to separate the acidic OTA. After 1 mol/ dm$^3$ hydrochloric acid addition and chloroform extraction, reconstituted sample was spotted on TLC plate next to the standard, and then it was examined under ultraviolet light (B a l z e r et al., 1978).

The same sample was analyzed by the HPLC method after extraction with chloroform and 0.1 mol/dm phosphoric acid, filtration, evaporating and degreasing (S o l f r i z z o et al., 1998). The equipment consisted of an LC system – BioRad 2800 with Supelcosil™ LC-18-DB column (250 x 4.6 mm id, particle size 5 µm) with a fluorescence detector Hewlett Packard 1046A. Wavelength of excitation radiation was 330 nm and emission 460 nm. A mobile phase consisted of a mixture of acetonitrile–water–acetic acid (50:50:1), at a flow–rate of 1 ml/min. Chromatographic data were collected and processed using ValueChrom® Chromatography Software (Bio-Rad, USA). Calibration curve was constructed on the basis of the area under the chromatographic peak using five OTA working standard solutions. The linearity of the method was assessed by the standard, ranging from 0.3 to 3.0 µg/ml (Fig. 1B). Recovery of the method was determined using blank maize sample spiked with 1000 µg/kg.


## RESULTS AND DISCUSSION

OTA is a mycotoxin undesirable in cereals. Nowadays, most laboratories apply ELISA method for the determination of OTA. Although this method has a number of advantages, it is not considered as standard method. In some cases, false positive results are possible and this is why, standard methods for confirmation are advisable (A n k l a m  et al., 2002).

Lab-organizer prepared maize sample naturally contaminated with OTA for this study. In this sample, OTA was determined and confirmed by two standard methods before interlaboratory comparison.

Although TLC used in this study is a standard method (B a l z e r et al., 1978), fluorescence of OTA spots on thin layer plates was assessed visually, and thus, only semi quantitative results were obtained. After comparing the intensity of sample spots with a series of standard solution spots and taking into account the sample dilution, the obtained OTA content in the sample was 900 µg/kg.

Since the sample preparation for the applied HPLC method included liquid-liquid extraction instead of solid phase extraction, the recovery study using spiked maize sample was carried out (Fig. 1C). Recovery achieved by this method was very poor, only 42%, which was not enough for quantitative standard method, according to the regulations (EC, 2006). However, having
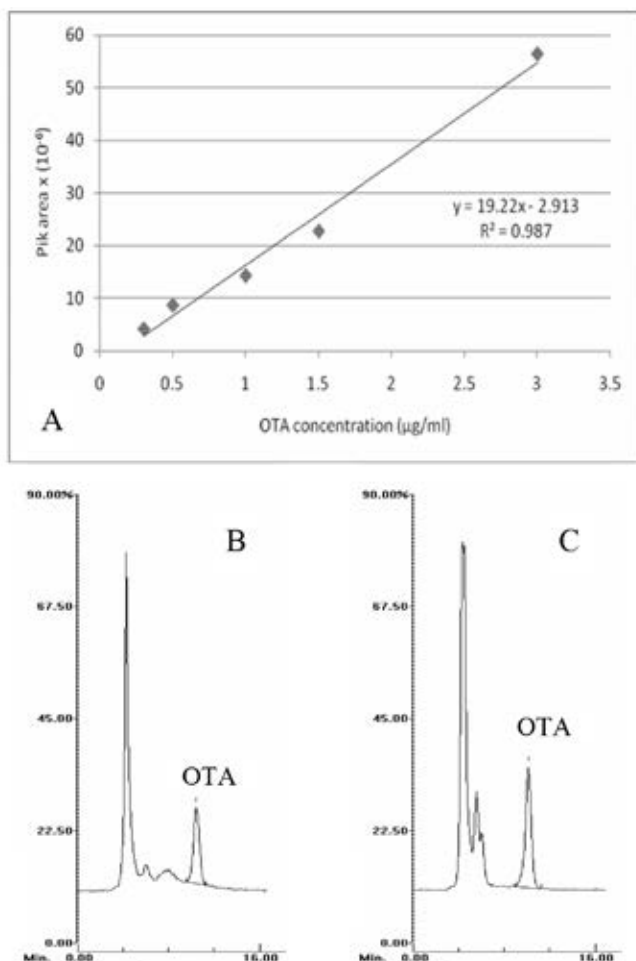
Fig. 1 – A Calibration curve for OTA determination by HPLC method;
B Naturally contaminated maize sample; C Blank maize sample spiked with 1000 μg/kg.

in mind this recovery, OTA content determined by HPLC in the maize sample (Fig. 1B) was 660 μg/kg.

Qualitative OTA confirmation included yellow florescence of OTA spots, i.e. blue fluorescence of OTA spots after treatment with ammonia vapors. Other qualitative evidence was retention time of OTA peak in the maize sample on HPLC chromatogram, which matched the peak of OTA standard. After this semi quantitative and qualitative confirmation of OTA in the maize sample, it was chosen for interlaboratory study. Laboratories that were participating in this interlaboratory comparison submitted the test results to the organizer-lab in predefined time. Since the number of tests in all series was the same, the estimation of inconsistent variance values was performed using

the Cochran´s C test (I S O  5 7 2 5 - 2, 2002; A t a n a s i j e v i ć et al., 1994). Furthermore, since the calculated Cochran´s coefficient was lower than critical value for comparing more than two series (0.55 < 1.44; H a d ž i v u k o v i ć, 1973), all variances were equal. Subsequently, the results were evaluated by calculating the deviation of the results, obtained in each particular laboratory, from the prescribed value. The prescribed value was determined based on a consensus-value of participating laboratories. The results were classified according to the recommendations of international norms (I S O / I E C  1 7 0 4 3, 2010; I S O  1 3 5 2 8, 2005) and are expressed as z–scores (Table 1). The expanded measurement uncertainty (k = 2) calculated from the standard deviation of bias based on proficiency testing was 19 µg/kg. The maize sample used in this study could then be used for interlaboratory internal review since it received consensual value and measurement uncertainty in described intralaboratory check (I S O  G u i d e  3 5, 2006).

Figure 2 shows the comparison between ELISA and standard methods. It can be concluded that ELISA tests gave somewhat better results in OTA determination in comparison to TLC and HPLC.

Tab. 1 – Results of interlaboratory comparison and z–score of participating laboratories

| Laboratory | Lh0 | Lh1 | Lh2 |
|---|---|---|---|
| Results ± SD (µg/kg) | 1073 ± 256 | 1039 ± 289 | 1082 ± 56 |
| Attributed consensual value | 1065 ± 23 | | |
| CV (%) | 23.9 | 27.8 | 5.18 |
| X max (µg/kg) | 1250 | 1370 | 1089 |
| X min (µg/kg) | 780 | 837 | 1023 |
| N | 3 | 3 | 3 |
| *z–score* | **+0.35** | **-1.13** | **+0.74** |
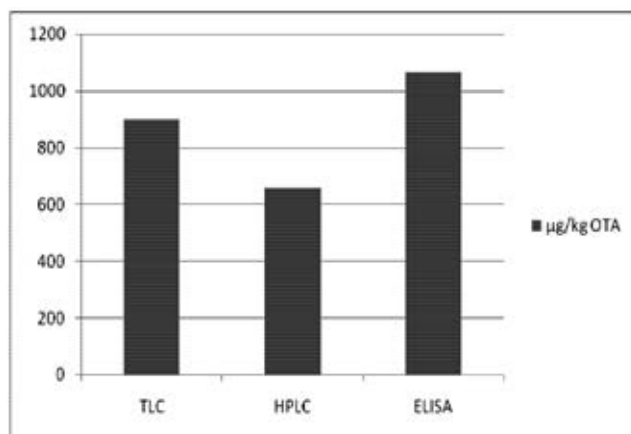


Fig. 2 – Content of OTA in naturally contaminated maize sample: interlaboratory ELISA result and standard methods results

Maximum permitted amount of OTA in animal feed was in the range of 0.1 to 1 mg/kg, depending on the types and categories of animals (S l. G l a - s n i k R S, 2010). These values were far outside the range of calibration ELISA and required multiple dilution of the sample. Our paper shows the possibility of using ELISA method in the case of highly contaminated samples at concentrations relevant to poultry feeding.

## CONCLUSION

Although PT schemes organized by accredited providers are required for accredited laboratories, in some cases, when PT is not available, it is very useful for laboratories to participate in ILC. It is cheaper, faster and easier way to control the laboratory quality. In the comparison described in this paper, laboratories seized the opportunity to check their methods for determination of higher OTA concentration. In this way, the robustness of the methods was verified. By processing the results of tests for OTA content, and analysis of z–values for all three laboratories, it was concluded that $z < \pm 2$ was the acceptable result.

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## МЕЂУЛАБОРАТОРИЈСКО ПОРЕЂЕЊЕ РЕЗУЛТАТА ОДРЕЂИВАЊА ОХРАТОКСИНА А ELISA МЕТОДОМ У КУКУРУЗУ

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### Резиме

Учешће у међулабораторијским поређењима и шемама за испитивање оспособљености је важно за контролу квалитета рада лабораторије. У овом раду су дати резултати одређивања охратоксина А (ОТА) у природно контаминираном узорку кукуруза од стране три лабораторије у Србији. Лабораторије учеснице су за одређивање ОТА користиле ензимску имуноафинитетну методу (ELISA), а избор ELISA кита је био слободан. Међулабораторијска прецизност је била задовољавајућа, што је доказано Кохрановим критеријумом. Такође, анализом z-вредности је код све три лабораторије добијено z < ±2, што представља прихватљиве резултате. У истом узорку је ОТА одређен и стандардним методама – танкослојном (TLC) и течном хроматографијом под високим притиском (HPLC) са флуоресцентним детектором. Резултати добијени ELISA, TLC и HPLC методама су били упоредиви.

КЉУЧНЕ РЕЧИ: међулабораторијско поређење, ELISA, охратоксин А, кукуруз, стандардне методе

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# LEVEL OF SEED INFECTION OF CULTIVATED SORGHUM WITH FUNGI FROM GENUS *FUSARIUM*

ABSTRACT: During the year of 2011, the level of fungi infection with the genus *Fusarium* was examined in seven genotypes of grain sorghum (Gold F1, A 28, B 28, Alba F1, A 73, B 73, Re 236) grown in six localities: Srbobran, Futog and four localities nearby Bački Petrovac 1 (2, 3 and 4).

The rate of seed infection with fungus *Fusarium* in the investigated period ranged from 0 to 8.5%. The infection did not occur in the genotype B73 (Bački Petrovac 1), while a low rate of infection of 0-3% was detected in the genotype B 28 (Srbobran). Medium rate of infection was recorded for the genotype A 28 (Srbobran) and A 28 (Futog) with 4.25% and for Gold F1 (Bački Petrovac 2) with 5.5%. High rate of infection was recorded for the genotype A 73 (Futog), A 73 (Bački Petrovac 2) (6.25%), Alba F1 (Bački Petrovac 2) (8%) and Re 236 (Futog) (8.5%).

One isolate from each *F. solani, F. graminearum, F. subglutinans* and *F. verticillioides* was selected for the pathogenicity investigation. Thirty plants were inoculated from each genotype of grain sorghum (varieties Alba F1 and Gold F1) and broomcorn (var. Reform and Prima). The spraying inoculation with the suspension of mycelium and conidia was performed when sorghum was at the end of blooming on June 19, 2012. The intensity of the infection on the sorghum panicles was reached in the phenophase of milky-wax maturity on July 8, 2012 and the scale of S h a r m a et al., (2010) was used. The symptoms of the artificial inoculation appeared only in the broomcorn. Among all isolates, the isolate of the species *F. graminearum* manifested the highest pathogenicity on the variety Reform with average score of 4.43 and on variety Prima with 4.17.

KEY WORDS: broomcorn, *Fusarium,* grain sorghum*,* disease intensity score

## INTRODUCTION

Sorghum (*Sorghum* spp.) is one of the oldest crops and by the area, it is fourth after rice, wheat and corn (K a z a n a s and F i e l d s, 1981). Based on the surfaces where broomcorn is grown, Serbia is one of the leading manufacturer in Europe and in the world. Over the last few years, this industrial crop has been cultivated mainly in the north and south of Vojvodina (B e r e n j i, 1990).

Grain sorghum is cultivated because of the seed which is very rich in proteins and starch. It is extremely energetic feed with more digestible proteins than corn seed.

In underdeveloped countries of Africa, Asia and Central America, grain sorghum is used as a staple food (D o g g e t t, 1988). However, in developed countries, there are no food products made from sorghum.

The aim of this work was to establish the composition of fungi on sorghum seed, the intensity of seed infection with fungi and to establish the pathogenicity of 7 sorghum genotypes in conditions of artificial infection with fungi *Fusarium graminearum, F. solani, F. verticlliodes* and *F. subglutinans.*

## MATERIAL AND METHODS

For this research, 11 samples of sorghum were used: Gold zavod 2011; Alba majka Planta 2011; Gold majka Planta 2011; Alba zavod 2011; Alba majka DDP 2011; Alba B. Petrovac 2 Milinka; Alba and Gold oprašivač Planta 2011; Gold oprašivač Srbobran 2011; Alba B. Petrovac 1 Miroslav; Alba održivač DDP 2011 and Gold majka Srbobran 2011. These seeds belong to 7 different genotypes that were obtained from 6 localities: 1) Zavod= Experimental field of the Institute for Field and Vegetable Crops in Bački Petrovac, 2) Planta= Agricultural Organization Planta from Futog, 3) Srbobran= Experimental field of the Institute for Field and Vegetable Crops in Srbobran, 4) DDP= Agricultural Organization "Petrovec" from Bački Petrovac, 5) Srbobran, 6) Futog.

Twenty-five seeds were randomly chosen from every sample in 4 replications. The method of wet filter paper (P i t t and H o c k i n g, 1985) was used to establish the mycobiota of the seeds. The sorghum seeds were incubated on wet filter paper for 6 days at the temperature of 25°C, and from the infected seeds, the fungi from genus *Fusarium* were subcultured on a Carnation Leaf-piece Agar-CLA. After the monosporic isolation of the selected isolates, they were identified on the basis of morphological and breeding characteristics using the determinator of L e s l i e and S u m m e r e l l (2006), N e l s o n et al. (1983) and L e v i ć (2008). One isolate from each *F. solani, F. graminearum, F. subglutinans* and *F. verticilliodes* was selected for the pathogenicity investigation. Artificial inoculation was performed on the sample plots of the Institute of Field and Vegetable Crops in Bački Petrovac on the sorghum genotypes Reform and Prima (broomcorn) and Alba and Gold (grain sorghum). Thirty plants were inoculated with every sorghum genotype. The spraying inoculation with the suspension of mycelium and conidia was performed when sorghum was at the end of blooming on June 19, 2012. The intensity of the infection on the sorghum panicles was reached in the phenophase of milky-wax maturity on July 8, 2012 and the scale of S h a r m a et al. (2010) was used: 1= no symptoms, 2=1-5%; 3=6-10%; 4=11-20%; 5=21-30%; 6=31-40%; 7=41-50%; 8=51-75%; 9=76-100%.

After inoculation, every plant was covered with nylon and paper bag and these were taken off after 24 hours. Standard statistical methods were applied.

# RESULTS

The seed inoculation with fungi ranged from 0- 8.5%. The infection did not occur in the genotype B73 (Bački Petrovac 1), low rate of infection (0-3%) was recorded for the genotype B28 (Srbobran), medium level of infection (4.25%) was recorded for the genotype A28 (Srbobran) and A28 (Futog) and Gold F1 (Bački Petrovac 2) (5.5%). High rate of infection was recorded for A73 (Futog), A73 (Bački Petrovac 2) (6.25%), Alba F1 (Bački Petrovac 2) (8%) and Re 236 (Futog) (8.5%). The percentage of determinated species from genus *Fusarium* can be seen in Figure 1. The dominant species was *F. graminearum*.

Among the investigated isolates, *F.graminearum* manifested the highest pathogenicity with average score of 4.43 in the sorghum genotype Reform and 4.17 in Prima. Average score in the plants, which belonged to the genotype Reform inoculated with fungus *F. solani,* was 2.4; in case of *F. subglutinans* it was 2.3; and in case of *F. verticilliodes* it was 2.36. In the variety Prima, these scores were 3.53 (*F. solani*); 2.34 (*F. subglutinans*) and 2.4 (*F. verticilliodes*).


# DISCUSSION

In this research, it was found that the highest seed pathogenicity with fungus from genus *Fusarium* was in the genotype Re 236 (Futog) and there was no statistical significance in comparison to the other genotypes.

According to L e s l i e et al. (2005), the highest pathogenicity on the sorghum was manifested by *F. thapsinum* in comparison to the other species that were included in the experiment. Although *F. verticilliodes* is primarly a maize pathogen, it can also be found in sorghum (L e s l i e et al., 1992), but it was less present than *F. thapsinum* (J a r d i n e and L e s l i e, 1992; M a n s u e t u s et al., 1997).

Agroecological conditions in Serbia are favorable for wide range of pathogens and toxigenic species from genus *Fusarium.* The total number of species was 63, out of which 35 varieties (var.) and 19 specialised forms (f.sp.) of basic species, particularly of *F. oxysporum* (4 var. and 12 f. sp.) and *F. solani* (7 var. and 3 f. sp.) were identified (L e v i ć et al., 2009). In Serbia, these species have been isolated from over 100 plant species and, from economical aspect, they are the most common causal agents of fusariosis of corn and wheat (L e v i ć, 2008).

*Fusarium* species produce several toxins, out of which the most important are trichothecenes, deoxynivalenol, fumonisins, moniliformin and others. The toxins often inhibit host protein production and act in defense against other fungi (N i s h i u c h i et al., 2006). Actually, transgenic plants bearing the trichothecene pump from of the *Fusarium* protects barley against *Fusarium* (M a n o h a r a n et al., 2006).

Similar to maize, sorghum is susceptible to various *Fusarium* spp. Mycotoxins such as aflatoxin, ochratoxin, zearalenone, deoxynivalenol or fumonisins may thus occur. However, fungal attacks on sorghum are less frequent than on maize since sorghum grows in warmer and drier climates in comparison to maize. Moreover, when *Fusarium* occurs in sorghum it produces much less fumonisin than in maize (V i s c o n t i et al., 1994).

*Fusarium* species have been and still are the most important in crop production. The basis of selection was to establish the level of seed infection and it was also important for taking some measures to prevent contamination of forage food.
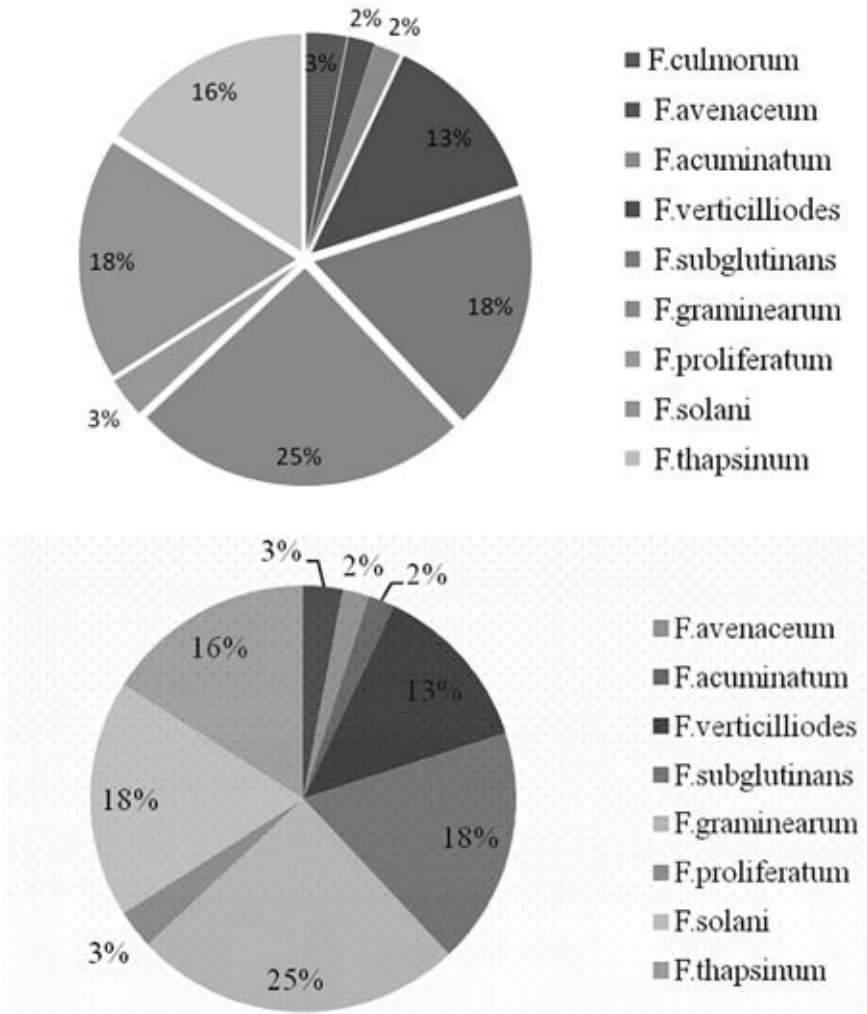


Chart 1. – Percentage of *Fusarium* fungus presence in sorghum seeds

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# СТЕПЕН ЗАРАЗЕ СЕМЕНА ГАЈЕНОГ СИРКА ГЉИВАМА ИЗ РОДА *FUSARIUM*

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## Резиме

Током 2011. године испитан је степен заразе гљивама из рода *Fusarium* 7 генотипова сирка за зрно (Gold F1, A 28, B 28, Alba F1, A 73, B 73, Re 236) из шест локалитета: Србобран, Футог, и четири локалитета у близини Бачког Петровца (Бачки Петровац 1, 2, 3 и 4).

Семе сирка је инкубирано на влажном филтер папиру током 6 дана на 25°C, а након тога је са заражених зрна извршено пресејавање гљива из рода *Fusarium* на подлогу од листа каранфила – CLA. Након моноспорне изолације одабраних изолата, а на основу морфолошких и одгајивачких карактеристика изолата извршена је детерминација на нивоу врсте коришћењем детерминатора Leslie and Summerell (2006) и монографије Левић (2008).

Зараза семена сирка гљивама из рода *Fusarium* се у испитиваној години кретала од 0 до 8,5%. Зараза није ни утврђена код генотипа B73 (Бачки Петровац 1), док је низак степен заразе од 0-3% утврђен код генотипа B 28 (Србобран). Средњи ниво заразе је утврђен код генотипа A 28 (Србобран), A 28 (Футог) од 4,25% и Голд F1 (Бачки Петровац 2) од 5,5%, а висок код узорака A 73 (Футог), A 73 (Бачки Петровац 2) (6,25%), Алба F1 (Бачки Петровац 2) (8%) и Re 236 (Футог) (8,5%).

У циљу испитивања патогености на метлици сирка за зрно и сирка метлаша одабран је по је један изолат гљива *F. solani, F. graminearum, F. subglutinans* и *F. verticillioides.* Са сваким изолатом инокулисано је по 30 биљака сирка метлаша, сорте Реформ и Прима, као и сирка за зрно сорте Алба F1 и Голд F1. Инокулација је извршена прскањем суспензијом конидија и мицелије у фенофази прецветавања метлица 19. јуна 2012. године. Оцена је извршена у фенофази млечно-воштане зрелости зрна 8. јула. За оцену интензитета обољења коришћена је скала према S h a r m a et al., 2010. Симптоми обољења на вештачки инокулисаним биљкама су се испољили само на сортама сирка метлаша, али не и на сирку зрнашу. Међу испитиваним изолатима највећу патогеност је испољио изолат врсте *F. graminearum* са просеком оцена 4,43 на сорти Реформ и 4,17 на сорти Прима.

КЉУЧНЕ РЕЧИ: *fusarium,* сирак за зрно, сирак метлаш, оцена интензитета

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# PREVENTION, CONTROL AND DETECTION OF FUSARIAL TOXINS

ABSTRACT: The past couple of decades have provided considerable details on fungi and the toxins that they produce, as well on the mechanism of toxin action, toxicity and effects on animal and human health. But, since they are natural contaminants, their presence is often inevitable. *Fusaria* are widespread in all cereal-growing territories of the world, but they are especially common in our geographic area. Therefore, special attention is paid to the prevention and control, and also to the improvement of methods for their detection. Although all collected data were critical for understanding this worldwide problem, managing the impact of these toxins on the feed and food safety is still great practical challenge. There are a number of approaches that can be taken to minimize mycotoxin contamination in this chain: prevention of fungal growth and thus mycotoxin formation, strategies to reduce or eliminate mycotoxins from contaminated feedstuffs or diverting the contaminated products to low risk uses. A control program for mycotoxins from field to table should involve the criteria of an HACCP (Hazard Analysis Critical Control Points) approach. It requires an understanding of the important aspects of the interactions of the toxigenic fungi with crop plants, the on-farm production and harvest methods for crops, the production of livestock using grains and processed feeds, including diagnostic capabilities for mycotoxicoses, and all the way to the development of processed foods for human consumption, as well as understanding the marketing and trade channels including storage and delivery of foods to the consumer's table. A good testing protocol for mycotoxins is necessary to manage all of the control points and in order to be able to ensure a food supply free of toxic levels of mycotoxins for the consumer.
KEY WORDS: *Fusarium* toxins, mycotoxin detection, mycotoxin prevention

## INTRODUCTION

Fungi are normal part of the microflora of standing crops and stored feeds, but the production of mycotoxins depends on the fungi presence, agronomic practices, the composition of the commodity and the conditions of harvesting,

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handling and storage (B r y d e n, 2009). Mycotoxins that adversely affect human or animal health are found mainly in post-harvest crops such as cereal grains or forages. These toxins are produced by saprophytic fungi during storage or by endophytic fungi during plant growth.

The genus *Fusarium* was established by Link more than 200 years ago and currently contains over 20 species (D e H o o g et al., 2000). They produce long, multicellular, canoe-shaped or banana-shaped macroconidia. These large asexual conidia are the defining morphological characteristic of the genus. Many species will also produce small, generally single-celled microconidia that range in shape from fusiform, oval to spherical. Additionally, some species produce thick-walled resistant chlamydospores important for long-term survival. Microconidia and macroconidia are important for wind and splash dispersal of the fungi. The conidia are also generally the propagules that result in infection of host plants.

*Fusarium* species are diverse in their host-associations and mycotoxin profiles, clearly distinguishing one species from another based on the range of morphological, molecular, and metabolic data. They cause root, stem and ear rot with severe crop yield reduction of economic relevance, often estimated to be between 10 and 30%. *Fusaria* are widespread in all cereal-growing areas of the world, but there are some geographical differences in the natural distribution of the *Fusarium* species, as well as of their corresponding mycotoxins, which are influenced primarily by the environmental conditions, crop production and storage methods (B a t t i l a n i et al., 2009; L o g r i e c o et al., 2002).


## FUSARIAL TOXINS

*Fusarial* toxins are produced in cereal grains under high moisture conditions during harvest. Wheat, triticale and maize grains are especially vulnerable for *Fusarium* infection and are also frequently highly contaminated with their secondary metabolites (D ö l l and D a n i c k e, 2011). The amount of produced toxin will depend on physical factors (moisture, relative humidity, temperature and mechanical damage), chemical factors (carbon dioxide, oxygen, composition of substrate, pesticide and fungicides), and biological factors (plant variety, stress, insects, spore load). Moisture and temperature have a major influence on mold growth and mycotoxin production. Although water activity is the most useful expression of the availability of water for microorganism growth, it is convenient to express the water content of a feed commodity as moisture percentage. Pathogenic fungi that invade crops prior to harvest usually require higher moisture levels (200–250 g/kg) for infection than fungi that can proliferate during storage (130–180 g/kg). Therefore, most feedstuffs with moisture contents above 130 g/kg are susceptible to mold growth and mycotoxin formation (J a k i c - D i m i c and N e s i c, 2009; J a k i c - D i m i c and N e s i c, 2011b; B r y d e n, 2012). In temperate climates, the *Fusarium* toxins are common contaminants of cereal crops. Due to their stability at high temperatures and during storage, milling, processing and cooking of food and

feed, humans and animals are, to a certain extent, always exposed (J a k i c - D i - m i c et al., 2009; EFSA, 2011a, b). J a k i c-D i m i c et al. (2010) observed the highest level of contamination and the most frequent occurrence of zearalenone in poultry feed in Serbia, while the studies of J a k š i ć et al. (2011; 2012) also indicated the presence of fumonisins and deoxynivalenol, as well as other trichotecenes, in Serbian cereals. Detected concentrations were usually lower than the maximal concentrations prescribed by state regulation (Službeni Glasnik RS, 4/2010).

## PREVENTION AND CONTROL OF FUSARIAL TOXINS

There are a number of approaches that can be taken to minimize mycotoxin contamination in the feed chain. They include prevention of fungal growth and thus mycotoxin formation, strategies to reduce or eliminate mycotoxins from contaminated feedstuffs or diverting contaminated products to low risk uses.

Agricultural practices such as crop rotation and soil tillage are recommended to control plant contamination with *Fusarium* spp., even though these techniques are not always recognized as efficient. Removal, burning or burial of crop residues is likely to reduce *Fusarium* inoculum on a crop (J o u a n y, 2007).

As the contamination by *Fusaria* is most likely during the crop flowering stage at the time of spore release, earlier planting dates in temperate areas will often result in a lower contamination level in maize, even though annual weather changes can challenge this potential advantage (B l a n d i n o et al., 2009). In wheat and barley, winter varieties develop and mature earlier than spring varieties and consequently they have a reduced risk of *Fusarium* infection (J o u a n i, 2007).

The harvest and post-harvest control of pathogens is connected with the period of harvest because, generally, earlier harvest results in lower concentrations of mycotoxins (J o n e s et al., 1981). The cutting height is another important factor for the prevention of post-harvest contamination. Post-harvest, damaged grains should be eliminated and the moisture content for the kernels must be lowered in order to reduce the possibilities of infection and the production of toxins by fungi. A water activity lower than 0.65 and a humidity level under 14% in cereals are usually considered as limiting factors for fungal growth; effectively, *Fusarium* spp. need 17-19% humidity to grow. The temperature of storage has an effect on fungal growth too and its control is relevant especially in silo storage: combined cooling and drying operations associated with ventilation systems are necessary to avoid the worsening of contamination during storage (J o u a n y, 2007).

One of the strategies for the control of toxin production is connected with the possibility of limiting the infection by using varieties that have proved to be more resistant to *Fusarium* spp. and insects injuries. Fungal geneticists have unraveled the pathways and the genes responsible for the synthesis and regulation of mycotoxin production, especially aflatoxin and the trichothecenes (B h a t n a g a r et al., 2008) and this may assist in the development of plants

that are resistant to toxin accumulation. In some respects, this is demonstrated by the success of Bt maize hybrids which have been developed as a mean of transgenic insect protection (W u et al., 2004). The transgenic Bt maize contains a gene from the soil bacterium *Bacillus thuringiensis* which encodes for a protein that is toxic to common lepidopteran maize pests. These hybrids offer a new tool for mycotoxin management because insect damage is often a major aetiological factor in facilitating toxigenic fungal infection of crops. Bt maize is effective in reducing the incidence of fumonisin contamination but less effective in reducing deoxynivalenol contamination. This reflects different disease patterns and pathogens since deoxynivalenol is associated with Gibberella ear rot, whereas fumonisin production is associated with Fusarium ear rot and, the occurrence of Gibberella ear rot is not as strongly influenced by insect damage as it is by fumonisin accumulation.

Chemical control of the pathogen is difficult because, in order to be efficient, the fungicides must be totally lethal to *Fusarium* spp.; if not, they stimulate mycotoxin production *in vitro* (D'M e l l o et al., 1998). Biological control with microbial antagonists or competitors to *Fusarium* spp can be integrated in contamination control strategies by spraying it on plants at flowering stage to eradicate or limit the growth of toxin producers (J o u a n y, 2007). Some biological agents, such as some strains of *Bacillus subtilis, Bacillus thuringensis, Candida, Pseudomonas or Trichoderma* spp., have already been included in the pesticide database of the European Union.

There are different possibilities of the post-harvest decontamination strategies: biological approach or application of physical or chemical methods. Many of them have still been analyzed. Farm feed storage and on-farm feeding systems can also contribute to mycotoxin exposure of the animals being fed. Simple measures can significantly reduce the risk of mycotoxin exposure on farm. Storage of grain with appropriate moisture content (below 130 g/kg), inspection of grain regularly for temperature, insects and wet spots will limit the possibility of fungal development in feeds and feedstuffs. The risk of feed contamination will be reduced in animal units with rapid turnover of feed because there will be less time for fungal growth and toxin production. A recent survey of M o o r e et al. (2008), who investigated the on farm occurrence of aflatoxin, deoxynivalenol and zearalenone in cereal grains, forage and straw, showed that those three mycotoxins were found in all commodities, with zearalenone being the most common. Interestingly, grains had the lowest frequency of contamination but they were often the only source of mycotoxins when examining a field of toxicosis. These results highlighted the potential risk of contamination of feedstuffs and forages other than grain used in animal production. Moreover, the contamination of straw, which may be used as a roughage source for horse and ruminant diets or as bedding for pigs, poultry and horses, may also be a source of mycotoxin exposure on farm, similar to grain dust.

Important way of mycotoxin control is to alleviate and/or prevent harmful effects of mycotoxins already present in feed. In order to minimize their impact, there is the approach of dilution of feed with uncontaminated feedstuffs. Dilution of mycotoxin-contaminated grain with uncontaminated grain

is one of the simplest and most widely utilized methods for improving feed intake and weight gains of animals. However, the success of this approach depends on the degree of contamination, the dilution achieved and the availability of a source of uncontaminated grain. In some countries this practice is not permitted.

There is also the possibility of using various feed additives which either adsorb mycotoxins on their surface or they provide enzymatic degradation of mycotoxins. Efficacy of alleviating harmful effects depends mostly on chemical structure of adsorbent, as well as on the type of mycotoxin. These are substances nonresorbable from the gut which can physically bind some chemicals and thus block their resorption. Mineral adsorbents are commonly used (active charcoal, hydrated sodium calcium aluminosilicate, sodium bentonit, dietary clay and zeolites). The feasibility of utilizing organic adsorbents was also examined, particularly esterified glucomanane which was isolated from the inner layer of yeast cell wall and which possesses significant capability of mycotoxin adsorption (N e s i c, 2003; N e s i c et al., 2008a,b). Recently a new type of additive has been developed and it contains microorganisms with the ability to inactivate mycotoxins by enzyme modification of its structure (N e s i c et al., 2011; N e s i c et al., 2012).

A control program for mycotoxins from field to table should involve the criteria of an HACCP (Hazard Analysis Critical Control Points) approach. It requires an understanding of the important aspects of the interactions of the toxigenic fungi with crop plants, the on-farm production and harvest methods for crops, the production of livestock using grains and processed feeds, including diagnostic capabilities for mycotoxicoses, and all the way to the development of processed foods for human consumption, as well as understanding the marketing and trade channels including storage and delivery of foods to the consumer's table. Good testing protocol for mycotoxins is necessary to manage all of the control points for finally being able to ensure a food supply free of toxic levels of mycotoxins for the consumer (R i c h a r d, 2007; J a k š i ć et al., 2011).


DETECTION OF MYCOTOXINS


Mycotoxins present a major analytical challenge due to the range of chemical compounds that they represent and the vast array of feed matrices in which they are found. Analysis is essential for determining the extent of mycotoxin contamination, for risk analysis, for the confirmation of the diagnosis of mycotoxicosis and for monitoring mycotoxin mitigation strategies. Quantification of these compounds requires sophisticated laboratory equipment including high performance liquid chromatography, gas chromatography, gas chromatography/mass spectrometry or liquid chromatography/mass spectrometry (K r s k a et al., 2008; R a h m a n i et al., 2009).

There are still a number of areas that require further study and refinement, including commodity sampling techniques, conjugated toxin determination and field or feed mill screening of feedstuffs. Sampling is the greatest source of

errors in quantifying mycotoxin contamination because of the difficulty with obtaining feed samples representative which may have caused a mycotoxicosis or for regulatory purposes from large grain consignments. These difficulties arise because of the uneven distribution of toxin within a commodity at which mycotoxins occur (J a k i c-D i m i c and N e s i c, 2011a).

Connection between 'masked', 'hidden', 'bound' or conjugated mycotoxins in feedstuffs and the potential for poor animal performance has recently become evident. These compounds may be formed as a result of plant metabolism, but they were not detected with conventional analytical procedures. For example, zearalenone-4-glucoside a conjugate of zearalenone and deoxynivalenol-3-glucoside a conjugate of deoxynivalenol can constitute up to 20% of the total content of the precursor mycotoxin in a feedstuff. It is likely that these conjugates will be hydrolyzed following ingestion and thus increasing the exposure to the precursor toxin. It is also evidence that ochratoxin A and fumonisins are conjugated by plants and fumonisins may also be conjugated with sugars and proteins during food processing (H u m p f and V o s s, 2004). B e r t h i l l e r et al. (2009) analyzed the formation and determination of conjugated mycotoxins.

The development of immunological methods for mycotoxin detection (P e s t k a, 1994), especially enzyme-linked immunosorbent assays (ELISA), although only semi-quantitative, was a major step towards the development of rapid, repeatable and sensitive assays. These assays are suitable for field use and screening of feed commodities in feed mills. There are a number of other approaches, still experimental, that show the possibility for rapid mycotoxin analysis without the need of sophisticated equipment (M a r a g o s and B u s m a n, 2010).


CONCLUSION


By combining Good Agricultural Practice, Good Manufacturing Practice, Good Storage Practice, and applying the seven HACCP principles within the framework of the quality systems, it is possible to introduce the most cost--effective prevention of mycotoxicosis. The corrective actions include implementation of all available measures for combating mold and preventing toxin production so that a food supply free of toxic levels of mycotoxins can be provided to the consumer. The methods for mycotoxin detection have a significant role in prevention and handling of this problem and therefore they must meet the highest standards.


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# ПРЕВЕНЦИЈА, КОНТРОЛА И ДЕТЕКЦИЈА FUSARIUM МИКОТОКСИНА

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Резиме

Током протеклих неколико деценија утврђено је много детаља о плеснима и токсинима које они производе, као и о механизму њиховог деловања, токсичности и ефектима на здравље животиња и људи. Међу многобројним врстама, плесни рода *Fusarium* распрострањене су широм света, али су уобичајене и на нашем географском подручју често предоминантне. Упркос многим прикупљеним подацима од критичне важности за разумевање овог глобалног проблема, утицај микотоксина на безбедност хране је и даље велики практични изазов, а како се ради о природним контаминентима, често и неизбежно присутан. Стога, неопходно је посветити посебну пажњу превенцији и контроли, као и унапређењу метода за детекцију.

Постоји низ приступа који се могу предузети да се смањи загађење микотоксинима: спречавање раста плесни и последично синтезе њихових токсичних секундарних метаболита, развој стратегије за смањење или елиминисање микотоксина већ присутних у храни за животиње, или усмеравање контаминираних производа на употребу нижег ризика. Програм контроле за микотоксине „од њиве до трпезе“ треба да укључи критеријуме HACCP (Hazard Analysis Critical Control Points) приступа. То захтева разумевање битних аспеката интеракције токсигених плесни са ратарским културама, одговарајуће агротехничке мере, стандардизовану производњу хране за животиње, контролу на фармама и адекватне поступке за дијагностику микотоксикоза, контролисану производњу хране за људе, обухватајући и трговинске канале, складиштење и промет, све до стола потрошача. За адекватно управљање контролним тачкама неопходни су добри тест протоколи за микотоксине како би се обезбедило снабдевање храном без токсичних нивоа микотоксина.

У раду су обједињени подаци и указано је на различите могућности за превенцију, контролу и детекцију секундарних метаболита *Fusarium* плесни.

КЉУЧНЕ РЕЧИ: *Fusarium* микотоксини, детекција микотоксина, превенција микотоксина

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# PRESENCE OF ZEARALENONE IN THE MOST COMMONLY GROWN WHEAT CULTIVARS IN SERBIA

ABSTRACT: A total of 45 samples of wheat from three different locations in Vojvo-dina were analyzed for the presence of zearalenone. Analytical methods based on clean-up by solid-phase extraction (SPE) columns and detection by liquid chromatography were used after validation. Limit of detection for ZEA in wheat was 18.6 μg/kg and the limit of quan-tification was 56.5 μg/kg. Recovery values ranged between 86% and 97%. The occurrence of ZEA in wheat was rather high with 53.3% of positive samples with the average value of 330 μg/kg. Incidences were found from 68 μg/kg to 1079 μg/kg. Contamination levels were above the established maximum limit for unprocessed cereals, other than maize, in as many as seventeen samples. These results were compared to the results of investigation of deox-ynivalenol and fumonisin content, established in our previous work on the same samples. The results obtained were also compared to those of the neighboring countries where the relevant data existed and to the data of previous studies in our country.
KEY WORDS: HPLC, wheat, zearalenone

INTRODUCTION

Fusarium molds appear as the most common contaminants of grains in the mild climatic zone. As a result, mycotoxins are often found in such substrates in larger or smaller concentrations, depending on storage and microclimatic conditions (K l a r i ć, 2008). The main groups of Fusarium toxins commonly found in grains are trichothecenes (including deoxynivalenol, nivalenol, T-2

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and HT-2 toxins), zearalenone (ZEA) and fumonisins B1 and B2. Fusarium toxins pose safety concerns for grains intended for direct consumption due to their harmful impact on human health.

*Fusarium*, *Penicillium* and *Aspergillus* genera are the most frequently isolated fungi contaminating cereals, feedstuffs, vegetables and fruit in Serbia (L e v i ć et al., 2004). Fungi from the *Fusarium* genera are especially present, and with them *Fusarium* mycotoxins, first of all zearalenone, which presence is mainly associated with *F. graminearum* and it is the highest in years with abundant precipitation and lower temperatures at the end of summer and the beginning of autumn (L e v i ć et al., 2004).

In Serbia, grain production, especially wheat and maize, represents an important economic factor considering the fact that our country is mostly agricultural. Out of 3.099.000 ha of area sown with major crops, 493.000 ha was sown with wheat in 2010 (Statistical Office of the Republic of Serbia, 2012). In our country, main crops are wheat, barley and maize which are particularly important as food, feed and raw materials for breweries and distilleries. Grain-based products represent the main part of human diet and infants, in particular. Babies and young children are exposed to higher doses of toxins per body weight unit compared to adults because of their proportionally higher consumption of cereals. This poses concern for consumer health and requires continuous control of foodstuffs and foods for chemical and biological contaminants.

The Commission of the European Communities (Commission Regulation 1126/2007) established the following tolerance values for ZEA in cereals and cereal-based products: unprocessed cereals other than maize (100 μg/kg), unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling (350 μg/kg), cereals intended for direct human consumption, cereal flour, bran and germ as end products marketed for direct human consumption (75 μg/kg), refined maize oil (400 μg/kg), bread (including small bakery commodities), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereal (50 μg/kg), maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals (100 μg/kg), processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children (20 μg/kg) and processed maize-based foods for infants and young children (20 μg/kg). With respect to the aforementioned cereals and their products, Serbian regulation (Službeni glasnik, 2011) established the same tolerance levels as Commission Regulation did, and for feed (Službeni glasnik, 2010) the following tolerance levels were set: in complete and supplementary mixtures for piglets, young mails and gilts is 500 μg/kg, in complete and supplementary mixtures for other categories of pigs is 1000 μg/kg while in complete and supplementary mixtures for cattle, sheep and goats is 3000 μg/kg.

In Serbia, available data for the distribution of *Fusarium* mycotoxins in cereal production are still limited. There is no national data base established for the collection of this kind of data in order to use them for prediction and prognosis of annual mycotoxin risk exposure of the local population.

Considering the aforementioned facts, the primary aim of this study was to gain an insight into ZEA presence in Serbia on the basis of analysis of 45 wheat samples from the 2010 harvest and to compare the results of investigation of deoxynivalenol and fumonisin content, established in our previous work on the same samples. The samples were taken directly from the fields immediately after the harvest before entering further the food chain. The levels of ZEA were determined after SPE clean-up by liquid chromatography with DAD, allowing detection limits in the ppb (18.6 µg/kg) range.

## MATERIALS AND METHODS

### *2.1. Materials*

Acetonitrile and methanol (both HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Water (HiPerSolvChromanorm, HPLC grade) was purchased from Sigma (St. Louis, MO, USA). Zearalenone mycotoxin was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). MycoSep® 224 (AflaZon) clean-up columns were obtained from Romer (Romer Labs. Inc., Union, MO, USA) and paper filter (Whatman No. 4) was obtained from Whatman (Maidstone, UK).

### *Samples*

A total of 45 samples of wheat, from three different locations in Vojvodina, were analyzed for the presence of zearalenone. They were collected from the most important agricultural area in the country – Vojvodina, with 3 different regions: north-western (Bačka), north-eastern (Banat) and south-western region (Srem). The samples of wheat represent the most common cultivars grown in Vojvodina.

Due to the irregular mycotoxin distribution among the crops and kernels, a proper sampling was ensured according to EU requirements (Commission Regulation 401/2006). Manual sampling was performed by trained inspectors with grain probes which are authorized for official control of contaminants. Depending on the weight of bulk lot, from 3 to 100 incremental samples of 100 g or more, were taken randomly and combined into a representative sample of 1–10 kg weight. Each sample was transported to the laboratory immediately and was stored at low temperature in a dark place. All samples were milled on a laboratory mill and a portion was taken for the analysis of contaminants.

### *Extraction and clean-up*

Twenty-five grams of ground sample were extracted with 100 ml acetonitrile:water (84:16, v/v) by high speed blending for 3 min. The extract

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was filtered through paper filter, acidified with 50 μl acetic acid and 2 ml was cleaned-up by Mycosep® 224 (AflaZon) columns. The cleaned-up extract was evaporated to dryness.

### *Liquid chromatographic analysis*

The equipment consisted of an Agilent 1260 Infinity Liquid Chromatography system, equipped with a μ-Degasser (G1379B), 1260 binary pump (G1312B), 1260 standard autosampler (G1329B), 1260 thermostated column compartment (G1316A), 1260 diode array and multiple wavelength detector (G1315C), and a column Hypersil ODS (100 x 4.6 mm i.d., particle size 5 μm, Agilent Technologies, USA).

After evaporation, the residue was redissolved in 400 μl methanol, and a 15 μl aliquot of the solution was injected into the LC system. A mobile phase consisting of a mixture of methanol–water (70:30, v/v) was used at 0.5 ml/min with UV detection at 236 nm. The mobile phase was filtered through a 0.45 μm pore size cellulose filter membrane (Agilent Technologies, USA).

## RESULTS AND DISCUSSION

Validation parameters of method for ZEA determination were estimated using the recovery tests of samples of the studied wheat matrix. The accuracy of the method was determined with the recovery of fortified blank grain samples at three levels with three replicates for each level and three injections for each replicate (nine injections per level). Recoveries obtained for wheat were in the range 86 – 98%. These recoveries comply with the requirements of European Commission concerning analytical methods development (Commission Regulation 657/2002).

The limit of detection (LOD) and the limit of quantification (LOQ) are determined on the basis of standard deviation of the response and the slope of the linearity plot (R e a s o n, 2003). The LOD was calculated as $3.3\alpha/b$ and LOQ as $10\alpha/b$, where $\alpha$ was the standard deviation of the response about the line of the best fit and $b$ was the slope of the calibration curve. Detection limit (LOD) for wheat was below 18.6 μg/kg while limit of quantification (LOQ) was close to 56.5 μg/kg. The obtained results showed that the proposed analytical method fitted well for the purpose of contrl of ZEA in grain samples.

Zearalenone content was determined in fifteen cultivars of winter wheat which have been the most commonly grown cultivars in Serbia since 1955. The occurrence of ZEA in these samples originated from the trial fields of the Institute of Field and Vegetable Crops and it is summarized in Table 1. Banatka is a native population, San Pastore and Libellula are Italian cultivars, Zlatna Dolina is a Croatian cultivar. Serbian cultivars used in the study were created at the Institute of Field and Vegetable Crops, Novi Sad (Sava, Novosadska rana 2, Balkan, Lasta). All the mentioned cultivars are presently grown

in Serbia. Serbian cultivars that are most widespread and encompassed by this study are Novosadska rana 5, Renesansa, Pesma, Cipovka, Dragana, Simonida, and Zvezdana. Grain samples were obtained from 15 winter wheat cultivars grown in 2010 at the following Serbian locations: Novi Sad (Bačka), Sremska Mitrovica (Srem) and Pančevo (Banat). The locations are characterized by semiarid conditions, with dry, hot spring and summer, neutral autumn and moderately cold winter. At each location, the plots were rotated with soybean. Positive samples were contaminated with ZEA at levels from 68 – 1079 µg/kg. According to the data in Table 1, more than half of the analyzed wheat samples were ZEA-contaminated at mean level of 330 µg/kg. The contamination levels in as many as seventeen samples were above the maximum limit of 100 µg/kg, a level set in Europe (Commission Regulation 1126/2007). Contamination was found in samples with visually healthy (asymptomatic) kernels.

Tab. 1. – Occurrence of zearalenone in wheat samples in Serbia from the 2010 harvest

| Cultivar | Location/Concentration of ZEA (µg/kg) | | | |
|---|---|---|---|---|
| | Pančevo (Banat) | Novi Sad (Bačka) | Sremska Mitrovica (Srem) | |
| Banatka | 92 | ND | 231 | |
| San Pastore | ND | ND | 821 | |
| Libellula | 214 | ND | ND | |
| Zlatna Dolina | ND | ND | 351 | |
| Sava | 73 | ND | 327 | |
| Novosadska rana 2 | 68 | 71 | 158 | |
| Balkan | ND | ND | 301 | |
| Lasta | 181 | 467 | 143 | |
| Novosadska rana 5 | ND | ND | 944 | |
| Renesansa | 241 | ND | 356 | |
| Pesma | 88 | ND | 734 | |
| Cipovka | ND | ND | 293 | |
| Dragana | 71 | ND | 1079 | |
| Simonida | ND | ND | 139 | |
| Zvezdana | ND | ND | 469 | **Total** |
| No. of positive samples (%) | 8 (53.3) | 2 (13.3) | 14 (93.3) | **24 (53.3)** |
| Average (µg/kg) | 129 | 269 | 453 | **330** |
| Range (µg/kg) | 68 – 241 | 71 – 467 | 193 – 1079 | **68 – 1079** |
| Median (µg/kg) | 90 | 269 | 339 | **236** |

The incidence and level of contamination of studied cereal samples with ZEA did not show comparable levels as it was reported for previous harvest years in the country. The frequency of contamination seems to be highly dependent of weather conditions. The analysis of wheat samples for human consumption in 1995 (P a r r y et al., 1995) showed a widespread contamination (70% incidence) with low to medium levels of ZEA (V r a b c h e v a, 1996;

N e n o v, 1997; V a l c h e v a et al., 2003; V r a b c h e v a et al., 2004). As regards the occurrence of ZEA in wheat in neighboring countries, V r a b c h e v a (1996) reported 69% of positive samples in Bulgarian wheat, while M l a d e n o v a and M a n o v a (2009) obtained quite lower number of positive wheat samples (1.9%). In Romanian wheat, during the period from 2008-2010, ZEA occurred in 10% of wheat samples (G a l b e n u et al., 2011). Š k r b i ć et al. (2011) found no ZEA in wheat samples collected during the harvest of 2007.

In our previous study on the same samples, the contents of deoxynivalenol (DON) and fumonisins (FUMs) were determined (J a k š i ć et al., 2012). We compared these results to those obtained for ZEA, in terms of better estimation of mycotoxin contamination (Table 2). As it can be seen, the number of contaminated samples was quite similar for all toxins found in the samples from Banat region, while the number of fumonisin contaminated samples from Bačka and Srem regions was lower than in the other two. The highest number of positive samples was established in Srem region where the highest concentration of all three toxins was obtained. Also, it can be said that the distribution of DON and ZEA contamination is well correlated unlike the distribution of fumonisin contamination.

Tab. 2. – Comparison of occurrence of deoxynivalenol, fumonisin and zearalenone in crops in Serbia from the 2010 harvest

| Cultivar | Location/content μg/kg | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pančevo (Banat) | | | Novi Sad (Bačka) | | | Sremska Mitrovica (Srem) | | |
| | **FUMs** | **DON** | **ZEA** | **FUMs** | **DON** | **ZEA** | **FUMs** | **DON** | **ZEA** |
| Banatka | ND | 229 | 92 | ND | ND | ND | 28 | 392 | 231 |
| San Pastore | ND | ND | ND | ND | ND | ND | ND | 352 | 821 |
| Libellula | 81 | 312 | 214 | ND | 144 | ND | 27 | 136 | ND |
| Zlatna Dolina | ND | ND | ND | 38 | ND | ND | ND | 312 | 351 |
| Sava | 427 | 104 | 73 | 359 | ND | ND | 414 | 356 | 327 |
| Novosadska rana 2 | ND | 132 | 68 | ND | 156 | 71 | ND | 364 | 158 |
| Balkan | 419 | ND | ND | ND | ND | ND | ND | 316 | 301 |
| Lasta | ND | 288 | 181 | ND | 384 | 467 | 263 | 164 | 143 |
| Novosadska rana 5 | 68 | ND | ND | 55 | ND | ND | 37 | 864 | 944 |
| Renesansa | 351 | 196 | 241 | 417 | ND | ND | 525 | 660 | 356 |
| Pesma | 72 | 292 | 88 | 84 | ND | ND | 78 | 984 | 734 |
| Cipovka | 401 | ND | ND | ND | ND | ND | ND | 288 | 293 |
| Dragana | 36 | 232 | 71 | ND | ND | ND | ND | 308 | 1079 |
| Simonida | ND | ND | ND | 441 | ND | ND | 394 | 292 | 139 |
| Zvezdana | ND | ND | ND | ND | ND | ND | 52 | 692 | 469 |
| No. of positive samples (%) | 8 (53.3) | 8 (53.3) | 8 (53.3) | 6 (40.0) | 3 (20.0) | 2 (13.3) | 9 (60) | 15 (100) | 14 (93.3) |
| Average (mg/kg) | 232 | 223 | 129 | 232 | 228 | 269 | 202 | 432 | 453 |
| Range (mg/kg) | 36-427 | 104-312 | 68-241 | 38-441 | 144-384 | 71-467 | 27-525 | 136-984 | 193-1079 |
| Median (mg/kg) | 216 | 230 | 90 | 221 | 156 | 269 | 78 | 352 | 339 |

The obtained results for wheat harvested in 2010 confirmed that this crop should be continuously controlled in order to protect the population against unallowable risk of mycotoxin contamination. The results of the present study are particularly important for the wheat production which should be regarded as a potential source of *Fusarium* mycotoxins. This requires increased control measures for agricultural production starting from the field, before foodstuffs enter the manufacturing process and food chain.

## CONCLUSIONS

Samples of wheat grown in Serbia were tested for zearalenone, produced by common *Fusarium* species. The incidence of ZEA contamination of wheat (53.3%) was found to be quite high, especially in Srem region (93.3%). It should be noted that the average value (330µg/kg) in the examined wheat samples was very high, and in 17 out of 45 samples ZEA content exceeded the maximum tolerable level set by EU and Serbian regulations. By comparing these data to DON and FUMs content in the same samples, it can be said that ZEA distribution responds to DON distribution but not to distribution of FUMs. This study indicates the existence of a serious risk related to the occurrence of DON in the food chain in Serbia and importance of frequent monitoring of this mycotoxin. Since the new Serbian regulation for control of mycotoxins in food was adopted and harmonized with EU regulations in April 2011, it is expected that more frequent control of ZEA will be conducted and that more data on its occurrence in Serbia will be available. In the future, based on the obtained results, it will be necessary to form a national database of ZEA occurrence in the food chain in Serbia.

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# ПРИСУСТВО ЗЕАРАЛЕНОНА У НАЈЧЕШЋЕ УЗГАЈАНИМ СОРТАМА ПШЕНИЦЕ У СРБИЈИ

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Резиме

На присуство зеараленона анализирано је укупно 45 узорака пшенице са три различите локације у Војводини. Коришћене су аналитичке методе засноване на пречишћавању екстракцијом на чврстој фази, те квантификација течном хроматографијом, након валидације методе. Граница детекције за зеараленон у пшеници је износила 18,6 µg/kg, а граница одређивања 56,5 µg/kg. Ефикасност методе је била у опсегу од 86% до 97%. Зеараленон је био присутан у 53,3% испитиваних узорака, са просечним садржајем од 330 µg/kg. Добијене вредности садржаја зеараленона су биле у опсегу од 68 µg/kg до 1079 µg/kg. У чак седамнаест узорака је пронађена концентрација овог токсина која превазилази максимални дозвољени садржај зеараленона у нетретираним житарицама. Ови резултати су упоређени са вредностима садржаја деоксиниваленола и фумонизина у истим узорцима добијеним у нашим претходним истраживањима. Резултати су такође упоређени са доступним резултатима добијеним у нашој и суседним земљама током претходних година.
КЉУЧНЕ РЕЧИ: HPLC, пшеница, зеараленон

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# MOLDS AND MYCOTOXINS IN
# FRESHLY HARVESTED MAIZE

ABSTRACT: Incidence of toxigenic fungi (molds) and concentration of mycotoxin aflatoxin B1 (AFB1), deoxynivalenol (DON), zearalenone (ZON) and fumonisin ($FB_1$) were studied in the maize grains collected immediately after harvesting in 2012. A total of 29 maize samples were analyzed and the highest incidence was determined for fungal species of *Rhizopus* (56.41%), *Aspergillus* (43.66%) and *Fusarium* (14.97%) genera. Significantly lower incidence was obtained for species of genus *Penicillium* (3.31%), and especially for species of genera *Acremonium* (1.38%), *Alternaria* (0.75%) and *Cladosporium* (0.14%). Among toxigenic fungi *Aspergillus flavus* (36.69%) was the most common species of *Aspergillus* genus, whereas the *Fusarium verticillioides* with 14.69% of incidence was the predominant species of *Fusarium* genus. In all studied maize samples, the presence of $AFB_1$, ZON and $FB_1$ mycotoxins was established, except for DON which was established in 75.86% samples. $AFB_1$ was detected in average concentration of 13.95 µg kg$^{-1}$ for 44.83% of samples, and average concentration higher than 40 µg kg$^{-1}$ for 55.17% of samples. The average concentrations of DON which was detected was 235 µg kg$^{-1}$, while it was 98.38 µg kg$^{-1}$ and 3590 µg kg$^{-1}$ for the presence of ZON and FB1, respectively. Moderate positive correlation was obtained between concentrations of AFB1 and FB1 (r=0.35), while weak positive correlation was established between concentrations ZON and DON (r=0.02).

KEYWORDS: maize grains, molds, mycotoxins

## INTRODUCTION

Maize is one of the major crops in Serbia, and it plays an important role in rotation with wheat, in animal feed and direct human consumption. Maize grain as energy source is widely used in both human and animal nutrition (cows, sheep, goat, poultry and fish etc.). The nutritive value of maize grain depends on the nutrient contents and digestibility (A l p t e k i n et al., 2009). Maize grains are subject to infection by a variety of toxigenic fungi, most

commonly *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. and *Penicillium* spp. (K r n j a j a et al., 2006, 2007, and 2011). Toxigenic *Alternaria* and *Fusarium* species are often classified as field fungi, while *Aspergillus* and *Penicillium* species are considered as storage fungi (L o g r i e c o et al., 2003).

Although contamination with fungi diminishes the quality of grain, toxigenic fungi species can produce highly toxic compounds known as mycotoxins. Fungal growth and toxin production in maize have been found to depend on several interacting factors which stress maize plants. Stress factors include low moisture content of soil, high daytime maximum temperatures, high nighttime minimum temperatures, and nutrient-deficient soils (A b b a s et al., 2006).

The fusariotoxins (fumonisins, zearalenone – ZON and deoxynivalenol – DON) and aflatoxins have an important economic impact on the grain industry. Fumonisins are mycotoxin group produced by *F. verticillioides*, *F. proliferatum* and *A. niger* (M o g e n s e n et al., 2009) while ZON and DON were produced by *Fusarium* species, mainly of *F. graminearum* and *F. culmorum* (Y l i – M a t t i l a, 2010). Aflatoxins are potent carcinogenic and toxic metabolites produced by fungal species *A. flavus* and *A. parasiticus* (M e d i - n a – M a r t í n e z and M a r t í n e z, 2000).

The negative effect of mycotoxins on the growth and health of livestock make them a major problem for many production systems. Food and feed safety and hygiene represent a significant problem, and great attention is directed towards diseases that are closely related to different mycotoxicoses. Mycotoxins cause a whole range of disorders in the body of animals, ranging from biochemical changes, through the functional and morphological damages of different tissues and organs, to the appearance of clinical signs of mycotoxicoses with even possible fatal consequences (J a k i ć – D i m i ć et al., 2009).

The objectives of this study were to identify the fungi with special focus on *Aspergillus* and *Fusarium* species in freshly harvested maize grains and to quantify the associated mycotoxins.

## MATERIAL AND METHODS

A total of 29 maize grain samples were collected in the experimental maize fields of the Institute for Animal Husbandry (Belgrade) immediately after the harvest in 2012. Samples were collected according to the Commission Regulation (EC) No 401/2006 (E u r o p e a n C o m m i s s i o n, 2006). The total moisture content was determined according to the drying method.

For mycological analysis, kernels were surface-sterilized for 5 min in 5% NaOCl and rinsed three times in sterile water. Fifty kernels (5 kernels per plate 90 mm) from each sample (1450 kernels in total) were transferred to 2% water agar (WA) and the agar plates were incubated at 25ºC in the dark for 5-7 days. The morphological characteristic of isolated genera and species were

identified based on macroscopic (colony appearance) and microscopic (spores size and shape) traits (W a t a n a b e, 1994).

The presence of ZON, DON, FB1 and AFB1 was tested by *enzyme-linked immunosorbent assay* (ELISA) method. Five grams of sample were mixed with 1 g of NaCl and homogenized in 25 ml of 70% methanol in a 250 ml Erlenmeyer flask on the orbital shaker (GFL 3015, Germany) for 30 minutes. Homogenate was filtered through a Whatman filter paper 1. The filtrate was further analyzed according to the manufacturer's instructions Celery Techna ® ELISA kits. Absorbance was measured at a wavelength of 450 nm on an ELISA reader spectrophotometer (Biotek EL x 800TM, USA).

The incidence (I) of certain fungi was estimated according to L e v i ć et al. (2012): I (%) = (number of seeds in which a species occurred/total number of seeds) x 100.

Correlation between the concentrations of the investigated mycotoxins was determined using Pearson's correlation coefficient.

## RESULTS AND DISCUSSION

In mycological study of 1450 maize kernels (50 kernels / sample) with average moisture content of 11.74%, seven fungal genera, *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Rhizopus* were identified. The most common isolated species were from the genera *Rhizopus* (66.41%), *Aspergillus* (43.66%) and *Fusarium* spp. (14.97%), followed by species of genera *Penicillium* (3.31%), *Acremonium* (1.38%), *Alternaria* (0.75%) and *Cladosporium* (0.14%) (Table 1). From the genus *Aspergillus*, species *A. flavus* was more dominant (36.69%) than *A. niger* (6.97%), while from the genus *Fusarium*, two species were identified, *F. verticillioides* with presence of 14.69% which was more dominant than *F. graminearum* (0.28%) (Table 2).

Tab. 1. – Incidence of toxigenic fungi in maize grain samples

| Fungal genera | Number of infected kernels/Total number of investigated kernels | % infected kernels |
|---|---|---|
| *Acremonium* spp. | 20/1450 | 1.38 |
| *Alternaria* spp. | 11/1450 | 0.75 |
| *Aspergillus* spp. | 633/1450 | 43.66 |
| Cladosporium spp. | 2/1450 | 0.14 |
| *Fusarium* spp. | 217/1450 | 14.97 |
| *Penicillium* spp. | 48/1450 | 3.31 |
| *Rhizopus* spp. | 818/1450 | 56.41 |

Tab. 2. – Incidence of toxigenic species from *Aspergillus* and *Fusarium* genera in samples of maize grain

| Fungal species | Number of infected kernels/Total number of investigated kernels | % infected kernels |
|---|---|---|
| *Aspergillus flavus* | 532/1450 | 36.69 |
| *Aspergillus niger* | 101/1450 | 6.97 |
| *Fusarium verticillioides* | 213/1450 | 14.69 |
| *Fusarium graminearum* | 4/1450 | 0.28 |

According to the data from the Republic Hydrometeorological Service of Serbia for 2012, extremely high temperatures (daily temperatures above 25°C) and mostly dry weather with little precipitation (49 mm) were recorded during the reproductive period of maize. These climatic conditions were favorable for the development of some fungal species on maize grain, especially species from the *Aspergillus* and *Fusarium* genera.

Mycotoxicological study of 29 samples of maize grains revealed natural occurrence of four mycotoxins, aflatoxin $B_1$ ($AFB_1$), zearalenone (ZON), deoxynivalenol (DON) and fumonisin $B_1$ ($FB_1$). Average concentrations of fusariotoxins were 98.38 µg kg$^{-1}$ for ZON in 100% positive samples, 235 µg kg$^{-1}$ for DON in 75.68% positive samples and 3590 µg kg$^{-1}$ for 100% $FB_1$ positive samples (Table 3). All investigated samples of maize grain were 100% $AFB_1$ positive and 26 of total 29 samples of maize grain showed concentration >5 µg kg$^{-1}$. $AFB_1$ was detected in 44.83% of the samples with an average concentration of 13.95 µg kg$^{-1}$, while in other samples (55.17%) $AFB_1$ was present in average concentrations of >40 µg kg$^{-1}$.

Tab. 3. – Concentration of mycotoxins in samples of maize grain

| Item | $AFB_1$ | ZON | DON | $FB_1$ |
|---|---|---|---|---|
| Sample size[a] | 13/29+16/29 = 29/29 | 29/29 | 22/29 | 29/29 |
| Incidence (%) | 44.83 + 55.17 = 100 | 100 | 75.86 | 100 |
| Average (µg kg$^{-1}$)[b] | 13.95 + >40 | 98.38 | 235 | 3590.00 |

[a] Number of positive samples/Number of total samples
[b] Average concentration in positive samples

Positive but not significant (r=0.35) co-occurrence of $AFB_1$ and $FB_1$ was established and it was weaker between ZON and DON (r=0.02).

Based on the investigations carried out over the last decade in Serbia, there are more data about natural occurrence of fusariotoxins (J a j i ć et al., 2008a,b; K o k i ć et al., 2009; S t a n k o v i ć et al., 2011), than of $AFB_1$ in maize grains. This can be explained with the fact that *Aspergillus* spp. was not so common in freshly harvested maize during the investigated period. Thirty years ago, there were no significant changes in the distribution of genera *Aspergillus* (5.0%), *Fusarium* (41.0%) and *Penicillium* (46.0%) in maize grain in Serbia (L e v i ć et al., 2004), except in the species composition and its frequency. In recent years, the genus *Fusarium* has been the most common

fungus isolated from freshly harvested maize grains, followed by species from genera *Alternaria*, *Aspergillus*, *Rhizopus* and *Penicillium*. It was reported that among the *Fusarium* species the most dominant were *F. verticillioides* and *F. graminearum* (K r n j a j a et al., 2006, 2011).

In Serbia, there are numerous data about the high presence of *Aspergillus* spp. isolated from different kinds of feed which contained maize. From the samples for feeding dairy cattle K r n j a j a et al. (2008), *Aspergillus* spp. and *Fusarium* spp. were isolated in ground maize samples in the highest percentage (85.70%, 71.40%), while in samples of maize grain this percentage was lower for these fungal species, *Aspergillus* spp. (52.90%) and *Fusarium* spp. (64.70%). The most common species was *A. flavus* with 71.40% in samples of ground maize and 47.10% in samples of maize grain (K r n j a j a et al., 2008). Furthermore, from the samples of feed for dairy cows, the widest spectrum of fungi species was isolated in autumn and the most present were species of *Aspergillus* genus (68.0%), whereas *Penicillium* species were mostly present in summer (94.0%) and winter period (68.0%) (Š k r i n j a r et al., 2008). According to these authors, the presence (56.0%) of species from genera *Fusarium*, *Mucor* and *Penicillium* was equal in spring.

According to the National Regulation in the Republic of Serbia, all tested samples did not show an increase in average concentrations of ZON and DON above the maximum allowed limits, while concentration of FB1 was rather high comparing with maximum allowed limit (4000 μg kg⁻¹) in unprocessed maize. In addition, according to the Regulation on Amendments to the Regulation on the maximum allowed levels of residue of pesticides in food and animal feed, for which maximum allowed quantities of residue of products for plant protection are regulated (Sluzbeni Glasnik RS, 2011), concentrations of AFB1 in 26 of total 29 maize samples exceeded the maximum allowed limit (>5 μg kg⁻¹) in unprocessed maize.

In the first report on DON content in crops in Serbia, J a j i ć et al. (2008a) detected its presence in 44.7% of 76 investigated maize samples from the harvest in 2004, with a range of 40-2460 (on average 536 μg kg⁻¹). In only three samples concentrations were above the maximum level adopted by the European Commission. During the harvest from 2005 to 2007, there were 32.4% DON positive samples in a range of 27-2210 (on average 223 μg kg⁻¹) (J a j i ć et al., 2008b). Fumonisins and DON were found in 75 and 25% of the maize samples collected in 2009, respectively, but none of the samples was contaminated with aflatoxins, ochratoxins and ZON (K o k i ć et al., 2009). FB1 in a range of 750-4300 (on average 1225.7 μg kg⁻¹) were detected in 72% of 203 maize samples collected during 2006-2009 (S t a n k o v i ć et al. 2011). In Turkey, it was reported that incidence of *Penicillium* spp. was significantly higher than *Fusarium* and *Aspergillus* spp. in 28 tested maize samples. Twelve samples demonstrated the level of total aflatoxins of >5 μg kg⁻¹. Aflatoxin levels ranged from 7.70-108.86 μg kg⁻¹. To summarize, 43% of samples were contaminated with AFB1 (A l p t e k i n et al., 2009). Based on the data from R e d d y et al. (2007) and according to the USA Regulation, aflatoxin was above the regulatory limit ([3]20 μg kg⁻¹) in maize grain which was harvested in

2004, while fumonisin showed even higher level (4000 µg kg$^{-1}$). According to the data from R o i g é et al. (2009), *Penicillium* (70%), *Fusarium* (47%) and *Aspergillus* (34%) were the most frequent and abundant genera in maize grains produced in the Southeastern region of the Buenos Aires province. In all samples, DON levels ranged from 240 to 1000 µg kg$^{-1}$, while ZON ranged from >100 to 1560 µg kg$^{-1}$ in six positive samples and AFB1 was detected in two of 58 maize samples (R o i g é et al., 2009). In Malaysia, *A. flavus* (87%), *A. niger* (83%), *F. verticillioides* (47%), *F. graminearum* (43%), *F. proliferatum* (42%), *F. equiseti* (30%) and *Penicillium* spp. (5%) were the prevalent fungi in 80 maize grain samples used for animal feeds. Eighteen (22.5%) samples exceeded AFB1 above the international regulatory limits (legal limits of Argentina, Australia, Brazil, India, Netherlands and USA) of animal feeds (>20 µg kg$^{-1}$) ranging from 20.6-135 µg kg$^{-1}$. Fumonisins were detected in all maize samples (100%) ranging from 261-2420 µg kg$^{-1}$ (R e d d y and S a l l e h, 2011). In Argentina average values of AFB1 for freshly harvested samples of maize were between 0.38 and 2.54 µg kg$^{-1}$ during the period from 1999 to 2010. The average values and frequency of contamination with ZON and DON were low for all the investigated years. The average ZON and DON contaminations showed values from 0 up to 83 µg kg$^{-1}$ (ZON) and from 0 up to 140 µg kg$^{-1}$ (DON) (G a r r i d o et al., 2012).

The co-occurrence of mycotoxins is very important because the combined effects of the toxins can have synergistic or antagonistic effects on animals. In this study, relatively low co-occurrence of AFB1 and FB1 can be explained because *A. flavus* and *F. verticillioides* infect maize ears by different routes. *A. flavus* is a nonpathogenic fungus colonizing through the silk ears and cracks in the pericarp of maize grain and do not need to be seedborne. *F. verticillioides* is endophytic to maize, entering the grain through the pedicle to occupy the internal space distal to the tip cap and is primary seedborne (M e d i n a – M a r t í n e z and M a r t í n e z, 2000).

Weather conditions influence aflatoxin and fumonisin contamination of maize, although *Fusarium* spp. are found in a wider range of climate conditions. Heat stress during the period of kernel development, particularly nighttime temperatures of above 20ºC, is a major factor in mycotoxin contamination (A b b a s et al., 2006). Aflatoxin production by the *Aspergillus* spp. is triggered by drought and high temperature during grain fill. Nitrogen deficiency, excessive plant population, poor root development and insect damage of grains may also induce aflatoxin production in the field. When the weather conditions are favorable for the development of fungi, the fungus may produce aflatoxins at any stages of production and transformations (A l p t e k i n et al., 2009). The optimal conditions for *Fusarium* species, which causes maize ear rot, tend to be hot and dry weather (D o o h a n et al., 2003). According to M i l l e r (2001), the incidence of *Fusarium* kernel rot (*F. verticillioides* and *F. prolifer*atum) is higher in warmer climates under dry conditions. In such environments, insect damage is well recognized as a collateral factor (M i l l e r, 2001). There is no doubt that the climate is the principal factor triggering the fungal attacks, but several other factors may intervene and consequently affect

the incidence of the fungal infection in cereal crops over the regions such as the influence of location, cultivar properties and agricultural practices (crop rotation and management) (G u e r i f et al., 2001). Differences in geographical and environmental conditions might be responsible for differences in fungal distributions and concentration observed among different locations (R o i g é et al., 2009).

## CONCLUSION

The results obtained from this study showed that the most presented toxigenic fungi were from the genera *Aspergillus* and *Fusarium*. It could be assumed that their high incidence in the field resulted in high average concentration of AFB1 and FB1 in maize grain samples. This was also the consequence of exceptional favorable weather conditions during the period of maize maturation. Data obtained in this study indicated that all the subjects included in the technology of maize production should be responsible for the good quality of maize and its products.

Therefore, it is very important for farmers, feed manufacturers, agronomists, plant pathologists, and other human participants to be informed about the risks of consuming moldy maize and maize products. Emphasis should be put on designing the strategies that will develop awareness about the undesirable effects of contaminated grain in human and animal foods and feeds, and on organizing interventions with appropriate crop and commodity management methods to reduce the risk of contamination at the farm gate.

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## ПЛЕСНИ И МИКОТОКСИНИ У ЗРНУ КУКУРУЗА ПОСЛЕ БЕРБЕ

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### Резиме

Учесталост токсигених гљива (плесни) и концентрација микотоксина афлатоксина $B_1$ (AFB$_1$), деоксиниваленола (DON), зеараленона (ZON) и фумонизина $B_1$ (FB$_1$) је проучавана у узорцима зрна кукуруза прикупљеним одмах после бербе у 2012. години. У испитиваних 29 узорака највећу заступљеност имају врсте из родова *Rhizopus* (56,41%), *Aspergillus* (43,66%) и *Fusarium* (14,97%). Значајно нижа учесталост установљена је за врсте рода *Penicillium* (3.31%), а посебно за врсте из родова *Alternaria* (0,75%) и *Cladosporium* (0,14%). Међу токсигеним врстама, *A. flavus* (36,69%) је била најучесталија врста из рода *Aspergillus*, док је *F. verticillioides* са присуством од 14,69% била доминантна врста рода *Fusarium*. У свим испитиваним узорцима кукуруза установљено је присуство микотоксина AFB$_1$, ZON-а и FB$_1$ (100%), осим DON-а који је био присутан у 75,86% узорака. AFB$_1$ је детектован у просечној концентрацији од 13,95 µg kg$^{-1}$ у 44,83% узорака и у просечној концентрацији од више од 40 µg kg$^{-1}$ у 55,17% узорака. Просечна концентрација DON-а је била 235 µg kg$^{-1}$, ZON-а 98,38 µg kg$^{-1}$ и FB$_1$ 3590 µg kg$^{-1}$. Средња позитивна корелација установљена је између концентрација AFB$_1$ и FB$_1$ (r=0,35), док је слаба позитивна корелација утврђена између концентрација ZON-а и DON-а (r=0,02).

КЉУЧНЕ РЕЧИ: зрна кукуруза, плесни, микотоксини

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# INFLUENCE OF MYCOTOXIN ZEARALENONE ON THE SWINE REPRODUCTIVE FAILURE

ABSTRACT: Reproductive failure in swine is often a difficult diagnostic problem. If diagnoses of infectious disease or management related problems are not obtained, feed quality and safety may be questioned. Mycotoxins are often present in swine feed in the amount that can have detrimental impact on production and reproduction. Problems are expressed only as alterations of the reproductive cycle, reduced feed intake, slow growth or impaired feed efficiency. In Serbia, generally speaking, high concentrations of mycotoxins were noticed, especially mycotoxin zearalenone. High presence of zearalenone in swine feed is probably due to climatic influence and should be monitored constantly. This paper includes field observations regarding the influence of moldy feed containing mycotoxin zearalenone on the occurrence of the reproductive failure in swine breeding categories (sows, gilts and boars).

The material for this research was obtained from four swine farms where certain reproductive disorders and health problems in breeding animals were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: anamnestic and clinical evaluation, pathomorphological examination, standard laboratory testing for detection of aerobic and anaerobic bacteria, and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by applying the method of thin layer chromatography.

On the basis of the obtained results, it could be concluded that mycotoxin zearalenone was detected in all examined feed samples. The presence of mycotoxin in feed was directly related to the reproductive failures in the examined swine categories (vulvovaginitis, endometritis, rebreeding, infertility). Swine reproduction represents the base for intensive swine production. The presence of mycotoxins in swine feed have influence on the reproduction and health status of pigs and under certain conditions may significantly disturb the production process.

KEY WORDS: mycotoxins, swine reproductive disorders, zearalenone

## INTRODUCTION

Mycotoxins are structurally a diverse group of mostly small molecular weight components, produced mainly by the secondary metabolism of some filamentous fungi or molds which, under suitable temperature and humidity

conditions, may develop on various food and feeds, causing serious risks for human and animal health. Today, more than 300 mycotoxins have been detected and scientific attention is focused mainly on those that have proven to be carcinogenic and/or toxic. Examples of mycotoxins that are considered to be of significance for public health and agro-economy include aflatoxins (AF), ochratoxins (OCT), trichothecenes, zearalenone (ZEA), fumonisins (F), tremorgenic toxins and ergot alcaloids (Z a i n, 2011). Zearalenone is a mycotoxin produced by *Fusarium graminearum* and other *Fusarium* molds using corn, wheat, barley, oats and sorghum as substrates. Swine are among the most sensitive species to this mycotoxin (D i e k m a n and G r e e n, 1992).

Reproductive failure in swine is often a difficult diagnostic problem. If diagnoses of infectious disease or management problems are not obtained, then the feed quality and safety may be questioned. Experimental studies conducted with the aim of supporting the role of mycotoxin in swine reproduction diseases are limited (O s w e i l e r et al., 1990). Sometimes, conclusions could be drawn from herd observations rather than planned experiments, and mycotoxins are often not measured in the feeds involved (E t i e n n e and J e m m a l i, 1982). This paper included field observations regarding the influence of moldy feed containing ZEA and some other mycotoxins on the occurrence of reproductive failure in swine breeding categories (sows, gilts and boars).

## MATERIAL AND METHODS

The material for this research was obtained from four swine farms where certain reproductive disorders and health problems in breeding animals (sows, gilts, boars) were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: anamnestic and clinical evaluation, pathomorphological examination, standard laboratory testing for detection of the presence of aerobic and anaerobic bacteria in the samples of reproductive organs derived from slaughtered sows and gilts, and microbiological feed testing, performed in order to examine the presence of fungi and mycotoxins by applying the method of thin layer chromatography.

## RESULTS AND DISCUSSION

On the first examined swine farm, based on the anamnestic data on sows that were excluded and reasons for their exclusion, the following reproductive disorders were discovered: rebreeding (27%), infertility (20%), anestrous (10.6%) and frequently observed endometritis. Due to the reproductive problems, the parity structure of the herd was altered. The highest percent of exclusion was related to the first litter sows, i. e. with the first and the second parity (43%) and in the herd the maiden gilts were introduced. Most of the rebreedings were in relation to the first and second expected oestrus. The oc-

currence of the increased number of dead born and mummified piglets was not detected. However, in certain number of boars high percent of litters with small piglets was evident. Besides this, the occurrence of neonatal diarrhea in suckling piglets was detected already in the first 3 days of life after farrowing. The most prominent clinical sign in large number of farrowed piglets was vulvovaginitis (swelling and reddening of the vulva). By patomorphological examination of genital organs (ovaria, uterus) from excluded females in the slaughter-house, the following lesions were discovered: 24% ovaries in the luteal phase, 16% ovaries in the follicular phase and 2% with pathological changes (cysts and fibrosis). Only 20% of the examined ovaries was recorded to have the ovulation rate 20 and above. Application of pathological control of reproductive organs of excluded sows, a significant percent of endometritis (24%) was discovered (presence of liquid muddy content in the uterus with small pieces of destroyed tissue or content that looked like a sour cream). Bacteriological tests of tissue samples from the dams genital organs showed the presence of *Streptococcus dysgalactiae subsp.equisimilis, Staphylococcus haemolyticus, Escherichia coli, Pasteurella aerogenes, Streptococcus uberis.* Having in mind the clinical and pathological symptoms observed, especially the signs of vulvovaginitis in just farrowed piglets, a justified suspicion on the presence of mycotoxins in feed was made. After laboratory tests a total number of fungi in the large number of microbiologically examined feeds was discovered: corn ($887x10^3$ *Aspergillus*, *Rhisopus*), feed for pregnant sows ($123x10^3$ *Penicillium, Fusarium*), feed for lactating sows ($526x10^3$ *Aspergillus, Penicillium,Mucor*) and feed for boars ($177 x 10^3$ *Aspergillus, Penicillium, Mucor*). The presence of mycotoxins was detected: ZEA in the feed for pregnant sows (0.72 mg/kg) and OCT (0.08 mg/kg) in the feed for boars.

Although the level of detected mycotoxins in the feed for pregnant sows and boars, obtained from the first examined farm, was lower when compared to the level set by the regulation, a cumulative effect of mycotoxins in organisms should be considered as well. Many toxigenic strains of molds can occur in grains without any production of mycotoxins, and there is little correlation between spore counts or degree of fungal growth and presence of mycotoxins. Conversely, absence of molds does not mean that feed is safe from mycotoxins (O s w e i l e r, 2006).

Combinations of some mycotoxins may potentiate the action of one another, or at least exert an additional effect. With respect to the known mycotoxins that are of clinical importance, the response is usually subacute or chronic and the presenting signs are often subtle and vague (P r o d a n o v et al., 2009). Problems are frequently expressed only as alterations of the reproductive cycle, reduced feed intake, slow growth or impaired feed efficiency (O s w e i l e r, 2006). Zearalenone affects the reproduction of livestock most seriously because it possesses estrogenic activity. Other mycotoxins affect the reproduction in livestock via indirect means such as reduced feed intake or reduced growth, or by damaging other vital organs of the body (D i e k m a n and G r e e n, 1992). We need to consider that feed samples available at the

time of anestrus or return to service may not represent the contaminated feed that initiated the problem (O s w e i l e r, 2006).

On the second examined swine farm, clinical examination of swines in the farrowing hause showed that the most prominent clinical sign in the piglets of different age was vulvovaginitis. The litter size varied significantly, whilst the small litter dominated in the recently farrowed sows and gilts. In about 30% of farrowed sows the mastitis-metritis-agalactiae syndrome was discovered. In weaned piglets and fatteners the vulvovaginitis in the almost all female pigs was noticed as well as low weight gain. By clinical examination in the mating and waiting place, the impression was that all animals were in the heat, while the boars were uninterested for the jump (weakened libido). Consequently, the lower number of sows was moved to the farrowing house. There were no rebreedings and dead born piglets because there were no pregnant females. The mating and waiting house was overcrowded and the number of fatteners progresively decreased. Organoleptic control of the farm storage space showed, approximately, 70–80% of moldy ear – corn. After laboratory tests of corn samples the presence of ZEA from 14 to 36 mg/kg, depending on the sampling place, was detected. Three weeks after the introduction of artificially dried corn in swine feed, the signs of vulvovaginitis became less evident, the mating was normalized but the farrowing house was empty. The initial number of females on the farm significantly decreased because there were no signs of heat and no pregnancy, and gilts and sows were slaughtered. Pathological control performed in the slaughter house on all examined females detected cystic degeneration of ovaries, enlargement of the uterus and hyperemic mucosa. Frequently, mucopurulent content in uterus was discovered.

Mycotoxin ZEA has a unique nonsteroidal resorcyclic acid lactone structure. This structure resembles steroid hormones and allows ZEA to bind to estrogen receptors, where it acts as an agonist and partial antagonist to estradiol. ZEA induces estrogenic effects, often reported as hyperestrogenism, in all species tested, particularly in pigs (M a l e k i n e j a d et al., 2007). ZEA inhibits the release and secretion of follicle stimulating hormone (FSH) to depress the maturation of ovarian follicles during the preovulation stage. This results in atresia of follicles becoming the prominent histological trait and resulting in atrophy of ovaries (D i a z – L l a n o and S m i t h, 2007). Clinical signs of ZEA mycotoxicosis vary with dosage and age of swine exposed. In prepubertal gilts, ZEA causes vulvovaginitis, which is characterized by tumescence and edema of the vulva and vagina and precocious mammary development. As with other estrogens, ZEA is luteotropic in swine and can induce anestrus in sows if consumed during the middle portion of the estrous cycle. Piglets born from sows receiving ZEA may have enlarged external genitalia and uteri (O s w e i l e r, 2006). ZEA and its metabolites, alpha and beta ZEA, can cross the placenta and be present in milk of exposed sows, risking the exposure of the embryo and neonate (M a l e k i n e j a d et al., 2007) and contributing to estrogenic effects in piglets (perinatal hyperestrogenic syndrom). Lower conception rate, increased number of repeated breeders, decreased litter size, increased number of stillbirths are frequently observed. Clinical signs

in neonatal gilts include swelling of the vulva and teats, edematous infiltration of the perineal region, ventral abdomen and umbilicus, usually accompanied by exudative, crusted inflammation and necrosis of the teats. An increase of splayleg and trembling piglets has been reported. Pathological lesions of hyperestrogenism included enlargement of the ovary and uterus, ovarian follicle maturation, glandular proliferation of the endometrium and epithelial proliferation of the vagina. Young boars may have reduced libido and decreased testicular size (O s w e i l e r, 2006).

In Serbia, generally speaking, alarmingly high concentrations of mycotoxins were noticed, especially increased concentration of ZEA. High presence of ZEA in swine feed is probably due to climatic influence and should be monitored constantly (P e t r u j k i ć et al., 2008).

The results from the third examined farm included prolonged gravidity period and farrowing time of sows, cases of agalactia (sudden loss of milk and lying on the udder), a small number of stillbirths and mummified piglets. The newborn piglets were described as weak, nonviable, with diarrhea. The diseased piglets lived only for 4 days after the birth. They probably died due to hypoglicemia because sows did not have enough milk or the piglets were too weak and did not have enough strength for milk suckling. Sporadically, the occurrence of splayleg was observed. The sows had good body condition in all production stages but an increase in body temperature was detected after parturition in large number of sows (48%). The abortions and rebreeding were not registered, and no connection of the above mentioned problems with the parity was observed. Conducted laboratory tests on feed showed that there was simultaneous presence of several mycotoxins (ZEA, AFB1, AFG1, OCT). Mycotoxins were detected in feed for pregnant and lactating sows (ZEA 0.8 mg/kg; AFB1 0.008 mg/kg; AFG1 0.02 mg/kg; OCT 0.2 mg/kg), in corn (ZEA 4 mg/kg; AFB1 0.008 mg/kg; AFG1 0.002 mg/kg; OCT 0.2 mg/kg), sunflower pellets (ZEA 4 mg/kg; AFB1 0.016 mg/kg; AFG1 0.008 mg/kg; OCT 1.0 mg/kg) and soyabean pellets (ZEA 2.0 mg/kg; AFB1 0.016 mg/kg; AFG1 0.008 mg/kg; OCT 1.0 mg/kg).

Mycotoxin mixtures, i.e. the combinations of several mycotoxins, can normally occur and they may affect the immunity in an additional or synergistic manner. The presence of mycotoxins in feed for pregnant sows causes the occurrence of embrional and fetal death and decreased immunological defense in piglets (P r o d a n o v – R a d u l o v i ć et al., 2011). Feeding lactating sows with grains naturally contaminated with *Fusarium* mycotoxins results in reduced feed intake and increased body weight loss in piglets, but no changes in milk composition or milk production are detected. The reduced feed intake and losses of body tissues tend to increase the weaning to estrus interval (D i a z – L l a n o and S m i t h, 2007).

Even though it is not clear whether ZEA consumption affects the onset of puberty, experimental results indicate that the estrogenic properties of ZEA are not permanently damaging and that gilts may successfully enter the breeding herd without subsequent reduction in fertility if they are given a 2 week withdrawal period. ZEA causes multiple reproductive dysfunctions in mature,

cycling gilts (constant estrus, pseudopregnancy, and ultimately, infertility) and if it is added to the diet of pregnant sows it can cause them to farrow smaller litters with smaller offspring (D i e k m a n and G r e e n, 1992).

Complex health problems, including significant reproductive disturbances, were observed on the fourth swine farm. By applying the control of anamnestic data, frequent cases of sows delivering mummified piglets, stillbirths and decreased litter size were observed. Also, the increased number of rebreeding sows at irregular intervals was discovered. The conception rate dramatically decreased and the problem with frequent abortions, occurring 2 months before farrowing, was intensified. Besides this, just farrowed piglets were non-viable and despite the medical treatment they lived only for 3-4 days after birth. Sporadically, the occurrence of severe yellowish diarrhea in piglets and apparent clinical signs of vulvovaginitis in just born piglets were evident. After conducted laboratory testing of swine feed samples the simultaneous presence of several mycotoxins was recorded: ZEA (6.4 mg/kg), AF (0.0064 mg/kg) and OCT (0.032 mg/kg).

The effects of ZEA in mature gilts and sows were related to the period of contaminated feed intake, i.e. mating period, the concentration in the diet and the duration of administration. The most apparent effect of feeding the mature gilts with low concentrations of ZEA was increased pseudopregnancy if the contaminated diet was fed prior to mating (Y o u n g and K i n g, 1986). ZEA ingestion by mature gilts may produce two different effects which are related to estrogenic properties of this mycotoxin. When females are not pregnant, ZEA induces a pseudopregnancy state characterized by uterine hypertrophy and corpora lutea maintenance on ovaries. Sows do not cycle and cannot be bred. These consequences may be of great economical importance because they can disrupt a breeding program. Secondly, when fed during gestation, ZEA reduces development of the uterus, placental membranes and fetuses. These effects may induce lower embryonic survival or higher rate of immature piglets at birth, which, are less able to suckle and may die within a few days after farrowing (E t i e n n e and J e m m a l i, 1982).

Main difficulty in assessing the risk of mycotoxins to animal health is the multiplicity of factors affecting the production or presence of mycotoxins in feeds. Presence of molds is not necessarily accompanied with the production of toxins. Thus, the demonstration of mold contamination does not easily prove that there are etiological agents in a given veterinary health problem. The incidence of mycotoxicoses may be more common than suspected (Z a i n, 2011). It is considered that pigs with the signs of ZEA mycotoxicosis should not be selected for reproduction, i.e. as breeding animals. Residues of ZEA in pork meat represent a potential danger for humans who are the consumers of contaminated meat. Animals that consumed feed contaminated with ZEA should be slaughtered 3-4 weeks after the withdrawal of feed (P o p o v i ć, 2007). The basic preventive protection measures for animals are the use of healthy feed and proper storage and management conditions for animals feed. Certainly, when mycotoxicosis occurs or is suspected, the first action should be to change the source of feed (P r o d a n o v et al., 2009).

## CONCLUSION

On the basis of the obtained results, it may be concluded that mycotoxin ZEA was detected in all examined feed samples. The presence of mycotoxin in feed was directly related to the reproductive failures in the examined swine categories (vulvovaginitis, endometritis, rebreeding, infertility). Swine reproduction represents the base for intensive swine production. The presence of mycotoxins in swine feed afects the reproduction and health status of pigs and, under certain conditions, it may significantly disturb the production process.

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## УТИЦАЈ МИКОТОКСИНА ЗЕАРАЛЕНОНА НА ПОРЕМЕЋАЈЕ У РЕПРОДУКЦИЈИ СВИЊА

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### Резиме

Поремећаји у репродукцији свиња представљају озбиљан дијагностички проблем. Уколико се не дијагностикују инфективно обољење или проблеми у менаџменту на фарми свиња, оправдано се поставља сумња на исправност квалитета и сигурност хране за исхрану свиња. У храни за свиње често су присутни микотоксини у количини која има штетан утицај на производњу и репродукцију. У великом броју случајева проблеми се бележе у виду репродуктивних поремећаја, слабије конзумације хране или прираста. У Србији је утврђено присуство високих концентрација микотоксина, нарочито зеараленона. Сматра се да је присуство високих концентрација зеараленона у храни за свиње вероватно последица утицаја климатских фактора, што захтева константну контролу. У раду су приказани резултати теренских испитивања утицаја плесниве хране која садржи микотоксин зеараленон на појаву репродуктивних поремећаја код приоплодних категорија свиња (крмаче, назимице, нерастови).

Материјал за испитивање је обухватао четири фарме свиња, на којима су регистровани одређени репродуктивни поремећаји и здравствени проблеми приплодних јединки. У зависности од специфичности испитиваног случаја и доступног материјала, примењене су следеће методе испитивања: анамнестичка и клиничка испитивања, патоморфолошки преглед, стандардне лабораторијске методе за утврђивање присуства аеробних и анаеробних бактерија у узорцима репродуктивних органа угинулих јединки и микробиолошко испитивање узорака хране у циљу установљавања присуства плесни и микотоксина, методом танкослојне хроматографије.

Постигнути резултати испитивања указују да је у свим испитаним узорцима хране за свиње установљено присуство микотоксина зеареленона. Присуство овог микотоксина у храни се директно доводи у везу са утврђеним репродуктивним поремећајима код испитиваних категорија свиња (вулвовагинитиси, ендометритиси, поваћање, инфертилост). Репродукција представља основу интензивне свињарске производње. Присуство микотоксина у храни утиче на репродукцију и здраствено стање свиња и у одређеним условима може знатно нарушити процес производње.

КЉУЧНЕ РЕЧИ: микотоксини, поремећаји у репродукцији свиња, зеараленон

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# PRESENCE OF T-2 AND HT-2 TOXINS IN MAIZE

ABSTRACT: Data on the occurrence of T-2 and HT-2 toxins in maize from Serbia and Europe are very limited. In this study a total of 50 maize samples harvested during September and October 2012 in Serbia were analyzed. Presence of T-2/HT-2 toxins was determined by enzyme-linked immunosorbent assay (ELISA) method. Among the 50 analyzed maize samples even 26 (52.0%) samples were contaminated with T-2/HT-2 toxins. Concentration interval between 25-60 μg/kg and 60-200 μg/kg were found in 46.0% and 6.0% of analyzed maize samples, respectively.
KEY WORDS: T-2/HT-2 toxins, maize, ELISA

## INTRODUCTION

T-2 and HT-2 toxins are members of a large group of fungal sesquiterpenes, commonly marked as type of A trichothecenes. They are produced by various *Fusarium* species, including *F. sporotrichoides*, *F. poae*, *F equiseti*, *F. acumninatum*, as well as species from the genera *Myrothecium*, *Cephalosporum*, *Verticimonosporum*, *Trichoderma, Trichothecium* and *Stachybotrys* (EFSA, 2011). One of the major producers of T-2 and HT-2 toxins is *F. sporotrichioides*, which can grow in temperature interval ranging from – 2 to 35 °C and with water activities above 0.88 (R i c h a r d, 2007; C r e p p y, 2002). Previous studies have shown that the presence of T-2 and HT-2 toxins is quite often related to grains such as wheat, maize, oats, barley, rice, beans, soya beans and their derived products (SCF, 2001). HT-2 toxin is a metabolite of T-2 toxin and it is formed in microbial transformation via deacetylation reaction (Z h o u et al., 2008). In general, trichothecenes are very stable compounds during storage, milling, processing, and cooking at high temperatures. T-2 toxin is more rapidly metabolized than HT-2 toxin which is also the main metabolite *in vivo* (E r i k s e n and A l e x a n d e r, 1998); also, they can be metabolized by various animals but can also be metabolized by plants and fungi (D o h n a l et al., 2008). T-2 toxin is one of the most toxic trichothecenes with potent inhibitory influence on DNA, RNA and protein synthesis, and it

also shows immunosuppressive and cytotoxic effects both *in vivo* and *in vitro*. T-2 toxin is a very potent cytotoxic and immunosuppressive toxin, which can cause acute intoxication and chronic diseases in both humans and animals (U e n o et al., 1983; P e r a i c a et al., 1999; Z h o u et al., 2008). The symptoms of acute intoxication are nausea, vomiting, abdominal pain, diarrhea, bloody stools and weight loss. The major effect of T-2 toxin is inhibition of protein synthesis which leads to secondary disruption of DNA and RNA synthesis (R i c h a r d, 2007; C r e p p y, 2002; B e n n e t t and K l i c h, 2003). The immune system is also a target of T-2 toxin and the effect includes changes in leukocyte count, delayed hypersensitivity, depletion of selective blood cell progenitors, depressed antibody formation, allograft rejection and blastogenic response to lectins (C r e p p y, 2002).

Recent data, collected by the EU member states in order to evaluate the risk of dietary exposure to *Fusarium* toxins, have shown that T-2 and HT-2 toxins are quite common contaminants in cereals in the EU (SCF, 2001). The joint FAO/WHO expert committee on food and additives (JECFA, 2001) established a permissible limit of tolerable daily intake (TDI) value of 0.06 µg/kg body weight for T-2 toxin and HT-2 toxin, alone or in combination.

Worldwide, 13 countries have reported legal maximum levels (MLs) or recommendations for T-2 and HT-2 toxins in food and feed products. The following European countries have prescribed regulations for T-2 and HT-2 toxins: in Armenia the maximum allowed level is 100 µg/kg for T-2 toxin in all types of food; in Hungary 300 µg/kg for T-2 toxin in milled products, and 300 µg/kg for the sum of T-2/HT-2 toxins cereal constituents of muesli; in Moldova 100 µg/kg for T-2 toxin in cereals and cereal flour; in Norway 100 µg/kg for T-2 and HT-2 toxins in cereals and cereal products, and 50 µg/kg for T-2 and HT-2 toxins in cereals and cereal products for infants and young children; in Russia 100 µg/kg for T-2 toxin in barley, and in Ukraine 100 µg/kg for T-2 toxin in grains, flour, wheat middlings, bread products, and all seeds used for immediate human consumption and for processing in the products for human consumption. As it can be seen, MLs for T-2 and HT-2 toxins in food are mainly regulated in Eastern Europe. Further, other countries (Canada, China, Croatia, Iran, Israel, Norway, Serbia, and Ukraine) have set maximum levels for T-2 toxin in feed products, mostly ranging from 25 to 1000 µg/kg (EFSA, 2011).

To protect the health of consumers, the commission of the European Communities established the maximum level for most studied mycotoxins in cereals (EC, 2006; EC, 2007), while regulations regarding HT-2 and T-2 toxins in cereals have not yet been established.

Maximum levels of T-2 and HT-2 toxins in food are not regulated even in Serbia, but it is regulated for compounds and complementary feed mixtures for chickens, piglets and calves, and compound and complementary feed mixtures for pigs and poultry with maximum level of T-2 toxin of 500 µg/kg, 1000 µg/kg, respectively. Also, the maximum allowed level for total trichothecenes is 300 µg/kg for feed mixtures for chickens, piglets and calves, and 600 µg/kg for feed mixtures for cows and poultry (Službeni Glasnik RS, 2010).

Due to the fact that there is no available data on the occurrence of T-2 and HT-2 toxins in maize from Serbia, the aim of this study was to investigate the presence of sum of T-2/HT2 toxins in maize for human consumption and animal feed products.

## MATERIALS AND METHOD

### *Samples*

A total of 50 maize samples were collected in Serbia. Samples were collected after harvest, during September and October, 2012. All samples were stored at 4 °C in a refrigerator before analysis.

### *Sample preparation*

After grinding, 5 g of maize sample was extracted with 25 ml of methanol/deionized water (70/30; v/v) solution and it was shaked vigorously for 3 minutes, followed by extract filtering through a filter paper (Whatman, Black Ribbon). The filtrate obtained was diluted 1:1 (e.g. 1 ml in 1 ml) with deionized water and it was mixed by Vortex.

### *Determination of sum of T-2/HT-2 toxins*

Content of T-2/HT-2 toxins was determined by the enzyme immunosorbent assay method (ELISA). All samples were analyzed in duplicate with Quantitative T-2/HT-2 Toxins test kit (Neogen Veratox®, Lansing, USA). Range of quantification for T-2/HT-2 toxins test kit was between 25 – 250 µg/kg and analysis were done according to the manufacturer´s description.

Free T-2 or HT-2 toxins in the samples and controls are allowed to compete with enzyme- labeled HT-2 toxin (conjugate) for the antibody binding sites. After a wash step, substrate was added and it reacted with the bound conjugate to produce blue color. More blue color meant less

T-2/HT-2 toxins. The test was read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls formed a standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of

T-2/HT-2 toxins. According to the manufacturer's description the detection limit for T-2/HT-2 toxins was 25 µg/kg.

## RESULTS AND DISCUSSION

In this study, 50 maize samples were analyzed to evaluate the sum of T-2/HT-2 toxins contamination. The obtained results (Table 1) showed that

T-2/TH-2 toxins were found in 26 (52.0%) samples with concentrations over 25 µg/kg. Concentrations of T-2/TH-2 toxins in the positive samples ranged from 25.3 to 185.2 µg/kg. Positive results were classified into two groups. Results for the sum of T-2/HT-2 showed that 24 (48.0%) of the analyzed maize samples contained less than 25 µg/kg, while concentrations that ranged from 25-60 µg/kg and 60-200 µg/kg were found in 23 (46.0%) and 3 (6.0%) samples, respectively. Mean level of all positive samples was 25.3 µg/kg.

Tab. 1. – Contamination frequency (CF), interval (CI) and mean level (CM±SD) of T-2/HT-2 toxins

|  | < 25 µg/kg | 25–60 µg/kg | 60–200 µg/kg |
| --- | --- | --- | --- |
| CF | 48.0 | 46.0 | 6.0 |
| CI | – | 25.3–57.7 | 122.9–185.2 |
| CM | – | 34.7 ± 7.95 | 154.1 ± 31.2 |

CF(%), CI (µg/kg), CM (µg/kg) and SD (standard deviation)

Given the fact that high mycotoxin concentrations are usually associated with climate changes, in particular humidity and temperature as the factors most critical for mould formation and mycotoxin production, the obtained medium average contamination levels observed in this study could be explained by the fact that in 2012 the investigated parts of our country were extremely warm and dry throughout the vegetation period of maize (Republic Hydrometeorological Service of Serbia, 2012).

Most of the previously mentioned countries that prescribed regulations for T-2 and sum of T-2/HT-2 toxins set 100 µg/kg as the maximum limit for cereals. According to the obtained results, 3 (6%) of the 50 analyzed maize samples contained concentration of T-2/HT-2 toxins that was greater than 100 µg/kg.

Due to the lack of available data on the occurrence of T-2/HT-2 toxins in maize from Serbia, we analyzed the results from the region. J a k o v a c – S t r a j n  et al. (2010) reported that analyzed grains from Slovenia were not contaminated with HT-2 and T-2 toxins. V u l i ć et al. (2011) investigated the presence of T-2/HT-2 toxin in 25 feed samples and 25 commodities from Croatia. They reported that 76% of analyzed feed samples were contaminated with T-2/HT-2 in the concentrations that ranged from 6.09 to 67.7 µg/kg. Out of all analyzed commodities oat, barley, wheat, corn and soya beans contained the highest concentrations which were 32.9, 23.1, 6.91, 5.02 and 3.17 µg/kg, respectively.

Furthermore, B i n d e r et al. (2007) reported that 1 out of 18 maize samples collected in different European farms between October 2003 and December 2005 was positive for T-2 toxin. Greater number of cereal samples (63 maize, 51 wheat, 34 barley and 33 oat samples) was analyzed by P l e a d i n et al. (2013). The examined samples were collected from different fields situated in one of the six Croatian regions, and analyzed by using ELISA method. T-2 toxin was detected in 36 (57%) analyzed maize samples with average concentrations of 24.0 µg/kg. The obtained concentration of T-2 toxin ranged from 5 to 42 µg/kg.

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Results obtained in this study are similar with the results obtained from the region regarding T-2/HT-2 occurrence in maize. Based on the results, it can be concluded that it will be necessary to continue the control of the presence of T-2/HT-2 in maize and other types of food and feed.

## CONCLUSION

Considering the high toxicity of T-2 and HT-2 toxins, there is a need for collecting more data on their occurrence in food and feed and setting the maximum limits for these mycotoxins in food from Serbia.

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ПРИСУСТВО Т-2 И НТ-2 ТОКСИНА У КУКУРУЗУ

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Резиме

Подаци о појави Т-2 и НТ-2 токсина у кукурузу су веома ограничени како у Србији тако и у Европи. У току истраживања анализирани су узорци кукуруза који су сакупљани на територији Србије непосредно након бербе 2012. године. Имуноафинитетном методом (ELISA) анализирано је присуство суме Т-2 и НТ-2 токсина у 50 узорака кукуруза. Од укупног броја анализираних узорака кукуруза у 24 (48,0%) није детектовано присуство Т-2 и НТ-2 токсина, док је 26 (52,0%) узорака било позитивно на присуство ових микотоксина. Од позитивних узорака 46,0% било је контаминирано концентрацијом Т-2 и НТ-2 токсина у опсегу од 25 до 60 μg/kg, док је 6,0% испитаних узорака кукуруза садржало концентрацију у опсегу од 60 до 200 μg/kg.
КЉУЧНЕ РЕЧИ: Т-2/НТ-2 токсини, кукуруз, ELISA

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# CLINICAL AND PATHOMORPHOLOGICAL DIAGNOSTICS OF MYCOTOXICOSIS IN PARENT POULTRY FLOCK CAUSED BY T-2 TRYCHOTECENE

ABSTRACT: The most relevant mycotoxin from the trychotecene group, T-2, causes prominent citotoxic effects. The toxin is a secondary product of fungi from the genus *Fusarium* that contaminates feed. Oraly intaken, T-2 is absorbed fast in the upper digestive system and within only 3 to 4 hours later reaches liver, kidneys and muscle tissue. Clinical and pathological changes are sometimes not obvious.

The case of mycotoxicosis in a breeder flock of chickens, here presented, is aimed to underline the significance of clinical and pathological diagnosis supported with laboratory analysis that gave an objective causative diagnosis.

On the farm, the disease occurred suddenly and with total cessation of feed consumption. First cases were recorded in the flock at the age of 42 weeks. Grouping, intensive breathing and lying with overstretched legs and extended neck were symptoms observed in birds. Evident necrosis of beak tips and painful multi-focal necrosis in oral cavity were recorded during the clinical examination. On section, dark unclothed blood was first observed. Other *postmortem* findings included: filled gizzard with mucosal erosions and easy-removable cuticle, enlarged congested liver with multi-focal necrosis and subcapsulary bleeding. The mortality rate increased by 4%, and the drop of laying rate was by about 18%. The fertility rate decreased by 22%. There was the increased number of rejected hatching eggs, 12%.

Culture of the complete diet resulted in approximately 150000 colonies per 1g of *Fusarium*. T-2 was detected by using ELISA in concentration of 480 µg/kg, which corresponded to the upper limit of maximum permitted concentrations for chickens, according to national legislations. This bylaw interpretation of "tolerable" concentrations of mycotoxins provokes controversy among experts and public.

KEY WORDS: clinical and pathological diagnostics, poultry, mycotoxins, T-2, legislative

## INTRODUCTION

Diseases caused by mycotoxins are non-contagious and usually feed related. Treatment with antibiotics or other drugs in general is not efficient. Due to their small molecular weight mycotoxins do not provoke immune response.

In practice, chronic intoxication occurs more frequently. Small concentration intake over a prolonged period and *vice versa,* higher concentration of mycotoxins for a short period of time have the same effects (R a f a i et al., 2000; N e š i ć et al., 2005).

Based on the target tissue, mycotoxins are classified as hepato-, nephro-, neuro- and cyto-toxins (S i n o v e c et al., 2000). Their biological effects are diverse: cancerogenic, mutagenic and theratogenic, immunomodulation and protein synthesis inhibition (S i n o v e c et al., 1993). The toxic effects and severity of the mycotoxin related disease depend primarily on the group and concentration of mycotoxins, relative duration of the exposure, the species, gender and age of poultry, general health and immune status, zoohygiene and zootechnology, nutrition and feeding regimen (Binder et al., 2007).

In the veterinary medicine, T-2 toxin that expresses prominent cytotoxic effect is one of the most relevant trychotecene toxins. It is produced by fungi from the genus *Fusarium* that contaminate crops in fields and feed during storage. Microorganisms grow under different environmental conditions and produce toxin at lower temperature range, from 4 to $8^0$C, but not at $32^0$C (Q u i n n et al., 1998; J a c o b s e n et al.. 2013).

Orally intaken, the resorption of T-2 in the upper digestive tract is fast and after 3 to 4 hours reaches liver, kidneys and muscle tissue (S i n o v e c and J o v a n o v i ć, 1993). Residual concentrations of T-2 can be detected in eggs and meat. In comparison to chickens, turkeys and geese are more sensitive to T-2 (S i n o v e c et al., 2006).

In poultry, alimentary and nervous system disorders occur in cases of acute intoxication with trychotecenes. The letargy of birds, hyperpnoe and loss of balance are present. Haemorhage is a regular finding in the alimentary tract and muscle tissue, as well as prominent focal necrosis and ulcerations in oral cavity and stomatitis. During the chronic course, decrease of feed consumption and body weight are recorded, and oral lesions and irregular moving are evident.

For several decades, mycotoxin related problems have represented a challenge for the researchers, veterinarians and farmers. Some confusion exists in relation to the clinical and pathomorphological diagnostics of the disease complex caused by mycotoxins (N e š i ć et al., 2011). In this paper, a case of mycotoxicosis in a parent poultry flock was presented in order to show how important it is to make diagnosis beforehand, clinically and pathologically. Finally, objective causative diagnosis was confirmed with laboratory findings.


## MATERIAL AND METHODS

The parent flock of heavy breed Ross 308 was relocated in winter to an exploitation farm with two separate houses, on the ground and the first floor, each containing 2200 birds. The complete diet produced for the heavy parent hybrid was purchased from one supplier.

The outbreak of the disease was observed simultaneously in both houses in 42 week old breeders which corresponded to the passed peak of production.

Based on the course, clinical observation and pathomorphological examination, the case of mycotoxicosis was suspected.

Feed was sampled and submitted for microbiological analysis. The samples were cultured on Sabouroud agar. After the incubation period, the grown colonies of fungi were purified and identified.

The content of T-2 was determined in feed using ELISA test, Ridascreen® (Art.No. R:3801, R-Biopharm, Germany), with detection limit 3.5 pp (R-biopharm, www.r-biopharm.de). The readings were processed in a software package Softv Rida®Soft Win (Art. No. Z9999, R-Biopharm, Germany) and the obtained results were interpreted according to the instructions provided by the manufacturer.


## RESULTS AND DISCUSSION

Weak activity and grouping along the side walls in the flock was first observed by the workers in the early morning. A veterinary clinical observation revealed the following:
- grouping of breeders in island-like formations close to the side walls leaving the central part of the house line feeders and drinkers almost empty,
- the feed consumption completely stopped,
- a minor part of the birds that were standing had difficulties to move, supporting themselves with extended wings (Fig.1),
- yellow to yellowish-green diarrhea and large quantity of feathers were observed on the litter,
- birds with retracted necks and poorly feathered were mostly lying with extended neck and overstretched legs, opened beak and accelerated breathing,
- beak necrosis and painful multi- focal necrosis in oral cavity were obvious (Fig. 2).

On necropsy, pathomorphological changes found were identical in all carcasses. They were all found in a characteristic position with extended neck, overstreched legs and with filthy feathers on abdomen and around the cloaca due to the yellowish to green diarrhea. Unclothed, dark colored blood was first observed at necropsy. Other *postmortem* findings included: gizzard filled with feed and mucosal erosions, and easy-removable cuticle (Fig.3), enlarged congested liver with multi-focal necrosis and subcapsulary bleeding (Fig.4).

The dynamics of mortality and production parameters presented in Table 1 contain data for the age of 41, 42 and 43 weeks. The mortality rate increased by 4%, and the drop of laying rate was by about 18%. The fertility rate was decreased by 22%. There was an increased number of rejected hatching eggs of 12.2%. Clearly, all production parameters were violated. Although slow improvements in the flock started, the secondary loss was reflected in poor vitality of the one day old chickens that caused an increased number of afterclaims.

Tab. 1. – Mortality rate and production in breeders including one week before and after the outbreak.

| Flock age (week) | Mortality rate (%) | Egg production (%) | Rejected eggs (%) | Fertilized eggs (%) | Hatched chickens (%) |
|---|---|---|---|---|---|
| 41 | 0 | 72.5 | 0.8 | 92 | 83 |
| 42 | 4.25 | 54.6 | 13 | 70 | 61 |
| 43 | Parent flock was excluded from the production | | | | |

The microbiological analysis – culture of complete diet, declared for nutrition of adult producing breeder chicken flock, was positive only to *Fusarium* fungi, in the calculated quantity of approximately 150000 colonies per gram. This result was at the upper limit of maximum permitted number of colonies for adult poultry (Službeni Glasnik, 2010). The T-2 concentration of 480 μg/kg was detected by ELISA, which was also at the upper limit of maximum permitted concentration for adult poultry (Službeni Glasnik, 2010).

Sudden clinical disease on the farm with high mortality and typical pathological changes, indicated that cumulative effects of T-2 were expressed. Numerous reports can be found on the death outcome in domestic animals due to consumption of the contaminated feed (P r o d a n o v et. al, 2011). In the literature, similar *postmortem* findings were described and it was noted that in comparison to chickens, species including turkey and goose were more susceptible to the toxin (S i n o v e c et al., 2006). Gross changes of the "X Disease" in turkeys, first described by W a n n o p (1961), were directed toward possible mycotoxicosis.

Until the laboratory confirmation was obtained, preliminary clinical and pathological diagnosis indicated taht the following procedures had to be undertaken: First was to empty the feeding lines and replace the existing diet with the reliable safe one. The second action was supportive therapy with commercial product *Evitaselen* containing vitamine E and selenium. The late replacement of diet was unable to moderate the disease because of irreversible pathological changes. Early and beforehand mycotoxin detection and exclusion of the particular diet may help to mitigate the detrimental effects (N e š i ć et al., 2005). On farms with veterinary staff employed, it is possible to prevent, avoid or repair such losses. In this case further exploitation of the flock was found economically unjustified and it was excluded.

The laboratory findings of 150000 colonies per gram of *Fusarium* in the diet and the detected T-2 concentration of 480 μg/kg were high, nevertheless acceptable for consumption in adult poultry, according to the national regulations (Službeni Glasnik, 2010). Such interpretation provokes strong controversy among the experts. There can be no threshold concentration for the negative effects caused by mycotoxins and clearly, daily tolerable concentrations are impossible to determine (S i n o v e c et al., 2000; J a k i ć – D i m i ć et al., 2010; J a k š i ć et al., 2011). The regulations on the content of such and similar harmful substances in feed need to be corrected, particularly for more susceptible species and categories of poultry.

Fig. 1. – Flock grouping in island – like formation.



Fig. 2. – Necrotic process in oral cavity.



Fig. 3. – Gizzard: cuticle and mucosal erosions.
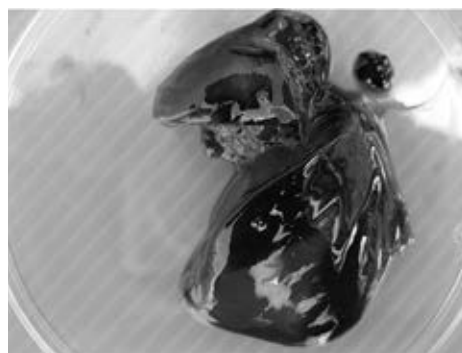


Fig. 4. – Liver: subcapsularry bleeding.

In spite of the fact that experienced veterinarians are able to recognize the symptoms without any difficulty and propose the therapy accordingly, further laboratory examinations are needed to confirm the diagnosis, including microbiological investigations and determination of mycotoxin content. In practice, cases of mycotoxicosis in poultry are frequently seen (K a p e t a n o v et al., 2012).

CONCLUSIONS

Clinical and pathological diagnostics is of great importance in the veterinary medicine due to efficient tools to note the disease, direct investigations toward specific laboratory tests and moderate the outcome. The results of numerous mycotoxin examinations indicate that, in many cases, the determined concentrations in feed do not exceed the levels regulated by the law. However, because of the cumulative effects and chronic exposure to mycotoxins, even in low doses, prompt expertise and action undertaken by the veterinarians are

inevitable, especially in more susceptible poultry species and categories. Additionally, in a number of feed plants and factories, components are obtained not in large quantities but rather capillary. In such circumstances a significant amount of complete mixtures may be delivered to poultry producers without the previous control, which indicates a need for more comprehensive feed monitoring.

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## УЛОГА КЛИНИЧКЕ И ПАТОМОРФОЛОШКЕ ДИЈАГНОСТИКЕ МИКОТОКСИКОЗА ИЗАЗВАНЕ Т-2 ТОКСИНОМ КОД РОДИТЕЉСКОГ ЈАТА У ЕКСПЛОАТАЦИЈИ

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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, чест извор недоумица чини процес клиничког и патоморфолошког дијагностиковања обољења изазваних микотоксинима. За ветеринарску медицину најважнији микотоксин трихотеценске групе је Т-2 који испољава најизраженије цитотоксично дејство. Циљ рада је да се, кроз приказ случаја микотоксикозе живине, родитељског јата узраста 44 недеље у експлоатацији, истакне значај постављања клиничке и патоморфолошке дијагнозе уз лабораторијску анализу како би се поставила објективна етиолошка детерминација.

На фарми родитељског јата у експлоатацији приказана је нагла клиничка појава болести праћена потпуним престанком конзумирања хране. Већина груписаних јединки је лежала а при покретању отежано се кретала. Клиничком опсервацијом јата уочавала се некроза врха језика и ограничена некротична жаришта слузокоже усне дупље која су на додир јединки стварали бол. Након отварања мишићног желуца уочавао се садржај хране, ерозије зида желуца и кутикуле која се лако скидала. Јетра угинулих јединки карактерисали су: конгестија, заобљеност режњева, мултифокална некротична жаришта и супкапсуларна крварења. Број угинулих јединки у току посматраног периода је био изнад технолошких норматива.

Натурални показатељи производње укључујући проценат снесених јаја, одабраних за инкубирање и лежања пилића били су далеко испод предвиђене технологије са тенденцијом опадања.

Добијене микробиолошке вредности потпуне смеше за исхрану родитељског јата у експлоатацији од 150 000 колонија плесни у 1 г рода *Fusarium* у чистој

култури и налаз трихотецена Т-2 од 450 μg/kg на граници максимално дозвоље-
не количине за пилиће како је регулисано постојећим законским актима изазива
општу полемику у стручним круговима.

КЉУЧНЕ РЕЧИ: клиничка и патоморфолошка дијагностика, живина, ми-
котоксини, Т-2 токсин, законска регулатива

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# MYCOBIOTA OF SERBIAN WHEAT GRAIN IN 2010

ABSTRACT: This research focused on the assessment of the infection level of sampled wheat grains with phytopathogenic fungi. The samples were taken from the localities Rimski Šančevi and Sombor. The research investigated the impact of localities to intensity of fungal infection by fungi from genus *Fusarium* and *Alternaria*. Isolates from genus *Fusarium* and *Alternaria* were determined to species level. Pathogenicity of *Fusarium* and *Alternaria* isolates from different localities to wheat seedlings was also established.

KEY WORDS: wheat, mycobiota, contamination, germination, determination, pathogenicity of isolates, impact of sites, *Fusarium, Alternaria*

## INTRODUCTION

Wheat grains are very suitable substrate for development of a large number of phytopathogenic and saprophytic microorganisms in the field and during storage. These organisms have negative effects on the quality of products and the majority of them produce mycotoxins which are harmful to human and animal health. This is the reason why it is very important to control the mycopopulation of grains in the production of healthy foods.

The aim of this work was to determine the mycopopulation of the sampled grains, evaluate the germination rate and fungal contamination of seeds, impact of localities and genotypes on the rate of germination and infection, and to establish the pathogenicity of several fungal isolates from genus *Fusarium* and *Alternaria* to wheat seedlings.

## MATERIAL AND METHODS

The wheat samples were taken after harvest from Rimski Šančevi and Sombor in 2010. Six genotypes of durum wheat were collected from Rimski Šančevi, and from both localities the same 15 early-maturing and 16 late-maturing wheat genotypes were collected (Table 1).

Tab. 1. – Wheat grain samples taken after harvest in Rimski Šančevi and Sombor in 2010.

| | Rimski Šančevi | | | Sombor | |
|---|---|---|---|---|---|
| | Durum | Early--maturing | Late--maturing | Early--maturing | Late--maturing |
| | 5 | 1 | 1 | 1 | 1 |
| | 6 | 2 | 2 | 2 | 2 |
| | 7 | 3 | 3 | 3 | 3 |
| | 8 | 4 | 4 | 4 | 4 |
| | 9 | 5 | 5 | 5 | 5 |
| | 10 | 6 | 6 | 6 | 6 |
| | | 7 | 7 | 7 | 7 |
| | | 8 | 8 | 8 | 8 |
| **Genotypes** | | 9 | 9 | 9 | 9 |
| | | 10 | 10 | 10 | 10 |
| | | 11 | 19 | 11 | 19 |
| | | 12 | 33 | 12 | 33 |
| | | 13 | 34 | 13 | 34 |
| | | 14 | 35 | 14 | 35 |
| | | 15 | 36 | 15 | 36 |
| | | | 37 | | 37 |

The evaluation of seed germination level and fungal infection intensity was established using the method of wet filter paper (P i t t and H o c k i n g, 1985). All data were processed by Statistica 10 software. Statistically significant differences of sample contamination between the same genotypes from different localities were detected using the Duncan's test.

As the fungi from genus *Fusarium* and *Alternaria* were the most important contaminants, they were indentified to the level of species on the basis of morphological and cultural characteristics using the determinator of N e l s o n et al. (1983), L e s l i e and S u m m e r e l l (2006) and S i m m o n s (2007). For the determination of *Fusarium* species the carnation leaf-agar (CLA) and potato-dextrose agar (PDA) were used. Fungi from genus *Alternaria* were identified on potato-carrot agar and tomato juice agar (V8).

Fungal suspension was prepared in order to test pathogenicity of *Fusarium* and *Alternaria* isolates. The grains of early-maturing wheat from Rimski Šančevi number 12 were kept for 24 hours in the suspension. After that, the grains were incubated in Petri dishes where the filter paper was soaked with the

same suspension. After 6 days, the level of seedling infection was evaluated. The percentage of disease index was established by McKinney's formula. The pathogenicity test results of different isolates were compared with the Duncan's test by statistical software Costat.

# RESULTS

In the year of 2009/10, the vegetation was extremely humid from the beginning of wheat tillering until harvesting, especially in the period from May to July on both localities (in Sombor, on June 22 the highest daily amount of rainfall was 171.8 mm). After this period, 3 very cold intervals with the average daily temperatures under 10°C were reported. Between the second and third period of cold weather, the temperatures were almost +35°C (June 10-17, 2010). These weather conditions had negative effect on yield and on some quality parameters although it was convenient for some pathogens and increased wheat fungal infection.

During the examination of health status of durum wheat, the infection level ranged from 33-70% and germination was between 12-69% depending on the genotype. In case of the samples from early-maturing wheat, the seed infection level was 33-61%, germination was 63-95%, while in the case of the same genotypes from Sombor, the seed infection level ranged from 52-82%, germination ranged from 73-99%. When the late-maturing wheat from Rimski Šančevi was considered, the seed infection level was 42-73%, germination 47-92%, while the rate of seed infection level in Sombor ranged from 50-73%, germination 82-98%. On the basis of these results, it was concluded that the localities significantly influenced the seed infection level and level of germination. The level of seed infection in Sombor was higher than that in Rimski Šančevi.

Infection of seeds with fungi from genus *Fusarium* was the highest with – 44% in the case of the late-maturing genotype number 35 from Rimski Šančevi and the lowest infection was recorded for the early-maturing genotype number 2 from the same locality. Infection of seeds with *Fusarium* was not detected in genotype 3 from the same location.

The highest rate of infection with *Alternaria* (63%) was detected in the early-maturing genotype number 5 from Sombor, while the lowest infection (12%) was established in the late-maturing genotype number 9 from Rimski Šančevi. On average, the infection with fungi from *Fusarium* genera was higher in durum wheat than the infection with *Alternaria,* while in early-maturing and late-maturing genotypes from both localities the infection with *Alternaria* was higher.

According to the assessment of the impact of genotypes and localities on the rate of grain infection, it was established that early-maturing and late-maturing genotypes and localities individually had statistically significant effect on the rate of seed infection with fungi from genera *Fusarium* and *Alter-*

*naria*. The interaction between genotypes and localities contributed to the presence of infection with fungi from genus *Fusarium*, but it did not show statistically significant effects on seed infection with *Alternaria*. Significant differences in seed infection level with fungi from genera *Alternaria* and *Fusarium* were found among the same genotypes but from different locations. These differences are shown in the Tables 2 and 3.

Tab. 2. – Level of seed infection with fungi from genus *Fusarium*

| Genotype | Locality | Rate of infection with fungi from genus *Fusarium* % | Level of significance 0.05 |
|---|---|---|---|
| Early maturing wheat 1 | Rimski Šančevi | 3 | abc |
| Early maturing wheat 1 | Sombor | 35 | k |
| Early maturing wheat 2 | Rimski Šančevi | 2 | ab |
| Early maturing wheat 2 | Sombor | 25 | j |
| Early maturing wheat 3 | Rimski Šančevi | 0 | a |
| Early maturing wheat 3 | Sombor | 24 | ij |
| Early maturing wheat 6 | Rimski Šančevi | 9 | bcde |
| Early maturing wheat 6 | Sombor | 19 | ghij |
| Early maturing wheat 9 | Rimski Šančevi | 25 | j |
| Early maturing wheat 9 | Sombor | 16 | efgh |
| Early maturing wheat 12 | Rimski Šančevi | 25 | j |
| Early maturing wheat 12 | Sombor | 16 | efgh |
| Early maturing wheat 13 | Rimski Šančevi | 8 | bcd |
| Early maturing wheat 13 | Sombor | 33 | k |
| Early maturing wheat 14 | Rimski Šančevi | 11 | def |
| Early maturing wheat 14 | Sombor | 22 | hij |
| Early maturing wheat 15 | Rimski Šančevi | 7 | abcd |
| Early maturing wheat 15 | Sombor | 16 | efgh |
| Lately maturing wheat 1 | Rimski Šančevi | 10 | ab |
| Lately maturing wheat 1 | Sombor | 36 | fghi |
| Lately maturing wheat 2 | Rimski Šančevi | 7 | a |
| Lately maturing wheat 2 | Sombor | 34 | fghi |
| Lately maturing wheat 3 | Rimski Šančevi | 15 | abcde |
| Lately maturing wheat 3 | Sombor | 37 | ghi |
| Lately maturing wheat 7 | Rimski Šančevi | 16 | abcde |
| Lately maturing wheat 7 | Sombor | 34 | fghi |
| Lately maturing wheat 9 | Rimski Šančevi | 12 | abcd |
| Lately maturing wheat 9 | Sombor | 32 | fghi |

On the basis of morphological and cultural characteristics, 6 genera of fungi were indentified in the grains: *Fusarium*, *Alternaria, Penicillium, Helmintho-sporium (Dreshlera), Mucor* and *Epicoccum*. The following species: *Fusarium graminearum, F. avenaceum, F. equiseti, F. semitectum, F. sporotrichoides, F. culmorum, F. sambucinum, F. oxysporum* and *F. tricinctum* were isolated

Tab. 3. – Level of seed infection with fungi from genus *Alternaria*

| Genotype | **Location** | **Rate of infection with fungi from genus *Alternaria* %** | **Level of significance 0.05** |
|---|---|---|---|
| Early maturing wheat 1 | Rimski Šančevi | 26 | abcdef |
| Early maturing wheat 1 | Sombor | 39 | ghij |
| Early maturing wheat 2 | Rimski Šančevi | 24 | abcde |
| Early maturing wheat 2 | Sombor | 54 | klmn |
| Early maturing wheat 4 | Rimski Šančevi | 15 | a |
| Early maturing wheat 4 | Sombor | 48 | ijklm |
| Early maturing wheat 5 | Rimski Šančevi | 48 | ijklm |
| Early maturing wheat 5 | Sombor | 63 | n |
| Early maturing wheat 7 | Rimski Šančevi | 31 | cdefg |
| Early maturing wheat 7 | Sombor | 50 | jklm |
| Early maturing wheat 8 | Rimski Šančevi | 16 | ab |
| Early maturing wheat 8 | Sombor | 39 | ghij |
| Early maturing wheat 9 | Rimski Šančevi | 21 | abc |
| Early maturing wheat 9 | Sombor | 46 | hijkl |
| Early maturing wheat 10 | Rimski Šančevi | 38 | fghij |
| Early maturing wheat 10 | Sombor | 60 | mn |
| Early maturing wheat 11 | Rimski Šančevi | 36 | efghi |
| Early maturing wheat 11 | Sombor | 59.5 | mn |
| Early maturing wheat 12 | Rimski Šančevi | 25 | abcde |
| Early maturing wheat 12 | Sombor | 55 | klmn |
| Early maturing wheat 13 | Rimski Šančevi | 23 | abcd |
| Early maturing wheat 13 | Sombor | 44 | hijk |
| Early maturing wheat 14 | Rimski Šančevi | 17 | ab |
| Early maturing wheat 14 | Sombor | 46 | hijkl |
| Early maturing wheat 15 | Rimski Šančevi | 35 | defgh |
| Early maturing wheat 15 | Sombor | 58 | lmn |
| Lately maturing wheat 6 | Rimski Šančevi | 17 | abc |
| Lately maturing wheat 6 | Sombor | 34 | def |

from genera *Fusarium*, but the dominant was *Fusarium graminearum* with 60%. Although a few species of genera *Fusarium* were indentified in Rimski Šančevi, the most common species in Sombor was *Fusarium graminearum*. *Alternaria tenuissima* appeared to be the most common species from genus *Alternaria* in the grains. According to the pathogenicity test with *Fusarium* isolates, the highest pathogenicity to wheat seedling was determined in the case of *Fusarium graminearum* islolates. By comparing the isolates from genus *Fusarium* and *Alternaria,* it was found that higher pathogenicity was exhibited by genus *Fusarium* (Tab. 4, Tab. 5). Localities did not influence pathogenicity of isolates because the isolates from both localities manifested similar percentage of pathogenicity to the seedlings.

Tab. 4. – Pathogenicity of isolates from genus *Fusarium* on wheat seedlings

| Isolates | Disease index (%) | Level of significance 0.05 |
|---|---|---|
| NSK$_1$5IVFS- *F. sporotrichoides* | 29,25 | f |
| NSR15IIIFS- *F. sporotrichoides* | 54 | c |
| SOK$_1$2IIFSe- *F. semitectum* | 41,75 | de |
| NSK14IIIFO- *F. oxysporum* | 57 | c |
| D9IVFA- *F. avenaceum* | 39,5 | e |
| SOR11IIIFG- *F. graminearum* | 50 | cd |
| D10IVFG- *F. graminearum* | 89,75 | a |
| NSR6IFG- *F. graminearum* | 74,75 | b |
| SOK$_1$6IVFG- *F. graminearum* | 85 | a |
| SOR14IIFG- *F. graminearum* | 85,75 | a |

*Fusarium* LSD 0.05 = 8,37

Tab. 5. – Pathogenicity of isolates of *Alternaria tenuissima* on wheat seedlings

| Isolates | Disease index (%) X | **Level of significance 0.05** |
|---|---|---|
| D7IIAT | 31 | ab |
| NSR13IVAT | 29,5 | b |
| NSK$_1$2IAT | 31 | ab |
| SOR13IIAT | 35,5 | a |
| SOK$_1$8IVAT | 30,5 | ab |

*Alternaria* LSD 0.05 = 5.32

# DISCUSSION

Fungi from genera *Fusarium* and *Alternaria* infected the wheat panicles and grains bcause of the unfavorable conditions during flowering in 2010. The infection with fungi from genus *Fusarium* was up to 44%, while in the case of *Alternaria* it ranged from 12-63%. In the Czech Republic, the internal infection of wheat seeds ranged from 30.6-45% depending on the climate conditions, growing technology, resistance of genotypes, methods of sampling, methods of mycological treatment and medium used for pathogen isolation (O s t r y and R u p r i c h, 2001).

The most common causal agent of wheat fusariosis in Serbia was *Fusarium graminearum* (B a g i, 1999) and it was confirmed during the determination of *Fusarium* species. Although dormant, the species *F. avenaceum, F. equiseti, F. semitectum, F. sporotrichoides, F. culmorum, F. sambucinum, F. oxysporum* and *F. tricinctum* were also determined. Fungi *Alternaria* could be found as dominant fungi in the wheat grains in the last decade (B a g i et al., 2004). The most common species were *Alternaria alternata, A. tenuissima, A. triticina* and *A. triticola* (S h a b a n a and K u m a r, 2000). According to this research *A. tenuissima* was the most frequent. Very high level of contamination (100%) was detected in some wheat samples from Argentina (P a t r i a c a et al., 2007).

The highest pathogenicity (89.7%) on seedlings was manifested by *F. graminearum*, as the most pathogenic species of genus *Fusarium*. This was proved by some other research (B r e n n a n  et al., 2003). In the mentioned work, the fungus caused reduction in coleoptil growth by 96% at the temperature of 25°C, but at even lower temperatures it caused up to 44% of reduction in growth.

Some different early-maturing and late-maturing genotypes and localities individually had statistically significant effect on the rate of seed infection with fungi from genera *Fusarium* and *Alternaria*. Although the interaction between genotypes and localities affected the infection level with fungi from genus *Fusarium,* it did not show statistically significant effect on seed infection with *Alternaria*. Significant differences in seed contamination with fungi from genera *Alternaria* and *Fusarium* were recorded in the same genotypes from different localities.

Current preventive measures that must be taken against wheat pathogens are timely sowing, sowing density, plowing of crop residues, crop rotation, balanced fertilization, and quality basic and pre-processing preparation of soil as well as the use of healthy seeds.

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# МИКОБИОТА ЗРНА ПШЕНИЦЕ У СРБИЈИ ТОКОМ 2010. ГОДИНЕ

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Резиме

У овом истраживању одређена је оцена клијавости и заразе фитопатогеним гљивама узоркованих зрна различитих сорти пшенице са два локалитета (Римски Шанчеви и Сомбор). Испитан је утицај локалитета на проценте клијавости и зараженостти генотипова пшенице, као и утицај локалитета и генотипова на проценат заразе зрна пшенице гљивама из родова *Fusarium* и *Alternaria* при чему су различити касностасни и раностасни генотипови и локалитети појединачно показали значајан утицај на проценат заразе зрна гљивама рода *Fusarium* и *Alternaria*, док је интеракција између генотипова и локалитета утицала на појаву заразе зрна врстама рода *Fusarium*, а није показала статистички значајан утицај на заразу гљивама рода *Alternaria*.

Извршена је и детерминација родова микобиота зрна пшенице, а у случају родова *Fusarium* и *Alternaria* и детерминација до нивоа врсте, при чему је утврђено да је најучесталија врста рода *Fusarium* са 60 % заступљености *F. graminearum*, док је из рода *Alternaria* детерминисана само врста *A. tenuissima*.

Такође је одређен интензитет патогености изолата гљива (родова *Fusarium* и *Alternaria*) према клијанцима пшенице и патогеност изолата са зрна пшенице из различитих локалитета. Локалитет није имао утицај на фитопатогеност изолата јер су изолати из оба локалитета испољили приближан проценат патогености на клијанцима. Највећу патогеност у оквиру рода *Fusarium* испољила је врста *F. graminearum*. У погледу патогености родова *Fusarium* и *Alternaria*, већу патогеност испољили су изолати рода *Fusarium*.

КЉУЧНЕ РЕЧИ: пшеница, микобиота, зараженост, клијавост, детерминирање, патогеност изолата, утицај локалитета, *Fusarium*, *Alternaria*

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# DEGREE OF MYCOTOXICOLOGICAL CONTAMINATION OF FEED AND COMPLETE FEED MIXTURES FOR PIGS AND POULTRY DURING THE PERIOD 2007–2012. ON THE TERRITORY OF THE REPUBLIC OF SERBIA

ABSTRACT: The most common producers of mycotoxins are fungi of the genera Aspergillus, Fusarium and Penicillium. Toxins are of extreme importance because it can be transmitted from animals to humans through milk and animal products, some of which are carcinogenic and teratogenic. Mycotoxins cause a health disturbance of all animals, but the effects are more noticeable in highly productive animals in the farm way of keeping considering the much greater consumption of concentrate feeds, although forages also can be contaminated with mycotoxins in a significant manner. Mycotoxicoses are the most common seasonal illnesses, and are an important diagnostic problem in veterinary practice, because its characteristics often resemble diseases caused by pathogens or nutritional deficiency or imbalance. The degree of health disturbances depends on the amount of toxins in feed and the length of intaking as on types and categories of animals.

The presence of mycotoxins in animal feed is inevitable and therefore testing of raw materials and products is necessary so that feed for humans and animals can be safe for use. Damages arising as consequences of mycotoxicosis in poultry and swine production, due to the direct loss because of animals lossor, more commonly, indirectly due to the fall productive and reproductive performances of animals, imposed the need for continuous monitoring of the hygienic quality of feed mixtures for feeding these animals.

During a five year period (2007-2012) were analyzed a total of 104 samples from the territory of Republic of Serbia intended for nutrition of all categories of poultry and mixtures for the initial and final fattening broilers (50 samples) and laying hens (54). The analysis included 57 samples of feed mixtures intended for all categories of swine – feed mixture for young (20 samples) and the old categories (37 samples) and 196 of the samples, which are commonly used in formulating rations for listed species (maize, soybean and sunflower meal). For the analysis of the samples was used thin layer chromatography and Elisa test. The results were compared with current Regulations on the quality of the feed

(Official Gazette of RS 41/09) in force since 1.05.2010. in the part where the maximum allowable quantities of hazardous substances (Article 99) gives the value of the maximum permitted levels of mycotoxins in animal feed. The number and types of mycotoxins vary depending on the feed, as well as on the year which can be directly related to climatic factors, and the average annual humidity. The fact that in the samples was not detected the presence of aflatoxin indicate that in our conditions toxin producing fungi do not find fertile ground for the production of toxins, as well as the absence of certain nutrients in the production of complete feed mixtures for pigs and poultry, which are the traditional sources of aflatoxin (peanut oil meals). The results are encouraging given the fact a relatively small number of defective mixtures and nutrients. However, the fact that only a limited number of feed samples we received for the analysis suggests further caution and constant monitoring of the presence of mycotoxins in animal feed.

KEY WORDS: Mycotoxins, pigs, poultry, feed

# INTRODUCTION

Mycotoxins are secondary toxic metabolites of a number of saprophytic molds that enters the body of animals and humans mostly through contaminated feed infested with spores, conidia and / or mycelium fragments. When toxins get into the body of animals and humans they cause intoxication, so called mycotoxicoses, given that they are related to feed, can take on a wide scale (U r a g u c h i and Y a m a z a k i, 1978).

It is assumed that the mycotoxins were present in food for animals and people from the beginning of life on Earth, and the first data on the harmful effects of the consumption of mycotoxins in China date back to 5000 years ago. Although the harmful effects of animals and humans nutrition with moldy food were well known since ancient times, a specific agent was not known for a long time. The emergence of unknown diseases ("Turkey X disease"), which is in England 1960th led to the deaths of more than 100,000 turkeys and about 20,000 other types of birds with signs of acute liver necrosis (L a n c a s t e r et al., 1961, B u t l e r, 1974), directed the research on determining the causal factors. A year later, from the imported peanut meal, raw material used for poultry feeding, was isolated culture of fungi Aspergillus flavus, as well as a few previously unknown compounds, which fluoresced very intense under UV light.

The discovery of these compounds, called aflatoxins, represents a milestone in the history of mycotoxins. Researchs over the past 40-odd years accumulated many data so that until now have been revealed several hundred mycotoxins (S m i t h and M o s s, 1985), of which only smaller number has been considered harmful (R i l e y, 1998), and only 20-30, by the incidence and adverse effects has medicinal, nutritional, environmental and economic importance. It is known that over 220 species produces mycotoxins and most of the toxin-producing species belongs to the genera of Fusarium, Penicillium and Aspergillus. It is known that mycotoxcoses are cause-effect related to the occurrence of a few very large-scale poisoning and death of hundreds of thousands of animals and people in Europe and other continents in the previous millennium (U e n o, 1983).

Contamination with molds and mycotoxins is a current global problem, according to FAO data today is about 25% of cereal production globally con-

taminated (D e v e g o w d a  et al., 1998). It is evident that molds and mycotoxins are a serious problem (S C O O P, 1996), not only in terms of the success of the harvest and grain quality, but also in relation to animal health and productivity (M a š i ć  et al., 2002b), and safety of feedstuffs of animal origin, aspects of health (M i l l e r  and  T r e n h o l m, 1994). It is believed that the economic losses caused by mycotoxins are almost immeasurable (D e v e - g o w d a  and  A r a v i n d, 2002).

Damage to livestock caused by mycotoxicosis manifest themselves in the form of direct losses due to loss of animals or, more commonly, indirectly due to falling productive and reproductive performances of animals. Mycotoxins cause a health disturbance of all animals, but the effects are more noticeable in highly productive animals in the farm way of keeping in view of the much greater consumption of concentrated feed eaven though, forages may be contaminated with mycotoxins in a significant manner (P a s t e i n e r, 1998, F i n k - G r e m m e l s, 2005). Diseases caused by mycotoxins are not contagious, they are related to feed and / or specific nutrients, similar to vitamin deficiencies are not treated with antibiotics or other drugs, in the body do not cause an immune response, because they are low molecular weight, and the animals are permanently unprotected from their effects (W y l l i e  and  M o r e h o u s e, 1977). Mycotoxicoses are the most common seasonal illnesses, and are an important diagnostic problem in veterinary practice, because by its characteristics often resemble the diseases caused by pathogens or nutritional deficiency or imbalance. Poisoning are manifested in the form of primary acute or chronic toxicosis, as well as in the form of secondary toxicosis (R i c h a r d  and  T h u r s - t o n, 1986; N u r r e d  and  R i l e y, 2001).

Changes caused by mycotoxins depend on the type and quantity of mycotoxins in feed, the length of ingestation, as well on genetic (species, breed, animal strain), physiological (gender, category, age, diet) and environmental (climatic conditions, keeping animals ) factors, and the presence of disease infectious and / or noninfectious nature (S m i t h  and  M o s s, 1985).

A particular issue is possibility that in the body of animals that consumed feed contaminated with mycotoxins can be found a mycotoxins in various amounts, so there may be a manifestation of adverse effects in humans. (O ž e - g o v i ć  and H l u b n a, 1981, O z e g o v i c, 1983). There must not be lost from the sight that main goal of feed production is to ensure the health of people who consume feeds of animal origin, and only then to meet the nutritional requirements of animals and the preservation of their health. Damages in the poultry and swine production caused by mycotoxicosis, direct losses due to loss of animals or, more commonly, indirectly due to falling productive and reproductive performance of animals, imposed the need for continuous monitoring of the hygienic quality of feed mixtures and different feed used in formulating rations for feeding these animals.

Considering the frequency of occurrence in the feed, in conjunction with conditions on our geo-climatic region, in the most important fungi can be classified Aspergillus and Fusarium species that contaminate feed in the fields and warehouses.

## Aspergillus mycotoxins

This group contains a number of mycotoxins (sterigmatocystin, citrulline, patulin), but aflatoxin B1 (AFB1) and ohratksin A (OTA) are certainly the most important representatives of this group.

## Aflatoxins

Aflatoxins, according to the structure of molecules, belong to a group of heterocyclic derivatives of bisfuranokumarin type, and aflatoxin B1 (AFB1) is the most toxic mycotoxin for both human and animal. In terms of afaltoksin toxicity with implications for human health certainly the most important metabolite is aflatoxin M1, while aflatoxin M2 and M4 are of minor importance (G o r e l i k, 1990).

The most common way of the aflatoxin absorption is through gastrointestinal tract, lungs and skin. Transport of chemicals through the cell membrane is in direct correlation with their liposolubility (K l a s s e n and R o s m a n, 1991).

Aflatoxin tends to be deposited in all soft tissues and fat depots of animals. The highest level of aflatoxin accumulation occurs in tissues which serve its biotransformation, such as the liver and kidneys (L e e s o n and S u m m e r s, 1995). Aflatoxins ingested by feed passes the gastrointestinal tract and reach the bloodstream within 30 minutes, and in the liver for 1 hour. Biodegradation of aflatoxin molecules in hepatocytes occurs in at least six ways (U e n o, 1983), and is considered among the metabolites of aflatoxin B1, aflatoksikol to be the most mutagen agent that causes cancer changes in the liver.

Aflatoxin residues can be found in the tissues and organs, as well as eggs from laying hens and milk from animals fed with aflatoxin contaminated feed (L e e s o n and S u m m e r s, 1995). Aflatoxins are detectable in all parts of the egg not before10 hours after ovulation. The amount of aflatoxin decreases in egg white after 48 hours, while the content in the egg yolk and shell increases (J a c o b s o n and W i s m a n, 1974). Aflatoxins, particulary AFB1, are highly toxic compounds (LD50 1-50 mg/kg) and in addition to acute toxicity exerts a very strong carcinogenic effect (Eaton and Groopman, 1994). The International Agency for Research on Cancer (IARAC) has classified AFB1 in group 1 of carcinogens, because the risk of possibility of primary human liver cancer is very high (H e n r y et al., 2001, 1999).

## Ohcratoxins

Ochratoxins, of which the most important type A (OTA), are by the chemical structure isocumarine derivatives (B e t i n a, 1984) and are absorbed relatively slowly from the digestive tract (U r a g u c h i and Y a m a z a k i, 1978). High affinity OTA to the plasma proteins is an important factor that facilitates passive absorption of non ionised form of toxins from the digestive

tract, but also makes difficulty its elimination from the organism by limiting glomerular filtration and renal excretion.

In the liver OTA hidrolisis forming the less toxic metabolites. Excreted via the bile into the intestine, and are subject to reabsorption (S i n o v e c et al., 1998). Feces excretion is 11% of unchanged OA, and about 23-33% metabolised mycotoxins in the form Oα.

Excretion is done efficiently and throughout urine (11% unchanged OTA), but OTA is subject to reabsorption in the renal tubules, which is the basis of residual effect of mycotoxins in the kidneys, and probably in the whole body (F u c h s, 1988). It is characteristic that mercury has the ability of efficiently and faster excretion of mycotoxins so that over a 90% of ingested OTA is excreted during 48-h (G a l t i e r et al., 1981).

There is a significant possibility of depositing OTA and its metabolites in the edible parts of pigs and poultry ( M a s i c, 1986; Z u r o v a c - K u z - m a n, 2001) especially in the kidneys and liver (F u c h s, 1988), and considerably lesser in muscle and adipose tissue (J o n k e r et al., 1999). Scandinavian countries introduced mandatory inspection of meat and kidney of pigs slaughtered in abattoirs, and WHO has prescribed the maximum permissible concentration of OTA in meat of slaughtered animals. Analyses of meat can show the presence of OTA residues in a large number of apparently healthy animals (up to 25%), and similar results were obtained in previous studies (M i l i c e v i c, 2004).

## Fusarium mycotoxins

Fusarium mycotoxins are commonly identified group of mycotoxins in feed. Some strains of Fusarium fungi can produce up to 17 mycotoxins at a time, and in this group, beside zearalenone (ZON) and T-2 toxin are classified other trichothecenes fall, fumonisins, moniliformin and fuzaric acid.

## Zearalenone

Zearalenone (F-2) belongs to a group of phytoestrogens and untill now has been identified 15 different products that have different biological activity (B e t i n a, 1984). Basically, they have a similar configuration (phenolic core) to estrogenic substances (estradiol, estriol and stilbestrol).

Zearalenone after the oral ingestion very well and quickly absorbs. It can be found in the plasma after 30 minutes from the moment of feed intake in the organism of pigs (O l s e n et al., 1981). It is believed that the F-2 is from less toxic then other metabolites of Fusarium fungi, and the relative toxicity (LD50) ranges from 1-10 mg/kg (T e r a o and O h t s u b o, 1991).

Most of the absorbed zearalenone is transported by the portal blood stream to the liver (U r a g u c h i and Y a m a z a k i, 1978), where is accumulated and metabolized by enzymes (reductase and esterase) creating more (up to 4 times) or less active metabolites than its predecessor. F-2 and its metabo-

lites are distributed primarily by target tissues as the uterus, intestine, testes, ovaries, and adipose tissue (R i l e y, 1998).

F-2 residues and the resulting products can be determined in the edible parts of the animals feeding with contaminated feed, mostly in the liver and muscles (and C i e g l e r  V e s o n d e r, 1983), but also in milk and eggs. Even in meat of clinically healthy animals may be determined the quantity of residues up to 10 mg/kg. The chickens meat is deposited with large amounts of metabolites (59-1200 mg/kg) more than in the meat of pigs with lower feed contamination (78-310 mg/kg). Residues are carcinogenic, and their biological effects are compared with the effects of dietilstilbestrola or estradiol.

## MATERIALS AND METHODS

During a five year period (2007-2012) was analyzed a total of 104 samples from the Republic of Serbia intended for nutrition of all categories of poultry – mixtures for the initial and final fattening broilers (50 samples) and mixtures for laying hens (54).). For the samples analysis were used thin layer chromatography and ELISA tests. The study included 57 samples of feed mixtures intended for nutriton of all categories of pigs – feed mixtures for young (20 samples) and the old categories (37 samples) and 196 of the samples, which are commonly used in formulating rations for listed species (corn, soybean and sunflower meal). The results were compared with current Regulations on the quality of the feed (Official Gazette of RS 41/09) in force since 1.05.2010. and in the part where the maximum allowable quantities of hazardous substances (Article 99) gives the value of the maximum permitted levels of mycotoxins in animal feed (table 1).

## RESULTS OBTAINED

From the total number of analyzed samples of feed mixtures intended for feeding all categories of pigs (57) there were 20 samples of feeding mixtures for young and 37 for elderly categories. The content of aflatoxin in feed mixtures for young varied in the range from 0.001 to 0.0092 mg/kg and a similar trend was noted for the adult category with a range of 0.001 to 0.018 mg/kg. Comparing the results with the actual national maximum allowable values for aflatoxin conclusion is that none of the samples were found above the permissible value.

The recorded values of zearalenone in feed mixtures for feeding young pigs varied in the range of 0.006 to 2.786 mg/kg. Of the total number of feed mixtures samples for feeding young (20) in the two samples the presence of zearalenone was above permitted level in the quantity of 2.786 and 0.819 mg/kg, which in the aggregate make up 10 percent of the total samples analyzed for this group of pigs and 3.51 % based on the total number of feeding mixtures samples intended for pigs (chart 1).

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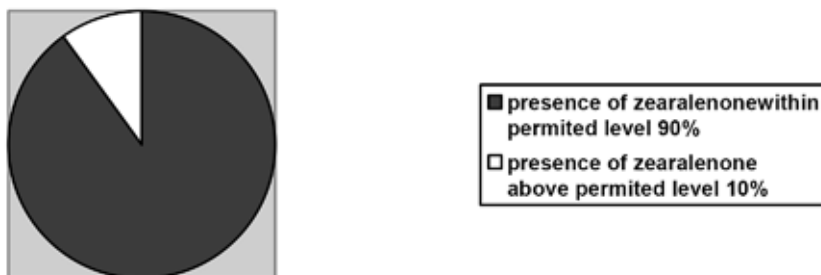
Chart 1 –
Samples of feeding mixtures intended for pigs nutrition with the presence
of zearalenone above and within permitted level

Analysis of feeding mixtures for pigs adult categories established values for zearalenone ranged from 0.01 to 0.35 mg/kg, which corresponds to the allowed values.

In the analysis of feeding mixtures for feeding young pigs established values for ochratoxin ranged from 0.001 to 0.10 mg/kg, which is consistent with the allowable values prescribed by regulation. The determined values for ochratoxin in feed mixtures for adult pigs categories were in the range from 0.002 to 0.2 mg/kg, also in accordance with the allowable values prescribed by regulation.

Total mycotoxicological analysis included 104 samples of feed mixtures intended for all categories of poultry : 50 samples of feed mixtures for the initial and final fattening of broilers and 54 samples of laying hens. The content of aflatoxin in feed mixtures for broilers varied in the range of 0.001 to 0.03 mg/kg and for adult categories ranging from 0.001 to 0.009 mg/kg. Comparing the results of the samples of feed mixtures for laying hens with applicable regulations of maximum allowable values for aflatoxin was found that none of the samples had detected values above the allowable. However, the determined value for aflatoxin in samples of feed mixtures for the initial and final fattening of broilers at five samples exceeded the allowed values which in the aggregate makes 10 percent of the total samples analyzed for this category of poultry or 4.81% based on the total number of samples of feed mixtures intended for poultry nutrition (chart 2).

In the analysis of feed mixtures for broilers detected values for zearalenone ranged from 0.013 to 1.257 mg/kg and for laying hens they were 0.013 to 0.54 mg/kg. The current regulations do not regulate the maximum permissible values of these mycotoxins in complete and supplementary feed mixtures for livestock.

The determined values for ochratoxin in feed mixtures for broilers ranged from 0.002 to 0.65 mg/kg, which is within the prescribed values same as the values determined in feed mixtures for laying hens from 0.004 to 0.10 mg/kg.
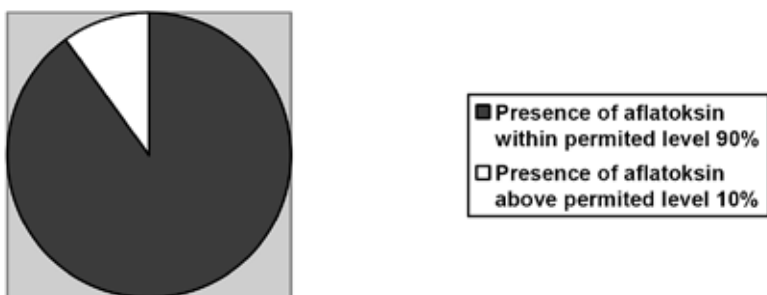
Chart 2 –
Samples of feeding mixtures intended for poultry nutrition with the presence
of aflatoxin above and within permitted level

During the mucotoxicological analysis of feeds we chose those materials that are widely used in pig and poultry diets and commonly used in formulating their meals: corn, soybean and sunflower meal. From a total of 196 samples of feeds not in one examined sample has been detected value of aflatoxin in excess of allowable values prescribed in the regulation except that the two nutrients had the maximum allowed value of 0.05 mg/kg. Presence of zearalenone was detected in the range of 0.001 to 0.62 mg/kg and ochratoxin 0.001 to 0.30 mg/kg with notice that currently applicable regulations do not regulate the maximum permissible value of these mycotoxins in feed, but only in complete and supplementary feed mixtures.

Tab. 1 – Maximally permissible levels of harmful substances (Official Gazette of RS 41/09 Article 99)

| Type | Feed and feeding mixtures | mg/kg (ppm) |
|---|---|---|
| **Aflatoxin** | Feeds | **0,05** |
| | Complete and supplemental mixtures for cattle, sheep and goats, with the exception of dairy cows, calves, lambs and kids | **0,05** |
| | Complete and supplemental mixtures for dairy cows | **0,01** |
| | Supplemental mixtures for pigs and poultry except offspring | **0,03** |
| | Complete and supplemental mixtures for pigs and poultry except offspring | **0,02** |
| | Complete and supplemental mixtures for calves, lambs, goats, pigs, chickens, little girl, ducklings | **0,01** |
| **Zearalenone and its derivatives** | Complete and supplemental mixtures for pigs, gilts to 50kg body weight | **0,50** |
| | Complete and supplemental mixtures for other categories of pigs | **1,0** |
| | Complete and supplemental mixtures for cattle, sheep and goats | **3,0** |
| **Ochratoxin A** | Mixtures for pigs | **0,1** |
| | Mixtures for fattening pigs and breeding sows | **0,2** |
| | Mixtures for poultry | **1,0** |
| | Mixtures for layers | **0,25** |

## DISCUSSION

Aflatoxin is, in our country during the period of 1999-2000. year, determined in amount of 20.1-21.6% of analysed samples of animal feed with an average content of 0:05 to 00:04 mg/kg, and some samples contained even 0.10 mg/kg (B o č a r o v - S t a n č i ć et al., 2000a). Tryals performed in 2002nd (M a š i ć et al., 2003) have showed that out of 585 samples 10.43% was contaminated with AFB1.

By analyzing the results of mycotoxicological analyses of 78 samples for poultry in the period 1990-1994. year (S h a f f e r et al., 1994a) was obtained that 6.41% of the samples for broilers (5/78) contained aflatoxin in allowable values, while in the same period this toxin was not detected in samples of feed for layers (0/62). In the next period (1994-1996.), the situation was much better because none of the 16 samples of feed for broilers and 27 samples of hens did not contain aflatoxin above the allowable limit (S h a f f e r et al., 1997, 1998). However, in the period of 1997-2003 (N e d e l j k o v i ć - T r a i l o v i ć et al., 2004) even 24.44% of poultry feed samples contained AFB1 above the allowable limit.

Retrospective analysis of results of mycotoxicological tests performed on 74 feed mixture for chickens and 88 mixtures for laying hens in the ten-year period (1995-2004.) showed that 17.6 and 18.2% of the samples contained AFB1 in amounts of $0.05 \pm 0.04$ and $0.04 \pm 0.03$ mg/kg , respectivelly, and that of the contaminated samples, 76.92% and 93.75 contain tested toxins above the allowable limit (S i n o v e c, 2005).

By analyzing the results of mycotoxicological examination of 87 feed mixtures for pigs, 35 for fattening pigs and 36 for breeding pigs in the ten-year period (1995-2004) has been demonstrated that 26.4, 22.9 and 25.0% of the samples contained AFB1 in quantities of $0.05 \pm 0.02$, $0.06 \pm 0.04$ and $0.06 \pm 0.04$ mg/kg respectivelly, and that 56.5, 100.0 and 22.2% of the contaminated samples contain tested toxins above the allowable limit (M a r k o v i c et al., 2005). The number and types of mycotoxins vary depending on the feed, as well as on the year which can be directly related to climatic factors, and the average annual humidity. The fact that the results of the tested samples, presented in this paper, showed no significant presence of aflatoxin, suggests that the toxin producing fungi in our environment do not find fertile ground for the production of this toxins, as well as the absence of certain nutrients in the production of complete feed mixtures for poultry and pigs, which are the traditional sources of aflatoxin (peanut oil meal). However, detected values for aflatoxin in samples of feed mixtures for the initial and final fattening of broilers were at the five samples above the allowed values which in the aggregate make 10 percent of the total analyzed samples for this category of poultry or 4.81% based on the total number of samples of feed mixtures intended for poultry feeding, suggesting the need for permanent monitoring and caution in nutrition of given poultry categories.

Ochratoxin (OTA) has a special importance because of its ties with Balkan endemic nephropathy of people, which is a chronic disease with a fatal

ending (R a d o v a n o v i c, 1991). In some trials (R a d i c et al., 1986) it was found that 56.6% of the tested serum samples obtained from people of the West nephropathyc area Posavina were positive for the presence of ochratoxin A. OTA is classified as potential carcinogenic for a population of people (group B), because, with a high content of OTA in feed (and C i e g l e r  V e s o n d e r, 1983), was noticed high incidence of renal adenomas and carcinomas (R a d o v a n o - v i c, 1991). The presence of OTA, as a natural contaminant, was first found in a sample of corn. OTA content in the feed and feedstuffs is usually lower than the 50 mg/kg, but in the incorrect storage may be identified significantly higher levels (J u r i c et al., 1999). In warmer regions, a significant number of samples usually contain very low amounts of OTA (<1 mg/kg), although and signifi- cantly higher levels can be detected (S p e i j e r s and E g m o n d, 1994).

OTA occurs naturally as a contaminant of various types of plant products such as cereals, flour, coffee, spices, pulses and dried fruit (S t u d e r - R o h r et al., 1995; K u i p e r - G o o d m a n and S c o t t, 1989). It was also found in wine, beer and fruit juices as a result of the use of contaminated raw materials for their production (J o r g e n s e n, 1998; K u i p e r - G o o d m a n, 1996).

In our country, during 1999-2000. year, ochratoxin was found in 41.2% of samples with an average content of 0.06 to 0.08 mg/kg, and some samples contained even 0,10 to 0,32 mg/kg (B o č a r o v - S t a n č i ć et al., 2000a). Tests performed 2002nd (M a š i ć et al., 2003) showed that out of 585 samples 15.56% was contaminated with OTA. In the period of 1990-1994. (S h a f f e r et al., 1994a) feed for broilers contained ochratoxin within acceptable limits, while 51.51% of feed samples for layers had OTA contamination above the allowable limit. In the next period (1994-1996.), the situation was much more favorable, because none of the 16 samples intended for broilers did not contain ochratoxin above allowable limits (S h a f f e r et al., 1997, 1998), while in the same period, feed for hens was defective in 37% (10/27). This period was characterized by the presence of ochratoxin in amounts up to 0.25 ppm in 49, or 63% of feed samples for broilers and laying hens, 0.25 to 0.50 ppm at 38, and 26% of cases and the 0.50 to 1.00 ppm in13, or 11% of the feed samples for broilers and laying hens, respectivelly. On the other hand, in the period of 1997-2003. (N e d e l j k o v i c - T r a i l o v i ć et al., 2004) even 33.33% of poultry feed samples contained OTA above the allowable limit.

Retrospective analysis of results mycotoxicological examinations of 74 feed mixtures for chickens and 88 mixtures for laying hens in the ten-year period (1995-2004.) found that 94.6 and 92.0% of the samples contained OTA in amounts of $0.26 \pm 0.15$ and $0.23 \pm 0.12$ mg/kg or that 10.00 and 46.91% of the contaminated samples contained tested toxins above the allowable limit (S i n o v e c, 2005).

By analyzing the results of 87 mycotoxicological examinations of feeding mixtures intended for pigs, 35 samples for fattening pigs and 36 for breeding pigs in the ten-year period (1995-2004) have showen that all samples contain OTA in amounts of $0.27 \pm 0.23$, $0.31 \pm 0.14$ and $0.27 \pm 0.11$ mg/kg and that 100.0, 82.9 and 19.4% of the contaminated samples contain tested toxins above the allowable limit (M a r k o v i c et al., 2005).

In the current conditions, the presence of OTA is the only relevant parameter for judging the contamination of feed mixtures for poultry fnutrition, considering he is the most toxic of all mycotoxins in poultry (L e e s o n et al.) In the presented work there was not in any of the tested sample determined data for ochratoxin that exceeded the maximum allowable values required by the applicable regulations. However, the analyses of feedstuff intended for pigs and poultry found maximum values of 0.30 mg/kg but currently applicable regulations do not regulate the maximum permissible value of these mycotoxins in feed, but only in complete and supplemental mixtures. If the same criteria for nutrients as well as for omplete and supplementary feed mixtures for pigs, breeding sows, fattening pigs and hens, could be applied then the number of samples that exceed the maximum permitted level would be much higher. It should be taken into account that the test feedstuff:corn, soybean and sunflower meals are also the most common raw material in formulating rations for this species and categories of animals and that must as well be considered in their use, especially with significant levels of detected mycotoxins.

Given the toxicity and frequency of occurrence in feed, zearalenone (ZON) is one of the most important mycotoxins of Fusarium fungis which are very widespread in nature and are very common in our geo-climatic region. Corn is the most commonly affected by contamination (K u i p e r - G o o d - m a n et al., 1987), and hybrid variety with long vegetation and high humidity at the time of harvest are suitable for mold growth. The amount of zearalenone in contaminated corn is very different and usually ranges on average from 2 to 4 mg/kg, although it may be 12 mg/kg and higher.

The incidence and degree of contamination of grain with zearalenone vary depending on the type of seeds, climatic conditions and the method of storage (P o z z i et al., 1995). Corn, wheat and barley are the nutrients that are most contaminated, while other types of feed grains seem to be less contaminated with small amounts of zearalenone. Generally, the mean value of ZON detected in barley are relatively high, low in wheat, while in corn they vary.

In areas of our country most often are contaminated feedstuffs for pig, especially maize, which may contain an average of over 10 ppm ZON (B o č a - r o v - S t a n č i ć et al., 1997a). The feed samples showed the presence of zearalenone in 70.7% of samples at amount of 0.2 to 20 mg/kg (B o č a r o v - S t a n - č i ć et al., 2000b). During the 1999. and 2000. the presence of zearalenone was detected in 72.3 and 74.5% of samples with an average content of 0.66 or 2.39 mg/kg, but some of the samples contained toxin at 3.2, or 12.8 mg/kg (B o č a - r o v - S t a n č i ć et al., 2000a). Trials performed 2002nd (M a š i ć et al., 2003) showed that out of 585 samples 15.04% were contaminated with ZON.

By analyzing the results of mycotoxicological examinations of 78 samples for poultry during the period 1990-1994. (S h a f f e r et al., 1994a) was obtained that 43.6% of the samples for broilers (34/78) and 51.6% samples for hens (32/62) contained ZON in allowed values. Also, in the next period (1994-1996.), none of the 43 poultry feed samples contained ZON in quantities above permissible (S h a f f e r et al., 1997, 1998), but the contamination in some samples was very high (0.72-10.70 ppm).

Retrospective analysis of results obtained from mycotoxicological examinations of 74 feed mixture for chickens and 88 mixtures for laying hens in the ten-year period (1995-2004..) showed that all samples contained ZON in amounts of $2.69 \pm 5.14$ and $5.29 \pm 2.61$ mg/kg, respectively within the permissible limits (M a r k o v i c et al., 2005).

Testing the content of mycotoxins in feed for pigs (212 samples) in 2000-2001 years (M a š i ć et al., 2002a), it was found that the contamination of a mixture intended for young animals with unpermitted levels of mycotoxins (70.2%) was almost identical to the same for adult animals ( 69.1%). It is pointed out that the most common cause of feeding mixtures contaminations is related to the presence of mycotoxin zearalenone (an average of about 10.7 mg/kg of feed DM). The results indicate a significantly higher contamination of mixtures in comparison to previous studies carried out in the period 1988-1993. (S h a f f e r et al., 1994b) where was found to be 97.6% of samples of feeding mixtures for young contaminated with a zearalenone, or 81.0% of the samples for adult animals.

Retrospective analysis of results of mycotoxicological examinations 87 feeding mixtures for pigs, 35 for fattening pigs and 36 for breeding pigs during the ten-year period (1995–2004.) showed that all samples contained ZON in quantities of $5.06 \pm 2.74$, $3.97 \pm 2.33$ and $5.25 \pm 3.20$ mg/kg and that 94.3, 85.7 and 94.4% of the contaminated samples contain tested toxins above the allowable limit (M a r k o v i c et al., 2005).

In this work examination of feed mixtures for broilers established for zearalenone values ranged from 0.013 to 1.257 mg/kg and for the hens from 0.013 to 0.54 mg/kg. The current regulations do not regulate the maximum permissible value of these mycotoxins in complete and supplementary feed mixtures for poultry. Relatively high amount of zearalenone in the feed mixtures samples for poultry nutrition does not need much to concern in view of the higher resistance of poultry compared to other species, but suggests caution because it as an indicator of ongoing contamination of complete feeding mixtures by toxin producing fungi. On the other hand the presence of zearalenone in feed as well in a complete feed mixture for pigs is a permanent threat in view of their sensitivity to this mycotoxin and a wide spectrum of clinical manifestations that Zearalenone causes. Of the total number of feed mixtures samples for young (20), examined in this paper, for the two samples the presence of zearalenone was above permitted level and the quantity was 2.786 and 0.819 mg/kg, which in the aggregate make 10 percent of the total analyzed samples for this group of pigs or 3.51% based on the total number of feeding mixture samples intended for pig nutrition.

CONCLUSION

Generally speaking, the results are encouraging in view of the fact that relatively small number of faulty mixture was detected. However, the fact that only a limited number of feed samples we received for analysis and which

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were already during the sensory examination suspicious for the presence of mycotoxins, as well as the history and clinical picture of animals fed with examined feed, suggest caution and mycotoxin presence. Early or timely determination of the presence of mycotoxins in feed and subsequent elimination from the use of contaminated feed and / or possible dilution or mixing with feed free of mycotoxins can mitigate the negative effects, but it requires a certain period of time for elimination of resorbed quantities of mycotoxins and adverse effects. Therefore, in the production conditions it must be practiced continuous and multistage monitoring of hygienic quality of feed in order to respond quickly and efficiently as, currently, the only successful way to prevent harmful effects of mycotoxins.

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## СТЕПЕН МИКОТОКСИКОЛОШКЕ КОНТАМИНАЦИЈЕ ХРАНИВА И ПОТПУНИХ КРМНИХ СМЕША ЗА ИСХРАНУ СВИЊА И ЖИВИНЕ ТОКОМ ПЕРИОДА 2007–2012. ГОДИНЕ НА ТЕРИТОРИЈИ РЕПУБЛИКЕ СРБИЈЕ

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Резиме

Микотоксине најчешће производе гљивице из родова *Aspergillus*, *Fusarium* и *Penicillium*. Токсини су од изразите важности јер се могу пренети са животиња на људе путем млека и животињских производа, а неки од њих су канцерогени и тератогени. Микотоксини доводе до поремећаја здравственог стања свих животи- ња, али су ефекти уочљивији код високо производних животиња у фармском на- чину држања с обзиром на знатно већу конзумацију концентрованих хранива иако и кабаста хранива могу да буду контаминирана микотоксинима у значајнијем сте- пену. Микотоксикозе су најчешће обољења сезонског карактера, а представљају значајан дијагностички проблем за ветеринарску праксу, јер по карактеристика- ма често личе на обољења изазвана патогеним микроорганизмима или нутритив- ним дефицитом или дисбалансом. Степен здравствених поремећаја зависи од ко- личине токсина у храни и дужине његовог уношења у организам као и од врсте и категорије животиња.

Присутност микотоксина у храни за животиње је неизбежна па је неопходно тестирање сировина и производа да би храна за људе и животиње била сигурна за употребу. Штете у живинарству и свињарству које настају услед микотоксикоза, услед директних губитака због угињавања животиња или, још чешће, индиректне због пада производних и репродуктивних способности животиња, наметнуле су потребу за континуираним мониторингом хигијенске исправности крмних смеша за исхрану ових животињских врста.

Током петогодишњег периода (2007–2012) анализирана су укупно 104 узорка са територије Републике Србије намењена исхрани свих категорија живине и то смеше за почетни и завршни тов бројлера (50 узорака) и за кокоши носиље (54). Анализом је обухваћено и 57 узорака крмних смеша намењених исхрани свих категорија свиња и то смеше за исхрану младих (20 узорака) и старих категорија (37 узорака) као и 196 узорака хранива која се најчешће користе приликом формулисања оброка за наведене животињске врсте (кукуруз, сојина и сунцокретова сачма). За анализу узорака коришћени су метода танкослојне хроматографије и елиса тест. Добијени резултати су поређени са тренутно важећим Правилником о квалитету хране за животиње (Службени Гласник РС 41/09) који се примењује од 1.05.2010. године и где се у делу о максимално дозвољеним количинама штетних материја (члан 99) износе вредности о максимално дозвољеној количини микотоксина у храни за животиње. Број и врста микотоксина варира у односу на врсту смеша, као и у односу на поједине године што се може довести у директну везу са климатским факторима, односно просечном годишњом влажношћу. Чињеница да у испитиваним узорцима није утврђено присуство афлатоксина указује да у нашим условима токсинпродукујуће гљивице не наилазе на погодно тле за продукцију овог токсина, као и на одсуство појединих хранива у производњи потпуних крмних смеша за свиње и живину које представљају традиционалне изворе афлатоксина (кикирикијева уљана сачма). Добијени резултати представљају охрабрујућу чињеницу с обзиром на релативно мали број неисправних смеша и хранива. Међутим, чињеница да се ради о ограниченом броју узорака хране које смо добијали на анализу упућује на опрез и даљи константан мониторинг присуства микотоксина у храни за животиње.

КЉУЧНЕ РЕЧИ: микотоксини, свиње, живина, сточна храна

*E m i l i j a   I v a n o v a\*,   N a t a l i j a   A t a n a s o v a – P a n č e v s k a,
D ž o k o   K u n g u l o v s k i*

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# ANTIMICROBIAL ACTIVITIES OF LABORATORY PRODUCED ESSENTIAL OIL SOLUTIONS AGAINST FIVE SELECTED FUNGAL STRAINS

ABSTRAKT: It is well known that essential oils possess significant antimicrobial activity. This study was conducted to estimate the antimicrobial activity of various types of Biokill, a laboratory produced solution composed of several essential oils (Biokill dissolved in 96% ethanol; Biokill 96% further dissolved in DMSO; Biokill dissolved in 70% ethanol and Biokill 70% further dissolved in DMSO). The antimicrobial activity was evaluated against five selected fungal strains, *Candida albicans ATCC 10231*, *Saccharomyces cerevisiae ATCC 9763*, *Aspergillus niger I.N. 1110*, *Aspergillus sojae CCF* and *Penicillium* spp. *FNS FCC 266*. A variation of the microtiter plate-based antimicrobial assay was used in order to assess the antimicrobial activity of the solutions. By applying this assay minimal inhibitory concentrations (MIC) of the Biokill solutions were determined for each strain of the selected test microorganisms. The results demonstrated that all variations of Biokill showed antimicrobial activity at concentrations lower than 2.5µg/mL. Biokill 70% further dissolved in DMSO showed the best antimicrobial properties against all the selected strains with MICs less than 1.25µg/mL. These results indicated that Biokill could find application in the pharmaceutical industry, in food preservation and conservation, in the prevention and treatment of plants infected by certain phytopathogens, etc.
KEY WORDS: Antimicrobial activity, Essential oils, Microtiter plate-based assay, Minimal Inhibitory Concentration.

## INTRODUCTION

The resistance that microorganisms have recently developed to antimicrobial agents, mainly as a result to their widespread use, has brought a lot of attention to the search of new compounds with antimicrobial properties from various sources. In this search the development of antifungal agents has certainly fallen behind the development of antibacterial agents. This notion is understandable considering the cellular structure of fungi, which are eukaryotic organisms with similar metabolic pathways as their hosts, as opposed to bacteria which

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are prokaryotic and present a number of structural and metabolic targets that differ from the ones employed in eukaryotes (D i x o n and W a l s h, 1996). The prevalence of resistance to antifungal agents has significantly increased in the past decade (A r i f et al., 2011). Some of the factors that contribute to the spread of fungal disease are immunosuppressive therapies, the common use of indwelling intravenous devices and the indiscriminate use of broad-spectrum antibiotics which eliminate or decrease the nonpathogenic bacterial populations that normally compete with fungi. Furthermore, unlike bacterial pathogens, fungal pathogens are more difficult to control (R a b a d i a et al., 2011). The limitations of current antifungal drugs, the increased incidence of systemic fungal infections, the difficulties in their treatment and the rapid development of resistance to antifungal agents have increased the research on therapeutic alternatives. Since most of the antibiotics available on the market are of natural origin, it can be inferred that natural products might be an excellent source of antifungal agents, either in their basic form or as template structures for more effective derivatives (B a r r e t t, 2002; J a c o b and W a l k e r, 2005). Plants produce a high diversity of bioactive secondary metabolites, great number of which serves to protect themselves against microbial attacks. Amongst these secondary metabolites, the antifungal properties of tannins, terpenoids, alkaloids and flavonoids have already been reported in numerous *in vitro* studies. It is believed that most of the 100,000 known secondary metabolites involved in the plant chemical defense systems seemed to have appeared as a response to the interactions with predators throughout the millions of years of co-evolution.

Essential oils have been long recognized for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties **(**B a s s o l e and J u l i a n i, 2012). S a r t o r a t t o et al. (2004) concluded that the presence of various chemical compounds in the essential oils was crucial for their antimicrobial properties. In many cases the complex interaction between the different classes of compounds such as phenols, ketones, alcohols, terpenes, esters or hydrocarbons can be the actual source of the antimicrobial activity of the essential oil. The use of combinations, either from whole essential oils or artificial mixtures of purified main components, is a new approach which aims to increase the efficacy of the essential oil by taking advantage of the synergistic and additive properties that these components can exhibit. Several bioactive chemical compounds can affect multiple target sites and thus affect multiple biochemical processes in the microorganisms, producing a plethora of interactive antimicrobial effects. Generally, compounds with similar structures exhibit additive or less often synergistic effect. For example, the occurrence of additive interaction between *Origanum vulgare* L. and *Thymus vulgaris* has been related to their main bioactive phenolic compounds, carvacrol and thymol (L a m b e r t et al., 2001).

Bioassays can be defined as the use of a biological system to detect properties, such as antibacterial, antifungal, anticancer, antiviral and similar, of a crude extract, a chromatographic fraction, a mixture or a pure compound. For screening natural products which are most often found in small quantities,

especially their purified compounds, the application of a reliable and economically efficient *in vitro* assay can be a limiting factor in any viable screening. Usually, the most common methods employed in antifungal screening are: the disc diffusion method and the broth micro dilution method, which can both be time consuming and require significant quantities of the test material. The variation of the micro-titer plate based method that we have developed for this study, successfully surpassed all of the abovementioned problems and fulfilled all of the conditions that qualify an excellent scientific method: it is simple, safe, sensitive, efficient, easily reproducible, time saving and cost-effective.

The objective of this study was to employ our variation of the microtiter plate-based method in order to assess the antifungal activities of various solutions of Biokill, a laboratory produced mixture of several essential oils and their active components against five fungal strains: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Aspergillus niger* I.N. 1110, *Aspergillus sojae* CCF and *Penicillium* spp. FNS FCC 266. All the ingredients that were a part of the composition of Biokill were previously investigated in the laboratory and had already been confirmed to have significant antifungal activities. In this study, the aim was to investigate the influence of the solvent on the antifungal properties of the mixture and to establish the solvent that enables the highest antifungal activity of Biokill. For that purpose we used four different solutions of Biokill by using combinations of the following solvents: 70% ethanol, 96% ethanol and dimethyl sulfoxide (DMSO).

MATERIALS AND METHODS

**Biokill solutions:** Biokill original represents a laboratory produced mixture composed of several essential oils with already investigated antifungal properties. Four variations of Biokill solutions were used in our study: *Solution 1*: 100μL of Biokill original dissolved in 1ml 96% ethanol; *Solution 2*: 100μL of Solution 1 dissolved in 1mL of dimethyl sulfoxide (DMSO); *Solution 3*: 100μL of Biokill original dissolved in 1mL 70% ethanol and *Solution 4*: 100μL of Solution 3 dissolved in 1mL of dimethyl sulfoxide (DMSO).

**Fungal Strains and Cultures:** The test microorganisms used in this study included five strains of fungi: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Aspergillus niger* I.N. 1110, *Aspergillus sojae* CCF and *Penicillium* spp. FNS FCC 266. All the fungal strains were derived from stock cultures, property of the Institute of Biology at the Faculty of Natural Sciences in Skopje, Macedonia.

All the strains were identified according to their macroscopic and microscopic morphological properties. The mediums for growth and maintenance of the fungal cultures were Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA). The cultures were incubated at room temperature and were transferred to fresh media every 3-5 days for the yeasts and every 5-7 days for the molds.

***Preparation of Fungal Suspensions:*** For preparation of the fungal suspensions, inoculum of the culture was suspended into sterile normal saline solution (0.90% w/v of NaCl) and the suspension was homogenized by gentle mixing in the hands. The turbidity of the fungal suspension was compared to a 0.5 McFarland standard. Inoculum of the culture or sterile normal saline solution was added until the fungal suspension matched the McFarland standard and the number of bacteria was $1.5 \times 10^8$ CFU/ml. From this initial solution, two additional serial dilutions were made to acquire a working solution of $1.5 \times 10^6$ CFU/ml.

***Resazurin solution:*** The resazurin solution was prepared by dissolving 270 mg of resazurin powder (Sigma-Aldrich GmbH, Germany) in 40 ml sterile distilled water. The solution was mixed on a vortex mixer until the powder was completely dissolved and the solution was homogenous.

***Microtiter plate based assay:*** The antifungal activity of the Biokill solutions was assessed using a modified version of the microdilution techniques described by Drummond and Waigh (2000). The antifungal assay was performed by using a sterile 96-well plate and the Minimal Inhibitory Concentration (MIC) value was determined for estimating the antifungal activity. All the assays were prepared under aseptic conditions. Resazurin was used as an indicator of growth for the yeast assays, while the growth in the mold assays was inspected visually.

The first step of the assay was adding 50 µL of sterile Sabouraud Dextrose Broth (SDB) into the first four and the last two rows of the 96-well plate. The first four rows were used for evaluation of the activity of the Biokill solutions, while the last two rows served as a positive and a negative control. The positive control confirmed the viability of the fungal culture, while the negative control verified the sterility of the working conditions and solutions. The second step was adding 50 µL of the first Biokill solution to the first well of the first row of the plate. Using sterile pipette tips, the contents of the first well of the first row were mixed and 50 µL were transferred to the second well of the same row. Serial dilutions were carried out until all the wells contained 50 µL of the solution under examination in descending concentrations. The procedure was repeated for the remaining Biokill solutions in the next three rows. Then, 5 µL of resazurin solution was added to each row, followed by the addition of 5 µL of fungal suspension.

Positive control (viability control) comprised of 50 µL of SDB, 5 µL resazurin (where necessary) and 5 µL of fungal suspension, while the negative control (sterility control) comprised of 50 µL of SDB and 5 µL of resazurin (where necessary). The microtiter plates were wrapped in sterile tinfoil in order to prevent contamination and were then incubated at room temperature for 3-5 days for the yeast assays and 5-7 days for the mold assays. A blue colored solution indicated the growth inhibition in the test wells, while pale pink to colorless solution indicated microbial growth or absence of inhibition. The mold assays were inspected visually: a clear solution indicated absence of growth

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while visual indication of mycelia indicated microbial growth or absence of inhibition. All the tests were performed in triplicate.

## RESULTS

Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by the oxidoreductase enzymes within viable cells. Resorufin can be further reduced to hydroresorufin which is colorless and nonfluorescent. The antifungal activity was assessed by the MIC which was defined as the lowest concentration at which substance that prevents change in color occurred. A microtitre plate based assay was carried out for each fungal strain and the results for each fungal strain are shown in the Tables below.

According to the results, all of the Biokill solutions exhibited a broad anti-fungal spectrum of activity and caused inhibition on the mycelial growth of the fungal strains at minimal inhibitory concentrations lower than 2.5 µg/mL.

The results showed that Solution 4 (Biokill 70% further dissolved in DMSO) was the best solvent to potentiate the antifungal activity of the Biokill mixture with MICs lower than 1.25 µg/mL. The most sensitive microorganism was *Aspergillus sojae* CCF which mycelial growth was completely inhibited at concentrations lower than 0.3125 µg/mL. Next in the order of sensitivity was *Penicillium* spp. FNS FCC 266 which MICs were lower than 0.625 µg/mL. The most resilient were the yeasts *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231 which showed identical results as MICs did, ranging from 1.25 – 2.5 µg/mL.

Tab. 1. – MICs of the four Biokill solutions for *Candida albicans* ATCC 10231

| *Candida albicans* ATCC 10231 | Concentration in µL/mL | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0.04 | 0.02 | 0.01 | 0.005 |
| Solution 1 | | | | | | | | | | | | |
| Solution 2 | | | | | | | | | | | | |
| Solution 3 | | | | | | | | | | | | |
| Solution 4 | | | | | | | | | | | | |
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| Positive control | | | | | | | | | | | | |
| Negative control | | | | | | | | | | | | |

Table 1 shows the bioassay results for *Candida albicans* ATCC 10231. The results in this table indicate that: Solution 1 and Solution 3 in which pure ethanol with different concentrations was used as a solvent, inhibited the growth of the yeast at MIC of 2.5 µg/mL. On the other hand, Solution 2 and

Solution 4 where ethanol dissolved in DMSO at a ratio of 1:10 (v/v) was used as a solvent, showed better antifungal properties and inhibited the mycelial growth at MICs at 1.25 µg/mL.

Tab. 2. – MICs of the four Biokill solutions for *Saccharomyces cerevisiae* ATCC 9763

| *Saccharomyces cerevisiae* ATCC 9763 | Concentration in µL/mL | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0.04 | 0.02 | 0.01 | 0.005 |
| Solution 1 | ▓ | ▓ | ▓ | | | | | | | | | |
| Solution 2 | ▓ | ▓ | ▓ | ▓ | | | | | | | | |
| Solution 3 | ▓ | ▓ | ▓ | | | | | | | | | |
| Solution 4 | ▓ | ▓ | ▓ | ▓ | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| Positive control | | | | | | | | | | | | |
| Negative control | | | | | | | | | | | | |

Table 2 displays identical results as Table 1. Solution 1 and Solution 3 inhibited the growth of the yeast at MIC of 2.5 µg/mL, and Solution 2 and Solution 4 at MICs of 1.25 µg/mL. It can be hypothesized that the same molecular mechanisms in *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763 are employed in the defense mechanism against the active ingredients in the solutions with antifungal properties. We believe this finding should be further studied on different strains of yeasts in order to dismiss coincidence and draw a correlation between the defense mechanisms which are active in yeasts against the biologically active compounds of essential oils.

It should also be noted that as a result of the active metabolism of yeasts the resazurin indicator was two times reduced from resazurin > resorufin > hydroresorufin which is a colorless compound as opposed to bacterial bioassay where the resazurin is only reduced once to resofurin which color was pink.

Tab. 3. – MICs of the four Biokill solutions for *Aspergillus niger* I.N. 1110

| *Aspergillus niger* I.N. 1110 | Concentration in µL/mL | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0.04 | 0.02 | 0.01 | 0.005 |
| Solution 1 | ▓ | ▓ | ▓ | ▓ | | | | | | | | |
| Solution 2 | ▓ | ▓ | ▓ | | | | | | | | | |
| Solution 3 | ▓ | ▓ | ▓ | ▓ | ▓ | | | | | | | |
| Solution 4 | ▓ | ▓ | ▓ | ▓ | ▓ | ▓ | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| Positive control | | | | | | | | | | | | |
| Negative control | | | | | | | | | | | | |

*Aspergillus niger* I.N. 1110 proved to be more sensitive to the solution in comparison to *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763, but more resilient than the other molds studied in this research. All the solutions under examination showed different MICs: the MIC of Solution 1 was 0.625 µg/mL, the MIC of Solution 2 was 1.25 µg/mL, the MIC of Solution 3 was 0.3125 µg/mL and the MIC of Solution 4, which showed the best antifungal activity against *Aspergillus niger* I.N. 1110, was 0.156 µg/mL.

Tab. 4. – MICs of the four Biokill solutions for *Aspergillus sojae* CCF

| *Aspergillus sojae CCF* | Concentration in µL/mL | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0.04 | 0.02 | 0.01 | 0.005 |
| Solution 1 | | | | | | | | | | | | |
| Solution 2 | | | | | | | | | | | | |
| Solution 3 | | | | | | | | | | | | |
| Solution 4 | | | | | | | | | | | | |
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| | | | | | | | | | | | | |
| Positive control | | | | | | | | | | | | |
| Negative control | | | | | | | | | | | | |

*Aspergillus sojae* CCF was the most sensitive microorganism to the studied solutions. Solution 1 and Solution 3 inhibited the mycelial growth at MICs of 0.156 µg/mL, Solution 2 showed lower efficacy with MIC of 0.3125 µg/mL, and Solution 4 showed the highest inhibition rate with MIC of only 0.039 µg/mL.

Tab. 5. – MICs of the four Biokill solutions for *Penicillium* spp. FNS FCC 266

| *Penicillium spp. FNS FCC 266* | Concentration in µL/mL | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.31 | 0.16 | 0.08 | 0.04 | 0.02 | 0.01 | 0.005 |
| Solution 1 | | | | | | | | | | | | |
| Solution 2 | | | | | | | | | | | | |
| Solution 3 | | | | | | | | | | | | |
| Solution 4 | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| Positive control | | | | | | | | | | | | |
| Negative control | | | | | | | | | | | | |

*Penicillium* spp. FNS FCC 266 was also very sensitive to the Biokill solutions. Table 5 shows that Solution 1 and Solution 2 inhibited the growth of *Penicillium* spp. FNS FCC 266 at MICs of 0.3125 µg/mL, Solution 3 inhibited the growth at 0.078 µg/mL and Solution 4 for the third time confirmed the best antimicrobial properties by the lowest MIC of 0.039 µg/mL.

From the results obtained in this study it can undoubtedly be concluded that the best solvent to potentiate the antifungal activity of the Biokill mixture is 70% ethanol further dissolved in DMSO at a ratio of 1:10 (v/v). It should be noted that this solution had the lowest concentration of ethanol in comparison with all three other solutions. Following this logic, it can be hypothesized that ethanol might be chemically reacting with some of the bioactive metabolites present in Biokill, hence lowering the concentration of the aforementioned ingredient and diminishing the total antifungal properties of the mixture.

## DISCUSSION

Recently, the scientific interest into biological properties of essential oils and natural products in general has been increased as a series of molecules with antimicrobial activity have been found in plants. Active research on the use of the biologically active secondary metabolites present in essential oils of plants such as phenols, flavonoids, alkaloids, terpenes, tannins and others, has been seen as a potential alternative to the conventionally used antifungal agents, and as means to control pathogenic fungi and fungal contamination. The response of different essential oils usually depends on the fungal species tested and may include ranges from resistant to various degrees of susceptibility (A m i n i et al., 2012), but the results in our study clearly showed that Biokill had a broad fungitoxic spectrum by inhibiting the mycelial growth of several very different strains of fungi.

The antimicrobial activity of plant oils and extracts has formed the basis for many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (G y o r g y, 2010, H a m m e r et al., 1999). Some studies have concluded that combinations of essential oils have greater antimicrobial activity than their individual components (M o u s a v i and R a f t o s, 2012).

Essential oils are natural plant products containing complex mixture of components, thus having multiple antimicrobial properties. Different components of essential oils can interact in order to either reduce or increase the antimicrobial efficacy (D e l a q u i s, 2002; B a s s o l e and J u l i a n i, 2012). The interaction between essential oil compounds can produce four possible types of effects: indifferent, additive, antagonistic, or synergistic effects (B a s s o l e and J u l i a n i, 2012; B u r t, 2004; P e i et al., 2009). In the preparation of Biokill, our aim was to take advantage of the synergistic effects of the mixture. The practical implications of this approach are in the use of lower concentrations of active compounds which are needed to yield a similar antifungal reaction, primarily in order to prevent the possible cytotoxic effects of high concentrations of biologically active compounds, as well as to provide a more cost-effective solution.

There are limited numbers of reports dealing with the molecular mechanisms of action of combinations of essential oils or their purified components on microorganisms. First of all, the hydrophobic nature of essential oils enables

them to partition the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of the cell components (P r a b u - s e e n i v a s a n, 2006). The phenolic components present in the oils may interfere with cell wall enzymes like chitin synthase, as well as with α- and β-glucanases of the fungus (A d a m s et al., 1996; D a S i l v a et al., 2012). Thymol, the major constituent of the *Thymus vulgaris*, in a study by Z a m - b o n e l l i et al. (2004) was correlated with damage to the cell as a consequence of the increase in vacuolization of the cytoplasm and an accumulation of lipid droplets, appearance of ripples in the plasmalemma and changes in the mitochondria and endoplasmic reticulum of *Colletotrichum lindemuthianum* and *Pythium ultimum* (D a S i l v a et al., 2012). R a s o o l i et al. (2006) observed severe hyphae collapsing, plasmatic membrane rupture and destruction of mitochondria in *Aspergillus niger* treated with essential oils of *Thymus eriocalyx* and *Thymus porlock* (D a S i l v a et al., 2012).

There are some generally accepted mechanisms of interaction that produce synergism. These include the sequential inhibition of a common biochemical pathway, inhibition of protective enzymes and use of cell wall active agents to enhance the uptake of other antimicrobials (S a n t i e s t e b a n – L o p e z, 2007). In the recent study of B a s s o l e and J u l i a n i (2012), synergism between carvacrol and some hydrocarbon monoterpenes (such as α-pinene, camphene, myrcene, α-terpinene and *p*-cymene) that typically show low antimicrobial properties have been observed. The reason for this synergism was hypothesized to be the capability of hydrocarbons to interact with cell membranes which facilitates the penetration of carvacrol into the cell.

Since our bioassay was constructed to examine the *in vitro* properties of the studied materials, further studies on the safe use of these substances should be conducted. Even though the results confirm that all Biokill solutions have significant antifungal properties *in vitro*, if they are to be used for medicinal purposes additional studies of *in vivo* parameters are required. In order to assess that, the molecular mechanisms, the stability, toxicity and efficacy of the active components present in Biokill need to be further studied and evaluated.


CONCLUSION


Relatively nontoxic natural products can exert beneficial effects through the additive or synergistic effects caused by combining several essential oils with their isolated bioactive metabolites. The main goal was to potentiate their efficacy by producing a mixture of chemically active compounds ready to attack multiple target sites and completely destroy the cells of the pathogenic microorganism. In the present study, the antifungal screening of four solutions composed of a mixture of several essential oils and their active components named Biokill and 4 different solvents: Solution 1 (Biokill dissolved in 96% ethanol), Solution 2 (Biokill 96% further dissolved in DMSO), Solution 3 (Biokill dissolved in 70% ethanol) and Solution 4 (Biokill 70% further dissolved in DMSO) were assayed *in vitro* according to a microtitre plate based

method by utilizing resazurin as an indicatior against five fungal strains: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Aspergillus niger* I.N. 1110, *Aspergillus sojae* CCF and *Penicillium* spp. FNS FCC 266. All of the Biokill laboratory solutions used in this research had excellent antifungal effects against all the selected fungal strains with MICs lower than 2.5 µg/mL. Since Solution 4 was the best antifungal agent with MICs lower than 1.25 µg/mL, 70% ethanol further dissolved in DMSO at a ratio of 1:10 (v/v) was concluded to be the best solvent for this laboratory produced mixture.

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# АНТИМИКРОБНЕ АКТИВНОСТИ ЛАБОРАТОРИЈСКИ ПРОИЗВЕДЕНИХ РАСТВОРА ИЗ ЕСЕНЦИЈАЛНИХ УЉА У ОДНОСУ НА ПЕТ ИЗАБРАНИХ ФУНГАЛНИХ СОЈЕВА

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Резиме

Добро је познато да есенцијална уља поседују значајну антимикробну активност. Ово истраживање је спроведено како би се проценила антимикробна активност различитих типова Биокила, лабораторијски произведени раствор сачињен од неколико есенцијалних уља (Биокил растворен у 96% етанол; Биокил даље растворен у DMSO; Биокил растворен у 70% етанол и Биокил 70% даље растворен у DMSO). Антимикробна активност је оцењена кроз пет одабраних гљивичних сојева, *Candida albicans ATCC 10231, Saccharomyces cerevisiae ATCC 9763, Aspergillus niger I.N. 1110, Aspergillus sojae CCF* и *Penicillium* spp. *FNS FCC 266.* Варијације на антимикробни есеј базиран на микротитарској плочи је био коришћен како би се оценила антимикробна активност на поменути раствор. Овим есејем је урађена минимална инхибиторска концентрација (MIC) на Biokill раствор за сваку врсту/ сој одабраних тест микроорганизама. Резултати су показали да све варијације на Биокил поседују антимикробну активност при концентрацији нижој од 2.5 μg/mL. Биокил 70% даље растворен у DMSO показао је најбоља антимикробна својства за све одабране сојеве и MIC нижа од 1.25 μg/mL. Ови резултати показују да би Биокил могао да нађе примену у фармацеутској индустрији, у презервацији и конзервацији хране, у заштити и третману биљака које су инфициране одређеним фитопатогенима итд.

КЉУЧНЕ РЕЧИ: антимикробна активност, есенцијална уља, микротитарска плоча, минимална инхибиторна концентрација

*V l a d i s l a v a  M.  Š o š o*[1], *M a r i j a  M.  Š k r i n j a r*[1],
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# ANTIFUNGAL ACTIVITY OF OLEORESINS USED IN
# MEAT INDUSTRY ON SOME TOXIGENIC *ASPERGILLUS* SPP.

ABSTRACT: Different spice oleoresins are widely used in meat industry. They contribute to the specific aroma and flavor of the end products, but they have also been reported to have strong antimicrobial activity. These properties open a plenty of possibilities to be used for defining the specific sensory profile of the product but also as natural food preservatives. This paper focuses on the antifungal activity of four oleoresins against different foodborne toxigenic *Aspergillus* species. Oleoresins used in the experiments were cayenne pepper, black pepper, garlic and rosemary oleoresins, and they were tested against following *Aspergillus* species: *A. clavatus, A. flavus, A. fumigatus, A. niger, A. ochraceus* and *A. versicolor.* Antifungal activity was tested using microtitre-plate-based assay incorporating resazurin as an indicator of cell growth and broth microdilution-method.
KEY WORDS: Oleoresins, antifungal activity, *Aspergillus* spp.

## INTRODUCTION

Nowadays, different meat products are commonly produced and consumed worldwide. Small-scale production of traditional meat products, which have been well recognized by consumers, still occurs, but large quantities are also produced at butchers' shops and in meat processing companies (P a p a - g i a n n i et al., 2007). Meat products are protected against microbial spoilage by different preservation methods in combination with controlled atmosphere or vacuum packaging or by cold storage throughout distribution chain (S ø - r e n s e n et al., 2008). However, molds periodically cause problems in this kind of food products, especially in the traditional ones (Š k r i n j a r et al., 2012).

In recent decades, the question of mold occurrence and toxicity has attracted attention, especially in the fields of agriculture and food industry (M i ž á k o v á et al., 2002, Š k r i n j a r et al., 2012). Microscopic filamentous fungi often contaminate vegetable and animal products, which become the source of diseases in man and slaughter animals. The reason for an increasing interest is the ability of molds to produce secondary metabolites – mycotoxins,

that have unfavorable effects, such as carcinogenesis, mutagenicity, teratogenicity, etc. (F r i s v a d and T h r a n e, 2002).

The difficulties 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 such possibility is the use of biopreservatives as antifungal additives (Č a b a r k a p a et al., 2011). Numerous 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 foodborne pathogens (Š k r i n j a r and N e m e t, 2009). Some biopreservatives that can be used in food processing are plant extracts, such as oleoresins. Spice oleoresins constitute the true essence of spices in their most concentrated form, containing both volatile essential oil and non-volatile resinous fraction (P o n c e et al., 2010). Oleoresins are applied in food, cosmetics and pharmaceutical industry as flavoring agents and antimicrobials (R a j a m m a et al., 2012). They are the most convenient substitutes for raw spices in foods since they are free of microorganisms and may be standardized to desired flavor (P o n c e et al., 2008). Antimicrobial potency of oleoresins is generally lower in food systems than *in vitro*, depending on the food composition, processing steps and storage temperature which could strongly influence the effectiveness of these antimicrobial agents (B u r t, 2004). Accordingly, larger amounts of oleoresins are required in food systems, which could seriously interfere with the food's finally sensory profile. Therefore, the applied concentrations have to be well tested and optimized, considering their impact on sensory properties in final product and antimicrobial effectiveness.

Among a number of oleoresins, few of them are of special interest for meat industry due to their flavor and aroma and the greatest potential for use in industrial applications. Those are oleoresins of garlic, rosemary, cayenne pepper, sweet pepper, black pepper, oregano, etc. (B u s a t t a et al., 2008).

The aim of the presented study was to evaluate the antifungal properties of some oleoresins commonly used in meat industry against foodborne *Aspergillus* species.

## MATERIALS AND METHODS

**Oleoresins**. Four different oleoresins were tested: garlic, rosemary, black pepper and cayenne pepper. They were obtained from Milex Ltd., Rumenka, Serbia. They were used in meat industry to enhance flavor, aroma and to substitute some conventional spices. They were added in small amounts to the additives that are normally used in meat industry, so their final concentration in the final product was relatively small.

Rosemary oleoresin was extracted from dried leaf of *Rosemarinus officinalis* L. It is viscous homogenic greenish liquid that contains approximately 11% of carnosic acid which was thought to have antimicrobial activity (W e c k e s s e r et al., 2007). Black pepper oleoresin was extracted from dried fruit *Piper nigrum* L. It is dark green viscous liquid with approximately 40% of piperine. Also this

oleoresin has a share of ethereal oil in amount of 20%. Garlic oleoresin contains around 10% of ethereal oil. It was extracted from cloves of *Allium satvium* L. Cayenne pepper oleoresin is also used as colorant in food industry. It is red-brownish liquid that was extracted from dried fruit from *Capsicum annum* L. or *Cappsicum fruitescence* L. It is also a potential antimicrobial agent since it contains capsaicin (cca 6%) (S i n g h and C h i t t e n d e n, 2008).

**Microorganisms**. The antifungal activity of oleoresins was evaluated on six different species from genus *Aspergillus*: *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus* and *A. versicolor*. 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, Himedia) slants and were stored at temperature of +4 °C.

**Antifungal assay**. Evaluation of the antifungal activity of the tested oleoresins against selected mold isolates was done by determining the minimal inhibitory concentrations (MIC). The MIC is defined as the lowest concentration, recorded in mg/l, of an antifungal agent that inhibits the growth of a fungus (R o d r i g u e z-T u d e l a et al., 2008). For MIC determination in vitro, two different methods were used: a) microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth and b) broth microdilution method.

a) Microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth; Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide, Himedia) allows the detection of microbial growth in extremely small volumes of solution in microtitre plates without the use of a spectrophotometer (S a r k e r et al., 2007). A stock solution was prepared by adding 0.01% of the resazurin sodium salt powder in sterile distilled water. It was filter-sterilized and kept at 4°C (H u s s a i n et al., 2011).

The suspension of the fungal isolates was prepared from the 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 $1\times10^8$ cells/ml. One ml of suspension was inoculated in each sterile Petri dish, poured with about 15 mL of SMA in triplicate. Petri dishes were left to incubate for 7 days at 25°C.

Oleoresins were diluted in propilenglicol (PG) to the test concentrations of 250, 125, 62.5, 31.25, 15.62 and 7.8 µl/ml.

Twenty microliters aliquots of all tested oleoresin solutions were added to 96-well microtitre plates in the abovementioned concentrations. After that, aliquots of 160 µl of Sabouraud maltose broth (SMB, Himedia) were added into each microplate. At the end, 20 µl of standardized fungal spore suspensions (approximately $10^5$ cfu/ml) were inoculated into each microplate. The test was performed in a total volume of 200 µl with final oleoresin concentrations of 25-0.78 µl/ml. Growth control contained 180 µl of SMB and 20 µl of standard spore suspension, while negative control had 180 µl of SMB with 20 µl of diluted oleoresin sample. The inoculated microtitre plates were incu-

bated at 25°C for two days. After the end of the incubation period, 10 μl of resazurin solution was added into each well and left to re-incubate overnight. A change of color from blue (oxidized) to pink (reduced) indicated the growth of molds. The MIC was defined as the lowest concentration of each substance that prevented this change in color.

b) Broth microdilution assay. The preparation of microtitre plates for broth microdilution method was the same as method under a). The difference was in the last step, where instead of adding resazurin solution in each well, test samples were sub-plated on SMA, taking 10 μl from each well into Petri dishes and then pouring SMA. Petri dishes were left to incubate for 7 days at the temperature of 25°C. The minimal fungicidal concentration (MFC) is defined as the lowest concentration of the oleoresins, at which 99.9% of the inoculated microorganisms were killed.

Reduction degree (RD [%]) is calculated in order to express antifungal efficiency of all oleoresins on selected fungal strains.

$$\text{RD [\%]} = \left(1 - \frac{S}{GC}\right) \cdot 100\%$$

where S is the number of colony forming units per Petri dish (CFU/P.d.) of every probe and GC is CFU/P.d. of growth control.

## RESULTS AND DISSCUSION

a) Microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth

Microtitre plate-based antifungal assay incorporating resazurin as an indicator of cell growth was applied to all oleoresins in order to investigate their potential antifungal activity. Due to the intensive color of cayenne paprika, black pepper and rosemary oleoresin, this method was not reliable. Therefore, it can be concluded that only antifungal activity of garlic oleoresin was evaluated according to this method.

Table 1 shows the results of antifungal activity of garlic oleoresin on all tested *Aspergillus* species.

The first six test concentrations of garlic oleoresins showed very strong antifungal effect on all fungal species used in this experiment. The absence of color change was observed in all wells, except in those that presenting the growth control. Considering these facts, it was concluded that garlic oleoresin was diluted to the final concentration of 0.015μl/ml in order to find MIC. For *A. flavus* and *A. ochraceus*, MIC was between 0.78 and 0.50 μl/ml. Minimal inhibitory concentration of garlic oleoresin for *A. niger* was 0.062 μl/ml, while for *A. versicolor*, *A. clavatus* and *A. fumigates,* new series of dilution did not determine MIC, so new series of dilution were required and prepared to the final concentration of 0.0002 μl/ml. New series of dilutions gave the following results: *A. clavatus* had MIC between 0.015 and 7·10⁻³ μl/ml. Garlic oleoresin had the strongest antifungal effect on *A. versicolor* and *A. fumigatus*, 7·10⁻³ μl/ml. These concentrations are significantly lower than those found in literature (B e n k e b l i a, 2004).

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Tab. 1. – Inhibitory effect of garlic oleoresin on some fungal species from genus *Aspergillus*

| Fungal-species | Final concentration of garlic oleoresin in microtiter plate wells [$10^{-1}\cdot\mu l/ml$] | | | | | | | | | | | | | | | | | | | |
| | NC | GC | 250 | 125 | 62.5 | 31.25 | 15.6 | 7.8 | 5 | 2.5 | 1.25 | 0.62 | 0.31 | 0.15 | 0.07 | 0.035 | 0.017 | 0.008 | 0.004 | 0.002 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A.niger | – | + | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + |
| A.versicolor | – | + | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + |
| A.clavatus | – | + | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + |
| A.flavus | – | + | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| A.fumigatus | – | + | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + |
| A.ochraceus | – | + | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |

GC – Growth control, NC- negative control

b) Broth microdilution assay

Figure 1 shows the results of activities of black pepper oleoresin against some food-borne fungi from genus *Aspergillus*. Results represent mean value of viable count of molds per Petri dish with standard deviation.
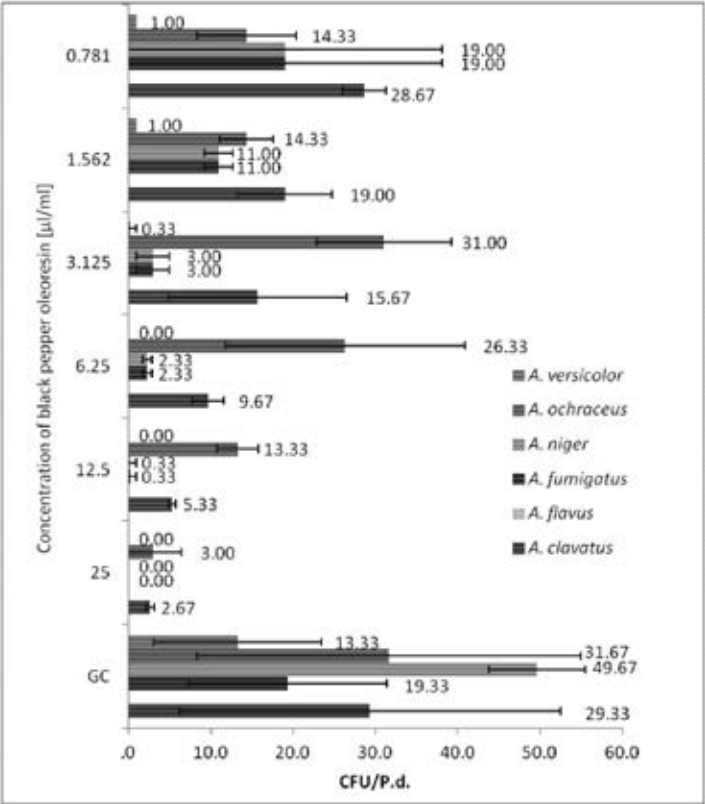


Fig. 1. – Influence of different concentrations of black pepper oleoresin on growth of some species form genus *Aspergillus*

Apart from black pepper oleoresin antibacterial activity (K a r s h a and L a k s h m i, 2010), it has also expressed antifungal activity by inhibiting the growth of *A. fumigatus*, *A. niger* and *A. versicolor* at concentrations of 25 μl/ml. In the case of *A. versicolor,* lower concentrations (12.5 and 6.25 μl/ml) were also efficient (Figure 2). *A. ochraceus* showed the highest resistance to black pepper oleoresin (Figure 1).
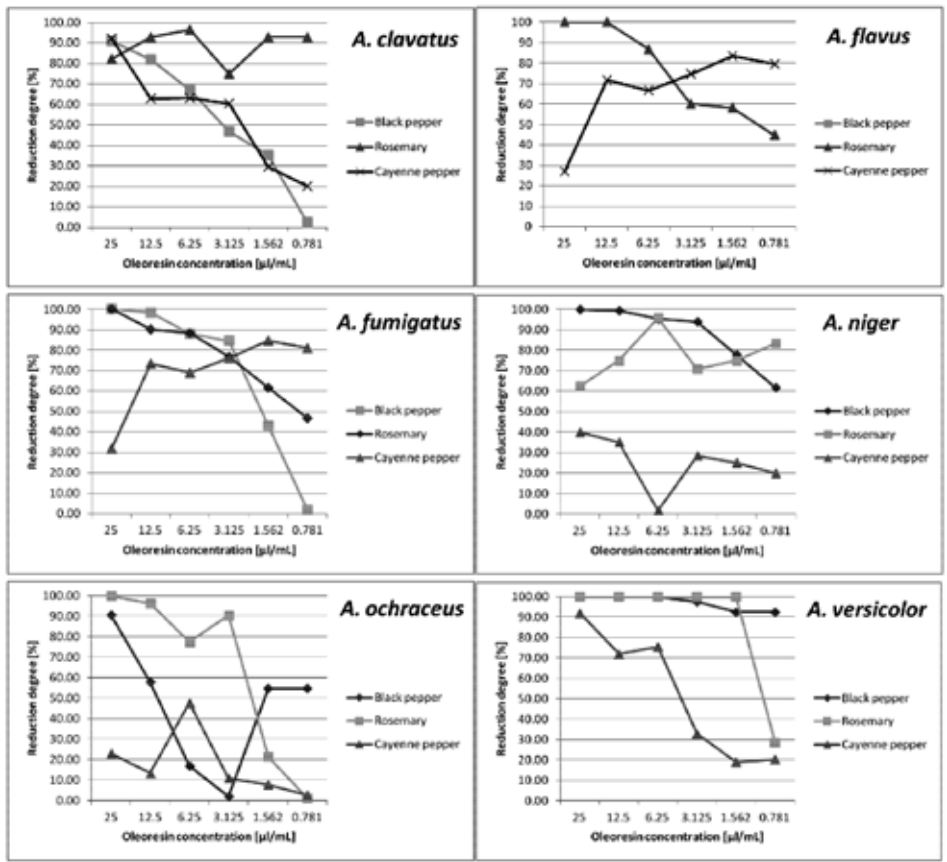


Fig. 2. – Reduction degree [%] of tested oleoresins in some species from genus *Aspergillus*

Figure 3 shows the results of rosemary oleoresin activities against the tested species from genus *Aspergillus*. Rosemary oleoresin, at concentration of 25 μl/ml, inhibited growth (MFC) of *A. flavus*, *A. fumigatus*, *A. ochraceus* and *A. versicolor*, and it had strong antifungal effect on *A. clavatus* (RD=90.91%) (Figure 2). In the case of *A. niger*, rosemary oleoresin did not show MFC, only the inhibition in the range of 62.5-91.2%. Figure 2 shows other values of reduction degree.
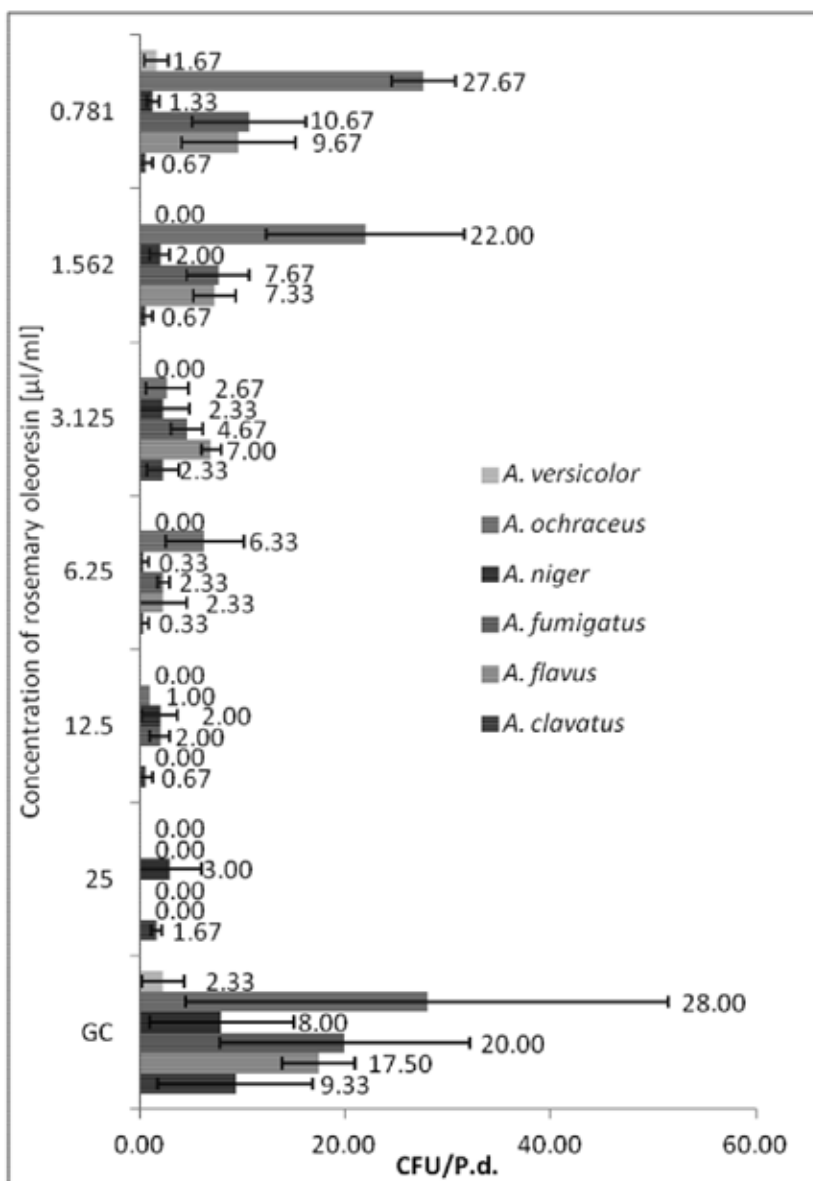
Fig. 3. – Influence of different concentrations of rosemary oleoresin on growth
of some species form genus *Aspergillus*

In comparison to the other two oleoresins, cayenne pepper oleoresin demonstrated low inhibitory effect on molds growth (Figure 4). It had a significant value of reduction degree for *A. clavatus* and *A. versicolor* species only at concentration of 25 μl/ml (Figure 2).
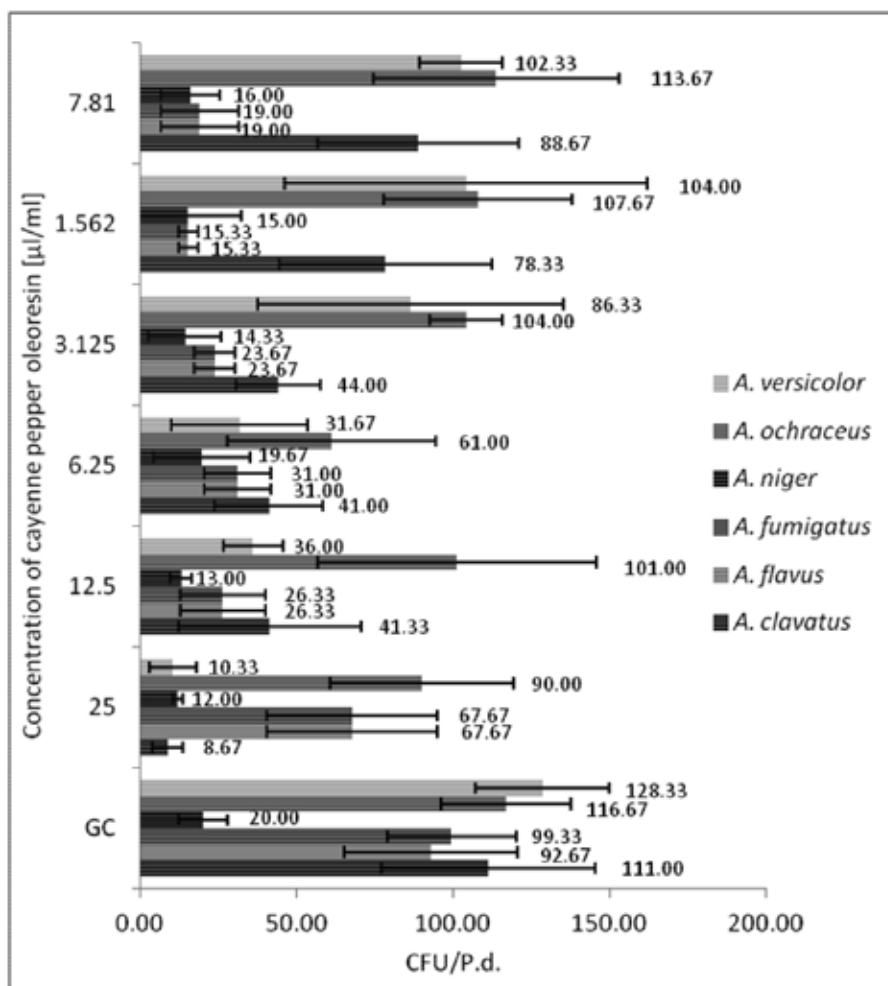
Fig. 4. – Influence of different concentrations of cayenne pepper oleoresin on growth of some species form genus *Aspergillus*

CONCLUSION

Garlic oleoresin was reported to have very high antifungal activity using resazurin as a growth indicator, hence the following concentrations of 0.78 µl/ml (*A. ochraceus* and *A. flavus*), 0.062 µl/ml (*A. niger*), 0.015 µl/ml (*A. clavatus*) and $7·10^{-3}$µl/ml (*A. fumigatus* and *A. versicolor*). Black pepper oleoresin had significant antifungal activity at concentrations of 25-12.5 µl/ml, while cayenne pepper oleoresin showed low antifungal activity, demonstrating growth inhibition at concentration of 25 µl/ml only in the cases of *A. clavatus* and *A. versicolor* species.

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## АНТИФУНГАЛНА АКТИВНОСТ ОЛЕОРЕЗИНА КОЈИ СЕ КОРИСТЕ У МЕСНОЈ ИНДУСТРИЈИ НА НЕКЕ ТОКСИГЕНЕ ВРСТЕ РОДА *ASPERGILLUS* SPP.

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Резиме

Различите врсте олеорезина имају широку примену у месној индустрији. Они доприносе специфичној ароми и укусу крајњих производа, али такође је показано да поседују и изузетну антимикробну активност. Ове особине омогућавају широк спектар разноврсних комбинација у циљу формирања новог сензорног профила производа, али такође могу да послуже као природни прехрамбени конзерванси. У овом научном раду је испитивана антифунгална активност 4 различита олеорезина на неколико токсигених врста из рода *Aspergillus.* Тестирани су олеорезини љуте паприке, црног бибера, белог лука и рузмарина. Као тест микроргнизми коришћене су следеће врсте рода *Aspergillus*: *A. clavatus, A. flavus, A. fumigatus, A. niger, A. ochraceus* и *A. versicolor.* Антифунгална активност је испитивана бујон-микродилуционом методом са додатком ресазурина као индикатора ћелијског раста, као и класичном бујон-микродилуционом методом.

КЉУЧНЕ РЕЧИ: Олеорезини, антифунгална активност, *Aspergillus* spp.

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# ANTIFUNGAL PROPERTIES OF *FOENICULUM VULGARE*, *CARUM CARVI* AND *EUCALYPTUS* SP. ESSENTIAL OILS AGAINST *CANDIDA ALBICANS* STRAINS

ABSTRACT: Aromatic plants are among the most important sources of biologically active secondary metabolites, with high antimicrobal potential. This study was carried out to examine *in vitro* antifungal activity of *Foeniculum vulgare* (Apiaceae), *Carum carvi* (Apiaceae) and *Eucalyptus* sp.(Myrtaceae) essential oils against three *Candida albicans* strains of different origin (laboratory-$CA^L$, human pulmonary-$CA^H$ and ATCC10231-$CA^R$). The essential oils were screened on *C. albicans* using disc and well-diffusion and microdilution method, and compared to Nystatine and Fluconazole as standard anti-mycotics. The activity of tested oils was expressed by inhibition zone diameter (mm), minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) (mg/ml). The results indicated that studied essential oils show antifungal activity against all three isolates of *C. albicans*. It was observed that each oil exhibits different degree of antifungal activity depending on the oil concentration applied as well as on analyzed strain of *C. albicans*. *Carum carvi* demonstrated the strongest antifungal effect to all tested strains, showing the lowest MIC values (0.03mg/ml for $CA^L$, 0.06mg/ml for $CA^H$, and 0.11mg/ml for $CA^R$, respectively). *Eucalyptus* sp. exhibited the lowest antifungal activity, with MIC values ranging from 0.11 mg/ml for $CA^L$ to 0.45 mg/ml for both $CA^H$ and $CA^R$.
KEY WORDS: antifungal activity, *Candida albicans*, *Carum carvi*, essential oils, *Eucalyptus* sp., *Foeniculum vulgare*.

## INTRODUCTION

Excessive use of commercial antibiotics has caused increasing resistance of pathogenic microorganisms to existing drugs. Therefore, there is a growing interest in finding new, natural antimycotic agents, with novel mechanism of action (H e m a i s w a r y a et al., 2008). Essential oils (EOs) are considered

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as a part of aromatic plants defense mechanism (R e i c h l i n g et al., 2009). These substances are complex, natural, volatile plants secondary metabolites, characterized by strong fragrance. They are mixtures of about 20-60 compounds, present at different concentrations. The composition of EOs includes two or three major components, predominantly terpenes, that are present in high concentrations (20-70%), while the other compounds are present in traces (B a k k a l i et al., 2008). EOs derived from aromatic medicinal plants are effective against many pathogenic microorganisms (R e i c h l i n g et al., 2009). Due to their antimicrobial properties and characteristic fragrance, EOs or some of their components are widely used in pharmaceutical, agronomic, food, sanitary, cosmetics and perfume industries (B a k k a l i et al., 2008). Various publications have documented the antimicrobial activity of EOs derived from different families of aromatic plants, including fennel, caraway and eucalyptus (D i P a s q u a et al., 2005; S h a h a t et al., 2011).

The objective of this work was to investigate the antimicrobial activity of commercial EOs derived from *Foeniculum vulgare*, *Carum carvi* and *Eucalyptus* sp. against three CA strains of different origin (laboratory, human pulmonary and referent strain ATCC10231).


## MATERIALS AND METHODS


**Essential Oils:**
Analyzed EOs of *F. vulgare* (fEO), *C. carvi* (cEO) and *Eucalyptus* sp. (eEO) were produced by Ireks aroma Ltd. Zagreb, Croatia. According to the manufacturer, these oils are 100% natural, with all necessary documentation (MADS – Material safety data sheet) and manufacturer specification (http://www.ireks-aroma.hr/xist4c/web/hrvatski_id_1588_.htm).

Analyzed strains and Inoculum Preparation:
Laboratory (CA$^L$) and referent ATCC10231 (CA$^R$) strains were obtained from the Culture Collection of Department of Biology and Ecology, University of Novi Sad, Serbia. The human pulmonary strain (CA$^H$) was obtained from the Institute of Public Health of Vojvodina, Novi Sad, Serbia.

All three strains were cultivated on Malt agar (Torlak) at –30 ° C. For the preparation of suspension, 24h old colonies were suspended in sterile 0.85% NaCl. Turbidity was estimated using photoelectric photometer (COLORIMETER MA 9504. Metrix) and 0.5 McFarland standard. The number of cells in tested inoculum was checked by CFU (Colony-forming unit) method. It was determined that initial density of the inoculum was 1.5x10$^6$ CFU.

**Antifungal Assay:** Investigated EOs were dissolved in Tween 80, for the purpose of making five initial working concentrations (5.00 mg/ml, 2.50 mg/ml, 1.25 mg/ml, 0.63 mg/ml, 0.31 mg/ml). Nystatine (Hemofarm, Vršac) (0.30 mg/ml, 0.25 mg/ml, 0.20 mg/ml) and commercial Fluconazole solution (Hemofarm, Vršac) (2 mg/ml) were used as a positive control, while 0.1% Tween 80 was used as a negative control. Tests were carried out in triplicate.

**Disc-diffusion method-** 100µl of CA inoculum was spread evenly on the surface of Malt agar plates (d=90.0 mm). Under aseptic conditions, sterile discs (Whatman, d=5.0 mm) were placed in the center of each Petri-plate. Discs were impregnated with 15.0 µl of each essential oil (EO) in 5 analyzed concentrations. The plates were incubated at 30°C for 48h.

**Well-diffusion method-** In the center of each Malt agar Petri-plate (d= 90.0 mm), the wells of 6.0 mm diameter were made. Each well was filled with 25.0 µl of EO, using previously mentioned working concentrations. The plates were left for 2h at room temperature to allow the diffusion of the oil, and than 100 µl of CA suspension was spread over the plates. The plates were incubated at 30°C for 48h.

Antimicrobial activity was evaluated by measuring the inhibition zone in mm (including diameter of disc/well) against the tested strains.

**Microdilution method-** This method was performed in order to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The experiment was conducted using 96/well microtiter plates (Spektar, Čačak, Serbia), with each well containing 200 µl of Malt Broth (Torlak), 2 µl of CA inoculum ($1.5x10^4$ final concentration in well) and 20 µl of EO. Final concentrations of tested oils in wells (mg/ml) were: 0.45, 0.23, 0.11, 0.06 and 0.03. MIC was determined as the lowest concentration of EO at which CA strains showed no visible growth. To determine MFC, 100 µl from each well was taken and inoculated on Malt agar Petri-plate. Plates were incubated at 30° for 48h. The results were recorded through number of yeast colonies.

**Statistical analysis-** conventional statistical methods were used in order to determine means and standard deviations (Microsoft Excel XP). Statistical analysis of results obtained in agar diffusion methods was performed using one way ANOVA (Statistica 10). Probability value at P≤0.05 was considered statistically significant (Duncan's test).

## RESULTS AND DISCUSSION

The antifungal effect of tested EOs varied in accordance with the oil concentration and investigated CA strain. The results obtained using disc-diffusion technique (Table 1.) indicated that, among the examined EOs, fEO had the strongest antifungal activity regarding CA$^L$ and CA$^R$, with inhibition zone ranging from 90.00-13.67mm for CA$^L$, and from 17.5-8.5mm for CA$^R$, depending on the oil concentration used. At the concentration of 5mg/ml cEO presented the strongest antifungal activity against CA$^H$ (14.50mm), but, unlike fEO, this oil was ineffective at a concentration of 2.50mg/ml against this particular strain.

The results obtained by well diffusion method (Table 2) indicated that cEO showed the highest antifungal activity against all three yeast strains, particularly CA$^L$, which was affected by *C. carvi* even at a concentration of 0.06 mg/ml (14.83mm inhibition zone). In both agar diffusion methods, eEo exhibited the lowest antifungal activity against all three tested strains.

Tab. 1. – Disc-diffusion method. Inhibition zone (mm±stdev) of essential oils and anti-mycotics against *C. albicans* strains.

| Concentration (mg/ml) | Essential oil | $CA^L$ | $CA^H$ | $CA^R$ |
|---|---|---|---|---|
| | *F. vulgare* | 90.00±0.00[a] | 12.33±0.76[b] | 17.50±0.87[a] |
| 5.00 | C. carvi | 44.33±0.76[b] | 14.50±0.87[a] | 13.83±0.29[b] |
| | *Eucalyptus* sp. | 12.50±1.32[e] | 7.17±0.76[d] | nd |
| | *F. vulgare* | 24.00±0.87[c] | 8.17±0.29[c] | 8.50±0.50[c] |
| 2.50 | C. carvi | 13.50±0.87[d] | nd | 7.33±0.29[d] |
| | *Eucalyptus* sp. | 8.67±0.58[f] | nd | nd |
| | *F. vulgare* | 13.67±0.76[d] | nd | nd |
| 1.25 | C. carvi | 7.50±0.50[g] | nd | nd |
| | *Eucalyptus* sp. | nd | nd | nd |
| | *F. vulgare* | nd | nd | nd |
| 0.63 | C. carvi | nd | nd | nd |
| | *Eucalyptus* sp. | nd | nd | nd |
| | *F. vulgare* | nd | nd | nd |
| 0.31 | C. carvi | nd | nd | nd |
| | *Eucalyptus* sp. | nd | nd | nd |
| **Anti-mycotic** | | | | |
| 2.00 | Fluconazole | nd | nd | nd |
| 0.30 | Nystatin | nd | 13.35±0.92 | 14.50±0.71 |
| 0.25 | Nystatin | nd | 11.90±0.14 | 12.25±0.35 |
| 0.20 | Nystatin | nd | 8.05±0.21 | 7.01±0.01 |

Strains: $CA^L$ (laboratory), $CA^H$ (human pulmonary), $CA^R$ ( reference ATCC10231).
nd= not detected inhibitory zone (d=0.00 mm)
Different letters in superscript along each column indicate significant differences (P≤0.05), Duncan′s test.

It can be noticed that in both disc and well diffusion methods, $CA^L$ was the most susceptible, while $CA^R$ exhibited the strongest resistance towards all three examined oils. According to D o b r e et al., agar-diffusion method is marked as prescreening procedure in the determination of EOs antimicrobial properties. However, agar-diffusion methods are limited, primarily due to different diffusion capacity of diverse EOs, which depend on oil chemical structure (H a m m e r et al.,1999). As it can be noticed from the results obtained (Table 1, 2), EOs had higher activity in screening by well-diffusion assay, comparing to disc-diffusion assay. Better sensibility of well-diffusion technique in comparison to the disc variant has been mentioned by many authors. This can be explained by the fact that, in well-diffusion method, EOs are in direct contact with agar, which facilitates the diffusion of oil. On the other hand, cellulose disc absorbs certain portion of oil, thus lowering the oil diffusion ability through the agar (V a l g a s et al., 2007).

Tab. 2. – Well-diffusion method. Inhibition zone (mm±stdev) of essential oils and anti-mycotics against *C. albicans* strains.

| Concentration (mg/ml) | Essential oil | CA$^L$ | CA$^H$ | CA$^R$ |
|---|---|---|---|---|
| | *F. vulgare* | 90.00±0.00$^a$ | 12.83±0.76$^b$ | 13.83±0.76$^b$ |
| 5.00 | C. carvi | 90.00±0.00$^a$ | 17.50±0.87$^a$ | 20.67±1.04$^a$ |
| | *Eucalyptus* sp. | 14.83±0.76$^e$ | 8.17±0.76$^b$ | 9.00±0.50$^c$ |
| | *F. vulgare* | 28.17±1.04$^c$ | 8.50±0.87$^b$ | 8.17±0.29$^d$ |
| 2.50 | C. carvi | 32.83±1.04$^b$ | 8.83±0.76$^b$ | 8.83±0.29$^c$ |
| | *Eucalyptus* sp. | 12.00±0.87$^f$ | nd | nd |
| | *F. vulgare* | 11.33±0.58$^f$ | nd | nd |
| 1.25 | C. carvi | 16.83±0.29$^d$ | nd | nd |
| | *Eucalyptus* sp. | nd | nd | nd |
| | *F. vulgare* | nd | nd | nd |
| 0.63 | C. carvi | 14.83±1.04$^e$ | nd | nd |
| | *Eucalyptus* sp. | nd | nd | nd |
| | *F. vulgare* | nd | nd | nd |
| 0.31 | C. carvi | nd | nd | nd |
| | *Eucalyptus* sp. | nd | nd | nd |
| **Anti-mycotic** | | | | |
| 2.00 | Fluconazole | nd | nd | nd |
| 0.30 | Nystatin | 11.00±0.57 | 18.00±0.71 | 18.70±0.99 |
| 0.25 | Nystatin | 9.05±0.35 | 15.10±0.14 | 13.75±0.35 |
| 0.20 | Nystatin | nd | 12.05±0.07 | 7.05±0.21 |

Strains: CA$^L$ (laboratory), CA$^H$ (human pulmonary), CA$^R$ (reference ATCC10231).
nd= not detected inhibitory zone (d= 0.00mm), Different letters in superscript along each column indicate significant differences (P≤0.05), Duncan′s test.

Tab. 3. – Antifungal activity of essential oils and anti-mycotics against *C. albicans* strains. Microdilution method. MIC and MFC values (mg/ml).

| strain | *F. vulgare* | | *C. carvi* | | *Eucalyptus* sp. | | Nystatin | | Fluconazole | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC[*] | MFC[**] | MIC | MFC | MIC | MFC | MIC | MFC | MIC | MFC |
| CA$^L$ | 0.06 | 0.11 | 0.03 | 0.06 | 0.11 | 0.23 | 0.01 | 0.02 | ↑2.00 | ↑2.00 |
| CA$^H$ | 0.23 | 0.45 | 0.06 | 0.06 | 0.45 | ↑0.45 | 0.01 | 0.02 | ↑2.00 | ↑2.00 |
| CA$^R$ | 0.23 | 0.45 | 0.11 | 0.11 | 0.45 | ↑0.45 | 0.01 | 0.02 | ↑2.00 | ↑2.00 |

Strains: CA$^L$ (laboratory), CA$^H$ (human pulmonary), CA$^R$ (reference ATCC10231), * – minimum inhibitory concentration; ** – minimum fungicidal concentration.

The results obtained by applying the microdilution method (Table 3) showed that fEO and cEO were found more effective comparing to eEO, which exhibited lower antifungal effect towards all three CA strains (MIC

0.11mg/ml for CA$^L$, and 0.45mg/ml for both CA$^H$ and CA$^R$). Investigated cEO demonstrated the strongest antifungal effect on all tested strains, showing the lowest MIC values (0.03mg/ml for CA$^L$, 0.06mg/ml for CA$^H$, and 0.11mg/ml for CA$^R$, respectively). Tested fEO showed stronger activity against CA$^R$ (0.23mg/ml), in comparison to the results of M a r t i n s et al. (2012), obtained by broth macro-dilution method, reaching the MIC at concentration higher than 1mg/ml. According to literature data, the major component of fEO is anethole (S h a h a t et al., 2011; M a r t i n s et al., 2012), while cEO consists predominantly of carvone and limonene (M e s h k a t a l s a d a t et al., 2012). These components were proven to be highly effective against CA (E r d o g a n et al., 2009). Within agar-diffusion and microdilution methods, anti-mycotic Nystatine showed stronger impact than EOs to all three CA strains (Table 1-3), while Fluconazole did not exhibit any activity against analyzed strains, even at the highest concentration applied (2mg/ml). According to D e v k k a t e et al. (2005), Fluconazole only partially inhibited CA, but total inhibition could not be reached.

## CONCLUSION

From the results gathered in the experiment, it can be concluded that fEO, cEO and eEO exhibited strain specific effect against CA, having the strongest effect to CA$^L$, then to CA$^H$ and CA$^R$, respectively. After this examination, more thorough work should be performed in order to describe in more detail the antifungal activities of tested EOs, especially of cEO, that demonstrated the strongest antifungal properties (MIC from 0.03mg/ml for CA$^L$ to 0.11mg/ml for CA$^R$). To obtain more comprehensive analysis of the tested EOs antifungal activity, it is important to perform phytochemical analysis in order to determine the active oil components, to expand the study to a larger range of *Candida* and other filamentous fungal strains, and to explore the oil activity *in vivo*.

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АНТИФУНГАЛНО ДЕЈСТВО ЕТАРСКИХ УЉА ВРСТА *FOENICULUM VULGARE*, *CARUM CARVI* И *EUCALYPTUS* SP. НА ИЗОЛАТЕ ВРСТЕ CANDIDA ALBICANS

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Резиме

Ароматичне биљке представљају један од најважнијих извора биолошки активних секундарних метаболита, са израженим антимикробним дејством. У оквиру овог рада испитана је, у *in vitro* условима, антифунгална активност етарских уља врста *Foeniculum vulgare* (Apiaceae), *Carum carvi* (Apiaceae) и *Eucalyptus sp.* (Myrtaceae) на три изолата врсте *Candida albicans* различитог порекла (лабораторијски-CA[L], хумани пулмонарни. -CA[H] и ATCC10231-CA[R]). За испитивање коришћене су диск-дифузиона, метода бунарчића, као и микродилуциона метода.

Као стандард употребљени су антимикотици нистатин и флуконазол. Активност етарских уља изражена је преко пречника инхибиторне зоне (mm), минималне инхибиторне концентрације (MIC) и минималне фунгицидне концентрације (MFC) (mg/ml). Добијени резултати показали су да етарска уља поседују анти-фунгалну активност на сва три тестирана изолата. Уочено је да свако етарско уље показује различит степен антифунгалне активности у односу на концентра-цију уља, као и у зависности од изолата *C. albicans*. Етарско уље врсте *Carum carvi* поседује најснажније антифунгално дејство, са најнижим MIC вредностима (0,03 mg/ml за CA$^L$, 0,06 mg/ml за CA$^H$ и 0,11 mg/ml за CA$^R$). *Eucalyptus* sp. поседује најслабију антифунгалну активност, са MIC вредностима у распону од 0,11 mg/ml за CA$^L$ до 0,45 mg/ml за CA$^H$ и CA$^R$.

КЉУЧНЕ РЕЧИ: антифунгална активност, *Candida albicans*, *Carum carvi*, етарска уља, *Eucalyptus* sp., *Foeniculum vulgare*

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# ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS OF *ORIGANUM VULGARE* AND *ROSMARINUS OFFICINALIS* AGAINST THREE C*ANDIDA ALBICANS* STRAINS

ABSTRACT: Due to general growing resistance and side effects to common antifungal drugs nowadays, there have been many studies reported on the use of herbal essential oils as antifungal agents in recent years. In this study, essential oils of *Origanum vulgare* and *Rosmarinus officinalis* (Lamiaceae) were examined for their *in vitro* antifungal activity against three *Candida albicans* strains (laboratory – $CA^L$, human pulmonary – $CA^H$, and reference ATCC10231-$CA^R$) in comparison to Nystatin (0.30 mg/ml) and Fluconazole (2 mg/ml) as standard antifungal agents.

The antifungal activity was evaluated by comparing inhibition zone diameters obtained both by disc-and well-diffusion assays, as well as by comparing MIC and MBC values detected by microdilution assay. Diffusion test results revealed stronger antifungal effect of *O. vulgare* against all analyzed *C. albicans* strains identifying $CA^L$ strain as the most susceptible one. Inhibition zones ranged from 12.65 to 25.10 mm depending on the concentrations applied. The highest concentrations of Rosemary essential oil (5.00 mg/ml) demonstrated activity against two strains: $CA^L$ and $CA^R$ ATCC 10231 in both diffusion assays applied, while no antifungal activity was recorded against $CA^H$ isolate.

Microdilution assay showed that both oils demonstrated the same MIC values for all tested strains (0.11 mg/ml), except MIC value against ATCC strain (0.23 mg/ml) obtained for Rosemary essential oil. The obtained results indicated that oregano and rosemary essential oils might be highly effective in the natural prevention treatment of candidiasis, although toxicity assays should be previously preformed.

KEY WORDS: Antifungal activity, *Candida albicans*, essential oil, nystatin, *Origanum vulgare*, *Rosmarinus officinalis*

## INTRODUCTION

*Candida albicans* (*CA*), as opportunistic commensal, is a member of standard gastrointestinal flora, respiratory system, female genital tract, and to a

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lesser extent of the skin and nails (D z a v e c et al., 1998; P a l m a – C a r l o s and P a l m a – C a r l o s, 2003). In the case when this yeast becomes a dominant member of flora it can cause a series of pathological conditions known as candidiasis (B e r m a n and S u d b e r y, 2002), which is considered to be the most common mycosis, with *C. albicans a*s the main species related, althgouh nonalbicans species (e.g. *C. tropicalis, C. parapsilosis, C. krusei, C. glabrata*) are becoming frequent among patients nowadays (P f a l l e r, 1995). Main predisposing factors for Candida infections are immune deficiency, diabetes mellitus, total exhaustion, intravenous drug addiction, venous and urethral catheterization, organ transplantation, corticosteroid therapy and the use of broad spectrum of antibiotics that alter the effects of normal bacterial flora (D z a v e c et al., 1998;

B e r m a n and S u d b e r y, 2002). Hence, increasing presence of autoimmune diseases caused by pathogenic yeasts led to serious medical concerns in the past decade (P f a l l e r et al., 2000; U w a m a h o r o and T r a v e n, 2010). Besides, essential oils derived from many plants, mostly members of Lamiaceae, Apiaceae, Asteraceae and Myrtaceae families are recognized as excellent natural sources of substances with antifungal activities. Due to a growing resistance and side effects to common antifungal drugs, a number of studies have recently demonstrated the use of herbal essential oils as antifungal agents (B a k k a l i et al., 2008; R e i c h l i n g et al., 2009).

In this study, essential oils (EOs) of Oregano (*Origanum vulgare*) and Rosemary (*Rosmarinus officinalis*), both belonging to Lamiaceae family, were examined for their *in vitro* antifungal activity against three *CA* strains (laboratory, human pulmonary and ATCC 10231).


## MATERIALS AND METHODS


**Essential oil**

Analyzed oils of Oregano (OEO) and Rosemary (REO) were commercial products produced by Ireks aroma Ltd. (Zagreb, Croatia) which were, according to the producer's notification, characterized as 100% natural and which was documented by all required documentation (MADS – Material Safety Data Sheet) and production specifications (http://www.ireks-aroma.hr/xist4c/web/hrvatski_id_1588_.htm). The EO solubility was enhanced with 0.1% Tween-80 solution to obtain five serial dilutions of oils in a range of 5.00 – 0.31 mg/ml.


**Antifungal activity**

Standard strains of *C. albicans* ATCC 10231 ($CA^R$) and laboratory isolates ($CA^L$) were used from the Culture Collection of Department of Biology and Ecology, University of Novi Sad, Serbia. The human pulmonary strain of *C. albicans* ($CA^H$) was isolated at the Institute of Public Health of Vojvodina (Novi Sad, Serbia).

All strains of *CA* used were grown on malt agar (Torlak, Belgrade, Serbia) at 30° C for 48 h. Inocula for the assays were prepared by diluting scraped cell

mass into 0.85% NaCl solution, adjusted to McFarland scale 0.5 amounted to $1.5 \times 10^6$ CFU. Turbidity was confirmed by photoelectric photometer (colorimeter MA 9504th Metrix), using red filter (E s p i n e l – I n g r o f f et al., 1998; CLSI M27-A2, 2002). Dilution series ($10^{-2}$ to $10^{-6}$) of 0.5 Mc Farland's initial inoculum were determined by applying classical CFU method in triplicate.

For diffusion assays the malt agar plates (d = 90 mm) were prepared and seeded with 100 μl suspension of $1.5 \times 10^6$ cfu ml$^{-1}$ using the inoculum of *Candida* strains prepared as described. Sterile Whatman discs (d = 5mm) or wells (d = 6mm) were placed in the middle of the malt agar, on which 15 μl (disc-diffusion method) and 25 μl (well-diffusion assay) of EOs were applied. In well-diffusion assay oil was left to diffuse through the agar for 2h at room temperature (25°C) before incubation. All the plates were incubated in triplicate for each concentration at 30° C for 48 h according to T y a g i and M a l i k (2010) while diameter of the zone of inhibition was measured including the diameter of the disc/well. The antifungal activity of EOs was compared to water solution of nystatin (0.30 mg/ml) and fluconazole (2 mg/ml) (Hemofarm, Vršac, Serbia) as positive controls.

Using 5 two-fold dilutions of EOs (0.45 mg/ml to 0.03 mg/ml) in the 96 wells microplates (Spektar, Čačak, Serbia) MIC and MFC values were determined according to M27-A2 of CLSI (CLSI, 2002). Total volume in the well was 222 μl: 200 μl of malt broth (Torlak, Belgrade, Serbia), 2 μl of *CA* inoculum ($1.4 \times 10^3$ CFU/ml at final concentration in well) and 20 μl of EO. The results were read after the incubation at 30° C for 48 h. The MIC (minimum inhibitory concentration) was determined as the lowest concentration of test substances that prevented visible growth of *CA* strains (J o r g e n s e n and F e r r a r o, 2009). From each well, in which blurring was not detected, 100 μl of culture was inoculated onto malt plates and incubated at 35°C for 48h. The plates were observed and the MFC (minimum fungicidal concentration) was determined as the lowest concentration of plant oil resulting in the death of 99.9% of the inoculums (D e v k a t t e et al., 2005). Nystatin and Fluconazole were used as positive and Tween 80 as negative control.

The obtained results were analyzed using Microsoft Excel XP (mean, standard deviation). The data for diffusion methods were analyzed by one-way ANOVA in Statistic. Statistically significant effects were further analyzed and means were compared using Duncan's test. Statistical significance was determined at $p \leq 0.05$.

## RESULTS AND DISCUSSION

The obtained inhibitory zones, after the application of EOs by using disc and well-diffusion assays, are presented in Tables 1-2. The results showed a decrease in the size of the inhibitory zones with decreasing EO concentrations in both assays performed. Nystatin showed inhibition zones at lower concentrations than the analyzed EOs (Tables 1, 2), while fluconazole exhibited none inhibition zone even at the highest concentration applied (2 mg/ml).

Tab. 1. – Inhibitory zones (mm±sd) of essential oils and antimycotics in disc-diffusion assay

| Concentration (mg/ml) | Essential oil | C. albicans[L] | C. albicans[H] | C. albicans[R] |
|---|---|---|---|---|
| 5.00 | O. vulgare | 25.10±1.01[a] | 19.40±0.36[a] | 17.70[a]±0.26[a] |
| | R. officinalis | 11.17±0.29[d] | nd | 7.10±0.36[d] |
| 2.50 | O. vulgare | 20.17±1.06[b] | 11.50±0.50[b] | 11.87±0.32[b] |
| | R. officinalis | nd | nd | nd |
| 1.25 | O. vulgare | 12.67±0.58[c] | nd | 8.00±1.00[c] |
| | R. officinalis | nd | nd | nd |
| 0.63 | O. vulgare | nd | nd | nd |
| | R. officinalis | nd | nd | nd |
| 0.31 | O. vulgare | nd | nd | nd |
| | R. officinalis | nd | nd | nd |
| | Antimycotic | | | |
| 2.00 | Fluconazole | nd | nd | nd |
| 0.30 | Nystatin | nd | 13.35±0.92 | 14.50±0.71 |
| 0.25 | Nystatin | nd | 11.90±0.14 | 12.25±0.35 |
| 0.20 | Nystatin | nd | 8.05±0.21 | 7.01±0.01 |

Strains: [L] (laboratory)-, [H] (human pulmonary)- and [R] (reference ATTC 10231), nd = not detected inhibitory zone (d=0 mm). Different letters in superscript along each column indicate significant differences (p<0.05), Duncan test.

After the application of OEO, the highest resistance was observed with $CA^H$ strain (Table 1) showing inhibition zones in a range of 19.4 – 11.5 mm, while the most susceptible strain was $CA^L$ which exhibited 25.10 – 12.67 mm.

Using the well-diffusion method, OEO at the concentration of 5.00 mg/ml had the greatest influence on $CA^L$ and it completely inhibited the growth of test organism on the entire Petri plate. The susceptibility of Candida strains was decreased in the following order: $CA^L$ (16.17–90.00 mm), $CA^H$ (8.33–44.25 mm) and $CA^R$ (8.07–38.17 mm).

In both diffusion methods, REO demonstrated higher antifungal activity against $CA^L$ with higher inhibitory zones than $CA^R$ strain, while no activity was shown against $CA^H$ (Tables 1, 2). The data obtained with microdilution method (Table 3) indicated that REO had the same MIC value as OEO against $CA^H$. Variations in the inhibitory zones caused by different diffusion rates of EOs on agar plates may lead to erroneous conclusions regarding their antifungal activity (D e v k a t t e et al., 2005).

The results obtained by using diffusion methods indicated that OEO had stronger activity against all tested Candida strains than REO. Due to the fact that inhibitory zones were obtained at lower concentrations of EOs by well-diffusion assay it can be concluded that this method is more appropriate than disk-diffusion method. This can be explained by the fact that cellulose discs absorb certain amount of EOs and prevent their diffusion through agar (V a l - g a s  et al., 2007).

Tab. 2. – Inhibitory zones (mm±sd) of essential oils and anti-mycotics in well-diffusion assay

| Concentration (mg/ml) | Essential oil | C. albicans[L] | C. albicans[H] | C. albicans[R] |
|---|---|---|---|---|
| 5.00 | O. vulgare | 90.00±0.00*[a] | 44.25±1.06[a] | 38.17±0.76[a] |
|  | R. officinalis | 16.00±0.50[c] | nd | 8.1±0.36[d] |
| 2.50 | O. vulgare | 21.33±0.58[b] | 24.00±0.70[b] | 20.00±0.50[b] |
|  | R. officinalis | nd | nd | nd |
| 1.25 | O. vulgare | 16.17±0.76[c] | 12.27±0.64[c] | 11.87±0.32[c] |
|  | R. officinalis | nd | nd | nd |
| 0.63 | O. vulgare | nd | 8.33±0.58[d] | 8.07±0.40[d] |
|  | R. officinalis | nd | nd | nd |
| 0.31 | O. vulgare | nd | nd | nd |
|  | R. officinalis | nd | nd | nd |
|  | Antimycotic |  |  |  |
| 2.00 | Fluconazole | nd | nd | nd |
| 0.30 | *Nystatin* | 11.00±0.57 | 18.00±0.71 | 18.7±0.99 |
| 0.25 | *Nystatin* | 9.05±0.35 | 15.10±0.14 | 13.75±0.35 |
| 0.20 | *Nystatin* | nd | 12.05±0.07 | 7.05±0.21 |

Strains: [L] (laboratory)-, [H] (human pulmonary)- and [R] (reference ATTC 10231); * = total inhibition zone; nd = not detected inhibitory zone (d=0 mm). Different letters in superscript along each column indicate significant differences ($p<0.05$), Duncan test.

Furthermore, the agar diffusion method can be recommended as a pre-screening method for a large number of EOs (H a m m e r et al., 1999; K a l e m b a and K u n i c k a, 2003), since these methods will only give an idea of the presence or absence of substances with antimicrobial activity (V a l g a s et al., 2007). Based on these results, the most active EOs may be selected for further analysis by means of more sophisticated methods, such as macro and micro-dilution methods (K a l e m b a and K u n i c k a, 2003; S c o r z o n i et al., 2007).

Microdilution assay gave the results that showed efficient EOs activity against all tested CA, reaching MICs/MBCs at 0.11–0.23/0.11–0.23 mg/ml, respectively (Table 3). OEO showed same MIC values for all tested strains and demonstrated fungistatic and fungicidal effects on CA[L] and CA[H] strains at 0.11 mg/ml which indicated equal susceptibility. After the application of microdilution method, REO and OEO demonstrated the same activity against *CA*[L], reaching MIC and MFC values at the same concentration (Table 3). The strain *CA*[L] was the most sensitive to REO, while the most resistant was *CA*[R] showing the same MIC and MFC value (0.23 mg/ml).

Based on their efficacy (MFC), we concluded that OEO had stronger antifungal activity than REO. Nystatin was effective for all investigated strains at lower MIC and MFC values (MIC/MFC=0.01/0.02 mg/ml) in comparison to the tested EOs. The sensitivity of the studied strains of *CA* to fluconazole was not recorded even at the highest initial concentration of 2 mg/ml. D e v k k a t e et al. (2005) found high resistance of *CA*[R] to fluconazole which could not reach complete inhibition, but partially inhibited at mg/ml.

Tab. 3. – MICs and MFCs of essential oils (mg/ml) and antimycotics against *C. albicans* strains in microdilution assay

| Strains | *O. vulgare* | | *R. officinalis* | | **Nystatin** | | **Fluconazole** | |
|---|---|---|---|---|---|---|---|---|
| | MIC* | MFC** | MIC | MFC | MIC | MFC | MIC | MFC |
| *C. albicans*[L] | 0.11 | 0.11 | 0.11 | 0.11 | 0.01 | 0.02 | nd | nd |
| *C. albicans*[H] | 0.11 | 0.11 | 0.11 | 0.23 | 0.01 | 0.02 | nd | nd |
| *C. albicans*[R] | 0.11 | 0.23 | 0.23 | 0.23 | 0.01 | 0.02 | nd | nd |

Strains: [L] (laboratory)-, [H] (human pulmonary)- and [R] (reference ATTC 10231) strain; nd = not detected; * – minimal inhibitory concentration; ** – minimal fungicidal concentration

Results presented here pointed to the importance of OEO and REO as antifungal agents that could enhance human health. Commercial EO products showed higher activity against $CA^R$ showing MIC/MBC at 0.11/0.23 mg/ml, respectively, when compared to other results obtained by (Hammer et al., 1999) that showed MIC value at 1.2 mg/ml, as well as by S a r t o r a t t o et al. (2004) that detected MIC value at even higher concentration, 2 mg/ml (by bioautographic method).

Furthermore, REO also showed better anticandidal activity with same fungistatic and fungicidal values for MIC and MFC (0.23 mg/ml) on $CA^R$ strain. In comparison to previous literature data in microdilution assay MICs were reached at the following concentrations: 2.5 mg/ml (Fu et al., 2007), 6 mg/ml (V u u r e n et al., 2009), $10 \leq$ mg/ml (K e s k i n et al., 2010) and 10 mg/ml by using agar-dilution method (H a m m e r et al., 1999).

CONCLUSION

Based on the results presented in this work, it can be concluded that EOs extracted from *O. vulgare* and *R. officinalis* have the potential to represent a good alternative for the treatment of candidiasis.

This study confirms that analyzed oils showed both fungistatic and fungicidal activities against all tested *CA* strains. However, if these EOs are planned to be used in medicinal purposes, issues of safety and toxicity will need to be addressed in the next research. Furthermore, the study should include larger number of standard and clinical strains in order to provide the final assessment of effectiveness of the studied EOs against *CA*. Also, more fluconazole/azole-resistant strains should be included in the future studies since EOs could be used as antifungal agents in the future. In general, standardized research assays, verification of compositions of EOs and choice of test organism(s) are necessary for more effective comparisons of the results obtained by various researchers.

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## АНТИФУНГАЛНА АКТИВНОСТ ЕТАРСКИХ УЉА ВРСТА *ORIGANUM VULGARE* И *ROSMARINUS OFFICINALIS* НА ТРИ ИЗОЛАТА *CANDIDA ALBICANS*

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Резиме

Услед повећане резистенције и нежељених ефеката уобичајених антифунгалних лекова у последње време, спроведене су многе студије у циљу коришћења етарских уља као антифунгалних агенаса. У оквиру овог рада су у *in vitro* условима истраживана антифунгална дејства етарских уља врста оригано (*Origanum vulgare*) и рузмарин (*Rosmarinus officinalis*) из фамилије уснатица (Lamiaceae) на три соја кандиде (*Candida albicans*) различитог порекла (лабораторијски, хумани пулмонарни и АТСС 10231), а у поређењу са стандардним антимикотицима – нистатин (0.30 mg/ml) и флуконазол (2 mg/ml). Антифунгална активност испитивана је поређењем инхибиторних зона употребом диск-дифузионе и методе бунара, као и на основу МIC и МВС вредноси забележених применом микродилуционе методе. Резултати дифузионе методе за сва три тестирана изолата показала су јаче антифунгално дејство етарског уља оригана, док је лабораторијски сој показао највећу осетљивост. У односу на коришћене концентрације, дијаметри инхи-

биторних зона варирали су између 12.65 и 25.10 mm. При највећим коришћеним концентрацијама етарског уља рузмарина (5,0 mg/ml) антифунгална активност забележена је коришћењем обе дифузионе методе само код два истраживана соја – на лабораторијском изолату и соју ATCC 10231. Антифунгална активност етарског уља рузмарина није забележена у експериментима са хуманим изолатом кандиде.

Употребом микродилуционе методе забележене су једнаке MIC вредности за сва три тестирана изолата (0,11 mg/ml), осим за MIC вредност етарског уља рузмарина на ATCC сој (0.23 mg/ml). Добијени резултати указују на то да употреба етарских уља оригана и рузмарина може бити веома ефикасан начин природног лечења и спречавања кандидијазе, иако би претходно требало проверити њихову цитотоксичност.

КЉУЧНЕ РЕЧИ: антифунгална активност, *Candida albicans,* етарска уља, нистатин, *Origanum vulgare*, *Rosmarinus officinalis*

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# CONTAMINATION OF CAKES WITH TOXIGENIC MOLDS

ABSTRACT: The total number of molds in cakes ranged up to $9.0 \times 10^2$ CFU/g. The highest number of molds was isolated on Dichloran 18% Glycerol Agar (DG18), and the lowest on Malt Yeast Extract 50% Glucose Agar (MY50G). Mycopopulation of cakes composed of species of genera *Acremonium, Alternaria, Aspergillus, Cladosporium, Eurotium, Mucor, Phialophora, Penicillium, Fusarium* and *Ulocladium*. Dominant species were *A. niger, P. aurantiogriseum, P. brevicompactum, P. hirsutum, F. proliferatum* and *A. alternata*. The most common potential producers were those of ochratoxin A (50.50%). The potential producers of fumonisins were present with 7.75%, moniliformin with 6.5% and sterigmatocystin with 0.75%. Potential producers of *Alternaria* toxins amounted to 4.5%. Aflatoxigenic molds were not isolated from the tested samples. Mycotoxicological analysis of the cakes did not determine the presence of aflatoxin, sterigmatocystin, ochratoxin A and zearalenone.

KEY WORDS: cakes, fungal contamination, frequency of toxigenic molds

## INTRODUCTION

Mold contamination of cakes and the possibility of mold growth can pose a serious health problem when there are species which have the ability to synthesize and actively secrete toxic metabolites. It is known that aflatoxins produced by *Aspergillus flavus* and *A. parasiticus,* ochratoxin A produced by *Aspergillus ochraceus* and some *Penicillium* spp., and some fusariotoxins produced by *Fusarium* spp., are strong cytotoxic and carcinogenic agents (D i a z, 2005; S i n o v e c et al., 2006; K o c i ć-T a n a c k o v and D i m i ć, 2012). Human population exposure to chronic mycotoxicosis is considered to be much more common than assumed. The fact that molds produce a large amount of spores that are easily disseminated provides explanation of the wide distribution in nature and herbal products. Cake-making includes various ingredients such as cereal flour, cereals, cocoa, nuts, dried fruit, spices which regularly contain molds (L e v i ć et al., 2004; S a m s o n et al., 2004; P i t t and H o c k i n g,

2009; K o c i ć-T a n a c k o v and D i m i ć, 2012). These microorganisms on cakes can come from the air, contaminated packaging materials and other sources. When water activity is below 0.90 $a_w$, mold appearance is a common type of microbial spoilage. Most molds are more resistant to drying, pH and osmotic pressure than bacteria and their presence always represents a potential risk of spoilage or diseases transmitted by food.

According to the available literature, there are very few data on mold and mycotoxin contamination of cakes. Given the importance of molds and their toxic metabolites in food products, the aim of this paper was to determine the presence of potentially toxigenic species in different types of cakes.


## MATERIALS AND METHODS

### *Samples of cakes*

Mycological and mycotoxic tests were carried out on samples of different types of cakes: petit fours (2 samples), petit fours from the market (4 samples), homemade petit fours (1 sample), magic cookies (1 sample), magic lean cookies (2 samples), wholemeal cookies (3 samples), various cakes (1 sample), various petit fours (1 sample), mix cap (1 sample) and vanilla-raspberry desert cups (1 sample). Samples were taken randomly in food stores and markets in the area of Novi Sad (Vojvodina, Serbia ). The analysis included 17 samples.


### *Isolation and determination of the total number of molds*

Isolation and determination of the total number of molds were performed on three different mycological media: Rose Bengal Chloramphenicol Dichloran Agar, DRBC (Merck, Darmstadr), Dichloran Rose Bengal Chloramphenicol Agar, DRBC (Merck, Darmstadr), Dichloran Glycerol Agar, DG18 (Merck, Darmstadr), and Malt Yeast Extract 50% Glucose Agar, MY50G. DRBC medium was used for the isolation and enumeration of molds that grow at $a_w > 0.95$, DG18 for the isolation of xerotolerant and xerophilic molds that grow at $a_w$ £ 0.95, while MY50G was used for the isolation of highly xerophilic species that grow at $a_w$ £ 0.89 (S a m s o n et al., 2004, P i t t and H o c k i n g, 2009). Addition of dichlorine in DRBC and DG18 media limits the mycelial growth of certain molds, such as *Mucor* spp. and *Rhizopus* spp., and the addition of chloramphenicol inhibits the growth of bacteria. Addition of glycerol (18%) in DG18 medium reduces water activity.

Under aseptic conditions, 20 g of cakes was homogenized in 180 mL of 0.1% sterile peptone water (0.1 g of peptone/100 mL of distilled water). After this, samples were shaken for 10 minutes at 200 rpm (Unimax 1010, Heidolph, Germany). Total number of molds was determined by the dilution method according to K o c h. For the preparation of dilution, a 0.1% sterile solution of peptone water was used. One milliliter of the prepared dilution ($10^{-1}$, $10^{-2}$, and $10^{-3}$) was

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transferred into Petri dishes (q 9cm), into which mediums were poured. The seeded mediums were incubated for 5-7 days at 25°C; the colonies were counted and expressed as CFU/g. Tests were conducted in triplicate.

## Mold identification

After determining the total number, conidia and hyphal fragments of the mold colonies were transmitted onto the Malt Extract Agar (MEA) (Merck, Darmstadr), Czapek Yeast Extract Agar (CYA), or the Potato Dextrose Agar (PDA) (Merck, Darmstadr). Based on macromorphological properties, these colonies were assumed to belong to genera *Penicillium*, *Aspergillus*, *Eurotium* and *Emericella*, and they were subcultured onto the CYA. Seeded mediums were incubated for 7 days at 25°C. Isolates which were presumed to belong to the genus *Fusarium* were grown on PDA, and than subcultured on Carnation Leaf Agar (CLA), in order to obtain monosporic culture (N e l s o n et al., 1983; L e v i ć, 2008). Monosporic cultures were incubated for 10-14 days in cyclic mode of 12 h combined light (fluorescent light and NUV – near ultraviolet light) and 12 h darkness at 25°C for the purpose of stimulating the formation of conidiogenous structures. Other isolates which were assumed to belong to genera *Acremonium*, *Alternaria*, *Cladosporium*, *Mucor*, *Philaphora* and *Ulocladium* were subcultured on MEA and incubated for 7 days at 25°C.

Criteria described by S a m s o n et al. (2004), S a m s o n and F r i s v a d (2004), and P i t t and H o c k i n g (2009), were applied so that species of the genus *Penicillium* could be identified, while *Aspergillus*, *Eurotium* and *Emericella* species were determined according to K l i c h (2002), S a m s o n et al. (2004), and P i t t and H o c k i n g (2009). *Fusarium* species were identified according to the keys for the determination, as described by N e l s o n et al. (1983), and L e v i ć (2008). Other isolated species were identified according to P i t t and H o c k i n g (2009) and S a m s o n et al. (2004).

Frequency and share of certain genera or species of molds in food samples were calculated according to the following equations:

$$\text{Frequency (\%)} = \frac{\text{number of samples where the genus were identified}}{\text{total number of samples x 100}}$$

$$\text{Share (\%)} = \frac{\text{number of isolates of a genus or species}}{\text{total number of isolates of all genera or species x 100}}$$

## Determination of aflatoxins, ochratoxin A and zearalenone

Determination of aflatoxins ($AB_1$, $AG_1$, $AB_2$, $AG_2$), ochratoxin A (OTA) and zearalenone (ZEA) was performed using the multi-mycotoxin method by B a l z e r et al. (1978). Extraction of mycotoxins from cake samples (25 g) was

performed using a mixture of acetonitrile and water in a ratio of 90:10, by shaking on the shaker for 1 h. After filtration, 50 mL of the filtrate was purified and defatted with 2 x 25 mL of *n*-hexane. Ochratoxin fraction as acidic was extracted with a saturated solution of $NaHCO_3$ (8 mL). After the separation of OTA, low-acid F-2 toxin fraction was extracted with 1 M NaOH (2 x 10 mL). A relatively neutral aflatoxin fraction remained in the solution. Each toxin fraction was filtered through anhydrous $NaSO_4$ and evaporated on the rotary vacuum evaporator at 60 °C. Evaporated toxin extracts were diluted in 1 mL of chloroform and along with the standards $AB_1$, $AG_1$, $AB_2$, $AG_2$, OTA, ZEA (Sigma Aldrich, Steinheim, Germany) were layered onto plates with type H silica gel. Chromatograms were developed in the system toluene:ethyl-acetate:formic acid (50:40:10 v/v/v) at room temperature until the solvent front height was 14 cm and after drying they were observed under a long-wave (365 nm) UV light (UV cabinet, Camag). Determination of mycotoxins was performed by comparing the intensity of fluorescence staining pattern with referent standards of mycotoxins. For the amplification of the fluorescence staining intensity, plates were sprayed with 25% solution of sulfuric acid in methanol (for aflatoxins), alcoholic solution of $NaHCO_3$ (for OTA) and alcoholic solution of $Al_2O_3$ (for ZEA). After spraying, plates were dried for 10 min at 130 °C (for aflatoxins and OTA) and 5 min at 130 °C (for ZEA).

The quantification of the mycotoxins was done by visual comparison of the intensities of both standards and samples. This involved the comparison of the fluorescence intensities of the spots with same retention factor (RF) of the mycotoxins in the samples with those of the corresponding standard and it was determined which of the sample spot matched the standards. The corresponding aliquot volumes were then recorded and the concentrations of the mycotoxins in the samples in μg/kg were then calculated as follows:

$$Mycotoxin\ content\ (μg/kg) = SxYxV/WxZ$$

Where:
S – volume of standard with same color intensity as sample (μL),
Y – concentration of mycotoxin standard (μg/mL),
V – volume of solvent required for the dilution of the sample contained in final extract (μL),
W – effective weight (g) of original sample contained in final extract,
Z – volume of spotted sample equivalent to standard (μL).

*Determination of sterigmatocystin*

The method according to v a n  E g m o n d et al. (1982) was used for qualitative determination of sterigmatocystin (STC). STC extraction from cake samples (50 g) was performed with a mixture of solution of acetonitrile (180 mL) and 4% KCl (20 mL), by shaking on the shaker for 1 h. After the extraction, the content was filtered and the acetonitrile portion (100 mL) was

defatted and purified with *n*-hexane (2 x 50 mL). After that, the STC extraction was performed with chloroform (1 x 50 mL and 1 x 25 mL). The chloroform fraction was added with 5 g of anhydrous $NaSO_4$ and left still for 30 min. This fraction was then filtered through the filter paper (W h a t m a n No. 1) and evaporated to dryness in a vacuum rotary evaporator at 35 °C. Dry residue was dissolved in 1 mL of chloroform, and along with the standard STC (Sigma Aldrich, Steinheim, Germany) was layered onto plates of silica gel type H. The mixture of toluene:glacial acetic acid (9:1 v/v) was used to develop the chromatograms. Chromatograms were developed until the solvent front reached the height of about 16 cm, and then dried for 15 min at room temperature. Visibility of STC was enhanced by spraying the plates with $AlCl_3$ solution (3 g $AlCl_3$ x $6H_2O$ was dissolved in 8 mL of $H_2O$ and 2 mL of glacial acetic acid was added). After spraying, plates were dried for 30 minutes at $70°C$.

The quantification of the STC was done by visual comparison of the fluorescence intensities of the standard and the samples. Concentration of STC in the samples of cakes was calculated with the equation:

STC content (µg/kg) = $V_1 x V_3 x S x Y / W x Z x V_2$

Where:
$V_1$ – total volume of the solvent used for the extraction of STC from samples (mL)
$V_2$ – volume of the filtrate after extraction (mL)
S – volume of standard with same color intensity as sample (µL),
Y – concentration of STC standard (µg/mL),
$V_3$ – volume of solvent required for dilution of sample contained in final extract (µL),
W – effective weight (g) of original sample contained in final extract,
Z – volume of spotted sample equivalent to standard (µL).

*Statistical analysis*

The relationship between the total numbers of molds on DG18, MY50G, and DRBC medium was tested by regression analysis.

RESULTS AND DISSCUSION

Based on the results obtained from three different media, a significant difference was found in the total number of molds in samples of cakes, from their absence up to 9 x $10^2$ CFU/g (Figure 1). Molds did not develop in the samples of vanilla-raspberry dessert cups (sample no. 5) and wholemeal cookies (samples no. 10 and 11). Light mold contamination was found in the samples of wholemeal cookies (sample 12) and homemade petit fours (sample 13) on MY50G and DRBC and in the samples of petit fours from the market 4 (sample 9) on MY50G.

A serious mold contamination was recorded in eight cake samples (1, 2, 6, 7, 8, 14, 15 and 16) on all three media, in a sample of lean magic cookies (4) and petit fours from the market 4 (9) on DG18 and DRBC and in the sample of various petit four (17) on DG18.
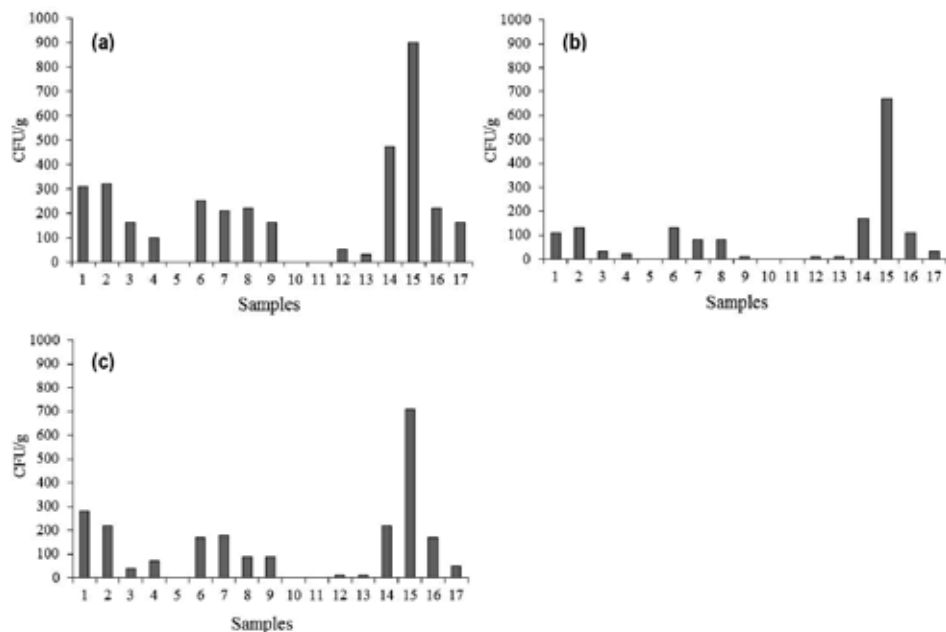


Fig. 1. – The total number of molds (CFU/g) in samples of cakes on DG18 Agar (a), MY50G Agar (b) and DRBC Agar (c) [1 – petit fours 1, 2 – magic cookies; 3 – magic lean cookies 1, 4 – magic lean cookies 2, 5 – vanilla-raspberry dessert cups, 6 – petit fours from the market 1, 7 – petit fours from the market 2 , 8 – petit fours from the market 3, 9 – petit fours from the market 4, 10 – wholemeal cookies 1; 11 – wholemeal cookies 2, 12 – wholemeal cookies 3; 13 – homemade petit fours; 14 – mix cap; 15 – petit fours 2, 16 – various cakes, 17 – various petit fours]

By comparing the data obtained for the number of molds on certain media, it can be concluded that the overall number was dependent on the type of the medium used for their isolation, and the presence of fungal contamination. Given that the most frequent species were from the groups of moderately xerotolerant molds (*Penicillium*, *Aspergillus*), the maximum number of colonies was noted on the DG18 medium, and the lowest on the MY50G (Figure 2).

There is a linear dependence with high values of correlation coefficients between the total number of molds on DG18, DRBC and MY50G medium (0.9307, 0.9004 and 0.9290) (Figure 3). This indicates that the determination of the total number of molds on one medium can with great reliability predict the total number of molds on the other two media for the tested samples of cakes.
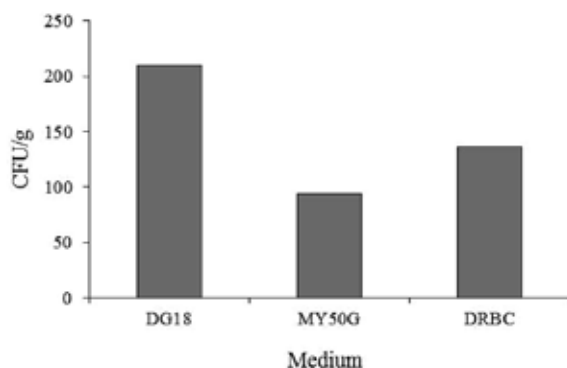
Fig. 2. – The average number of molds on DG18, MY50G and DRBC media
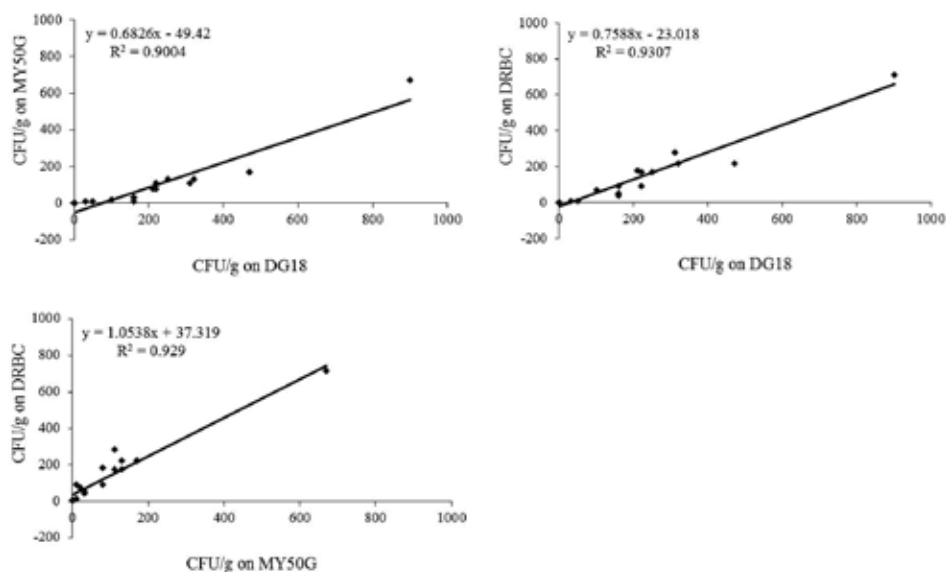


Fig. 3. – Linear correlation, regression equations and correlation coefficients
of the total number of molds on DG18, MY50G and DRBC media

Species of the genus *Penicillium*, which were the dominant mycopopulation, were most commonly isolated from these products (76.50%) (Table 1). Genus *Aspergillus* was isolated from 9 samples, with frequency of 53.0%. Significant presence of genera *Alternaria* and *Fusarium* was detected with frequency of 47.10%. The presence of *Cladosporium* and *Eurotium* species was found in 4 (frequency of 23.30%) and *Mucor* in 3 samples (frequency of 17.60%), while other genera (*Acremonium*, *Phialophora*, and *Ulocladium*) were isolated from one sample each (frequency of 5.90%).

In the mycopopulation of cakes the most numerous species were those of the genus *Penicillium* (19), which represented 51.25% of all isolates (Table 2).

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Tab. 1. – Frequencies (%) of mold genera in cake samples

| Genus | Number of tested samples / number of contaminated samples | Genus frequency (%) |
|---|---|---|
| *Acremonium* | 17/1 | 5.9 |
| *Alternaria* | 17/8 | 47.1 |
| *Aspergillus* | 17/9 | 53.0 |
| *Cladosporium* | 17/4 | 23.5 |
| *Eurotium* | 17/4 | 23.5 |
| *Fusarium* | 17/8 | 47.1 |
| *Mucor* | 17/3 | 17.6 |
| *Phialophora* | 17/1 | 5.9 |
| *Penicillium* | 17/13 | 76.5 |
| *Ulocladium* | 17/1 | 5.9 |

The dominant genus was also *Aspergillus* with a share of 29.75% in the total mycopopulation, with 6 isolated species. Genera *Fusarium*, *Alternaria*, *Cladosporium*, and *Eurotium* accounted for 7.75%, 4.50%, 2.75% and 1.50% of the total mycopopulation of cakes. Other genera (*Acremonium*, *Mucor*, *Phialophora*, and *Ulocladium*) were represented per one species and accounted to a smaller percentage (2.5%) of the isolated mycopopulation.

A total of 35 mold species were identified. *A. niger* was the species with the highest share (24.75%), followed by *P. aurantiogriseum* (16.75%), *P. brevicompactum* (8.50%), *P. hirsutum* (6.50%), *F. proliferatum* (4.75%) and *A. alternata* (4.50%) with a significant presence in the mycopopulation of these products (Table 2).

A total of 35 species were identified and 25 were potentially toxigenic (Table 3), which accounted for 86.61% of the total mycopopulation of cakes. *Penicillium* species which were isolated in the highest percentage had the largest share (43.11%). Secondary metabolites produced by these molds are of different nature and many of them can be listed as mycotoxins.

The most important mycotoxins are OTA (carcinogenic and nephrotoxic), citrinin (nephrotoxic), xanthomegnin, viomellein and vioxanthin (nephro and hepatoxic), nephrotoxic glycopeptides, verrucosidin (neurotoxin), patulin and penicillic acid (general mycotoxins) and penitrem A (neurotoxin) (S a m s o n et al., 2004). Potentially toxigenic *Aspergillus* species accounted for 29.75% of the total isolated mycopopulation. Prevailing species in this genera were the producers of OTA (*A. alliacus*, *A. carbonarius*, *A. niger*, *A. ochraceus*). *A. ochraceus* synthesizes ochratoxin A at temperatures of 12 to 37 °C and at 0.80 $a_w$ (P i t t and H o c k i n g, 2009), while psychrophile *Penicillium* spp. (e.g. *P. verrucosum*) can produce this toxin at temperatures ranging from 4 to 31 °C (S a m s o n et al., 2004). OTA was detected in corn, barley, beans, peanuts, fruits, vegetables, wine and beer (W e i d e n b ö r n e r, 2008). The main route of entry for humans is through contaminated grains, nuts, rice, coffee, wine, beer, olives, but also through meat products (Š k r i n j a r et al., 2005; K o c i ć-T a n a c k o v and D i m i ć, 2012).

Tab. 2. – The share of genera and species of molds in cake samples

| Genus | Genus share (%) | Species | Species share (%) |
|---|---|---|---|
| *Acremonium* | 0.25 | *A. butyri* (v. Beyma) W. Gams | 0.25 |
| *Alternaria* | 4.50 | *A. alternata* (Fr.) Keissler | 4.50 |
| *Aspergillus* | 29.75 | *A. alliacus* Thom & Church | 0.25 |
| | | *A. carbonarius* (Bainier) Thom | 1.25 |
| | | *A. niger* van Tieghem | 24.75 |
| | | *A. ochraceus* K. Wilh. | 2.50 |
| | | *A. wentii* Wehmer | 1.00 |
| *Cladosporium* | 2.75 | *C. cladosporioides* (Fres.) de Vries | 2.75 |
| *Eurotium* | 1.50 | *E. amstelodami* L. Mangin | 0.75 |
| | | *E. herbariorum* Link | 0.75 |
| *Fusarium* | 7.75 | *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Sheld.) | 1.25 |
| | | *F. proliferatum* (Matsushima) Nirenberg | 4.75 |
| | | *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas | 1.75 |
| *Mucor* | 1.00 | *M. plumbeus* Bon. | 1.00 |
| *Phialophora* | 0.25 | *P. fastigiata* (Legerb. & Melin) Conant | 0.25 |
| *Penicillium* | 51.25 | *P. aurantiogriseum* Dierckx | 16.75 |
| | | *P. brevicompactum* Dierckx | 8.50 |
| | | *P. chrysogenum* Thom | 3.75 |
| | | *P. citrinum* Thom | 0.25 |
| | | *P. citreonigrum* Dierckx | 0.25 |
| | | *P. crustosum* Thom | 3.25 |
| | | *P. dipodomyis* Frisvad, Filt. & Wicklow | 1.75 |
| | | *P. hirsutum* Dierckx | 6.50 |
| | | *P. freii* Frisvad & Samson | 0.25 |
| | | *P. fellutanum* Biourge | 2.25 |
| | | *P. funiculosum* Thom | 0.25 |
| | | *P. glabrum* (Wehmer) Westling | 0.50 |
| | | *P. olsonii* Bain. & Sartory | 1.50 |
| | | *P. polonicum* K. Zaleski, Bull. | 0.25 |
| | | *P. paneum* Frisvad | 2.50 |
| | | *P. raistrickii* G.Sm. | 0.25 |
| | | *P. solitum* Westling | 2.00 |
| | | *P. verrucosum* Dierckx | 0.25 |
| | | *P. viridicatum* Westling | 0.25 |
| *Ulocladium* | 1.00 | *U. chartarum* (Preuss) Simmons | 1.00 |

Potentially toxigenic species of the genus *Fusarium* were presented with 7.75%. The most frequent and most toxic fusariotoxins were from the group of fumonisins, trichothecenes and zearalenone. These mycotoxins and their producers are abundant as contaminants of grains (L e v i ć et al., 2009), pri-

Tab. 3. – Potential toxigenic molds isolated from cake samples and their mycotoxins (M o n t e - m u r r o and V i s c o n t i, 1992; S a m s o n et al., 2004; L e v i ć, 2008; P i t t and H o c k i n g, 2009; EFSA 2011)

| Species | Mycotoxin |
|---|---|
| *Alternaria alternata* | Alternariol, alternariol monomethyil ether, alterotoxin I and II, altenuene, tenuazonic acid |
| *Aspergillus alliacus* | Ochratoxin A |
| *A. carbonarius* | Ochratoxin A |
| *A. niger* | Naphtho- 4-pyrones, malphormins, ochratoxin A (few isolates) |
| *A. ochraceus* | Penicillic acid, ochratoxin A, B and C, xanthomegnin, viomellein, vioxanthin |
| *A. wentii* | Emodin, ventilacton |
| *Eurotium amstelodami* | Echinulin, physcion |
| *E. herbariorum* | Echinulin, physcion, sterigmatocystin |
| *Fusarium verticillioides* | Fumonisins B1, B2, B3, B4, fusaric acid, fusarins A, D, E, F, C, trichothecenes |
| *F. proliferatum* | Fumonisins B1, B2, B3, beauovericins, fusaroproliferin, fusaric acid, fusarin C, moniliformin, naphthoquinone pigments, fusapyrone |
| *F. subglutinans* | Moniliformin, fumonisin B1, fusaric acid, fusaproliferin, hami-dosporol, beauovericins, naphthoquinone pigments |
| *Penicillium aurantiogriseum* | Penicillic acid, verrucosidin, nephrotoxic glycopeptides, anacine, auranthine, aurantiomine, ochratoxin A |
| *P. brevicompactum* | Botryodiploidin, mycophenolic acid, Raistrick phenols, brevianamide A |
| *P. chrysogenum* | Roquefortine C, meleagrin, chrysogine, penicilline, ochratoxin A |
| *P. citrinum* | Citrinin, tanzawaic acid A |
| P. citronigrum | Citreoviridin |
| *P. hirsutum* | Roquefortine C, terrestric acid |
| *P. olsonii* | Verrucolone |
| *P. paneum* | Patulin, roquefortine C, marcfortines |
| *P. polonicum* | Peniciliic acid, verrucosidin, nephrotoxic glycopeptides, cyclopenin, cyclopenol |
| *P. raistrickii* | Griseofulvin |
| *P. rugulosum* | Rugolosin |
| *P. solitum* | Cyclopenin, cyclopenol, viridicatol, viridicatin, compactin |
| *P. verrucosum* | Ochratoxin A, citrinin, verrucolone, verrucins |
| *P. viridicatum* | Xanthomegnin, viomellein, vioxanthin, viridic acid, penicillic acid, brevianamide A |

marily corn, as well as grain-based food for humans and animals (K o c i ć - - T a n a c k o v et al., 2005, 2007; L e v i ć et al., 2004, 2008; W e i d e n b ö r n e r, 2008; N a y a k a et al., 2010). The best synthesis of fumonisin B$_1$ was achieved in the grains of corn which contained 27 to 32% humidity and at the temperature of 20 °C (L e B a r s et al., 1994).

*A. alternata*, the only species isolated from *Alternaria* genus, which relatively frequently occurred in the samples (47.1%), produces several toxins (alternariol, alternariol monomethyilether, tentoxin, tenuazonic acid, alterotoxin, *Alternaria alternata f. sp. lycopersici* toxins, stemphyltoxin III and altenuene) that adversely affect the organism of people and animals (EFSA 2011). *Alternaria alternata* toxins (AAT) are highly toxic metabolites of structure similar to that of fumonisins. The presence of *A. alternata* and its mycotoxins was detected in tomatoes, wheat, barley, corn, Chinese sugar cane, rapeseed, olives, apples, citrus fruits and spices (P i t t and H o c k i n g, 2009; D e s h p a n d e, 2002). Maximum production of alternariol, and its monomethyilether and altenuene by *A. alternata* was determined at 25 ºC and 0.98 $a_w$ (M a g a n et al., 1984), and tenuazonic acid at 0.90 $a_w$ and 25 °C (E t c h e v e r r y et al., 1994).

Two isolated species from the *Eurotium* genus (*E. amstelodami* and *E. herbariorum*) are potential producers of echinulin, physcion and sterigmatocystin. Species of this genus are extremely xerophilic molds (minimum $a_w$ of 0.70). They were isolated from grains, drupe fruits, dried fruits and vegetables, cheese, dried meat and fish (S a m s o n et al., 2004; P i t t and H o c k i n g, 2009).

Potential producers of OTA were present with the highest percentage (50.5%). Fumonisin producers were present with 7.75%, moniliformin with 6.5%. Potential producers of *Alternaria* toxins accounted for 4.5%. Aflatoxigenic molds were not isolated from the tested samples.

Regardless of the isolated potentially toxigenic mycopopulation, mycotoxicological analysis of the cake samples did not reveal the presence of $AB_1$, $AG_1$, $AB_2$, $AG_2$, OTA, ZEA, and STC.


CONCLUSION


Different types of cakes that are industrial or handmade, available on the market, were contaminated with potentially toxigenic mold species, however, mycotoxins $AB_1$, $AG_1$, $AB_2$, $AG_2$, OTA, ZEA and STC were not detected in the samples.

Mycopopulation of cakes primarily depends on the contamination of basic raw materials and additives used in their preparation. If raw materials and additives contain a higher initial number of microorganisms before the heat treatment, the greater is the chance of their survival. Particular problems are products that are not heat-treated. Mycopopulation introduced with raw materials and additives increases during storage and under inadequate conditions. Poor hygiene of workers, equipment and air also contributes to the total number of molds.

Improving the hygiene measures during production, addition of synthetic and natural preservatives, reduction of humidity and temperature during storage, as well as constant supervision can reduce mold growth in raw materials and cakes and the possibility of mycotoxin biosynthesis.

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# КОНТАМИНАЦИЈА ПОСЛАСТИЧАРСКИХ ПРОИЗВОДА ТОКСИГЕНИМ ПЛЕСНИМА

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## Резиме

Укупан број плесни у посластичарским производима кретао се до $9,0 \times 10^2$ CFU/g. Највећи број плесни изолован је на Дихлоран 18% глицерол агар (DG18), а најмањи на сладно квасном екстракту са 50% глукозе агару (MY50G). Микопопулацију посластичарских производа чиниле су врсте из родова *Acremonium, Alternaria, Aspergillus, Cladosporium, Eurotium, Mucor, Phialophora, Penicillium, Fusarium* и *Ulocladium*. Доминантане врсте су биле *A. niger, P. aurantiogriseum, P. brevicompactum, P. hirsutum, F. proliferatum* и *A. alternata*. У највећем проценту су били заступљени потенцијални продуценти охратоксина А (50,50%). Продуценти фумонизина били су заступљени са 7,75%, монилиформина са 6,5%, а стеригматоцистина са 0,75%. Потенцијални произвођачи *Alternaria* токсина чинили

су 4,5%. Афлатоксигене плесни нису изоловане из испитиваних узорака. Мико-токсиколошким испитивањима узорака посластичарских производа није конста-товано присуство афлатоксина, стеригматоцистина, охратоксина А и зеараленона.

КЉУЧНЕ РЕЧИ: посластичарски производи, фунгална контаминација, фреквентност токсигених плесни

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# DEVELOPMENT OF FORECASTING ELEMENTS FOR MINIMIZATION OF FUNGICIDE TREATMENT IN POTATO PROTECTION AGAINST EARLY BLIGHT IN MOSCOW REGION

ABSTRACT. Early blight of potato (the agent is imperfect fungus *Alternaria alternata* (Fr.) Keissl.) is a serious disease of potatoes under hot conditions. This disease is important in the regions of Eastern and Southern Europe, Asia and Africa. It is controlled with intensive application of fungicides commonly used against late blight. However, currently fungicides cause undesirable damage to humans and the environment. Elements of forecasting the early blight have been developed in order to minimize the dosage of fungicides. Primary symptoms (chloroses and necroses) and the potential of pathogen natural inoculum (determination of disease severity, indexes of formation of conidia and aggressiveness; as well as the current weather conditions) were registered and determined in the potato signal plots (no treatments or artificial inoculation) near main potato cropping. Seed tubers of main potato cropping were preliminary treated with plant growth regulator Circon and micro-fertilizer Siliplant produced by the Russian Company NEST-M. These preparations proved to be effective in acceleration process of potato growth and in delaying the early blight onset by 5-10 days. The same potato cultivar must be planted both in signaling plots and in the fields. Well-timed determination of primary symptoms and potential of pathogen inoculum allowed us to understand the damage that early blight can cause to potato natural inoculation, and hence to provide well-timed application of fungicides with optimum dosages in mixture with Circon or Silipant. This forecasting method can be helpful for the adequate management of early blight and decrease of the environmental damages.

KEY WORDS. *Alternaria alternata*, potato early blight, Circon, Siliplant, potato disease forecasting

## INTRODUCTION

Early blight of potato is a serious disease which spreads under hot conditions. This disease is important in the regions of Eastern and Southern Europe, Asia, and Africa.

The causal agents of potato early blight are *Alternaria solani* Sorauer and *A. alternata* (Fr.) Keissl. The first species is thought to be main pathogen which

causes potato early blight. *A. alternata* affects potato tissues as a secondary pathogen after *A. solani* development or other stressful factors such as nitrogen and boron shortage, as well as ozone influence (T u r k e n s t e e n L. J. et al., 2010). However, according to some alternative data, *A. alternata* is able to destroy potato tissues in rather autonomic regimen *in vivo* and *in vitro* (K a s p a J., O s o w s k i J., 2007; 2012). Thus, *A. alternate,* which was detected in potato blighted tissues of Moscow Region under dry and hot conditions, very often became an important point which attracted our attention.

Potato early blight is usually controlled with intensive application of fungicides normally used against late blight (oomycete *Phytophthora infestans* (Mont.) de Bary). However, currently fungicides cause urgent damage to human and environment. Elements for forecasting the early blight were developed in order for the dosage of fungicides to be minimized (P e n k i n, 2012). Another important point is the application of plant growth regulators, micro-fertilizers and plant extracts. Under the conditions unfavorable for pathogens, these substances can be effective in their suppression (S m i r n o v et al., 2011). The aim of our current investigation is development of forecasting elements which allow the application of plant growth regulators and micro-fertilizers for effective protection of potato against early blight.


## MATERIAL

*A. alternata* isolate Kart collected in 2010 in the Moscow Region, was used in all laboratory experiments. Also, fungicides such as Prestige (Bayer), Ridomil Gold MZ (Syngenta), plant growth regulator Circon and micro-fertilizer Siliplant (Nest-M) were used in laboratory experiments and field trials.


## METHODS

**Tests of conidia**. After the incubation for 14 days on potato-carrot agar, conidia of *A. alternata* isolate Kart were placed on 2% distilled water agar medium and incubated at temperatures -20, -15, -10, -5, 3, 18, 25, 30, 35, and 40º C (for negative temperatures during one month with following reanimation at 23-25º C, for positive temperatures during 7 days. Germination of conidia and formation of their chains was registered.

**Inoculation of detached leaves with mycelial plugs**. After the incubation for 14 days on potato-carrot agar, mycelial plugs or conidia of *A. alternata* isolate Kart were placed on injured cover of detached leaves of potato, tomato and different ornamental plants in moist chambers at room temperature (about 20º C). Development of chloroses and necroses with pathogen sporulation was registered for different variants of experiment which differ from each other based on the type of inoculation and treatment scheme.

**Determination of features of pathogen development on potato plants in field conditions**. Features DF, DS, IC and IA were determined for all vari-

ants of experiments which differ from each other based on treatment scheme applied to potato (cultivar Ilynsky) and tomato (cultivar Bely Naliv). Disease frequency (percentage of affected plants, DF) and severity (percentage of affected plant cover, DS) was determined. Blighted leaflets were placed into moist chambers and incubated for 6 days at room temperature (about $20^o$ C). Formation of $A.$ $alternata$ conidia per 10 fields of view of light microscope (10 mm$^2$) for every potato leaflet checked was registered: very rare (1-50), rare (51-150), moderate (151-200), frequent (201-250), very frequent (>250) (P e n k i n, 2012).

Index of formation of conidia (IC) was calculated as following:

IC= 0,05x VRC + 0,1x RC + 0,5x MC + 0,75x FC + VFC, where VRC, RC, MC, FC and VFC are percentages of samples (leaflets) with very rare, rare, moderate, frequent, very frequent conidia respectively (P e n k i n, 2012).

Index of aggressiveness (IA) was calculated as following:

IA=(DFx DSx IC)/10000 (as in S m i r n o v, K u z n e t s o v, 2006; 2009).

Ranges of IA for certain field $A.$ $alternata$ populations are presented in Table 1.

**Statistical analysis**. LSD$_{05}$ values were calculated by software STRAZ (version 2.1, Moscow Agricultural Academy). Correlation analysis was done by EXCEL (2007, Microsoft).

Tab. 1 – Ranges of field $A.alternata$ populations

| Main Gradation | IA | Sub-gradation | IA | Score for IA |
|---|---|---|---|---|
| L (Lowly aggressive) | ≤40.0 | L$_1$ (not aggressive) | 0 – 20.0 | 1 |
| | | L$_2$ (lowly aggressive) | 20.1 – 40.0 | 2 |
| A (Aggressive) | 40.1 – 100 | A$_1$ (moderately aggressive) | 40.1 – 60.0 | 3 |
| | | A$_2$ (aggressive) | 60.1 – 80.0 | 4 |
| | | A$_3$ (very aggressive) | 80.1 – 100 | 5 |

RESULTS

**Germination of conidia**. $A.$ $alternata$ conidia were able to actively germinate at all investigated positive temperatures. From third to seventh days, germinated conidia actively formed conidial chains from a few newly formed conidia, especially at temperatures 30 and $35^o$ C. After this, some mycelium pathogen was formed. In general, optimal germination for conidia was in the interval between 25 and $35^o$ C. At temperature of $3^o$ C formation of conidia was limited, but majority of them germinated. At temperature of $40^o$ C conidia could be formed and even germinate, but after this majority of them were decomposed and destroyed (Tables 2, 3; Fig. 1-3).

After a month incubation at negative investigated temperatures -5 and $-10^o$ C $A.$ $alternata$ conidia could be rather easily reanimated. So majority of them preserved their viability at these temperatures (Table 4). After a month of incubation at $-15^o$ C, only a few conidia were able to germinate and finally provide viable infective mycelium (see below). After a month of incubation at $-20^o$ C, no conidial germination was detected.

Tab. 2 – Formation and germination of *A.alternata* conidia at positive temperatures, per 10 fields of view of light microscope (10 mm$^2$) after incubation during one and three days on water agar

| Temperature, °C | First day | | Third day | |
|---|---|---|---|---|
| | General number of conidia | Number of germinated conidia | General number of conidia | Number of germinated conidia |
| 3 | 5.7 | 3.1 | 7.1 | 5.4 |
| 18 | 14.5 | 4.1 | 19.5 | 11.0 |
| 25 | 21.0 | 7.8 | 23.0 | 17.0 |
| 30 | 25.8 | 24.1 | –* | – |
| 35 | 32.4 | 24.8 | – | – |
| 40 | 20.3 | 8.2 | 0 | 0 |
| LSD$_{05}$ | 6.4 | 6.4 | 0.8 | 1.3 |

* All observed *A. alternata* conidia were in chains

Tab. 3 – Formation of *A.alternata* conidia in chains at positive temperatures, per 10 fields of view of light microscope (10 mm$^2$) after incubation during three, five, and seven days on water agar

| Temperature, °C | Third day | | Fifth day | | Seventh day | |
|---|---|---|---|---|---|---|
| | Number | | Number | | Number | |
| | conidia per chain | conidial chains | conidia per chain | conidial chains | conidia per chain | conidial chains |
| 3 | not observed | not observed | 10.1 | 7.4 | 12.0 | 13.0 |
| 18 | not observed | not observed | 16.3 | 8.0 | 22.0 | 7.6 |
| 25 | not observed | not observed | 15.7 | 14.3 | 24.3 | 14.5 |
| 30 | 5.6 | 3.6 | 8.1 | 14.7 | 11.0 | 15.0 |
| 35 | 7.8 | 15.5 | 8.5 | 18.2 | 13.2 | 21.5 |
| 40 | not observed | not observed | 0 | 0 | 0 | 0 |
| LSD$_{05}$ | 2.4 | 0.9 | 3.3 | 3.5 | 1.8 | 2.1 |



____ 25 μm
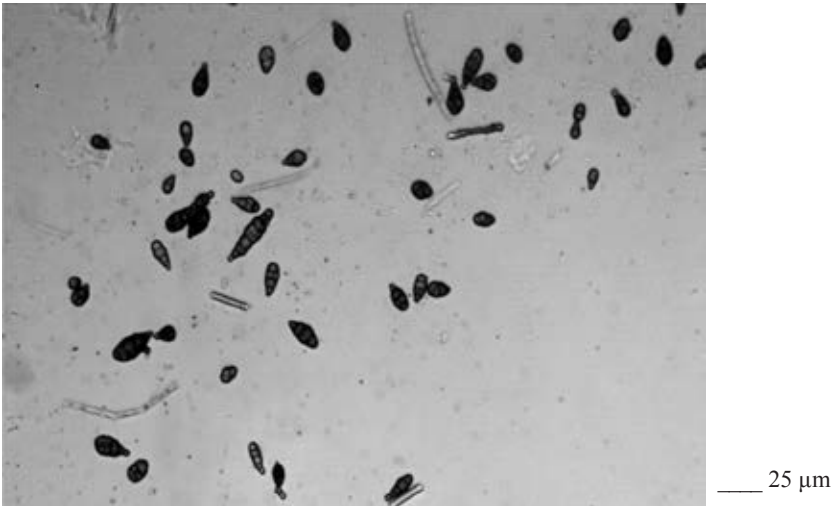
Fig. 1 – Conidia of *A. alternata* at 25° C, some of them begin to germinate and proliferate

___ 50 μm
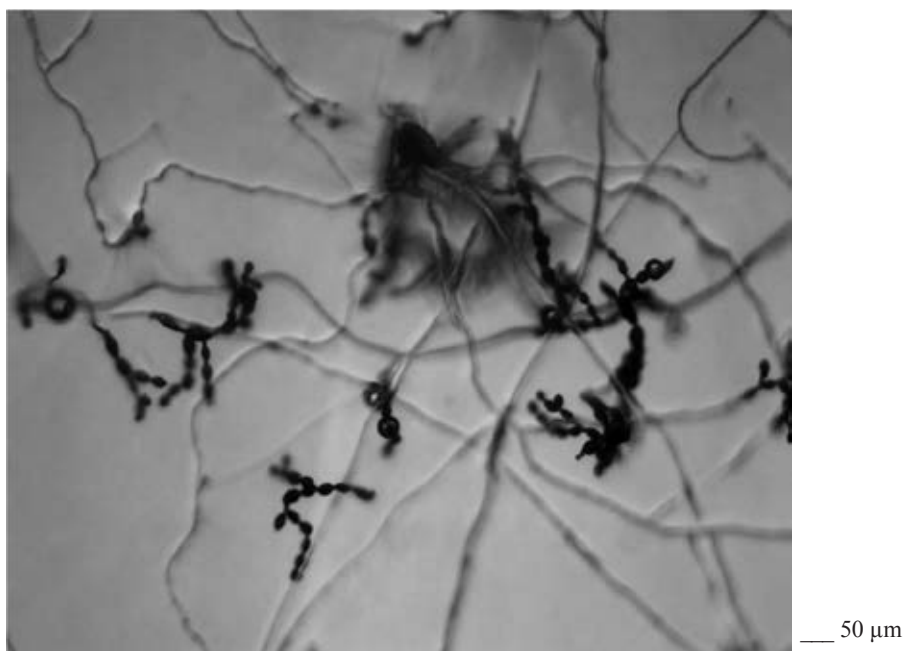
Fig. 2 – Conidial chains of *A. alternata* at 18º C



___ 50 μm

Fig. 3 – Conidial chains of *A. alternata* at 35º C

Tab. 4 – Formation and germination of *A.alternata* conidia at different negative temperatures, per 10 fields of view of light microscope (10 mm$^2$) after incubation during one month and consequent reanimation during one week at 3$^o$ C and one and three days at 23-25$^o$ C on water agar

| Temperature, $^o$C | First day | | Third day | |
|---|---|---|---|---|
| | General number of conidia | Number of germinated conidia | General number of conidia | Number of germinated conidia |
| -5 | 3.2 | 0 | 3.7 | 2.1 |
| -10 | 2.1 | 0 | 2.5 | 1.0 |
| LSD$_{05}$ | 1.2 | – | 1.1 | 0.9 |

**Infective potential of conidia and mycelium**. Inoculation with *A. alternata* mycelial plugs (isolate Kart) of leaves of different plants (potato, tomato, begonia, pelargonium, and geranium) was successful. Development of chlorosis, necrosis and sporulation was registered (Fig. 4). Chlorosis was the first symptom of outpaced development of *A. alternata* mycelium, caused by diffusion of pathogen toxins.

However, the same inoculation of tomato, preliminary treated with Siliplant or Circon or Rydomil Gold MZ, provided suppression of any development of *A. alternata* (Fig. 5).



Fig. 4 – Development of chlorosis, necrosis and sporulation on tomato leaves inoculated with *A. alternata* (isolate Kart) mycelial plug containing conidia

It is interesting and can be important to mention that symptoms of affection were observed at inoculation with *A. alternata* conidia (isolate Kart, 50 µl droplets, conidial concentration 25, 50 and 100 thousands/ml) of potato and tomato, but at the same time, inoculation of pelargonium typical hypersensitive reaction (HSR) was observed. Its manifestation was fully similar to the aforementioned suppression of any development of *A. alternata* treated with Siliplant or Circon or Rydomil Gold MZ (Fig. 5).

Fig. 5 – Suppression of development of chlorosis, necrosis and sporulation on tomato leaves inoculated with *A. alternata* (isolate Kart) mycelial plug containing conidia

**Development of early blight on potato without and after the treatment with Rydomil Gold MZ, Circon and Siliplant.** In 2010, during abnormally hot weather conditions in Moscow Region, development of early blight was very essential. Values of all features were very high, all investiageted *A. alternata* were aggressive with range A2 (Table 5). In general, in 2010 and 2011, development of early blight on potato, caused by A. alternata, looked very impressive.

Application of Circon and Siliplant was effective against *A. alternata*. The effect on necroses was not too strong. However, formation of conidia and consequent aggressiveness manifestation by field of *A. alternata* populations was highly suppressed by application of Circon and Siliplant (Table 6).

**Development of forecasting elements directed to decision support system (DSS) for effective protection of potato against early blight**. Our approach and obtained results enabled us to elaborate original methodology for forecasting the early blight development in large-scale potato fields. The basic point was to use adequate potato cultivar in signaling plots, untreated seed tubers, untreated potato plants located near main potato fields which needed to be protected. The plants from signaling plot provided first lesions of early blight and *A. alternata* natural inoculum after several days. It is also important that early blight in signaling plot occurs earlier than on the main cropping. This is possible due to the fact that seed tubers from main cropping are treated with fungicides and plant growth regulators (see above). Also the situation in the signal plot reflects real field partial resistance of planted potato cultivar, and it can be essentially different from the proclaimed resistance.

All steps of DSS are elucidated in Fig. 6. Detection of first lesions (chloroses and necroses) designates the time of preventive treatment of main potato field where disease symptoms do not yet appear but incubation period starts very early. Determination of range (scores) of aggressiveness (RA) of patho-

Tab. 5 – Features of *A. alternata* development in Moscow in 2010

| Date | Crop | DF (disease frequency) | DS (disease severity) | IC (index of formation of conidia) | IA (index of aggressivenes) | IA (sub-grada-tion) | IA (score) |
|---|---|---|---|---|---|---|---|
| 26.06 | potato | 100 | 81.0 | 95.0 | 77.0 | $A_2$ | 4 |
| 06.07 | potato | 100 | 75.0 | 98.3 | 73.7 | $A_2$ | 4 |
| 14.07 | potato | 100 | 74.2 | 100 | 74.2 | $A_2$ | 4 |
| 05.08 | potato | 100 | 62.4 | 65.0 | 40.6 | $A_1$ | 3 |
| 26.06 | tomato | 100 | 63.8 | 100 | 63.8 | $A_2$ | 4 |
| 06.07 | tomato | 100 | 61.8 | 98.3 | 60.7 | $A_2$ | 4 |
| 14.07 | tomato | 100 | 62.6 | 98.3 | 61.5 | $A_2$ | 4 |
| 05.08 | tomato | 100 | 65.0 | 95.0 | 61.8 | $A_2$ | 4 |

Tab. 6 – Features of *A. alternata* development in Moscow in 2011 without and after treatments with fungicides, Circon and Siliplant

| Variants | DS (disease severity) | IC (index of formation of conidia) | IA (index of aggressivenes) |
|---|---|---|---|
| Control. [Tubers without treatment. Vegetation – Rydomil Gold MZ 2 kg per hectare (standard dosage)] | 48.7 | 40.7 | 25.0 |
| Variant 1. [Tubers – Prestige 0.75 l/tn; Vegetation – Rydomil Gold MZ ½ standard dosage) | 56.7 | 43.3 | 25.0 |
| Variant 2. [Tubers – Prestige 0.6 l/tn+Siliplant 30 ml/ton; Vegetation – Rydomil Gold MZ ½ standard dosage + Siliplant 1 l per hectare) | 45.3 | 10.0 | 4.5 |
| Variant 3.[Tubers – Prestige 0.6 l/tn+Circon 10 ml/ton; Vegetation – Rydomil Gold MZ ½ standard dosage + Circon 10 ml per hectare)] | 36.7 | 10.0 | 3.5 |
| Variant4. [Tubers – Silplant 1 l/tn; Vegetation – Siliplant 1 l per hectare] | 35.3 | 9.8 | 3.3 |
| LSD$_{05}$ | 12.1 | 7.0 | 7.8 |
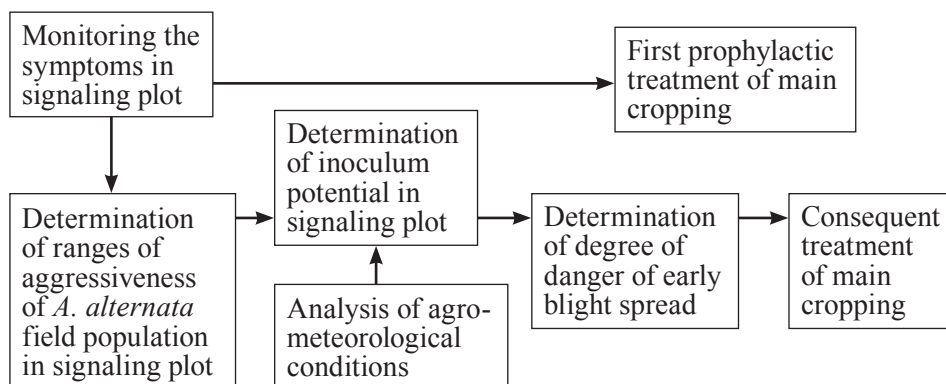


Fig. 6 – Milestones of Decision Support System (DSS) on potato treatments against early blight with use of signaling plot

gen population (Table 1) and simultaneously the range of agro-meteorological conditions (AMC) (scores on the base of ecological curve of normal distribution and our aforementioned data about the influence of temperature on the formation and germination of *A. alternata* conidia; Table 7; H a d d e r s, 2005) allows us to calculate scores of inoculum potential (IP):

$$IP_{scores} = RA_{scores} \times IP_{scores}$$

IP ranging enables distinguishing the different degrees (or different strength) of inoculum potential (Table 8).

Tab. 7 – Ecological gradations of agro-meteorological conditions on the base of ecological curve of normal distribution and data about the influence of temperature on the formation and germination of *A. alternata* conidia

| Air temperature, ºC | Relative air humidity, % | Ecological gradations of agro-meteorological conditions | Scores |
|---|---|---|---|
| > 40 | to 40 % | Maximum | 1 |
| 36 – 40 | 41 – 60% | Right pessimum | 3 |
| 25 – 35 | 61 – 75 % | Optimum | 5 |
| 20 – 24 | 76 – 90 % | Left pessimum | 3 |
| < 20 | 91 – 100% | Minimum | 1 |

Tab. 8 – Ranging *A. alternata* inoculum potentials

| Ranging inoculum potential | | Scores |
|---|---|---|
| Designation | Characteristics | |
| **IP1** | Very weak | 1-5 |
| **IP2** | Weak | 6-10 |
| **IP3** | Moderate | 11-15 |
| **IP4** | Strong | 16-20 |
| **IP5** | Very strong | 21-25 |

Also, in our additional investigation, the essential negative correlation was discovered between the inoculum potential and consequent field conditions (r= -0.57±0.25 (t=2.29, P<0.05). So, early blight development essentially influence consequent field condition but the influence of other factors also cannot be excluded. This allows the estimation of degrees of danger to main cropping in the nearest future, on the base of current inoculum potential in signaling plot (Table 9, Fig. 7). Consequently, well-timed determination of degrees of danger enables to plan cropping treatments with fungicides and/or plant growth regulators. This will be elaborated in consequent investigations basing the current legislative fungicides, micro-fertilizers and plant growth regulators.

Tab. 9 – Ranging degrees of danger of potential leaf and yield loss

| Ranging degrees of danger | | Expected rate of leaf necrotization and blight, % | Potential yield loss, % |
|---|---|---|---|
| Designation | Characteristics | | |
| DD1 | Absent | 1-5 | Not expected |
| DD2 | Very low | 6-10 | 1-5 |
| DD3 | Low | 11-30 | 6-15 |
| DD4 | High | 31-70 | 16-50 |
| DD5 | Very high | 71-100 | 51-100 |

| Ranges of Degrees of Danger | | | | | | |
|---|---|---|---|---|---|---|
| DD5 | | | | ▓ | ▓ | |
| DD4 | | | | ▓ | | |
| DD3 | | | ▓ | | | |
| DD2 | | ▓ | | | | |
| DD1 | ▓ | | | | | |
| | IP1 | IP2 | IP3 | IP4 | IP5 | Ranges of Inoculum potential (IP) |
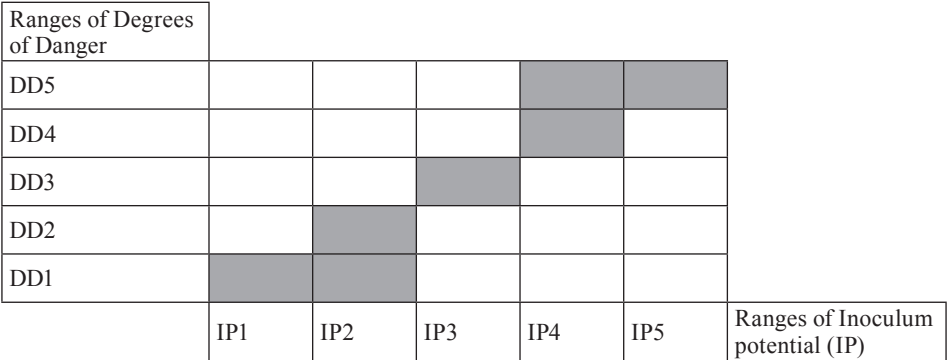
Fig. 7 – Nomogram of accordance between degree of danger for potato leaves and yield of potato cropping as well as inoculum potential of *A. alternata* in signaling plot

## DISCUSSION

*A. alternata* conidia proved to be structures which are universal for the realization of their viability. They preserve viability at temperatures from -15 to 35° C. Mycelial viability was higher and exceeded the aforementioned limits. However, conidia were destroyed by the pressure of soil bacteria and nematodes (P e n k i n, 2012; Z a i t s e v, unpublished data). Morphology of conidial chains strongly depends on temperature. This raises doubt over numerous assumptions about distinguishing similar 'small-conidial' species on the base of this really polymorphic feature. Teleomorpha corresponding to *A. alternata* was not detected in our investigation.

These data provide evidence about impossibility to distinguish different strategies of reproduction of *A. alternata*. Reproduction is performed by conidia formed with different intensiveness which can easily range. Both aggressiveness and preservation of *A. alternata* were also realized by conidia, and it was demonstrated in our investigation. Therefore, *A. alternata* conidia are really universal. For this pathogen, it is very difficult to distinguish short-term and long-term kinds of viability as they are realized by the same universal structures – conidia. This distinguishes many fungi of Hyphomycetes with multicellular conidia from, e.g., pseudofungi (*Phytophthora infestans*) which form

special structures responsible for different kinds of reproduction (asexual and sexual), short-term and long-term viability (S m i r n o v, K u z n e t s o v, 2006, 2009; P e n k i n, 2012).

Agro-meteorological conditions influence the development of potato early blight in many ways. Firstly, they influence the realization of primary infection: manifestation of first lesion and aggressiveness of *A. alternata* population being formed in signaling plot. Secondly, they influence the realization of this aggressiveness at the stage of secondary infection of potato plants in the field. In this way inoculum potential is formed and functioning.

Our investigation proved the significant effectiveness of plant growth regulator Circon and micro-fertilizer Silipant both *in vitro* (on detached tomato leaves) and *in vivo* (on potato fields). Additional test *in vitro* (P e n k i n, 2012) on potato-carrot agar amended with Circon and Siliplant did not reveal essential suppression of *A. alternata*. Only particular delay of mycelial growth and limitation of formation of conidia were registered. All these facts indicate that Circon and Siliplant increase immune response of potato plants and even of tissues, but fungicide effect may also be possible. Here it is interesting to mention that symptoms of early blight suppression look like hypersensitive response (HSR) (Fig. 5).

Under field conditions, first of all, it is necessary to minimize the potential of reproduction and aggressiveness of field *A. alternata* populations. This is main purpose and strategy which must be realized in large-scaled potato fields. But determination of aggressiveness potential and then of inoculum potential in isolated signalling plot near main potato cropping enables us to obtain true information about pathogen aggressiveness which in a few days can occur in potato main cropping.

Usual predicting systems and models primarily based on regression analysis of agro-meteorological conditions, often in real time and approbated many regions of the world like Dacom plant plus (H a d d e r s, 2005). Just a few models work with inoculum data, but often specially created artificial inoculum in signaling plots is used. All these ways can be effective and provide true time for potato treatments but in many cases they do not work well. They ignore natural inoculum potential which cannot be replaced by artificially created inoculums (without it the signaling plot was considered to be ineffective) which can essentially differ from natural inoculum. Also regression coefficients and dependences between weather and degree of danger of disease development can provide false recommendations because weather conditions vary from season to season. It is very difficult to note all specifics of it, especially having no information about real natural inoculum potential.

Our elaborating approach tries to decide on the aforementioned problems. Ranging current agro-meteorological conditions being adapted to early blight ecological gradations allows us to avoid bad reproducibility of the previous regression models. Also true information about inoculum potential is very useful for understanding real field data on early blight occuring a few days later. In general, this provides three advantages. Firstly, forecasting can be applied directly to local potato fields which needed to be protected, and all

initial information (first symptoms, aggressiveness of formed field *A. alternata* population in signaling plot, agro-meteorological conditions) can be in autonomous regimen obtained directly in the field. Secondly, biologization of forecasting system on the base of natural inoculum potential determination was provided, and this became the major point which basically distinguished our approach from other predicting systems based on agro-meteorological conditions. According to the previous points of view, disease development is mainly determined by agro-meteorological conditions. However, it can be true only to the end of disease development. The initial inoculum also plays an important role; at the beginning of disease development, contribution of inoculum potential is much stronger than that of agro-meteorological conditions. Thirdly, such approach enables minimization of fungicide application and prevention of unnecessary treatments. This can decrease the cost of potato production, improve its quality (concentration of fungicide residuals in production) and minimize the pollution of agrocenoses and the environment. Strong artificial inoculum should have much stronger effects than natural inoculum. This leads to starting fungicide application which can, however, be avoided without any problems for potato cropping. Of course, our approach also should be specified and verified. But it contributes to elucidation of problems of modern potato cropping.

## CONCLUSIONS

Our investigation has demonstrated that *A. alternata* is a very serious self-sufficient potato pathogen which is able to infect potato both by itself and in mixture with *A. solani*. Anyway, *A. alternata* should be moved from the shadow of *A. solani* as a true full-value agent of potato early blight and late blight.

*A. alternata* conidia proved to be universal structures which provide both short-term and long-term kinds of pathogen viability during temperature interval from -15º C to 35º C.

Plant growth regulator Circon and micro-fertilizer Siliplant from Russian company NEST-M manifested essential immune effect on potato against early blight. This effect was detected both at their autonomous application and at their joint application with fungicides at dosages of 100 and 50%.

Our investigations provide the basis for biologization for forecasting the potato early blight. Well-timed determination of primary symptoms and natural potential of pathogen inoculum in signaling plots (without treatments) allowed us to understand a degree of damage of potato inoculation with early blight and hence to provide well-timed application of fungicides with optimum dosages in mixture with Circon or Silipant. This forecasting approach can be helpful in adequate management of early blight and decreasing the risks for human health and environmental damage.

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## РАЗВОЈ ПРОГНОСТИЧКИХ ЕЛЕМЕНАТА ЗА МИНИМИЗАЦИЈУ ТРЕТМАНА ФУНГИЦИДИМА У ЗАШТИТИ КРОМПИРА ПРОТИВ РАНЕ ПЛАМЕЊАЧЕ У МОСКОВСКОМ РЕГИОНУ

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Резиме

Рана пламењача кромпира (узрочник је несавршена гљивица *Alternaria alternata* (Фр.) Keissl.) је озбиљна болест којој погодују врели климатски услови. Веома је значајна у регионима источне и јужне Европе, Азије и Африке. Контролише се интензивном применом фунгицида. Међутим, познато је да фунгициди имају штетан утицај на људе и околину. У циљу смањења количине фунгицида приликом третирања кромпира, развијен је систем који садржи елементе предвиђања појаве ране пламењаче. Примарни симптоми у виду хлороза и некроза и потенцијал природног инокулума патогена (одређивање јачине болести, индекса формирања колонија и агресивност као и временски услови) су одређивани на сигналним парцелама (нетретираним, без вештачке инокулације) у близини главних производних парцела кромпира. Кртоле су прелиминарно третиране регулатором

раста Circon и микро-фертилизером Siliplant руског произвођача NEST-M. За ове препарате је доказано да убрзавају раст кромпира и одлажу појаву ране пламењаче за 5-10 дана. Иста сорта кромпира треба да се посеје и на пољима и на сигналним парцелама. Благовремено уочавање примарних симптома и патогеног потенцијала инокулума омогућује сагледавање могућег степена оштећења на кромпиру узрокованих путем природне инокулације и благовремену употребу оптималних доза фунгицида у смеши са регулатором раста и минералним ђубривом са микроелементима. Овакав приступ може значајно да допринесе адекватном третирању ране пламењаче у усевима кромпира као и смањењу негативног утицаја третмана на животну средину.

КЉУЧНЕ РЕЧИ: *Alternaria alternata*, рана пламењача, кромпир, Circon, Siliplant, прогноза болести кромпира

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# FUNGAL DISEASES OF TREE STANDS UNDER URBANIZED CONDITIONS OF MOSCOW

ABSTRACT. Phytosanitary and ecological estimation of tree-stands has been conducted at the Forest Experimental Station of Moscow Agricultural Academy and parks of Northeast of Moscow in 2007-2011. *Fomes fomentarius* was proved to be a very serious pathogen of trees under conditions of Moscow, *Piptoporus betulinus*, *Phellinus igniarius,* and *Fomitopsis pinicola* also occurred and caused damage to trees. This rather bad phytosanitary situation depends on alarming ecological situation in Moscow. At the Forest Experimental Station of Moscow Agricultural Academy a number and cover of lichens decreased. In general, all trees in Moscow are in dynamic equilibrium with the urbanized environment. In connection with this, the following classification of tree-stands was proposed for the urbanized environment: 1 – healthy trees, 2 – affected trees which can be managed, 3 – dry woods, 3a – very diseased. Many tree-stands in investigated regions of Moscow are found to belong to the groups 2 and 3c. All tree-stands must be carefully monitored and managed in order to provide a well-timed decision on the support system for preservation of trees as 'lungs of city' and avoid unpredictable tree falling which put people and traffic at risk.

KEY WORDS: *Fomes fomentarius, Piptoporus betulinus*, *Phellinus igniarius, Fomitopsis pinicola*, trees, tree stands, fungal diseases of trees, Moscow

## INTRODUCTION

Tree stands perform important soil-protective, recreational, ecological, and sanitary-hygienic functions. Role of trees and their functions is especially significant in the area of big cities. Under urbanized city conditions it is necessary to control infectious and non-infectious diseases of tree stands as well as interrelations between them. This is due to the influence of the urbanized external environment which decreases immunity of tree stands and facilitates pathogen infection of host plant tissues.

However, the significance of this situation is not fully clear. Serious phytosanitary problems of tree stands in the urbanized environment of Moscow are a part of ecological situation. There is no enough attention paid to this problem because the main attention is directed to the forestry manufactures producing timber and lumber.

The objective of our investigation was to identify main fungal diseases of tree stands in some regions of Moscow and to provide an appropriate scale which reflects the condition of tree stands in the urbanized environment.

## MATERIAL AND METHODS

All observations were made in the period from 2006-2012 in the area of the Forestry Experimental Station (Moscow Timiryazev Agricultural Academy) (248.7 hectares) and some parks in the North and North-East of Moscow.

Diversity of tree stands on experimental plots (100m$^2$) was determined by means of index of Shannon (H): $H = \Sigma n_i \log_2(n_i/N)$, where: $n_i$ – number of trees of certain species, $N$ – general number of trees.

In order to estimate the ecological situation the occurrence of arboreal lichens and algae was observed. In order to estimate the phytosanitary situation the occurrence of arboreal mosses and polyporous fungi was observed (Table 1). Also, the categories of phytosanitary conditions were determined for all tree stands on the plot (Table 2). Recreational burden was determined for all forest and park plots as described in Table 3.

Cluster analysis of the obtained data was done by means of software STATISTICA 7.0 on the base of Euclidian distances and Ward's method.

Tab. 1 – Occurrence of arboreal lichens, mosses algae

|   | Range | Cover of lichens, mosses and algae per trunk, % | Cover of fruit bodies of polyporous fungi, % |
|---|---|---|---|
| 1 | Very rare | 1-2 | 1-2 |
| 2 | Rare | 3-5 | 3-5 |
| 3 | Moderate | 6-25 | 6-14 |
| 4 | Frequent | 26-50 | 15-30 |
| 5 | Very frquent | >50 | >30 |

Tab. 2 – Determination of categories of phytosanitary conditions of tree stands

|   | Range | Characteristics |
|---|---|---|
| 1 | Healthy | Normal tree stands without features of affection |
| 2 | Weakened | Local weakening of branches and affection with pests |
| 3 | Very weakened | Strong weakening of branches, small weakening of trunk, local features of affection with pests |
| 4 | Dried | Drying branches, strong weakening of trunk, large-scaled features of affection with pests |
| 5 | Dry woods of current year | Dried brunches and trunk, large-scaled features of affection with pests |
| 6 | Dry woods of last years | Dried destroying brunches and trunk, total development of pests |

Tab. 3 – Ranges in recreational burden

| | Range | Number of people per plot |
|---|---|---|
| 1 | Very rare | 1-5 |
| 2 | Rare | 6-15 |
| 3 | Moderate | 16-30 |
| 4 | Frequent | 31-60 |
| 5 | Very frequent | >60 |

# RESULTS

Analysis of index of Shannon distribution of tree species in the area of Forest Experimental Station indicated that majority of plots in the quarters 2, 3, 6, 7, 7, 8 and 10 manifested a limited biodiversion. Also, biodeversity in the area of Forest Experimental Station was heterogeneous (Fig. 1). This distribution was detected for the whole period of investigations.
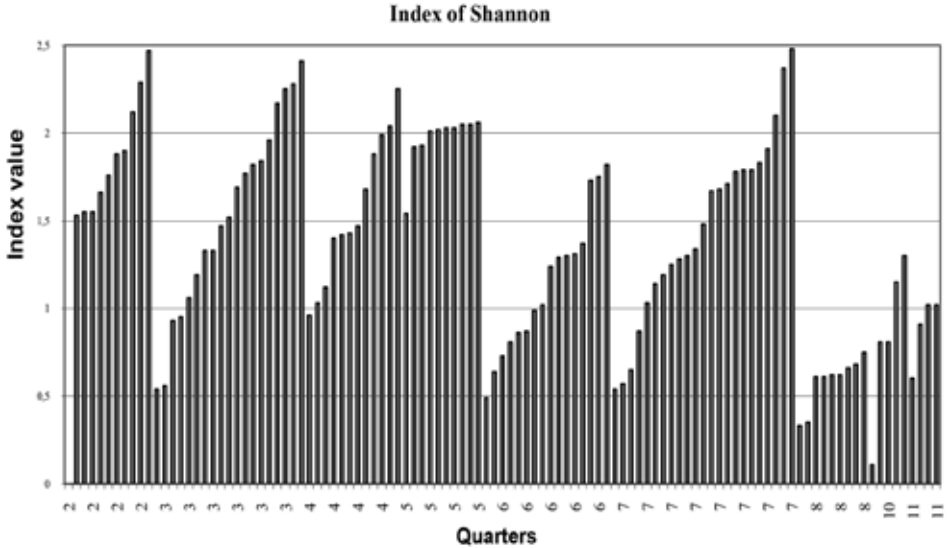


Fig. 1 – Diversity of tree species in the area of Forestry Experimental Station in 2006-2008.

In the period from 2006-2009, five species of lichens – *Parmelia aspera* Massal., *Hypogimnia physoides* L. Nyl., *H. tubulosa* Havaas, *Xanthoria parietina* (L.) Th., and *Cladonia coniocrea* (Elk.) Spreng. were detected in the area of the Forest Experimental Station and parks of Moscow with rare or very rare occurrence. At the beginning of 2010, the occurrence of lichens at the Forest Experimental Station was minimized. In the parks, occurrence of lichens was the same.

Tree stands of *Tilia* and *Acer* were covered with algae from the genus *Chlorococcus*, and those of Quercus – with *Trentepohlia*. Their occurrence in the area of Forestry Experimental Station was comparable with their development in natural forests.

During the whole period of investigation, *Fomes fomentarius* was a wide-spread arboreal fungus which destroyed many tree-stands of *Betula* both at the Forest Experimental Station and in the parks (Table 4). Occurrence of *Phellinus igniarius* on the *Quercus* increased. *Inonotus obliqus, Ganoderma aplanatum,* and *Pleurotus were* very rare. In parks, the groups of *Laetiporus sulfureus* were detected occasionally.

Tab. 4 – Occurrence of arboreal fungi in the area of Forestry experimental station

| Quarter number | Plot number | *Piptoporus betulinus* (Bull. ex Fr.) | *Fomes fomentarius* (L. ex Fr.) | *Fomitopsis pinicola* (Sow. ex Fr.) | *Phellinus igniarius* (L. ex Er.) | *Coriolus* spp.; *Stereum* spp. |
|---|---|---|---|---|---|---|
| | | *Betula* | *Betula* | *Betula, Pinus* | *Quercus* | *Pinus, Betula* |
| 4 | 3 | frequent | very frequent | – | – | – |
| 4 | 4 | very rare | – | – | – | very rare |
| 4 | 6 | – | – | – | – | very rare |
| 3 | 7 | – | – | – | – | very rare |
| 2 | 11 | – | rare | – | – | – |
| 2 | 13 | – | rare | – | – | – |
| 2 | 14 | – | – | very rare | – | – |
| 2 | 15 | – | very rare | – | – | – |
| 3/6 | 18 | – | very rare | rare | – | – |
| 3/4/6/7 | 19 | rare | rare | – | – | – |
| 6 | 22 | – | – | – | – | – |
| 5/6 | 23 | rare | rare | rare | – | rare |
| 5 | 24 | – | rare | frequent | – | – |
| 5 | 26 | – | very rare | – | – | very rare |
| 5/6/9/10 | 27 | – | – | – | – | – |
| 6/10 | 28 | very rare | very rare | – | – | very rare |
| 7/8 | 30 | – | – | – | – | – |
| 7/8 | 31 | – | – | – | very rare | very rare |

Predominant tree species were characterized by weakened and very weakened phytosanitary conditions from 2006-2008 (Fig. 2). Only a few *Quercus* tree stands were healthy. In the period from 2009-2012 the situation became worse. Occurrence of very weakened and dry tree stands of *Betula*, *Pinus*, and *Quercus* increased.

Cluster analysis revealed that about 50% of plots were characterized by weakened condition being attacked by arboreal fungi and mosses (Fig. 3). Certain relation between phytosanitary condition and land relief was revealed.
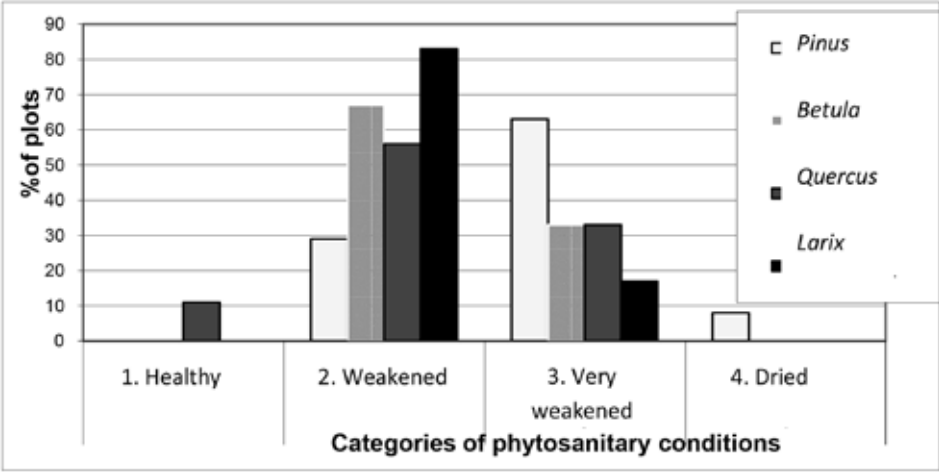
Fig. 2 – Distribution of categories of phytosanitary condition of tree species in the area of Forestry Experimental Station (2006-2008) (as per cent of number of plots with predomination of certain tree genera).



**1.Plain**. PSC: 3, Mosses: 3-5, Fungi: 0, RP:2

**2. Hill top**. PSC: 2, Mosses: 3, Fungi: 0, RP:2

**3. Valley slope**. PSC: 2, Mosses: 2, Fungi:2, RP:2

**3a. Plain**. PSC: 2, Mosses:2, Fungi: 0, RP:2

**4. Valley slope**. PSC: 2, Mosses: 3, Fungi: 5, RP:3

**5. Valley slope**. PSC: 3, Mosses: 3, Fungi: 3, RP:3

Tree Diagram for 31 Variables
Ward's method
Euclidean distances
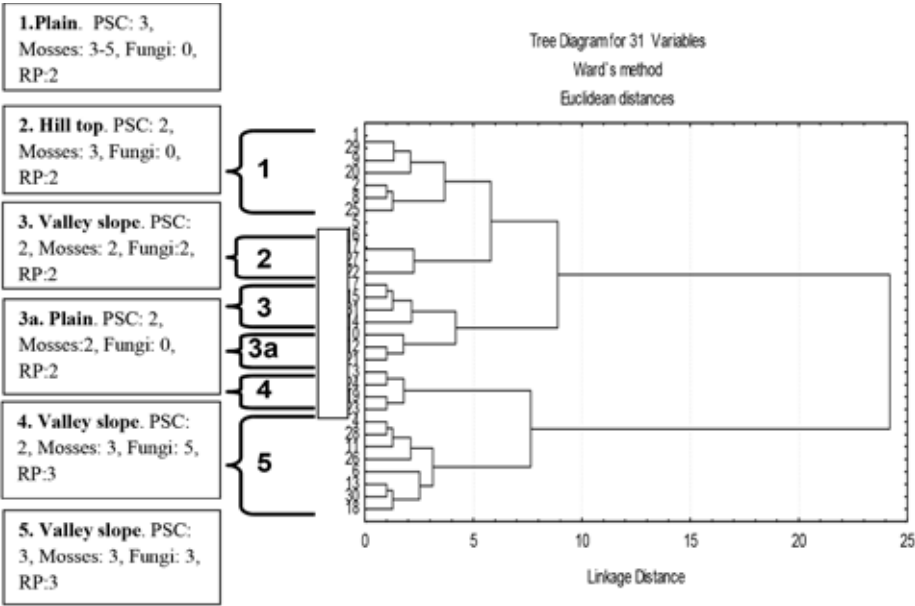
Note. PSC – phytosanitary condition, RP – recreational burden. Ranges are given in the Table 1-4.

Fig. 3 – Tree diagram for the similarity of plots of Forestry Experimental Station (2006-2008) based on the categories of phytosanitary conditions, tree crown density, affection with arboreal fungi and mosses.

The most weakened tree stands were found on valley slopes and plains. No clear connection between the phytosanitary condition and manifested recreational burden was revealed.

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## DISCUSSION

The present analysis revealed a very complicated situation with tree stands under urbanized conditions (B a r d a c h e v a, 2003). The cenosis of the Forest Experimental Station in Moscow was unbalanced (S m i r n o v a, 2010). Based on our data tree stands in Moscow are in dynamic equilibrium with the urbanized environment. The decrease in lichens and preservation of algae are alarming (T r a s s, 1973) but still not critical.

In the period from 2006 to 2012, synchronized deterioration of both ecological and phytosanitary situation of tree stands under urbanized conditions of Moscow was registered. These trends were highly interrelated. However, the land relief also strongly influenced the phytosanitary situation. These problems interrupted the functioning of 'green lungs' under urbanized conditions (B a r d a c h e v a, 2003). Hence, these problems and their solutions should be related to plant protection, instead of ecology (as it is now). *Fomes fomentarius* was proved to be a very serious leading pathogen of various tree species tolerant to the urbanized environment.

The scale for phytosanitary condition categories is appropriate for natural forests, but it seems to be unsuitable for the urbanized environment. Firstly, determination of different kinds of dried woods is not critical for cities. Secondly, the standard scale does not distinguish properly between very weakened tree stands which condition and viability can be improved, and very weakened tree stands which cannot be improved in this way.

According to the obtained data and their analysis, and with respect to the aforementioned problems, the following solutions relate to the categories of phytosanitary conditions of tree stands under urbanized conditions:

**Healthy:** requires careful monitoring and should be cut only in the case of exceeding growth and development.

**Weakened**: predominating in the cities; requires careful monitoring and personal management. They must not be cut as it is impossible to replace them.

**Dry woods**: at risk of easy falling and must be cut as soon as possible.

3a. **Weakened and affected**: unmanageable because of progressive drying out, in danger of easy falling especially in case of force majeure (strong wind). It should be cut.

This classification promotes phytosanitary cutting. With well-timed cutting the problems often related to legislative debates on abuse with fallen tree-stands could be avoided. These cases are not rare.

## CONCLUSIONS

The synchronized deterioration of both ecological and phytosanitary situation of tree stands under urban conditions of Moscow was analyzed. Genera *Betula*, *Quercus*, and *Pinus* often correspond to a weakened phytosanitary condition.

Fomes fomentarius was proved to be a very serious leading pathogen of various tree species tolerant to urban environment.

The scale for categories of phytosanitary conditions is not entirely appropriate for urban environment. The main criterion for distinguishing the categories is necessity for well-timed phytosanitary cutting.

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## ГЉИВИЧНЕ БОЛЕСТИ ДРВОРЕДА У УРБАНИМ УСЛОВИМА МОСКВЕ

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### Резиме

У периоду 2007–2011, спроведене су фитосанитарне и еколошке процене стања дрвореда у Шумарској експерименталној станици Московске пољопривредне академије и парковима у североисточној Москви. Потврђено је да се *Fomes fomentarius* јавља као веома озбиљан патоген дрвореда у урбаним условима Москве а поред тога је уочено присуство *Piptoporus betulinus*, *Phellinus igniarius* и *Fomitopsis pinicola* који су такође оштетили дрвореде. Ова веома лоша фитосанитарна слика је последица алармантне еколошке ситуације у Москви. У Шумарској експерименталној станици Московске пољопривредне академије утврђено је смањење бројности лишајева. Уопштено посматрано, стабла у Москви су у динамичкој равнотежи са градском околином. У складу са овим, предложена је следећа класификација дрвореда у градским условима: 1 – здрава стабла, 2 – угрожена стабла која се могу третирати, 3 – осушена стабла, 3а – веома оболела стабла. Већина стабала у испитиваним московским регионима припадала су групама 2 и 3а. Неопходно је организовати озбиљан мониторинг и на одговарајући начин благовремено третирати стабла како би се подржао систем очувања градских дрвореда као „плућа града" а истовремено спречиле непредвиђене ситуације попут ломљења стабала које озбиљно угрожавају безбедност људи и саобраћаја.

КЉУЧНЕ РЕЧИ: *Fomes fomentarius, Piptoporus betulinus, Phellinus igniarius, Fomitopsis pinicola*, стабла, дрвореди, гљивичне болести дрва, Москва

*Z o r n i t s a  B.  S t o y a n o v a*[1]*,  R o s s i t z a  M.  R o d e v a*[1*],
*I l i j a  K a r o v*[2]*,  B i l j a n a  K o v a c e v i k*[2]*,*
*V a s i l i s s a  I.  M a n o v a*[1]*,  R a l i t s a  G.  G e o r g i e v a*[1]

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# MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *COLLETOTRICHUM COCCODES* ISOLATED FROM PEPPER CULTIVATED IN BULGARIA AND MACEDONIA

ABSTRACT: *Colletotrichum coccodes* has been recognized as one of the causal agents of pepper fruit anthracnose in Bulgaria. Recently, this species has been recorded in pepper fruits in Macedonia. In Bulgaria, the fungus has also been isolated from roots of premature senescent pepper plants but in Macedonia it has not been isolated yet. The purpose of the investigation was to make comparative morphological, cultural and molecular characterization of *C. coccodes* isolates obtained from pepper fruits and roots in Bulgaria and Macedonia. Additionally, a technique was applied to differentiate among the *C. coccodes* isolates obtained from roots and other microsclerotia-producing fungi. On the host tissue, *C. coccodes* developed acervular conidiomata with cup-shaped fruiting bodies accompanied with dark-pigmented, unbranched, thick-walled sterile hyphae called setae. A slimy mass of hyaline, straight, unicellular, fusiform conidia appeared on nutrient media. In a short time, numerous small dark globose setose microsclerotia emerged in the colony starting from its centre and distributing proportionally throughout agar plates. Two PCR primer sets were used to sequences of the ribosomal internal transcribed spacer (ITS1 and ITS2) regions. Single products of ~450 bp and ~350 bp were amplified by the genus-specific (Cc1F1/Cc2R1) and the species-specific primers (Cc1NF1/Cc2NR1), respectively. Morphological, cultural and molecular characterization of the isolates from roots and fruits showed that root rot and fruit anthracnose of pepper were caused by one and the same causal agent determined as *C. coccodes*. The isolates from roots showed rapid mycelial growth, gave rise to numerous minute microsclerotia and produced conidia only on SSPA. SSPA supported significantly more mycelium growth and sporulation than all other media tested and could be recommended for production of large quantity of conidia. No pattern of genetic variation associated with the organ or geographic origin of the isolates was determined.
KEY WORDS: acervuli, fungal isolates, ITS, microsclerotia, pepper, plant pathogens

## INTRODUCTION

*Colletotrichum coccodes* (Wallr.) S.J. Hughes has been recognized as one of the causal agents of pepper fruit anthracnose in Bulgaria (R o d e v a

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et al., 2009a; 2009b). Recently, this species has been recorded in pepper fruits in Macedonia (R o d e v a et al., 2012). In Bulgaria, the fungus has also been isolated from roots of premature senescent pepper plants but in Macedonia it has not been isolated yet. The purpose of the work was to make comparative morphological, cultural and molecular characterization of *C. coccodes* isolates obtained from pepper fruits and roots in Bulgaria and Macedonia. The isolates from roots were of great interest for this investigation because, in Bulgaria, the causal agent was and still is recognized as *C. atramentarium* (Berk. & Broome) Taubenh. (B o b e v, 2000; 2009). The pathogen was often detected together with other root-infecting fungal species such as *Macrophomina phaseolina* (Tassi) Goidanich. For that reason, a technique was additionally applied to differentiate among the *C. coccodes* isolates obtained from roots and other microsclerotia-producing fungi.

## MATERIAL AND METHODS

### *Pathogen isolation*

Fruits with anthracnose symptoms as well as roots of premature dying plants were sampled from the experimental field, which is in the property of the Institute of Plant Physiology and Genetics (IPPG), Sofia and from commercial fields in the main pepper growing regions in Bulgaria. Infected fruits were also collected from farm fields and plastic greenhouses in pepper growing districts of East Macedonia, including Strumica, Kochani, Kavadarzi and Sveti Nikole. Isolations were made from diseased pepper fruits with typical anthracnose symptoms and from the roots of premature senescent pepper plants bearing microsclerotia. All experimental work was carried out at IPPG.

### *Morphological and cultural characterization*

Potato dextrose agar (PDA) was used for the initial isolations and storage of *C. coccodes*. Twelve isolates were included in the experiment and they were divided into three groups. Group A included isolates obtained from pepper roots in Bulgaria (B8-2, B12-9, B12-13 and K18-1, the latter one from early root infection); Group B included isolates from fruits in Bulgaria (B12-33, B12-45, B12-46 and B12-47) and Group C from diseased fruits taken from Macedonia (MK7-1, MK7-2, MK26-1 and MK26-2). Three nutrient media: PDA, sucrose soy protein agar (SSPA) (Y u et al., 1997) and water agar (WA) were selected in order for the study on morphological and cultural characteristics of the fungal isolates to be carried out. To determine colony growth and morphology, 4 mm plugs were taken from the periphery of actively growing colonies and transferred to the plate centre. Inoculated plates were incubated at 24 ºC. The diameter of each colony was measured 7 and 14 days after inoculation. There were five replicate plates. Isolates were morphologically charac-

terized on the basis of colony growth, color, mycelium density and the characteristics of microsclerotia, acervuli and conidia. Images were acquired using an Olympus BX41 Microscope. Cell$^{\wedge F}$ for Imaging Life Science Microscopy was the acquisition software. At least 30 microsclerotia and 100 conidia of each isolate were measured for cultures grown on the three nutrient media.

A technique for differentiation of some sclerotial fungi was additionally applied to *C. coccodes* isolates from roots (O s t a z e s k i, 1964). The experimental plot consisted of a Petri dish with WA growth medium onto which four 12-mm-discs of alfalfa leaves were placed. A 6-mm-plug of PDA growth medium colonized with fungal mycelium was inverted on the edge of each leaf disc so that half of the agar block could rest on leaf tissue and half on agar. *M. phaseolina* isolates were involved in the experiment in order to make comparison (R o d e v a et al., 2010). Four replicate plates of each isolate were incubated at 24 ºC.

### *Molecular characterization*

For molecular characterization, the *C. coccodes* isolates were grown in potato dextrose broth and gyrated at 125 rpm for 7-10 days at 24 ± 2 ºC. The mycelia were harvested by filtration, washed in sterile water and lightly squeezed in filter paper. Partially dried mycelium was stored at -80 ºC. Those samples were later used for DNA extraction. DNA was isolated from fungal mycelium by DNeasy Plant mini kit (Qiagen) according to the manufacturer's instructions. Controls included no DNA negative control (mQ water), but they included DNA from one *C. gloeosporioides* and two *C. acutatum* isolates. Briefly, fungal tissue was ground to fine powder in liquid nitrogen and then subjected to lysis and RNase treatment at 60° C for 15 min. Salt-precipitated proteins and polysaccharides were first pelleted by centrifugation for 5 minutes, the resulting supernatant was centrifuged through QIAshredder spin column and the cleared lysate was applied to DNeasy Mini spin column membrane. After 2-step washing, the total genomic DNA was eluted in AE buffer (10mM Tris-Cl, 0.5 mM EDTA, pH 9.0).

*C. coccodes* isolates were characterized by PCR amplification with both genus and species-specific primers, based on the ITS (internal-transcribed spacer) of ribosomal genes, as described by C u l l e n et al. (2002), with some modifications. PCR amplification was performed as follows: initial denaturation step at 95°C (3 min), 20 cycles at 95°C (45 s), annealing and elongation steps performed at 72°C for 2 min and 15 s, final elongation at 72°C for 5 min. The reactions were performed in 20 µl volume, containing 25 ng total genomic DNA, 1x reaction buffer A (Eurx), 200 µM dNTPs, 0.4 µM primers, 2.5mM $MgCl_2$ and 1.25U Color Taq (Eurx). A total of 2.5µl of each PCR reaction were loaded on 1.6% agarose gels (300ng/ml EtBr) and subjected to electrophoresis in 1xTAE buffer at 60V, for at least 2.5 h. The gels were visualized by UV transillumination, and their electronic images were captured by ImageQuant150 imager (GE Healthcare) and densitometrically analyzed with ImageQuantTL7

software (GE Healthcare) to determine the approximate length of the resulting PCR products.

### Statistical analysis

All experimental results were given as mean (M) and standard error of the mean (SEM). The data for colony growth were statistically processed by analysis of variance and calculation of the least significant difference (LSD).

## RESULTS

The diseased fruits of pepper showed typical anthracnose symptoms including soft, sunken, round or slightly elongated lesions bearing at first fungal fruiting bodies (acervuli), which extruded gelatinous conidial mass. The size of conidia on natural substrate was (14.9) 18.4±0.2 (22.4) x (3.2) 4.4±0.1 (5.2) µm. In a short time, small dark globose setose structures called microsclerotia emerged on and inside of the diseased pepper fruits (Fig. 1a). The below-ground symptoms caused by *C. coccodes* root attack appeared as large brown to grey lesions on roots covered with microsclerotia and sloughing of the root cortex (Fig. 1b). The above-ground symptoms of affected plants were expressed as chlorotic foliage, wilting and premature plant death. Under the climatic conditions in Bulgaria, the first isolations of *C. coccodes* from roots and fruits were made at the beginning of August. *C. coccodes* was detected on pepper roots either solely or as a disease complex with other root-infecting fungi such as *Verticillium dahliae* Kleb., *Fusarium oxysporum* Schlechtend.: Fr., *F. solani* (Mart.) Sacc., *Rhizoctonia solani* J.G. Kühn and *Macrophomina phaseolina*.
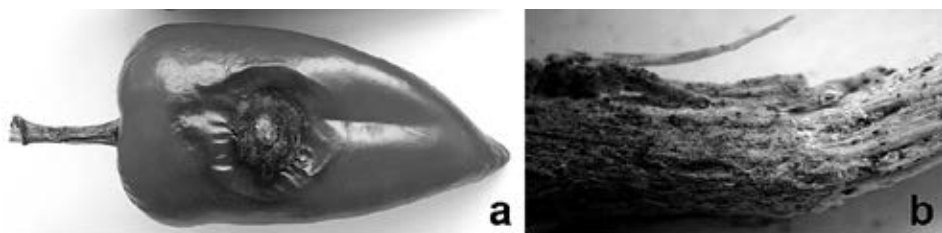


Fig. 1. – Symptoms caused by *C. coccodes*: a. on pepper fruit; b. on pepper root

Overlaying of alfalfa leaves on WA stimulated the development of abundant quantities of *C. coccodes* acervuli, which produced slimy mass of hyaline, straight, unicellular, fusiform conidia together with microsclerotia (Fig. 2a-h). The same technique applied to *M. phaseolina* isolates resulted in producing microsclerotia and great number of pycnidia extruding conidia (Fig. 3a-d).
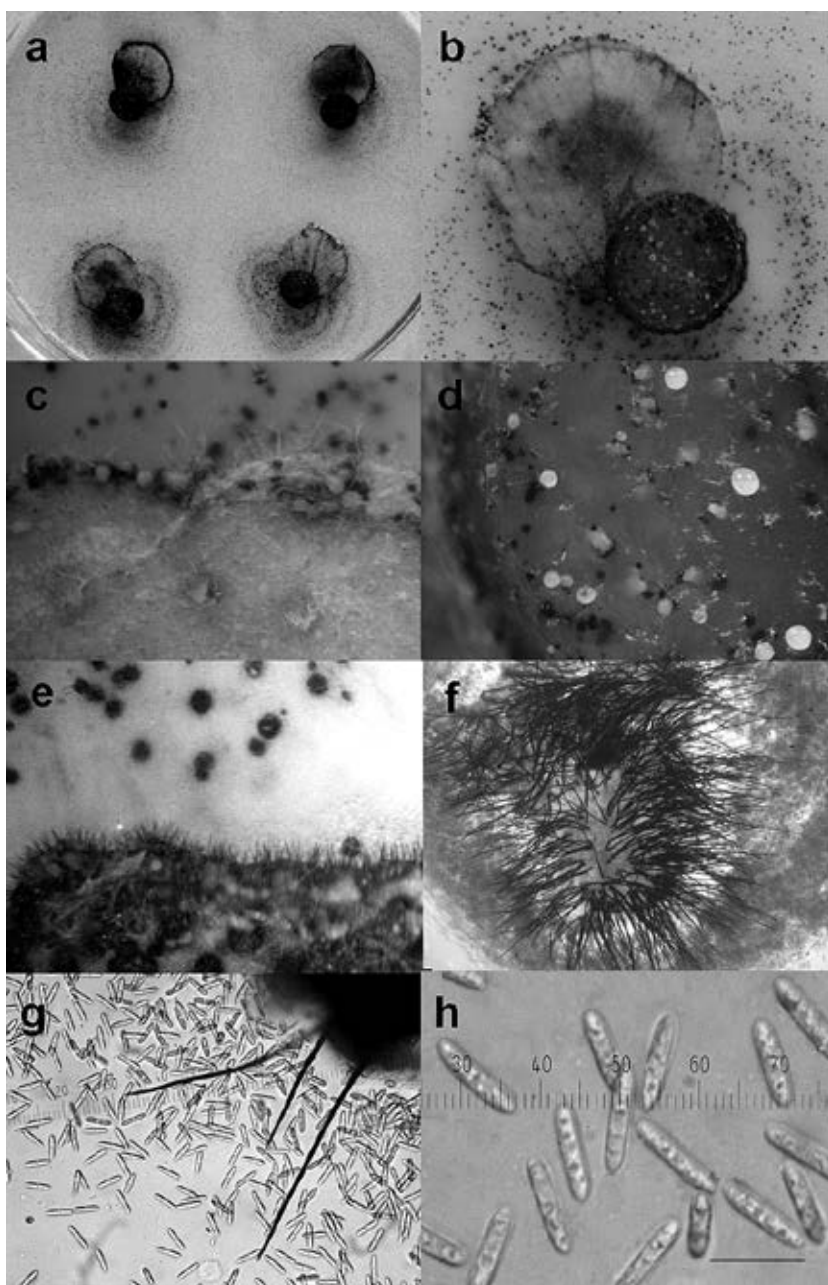
Fig. 2. – Sporulation of *C. coccodes* on alfalfa leaf discs: a. Experimental design on water agar plate; b. Acervuli and microsclerotia on and around the leaf disc and mycelial plug; c. Periphery of leaf disc with acervuli and slimy conidial mass; d. Reverse side of agar plug with droplets of conidial jelly; e. Increased number of acervuli and dark setae on the leaf disc; f. Acevulus accompanied with great number dark-pigmented setae; g. Acervulus, setae and conidia, x 160; h. Conidia, x 400, scale bar = 20 μm
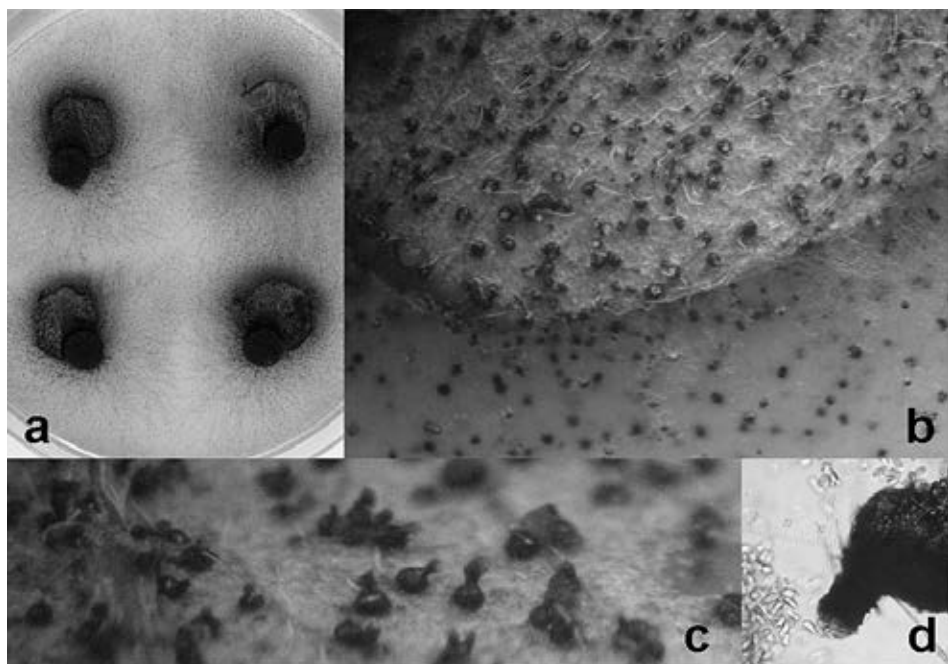
Fig. 3. – Sporulation of *M. phaseolina* on alfalfa leaf discs: a. Experimental design on water agar plate; b. Pycnidia and microsclerotia on and around the leaf disc; c. Pycnidia with short necks and ostioles; d. Pycnidium extruding conidia

All isolates grew quickly on PDA (Tab. 1, Fig. 4). Colonies of group A were dark gray with pale rose periphery; the reverse was dark gray. The mycelium was scarce. Numerous small microsclerotia were ordered in concentric rings darker in the colony centre. The isolate K18-1 showed well pronounced radial stripes. The growth of colonies belonging to group B was slower on PDA. The colonies were dark gray with pinkish to salmon periphery; the reverse was grayish with ochre to salmon periphery with well expressed concentric rings. Gray creeping mycelium that covered the colony surface with irregularly distributed light gray to white floccose patches (isolates B12-33 and B12-47). Microsclerotia showed to have bigger dimensions and were more aggregated. Colonies of group C had similar growth rate as those of group B. The isolates varied in morphology. Isolate MK7-1 gave rise to greenish gray, densely textured colonies with slightly expressed concentric and radial zones and pale rose irregular periphery; the reverse was light brown with dispersed aggregates of microsclerotia. Isolate MK7-2 showed well pronounced concentric rings and radial stripes like the isolate K18-1. A big central part was covered by gray velutina mycelium; the reverse was dark gray. Isolate MK26-1 grew quickly on PDA with well-defined concentric rings of microsclerotia and pale ochre to greenish periphery; the reverse was the same. Isolate MK26-2 was similar to the isolates of group B.

Fig. 4. – Colony morphology of the isolates of group A, B and C on potato-dextrose agar after 14 days of incubation: a. above side; b. reverse side

All colonies on SSPA grew very quickly, occupying the whole surface of Petri dish in 14 days (Tab. 1, Fig. 5). Each group expressed different morphology pattern. Colonies of group A had dark greenish-gray central part with microsclerotia in concentric rings, followed by a large pinkish to salmon actively sporulating zone; the reverse was the same. Colon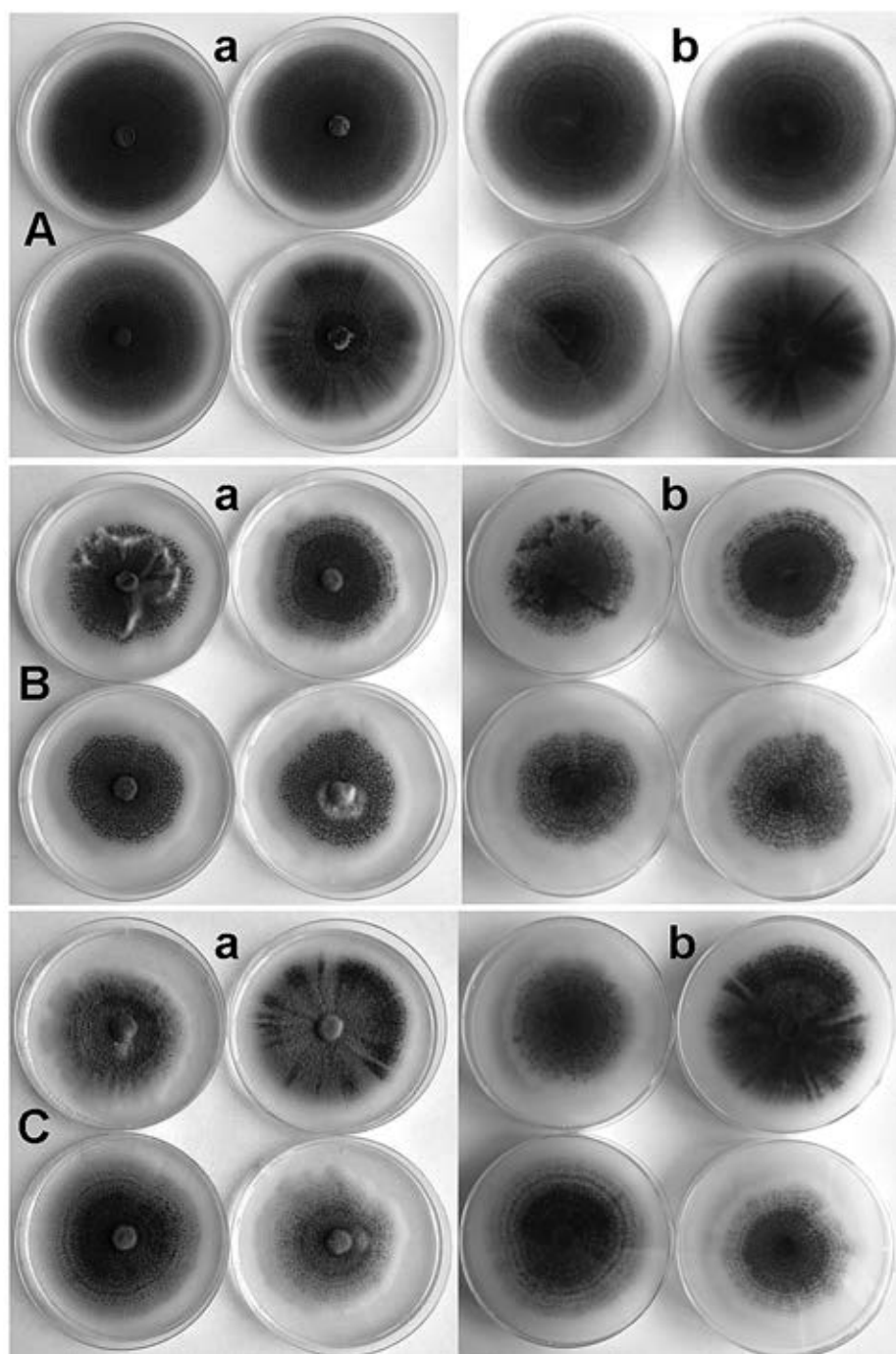ies of group B had dense texture and were spotted with sectors of light and dark gray mycelium. Big salmon drops of conidia were seen through the mycelium mainly around the colony centre. Colonia of group C developed less microsclerotia and less mycelium in irregular darker sectors. They produced big quantity of conidia, which appeared as large drops or flood of cream-colored jelly.

On WA, all colonies were loosely textured with similar characteristics (Tab.1, Fig. 6). They were transparent and consisted mainly of microsclerotia dispersed deeply in the media. No conidia were found. The isolates belonging to the group B and C had the same morphology (Figures are not shown).

Tab. 1 – The colony growth (mm) of *C. coccodes* isolates on three nutrient media (PDA, SSPA, WA) after 7 and 14 days of incubation

| Isolates (Factor A) | 7 days | | | Average per isolate | 14 days | | | Average per isolates |
|---|---|---|---|---|---|---|---|---|
| | Nutrient media (Factor B) | | | | Nutrient media (Factor B) | | | |
| | PDA | SSPA | WA | | PDA | SSPA | WA | |
| B8-2 | 50.0±0.7 | 54.0±0.4 | 31.6±1.9 | **45.2** | 80.0±0.0 | 85.0±0.0 | 80.0±0.0 | **81.7** |
| B12-9 | 49.6±0.6 | 54.6±0.8 | 33.4±1.2 | **45.9** | 80.0±0.0 | 85.0±0.0 | 66.4±2.5 | **77.1** |
| B12-13 | 48.0±0.6 | 52.8±0.4 | 29.2±0.5 | **43.3** | 80.0±0.0 | 85.0±0.0 | 50.0±0.0 | **71.7** |
| K18-1 | 49.8±0.2 | 51.0±0.4 | 31.0±1.1 | **43.9** | 80.0±0.0 | 85.0±0.0 | 73.2±2.0 | **79.4** |
| B12-33 | 36.2±2.0 | 58.4±2.8 | 28.2±1.0 | **40.9** | 60.8±2.1 | 85.0±0.0 | 75.2±1.1 | **73.7** |
| B12-45 | 38.6±1.4 | 52.4±0.3 | 32.4±0.4 | **41.1** | 59.4±2.3 | 85.0±0.0 | 48.4±1.3 | **64.3** |
| B12-46 | 37.4±1.8 | 60.8±1.0 | 28.8±0.5 | **42.3** | 59.8±1.0 | 85.0±0.0 | 55.0±1.1 | **66.6** |
| B12-47 | 36.4±2.4 | 53.2±1.2 | 34.6±0.4 | **41.4** | 58.8±2.0 | 85.0±0.0 | 51.2±1.4 | **65.0** |
| MK7-1 | 35.0±1.1 | 48.6±1.0 | 31.2±1.4 | **38.3** | 54.2±1.5 | 85.0±0.0 | 50.8±0.5 | **63.3** |
| MK7-2 | 47.4±0.8 | 54.4±1.2 | 29.4±0.7 | **43.7** | 77.2±1.0 | 85.0±0.0 | 53.6±1.1 | **71.9** |
| MK26-1 | 45.6±2.2 | 54.4±0.7 | 32.0±1.4 | **44.0** | 72.0±2.1 | 85.0±0.0 | 58.2±0.8 | **71.7** |
| MK26-2 | 35.2±0.5 | 50.2±0.7 | 30.2±0.9 | **38.5** | 56.2±1.2 | 85.0±0.0 | 62.0±2.1 | **67.7** |
| Average per media | 42.4 | 53.7 | 31.0 | | 68.2 | 85.0 | 60.3 | |
| LSD | A | B | A x B | | A | B | A x B | |
| 0.05 | 1.7 | 0.9 | 3.0 | | 1.6 | 0.8 | 2.8 | |
| 0.01 | 2.3 | 1.2 | 3.9 | | 2.1 | 1.0 | 3.7 | |
| 0.001 | 2.9 | 1.5 | 5.0 | | 2.7 | 1.3 | 4.7 | |

On PDA, all isolates from fruits (group B and C) produced hyaline, straight, unicellular, fusiform conidia with average size of 15.0 x 4.7 µm (Tab. 2). They appeared as a slimy mass mainly in the colony periphery, where the fungus was actively growing. In colonies of root isolates (group A), conidia were not found at the end of experiment. Pale rose periphery, where conidia
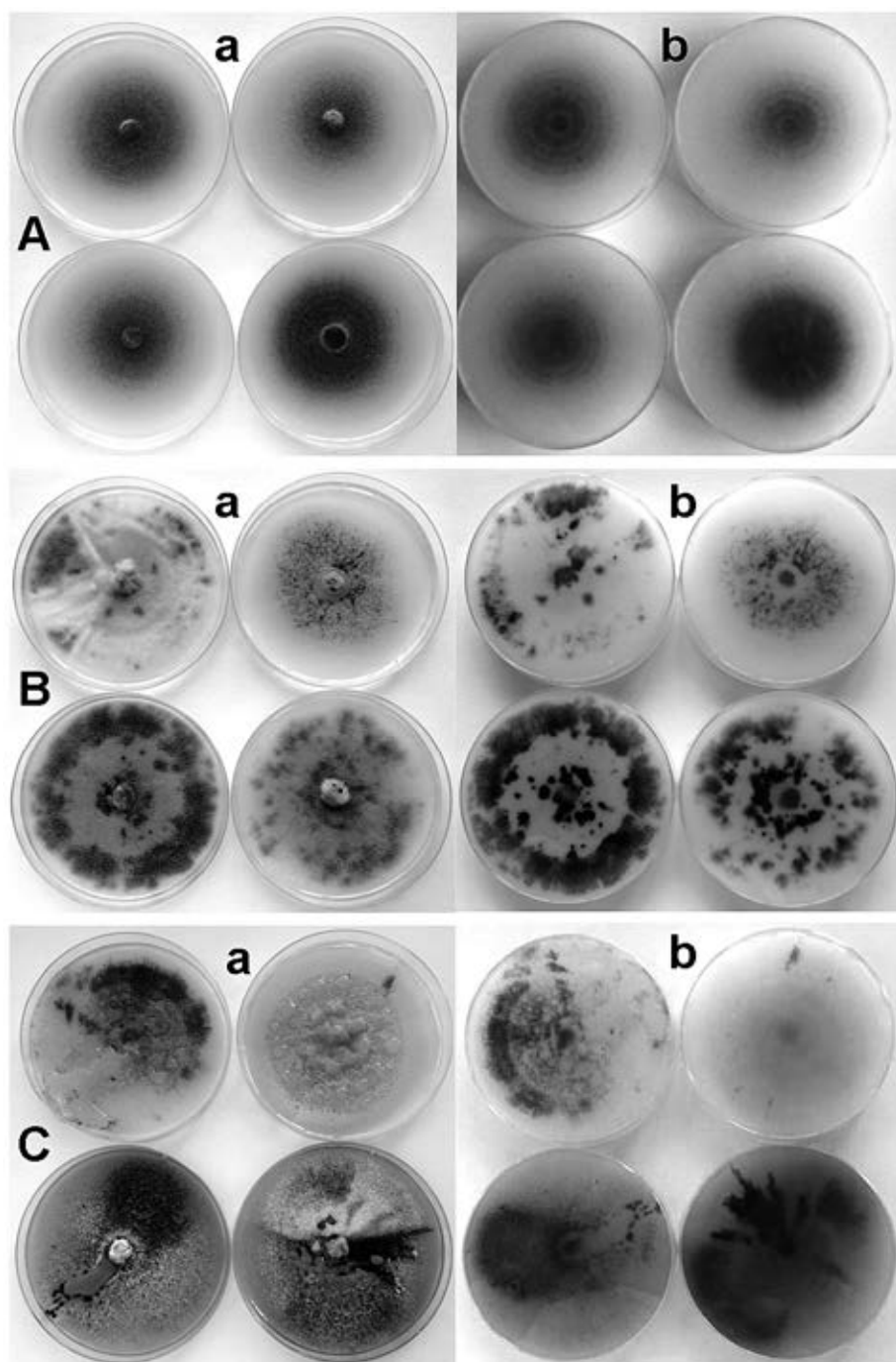
Fig. 5. Colony morphology of the isolates of group A, B and C on sucrose-soy protein agar after 14 days of incubation: a. above side; b. reverse side.
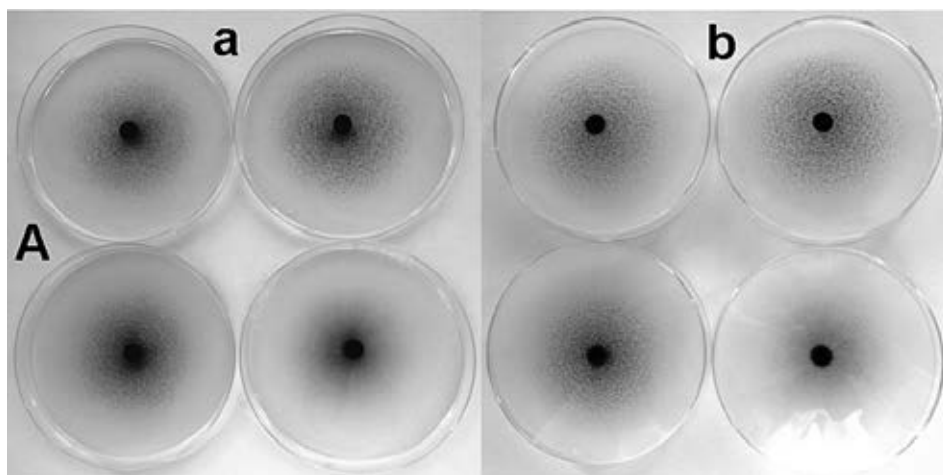
Fig. 6. – Colony morphology of the isolates of group A on water agar after 14 days of incubation: a. above side; b. reverse side.

could be found, was recorded in the first days of cultivation, but these colonies grew quickly occupying almost the whole surface of Petri dish after 15 days of cultivation. All isolates sporulated extremely well on SSPA. The conidial mass was accumulated in large drops or even flood of cream-colored jelly giving a strange yeast-like morphology to the colonies (group B and C). The average size of conidia was 13.3 x 4.7 μm. No conidia were found on WA.

Tab. 2 – The size (μm) of *C. coccodes* conidia on two nutrient media (PDA and SSPA)

| Isolates | Length | | Width | |
|---|---|---|---|---|
| | PDA | SSPA | PDA | SSPA |
| B8-2 | – | (9.0)**14.8±0.3**(19.8) | – | (4.0)**5.1±0.1**(6.4) |
| B12-9 | – | (10.7)**15.0±0.7**(16.6) | – | (4.5)**5.4±0.1**(5.9) |
| B12-13 | – | (11.8)**14.7±0.6**(22.2) | – | (4.6)**5.6±0.1**(7.4) |
| K18-1 | – | (9.1)**13.9±0.4**(25.9) | – | (3.6)**4.9±0.1**(6.1) |
| B12-33 | (11.0)**14.9±0.6**(19.2) | (8.5)**11.3±0.4**(20.7) | (4.2)**4.8±0.1**(6.1) | (3.6)**4.5±0.1**(5.4) |
| B12-45 | (11.9)**17.5±0.5**(23.0) | (10.6)**13.7±0.4**(16.3) | (3.2)**4.8±0.1**(6.6) | (3.5)**4.4±0.1**(5.4) |
| B12-46 | (10.3)**12.0±0.3**(14.3) | (8.3)**14.6±0.3**(22.0) | (4.0)**4.6±0.1**(5.3) | (3.7)**4.9±0.1**(6.1) |
| B12-47 | (11.2)**14.0±0.4**(18.6) | (7.6)**11.6±0.2**(18.6) | (3.8)**4.9±0.2**(6.2) | (3.2)**4.4±0.1**(6.3) |
| MK7-1 | (9.4)**15.8±1.0**(25.7) | (7.2)**12.7±0.4**(24.8) | (3.4)**4.2±0.1**(5.0) | (3.3)**4.5±0.1**(6.0) |
| MK7-2 | (8.0)**13.7±0.5**(21.2) | (8.8)**12.9±0.5**(19.2) | (3.4)**4.7±0.1**(5.9) | (2.9)**4.3±0.1**(5.4) |
| MK26-1 | (15.0)**16.8±1.1**(18.8) | (8.4)**12.0±0.3**(16.5) | (4.0)**4.9±0.4**(6.1) | (2.9)**4.2±0.1**(5.5) |
| MK26-2 | (10.8)**15.0±0.6**(19.2) | (9.5)**12.4±0.3**(20.0) | (3.6)**4.7±0.2**(5.8) | (3.2)**4.3±0.1**(6.0) |
| Average | 15.0 | 13.3 | 4.7 | 4.7 |

Tab. 3 – The size (µm) of *C. coccodes* microsclerotia on three nutrient media (PDA, SSPA, WA)

| Isolates | PDA | SSPA | WA | Average |
|---|---|---|---|---|
| B8-2 | (128) **175±4** (237) | (72) **107±2** (139) | (35) **58±2** (85) | 113 |
| B12-9 | (63) **100±2** (134) | (58) **95±3** (154) | (44) **67±3** (96) | **87** |
| B12-13 | (64) **96±2** (145) | (63) **92±3** (140) | (43) **72±3** (100) | **87** |
| K18-1 | (38) **58±2** (100) | (44) **84±5** (131) | (13) **21±2** (39) | **54** |
| B12-33 | (248) **307±8** (412) | (58) **105±6** (173) | (48) **79±4** (117) | 164 |
| B12-45 | (134) **223±10** (296) | (113) **148±6** 197 | (57) **98±5** (133) | **156** |
| B12-46 | (102) **137±4** (174) | (73) **108±4** (176) | (55) **84±3** (120) | **110** |
| B12-47 | (114) **150±5** (205) | (83) **114±6** (161) | (49) **92±7** (125) | **119** |
| MK7-1 | (153) **215±11** (309) | (46) **66±4** (113) | (45) **73±5** (129) | 118 |
| MK7-2 | (113) **159±7** (239) | (40) **65±4** (107) | (35) **50±3** (70) | **91** |
| MK26-1 | (218) **283±10** (393) | (67) **102±4** (174) | (38) **74±4** (123) | **153** |
| MK26-2 | (164) **192±8** (272) | (58) **73±1** (110) | (64) **94±4** (138) | **120** |
| Average | 175 | 97 | 72 | |

All isolates produced microsclerotia on all of the nutrient media (Tab. 3). Those were small dark globose setose bodies which emerged in the colony starting from its centre and distributing proportionally throughout agar plates. Culture media influenced the size of microsclerotia. On PDA, microsclerotia were more aggregated and bigger in size. On SSPA, they had intermediate size with the exception of isolate K18-1 and MK-1. The entire colonies on WA consisted of numerous, smallest in size, microsclerotia that immersed in the agar plate. The isolates of group A had the smallest microsclerotia, while the isolates from group B had the largest one.

PCR amplification with genus-specific primers (Cc1F1/Cc2R1) gave one single band of ~450 bp in all isolates analyzed (*C. coccodes, C. acutatum* and *C. gloeosporioides*) confirming that they belong to the genus *Colletotrichum* (Figure is not shown). The nested primer set Cc1NF1/Cc2NR1 amplified one single PCR band of ~350 bp only in the reactions containing DNA from *C. coccodes* isolates as a template and as it was expected from the literature (C u l l e n et al., 2002) (Fig. 7). When DNA isolated from *C. gloeosporioides* and *C. acutatum* was utilized as a template, no amplification band was visible on the gel because of the lack of homology between Cc1NF1 primer and the respective rDNA region in those species.

## DISCUSSION

As it is illustrated the present study, *C. coccodes* may, by itself, cause root rot and should be considered as a pathogen causing primary wilt disease. In mixed infections and in different combinations with vascular wilt agents
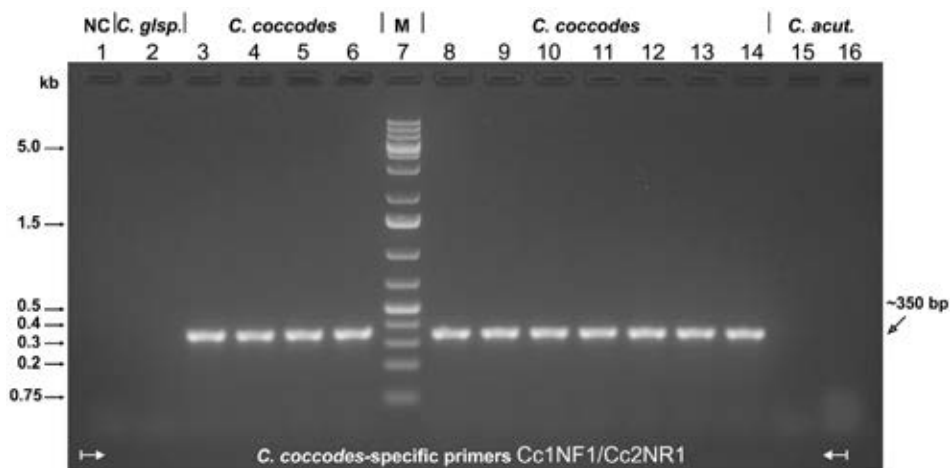
Fig. 7. – Identification of *C. coccodes* in genomic DNAs isolated from different *Colletotrichum* species. PCR amplification with *C. coccodes*-specific primers Cc1NF1/Cc2NR1: Lane 1: NC – Negative control mQ water; Lane 2: *C. gloeosporioides* isolate; Lanes 3-6: *C. coccodes* isolates from pepper roots; Lane 7: DNA marker GeneRuler 1kb+ (Fermentas); Lanes 8-14: *C. coccodes* isolates from pepper fruits; Lanes 15-16: *C. acutatum* isolates.

such as *V. dahliae* and *F. oxysporum* and other root-infecting fungi such as *F. solani*, *R. solani*, *M. phaseolina*, this pathogen could be responsible for the early dying syndrome of pepper. Morphological, cultural and molecular characterization of the isolates from roots and fruits showed that root rot and fruit anthracnose of pepper were caused by one and the same causal agent determined as *C. coccodes. C. atramentarium* could be considered only as an obsolete synonym. The isolates originated from roots showed rapid mycelial growth, gave rise to numerous minute microsclerotia and produced conidia only on SSPA.

Molecular identification of *C. coccodes* with species-specific primer was a successful method for the confirmation of species. Some morphological differences were observed between the isolates on PDA and predominantly on SSPA. ITS region was successfully amplified for the isolates of *C. coccodes,* but additional methods of identification were required in order for genetic diversity to be revealed.

SSPA supported mycelium growth and sporulation more significantly than all other media tested. Isolates from group B and especially from group C released extremely large quantity of conidia confirming the results of Y u et al. (1997). WA supported no conidia development and significantly less mycelium than all other media tested, but the isolates, especially those obtained from roots, produced more microsclerotia.

## CONCLUSION

Morphological, cultural and molecular characterization of the isolates from roots and fruits showed that root rot and fruit anthracnose of pepper were caused by one and the same causal agent determined as *C. coccodes*. The isolates from roots showed rapid mycelial growth, gave rise to numerous minute microsclerotia and produced conidia only on SSPA. SSPA supported mycelium growth and sporulation more significantly than all other media tested and could be recommended for the production of large quantity of conidia. No pattern of genetic variation associated with the organ or geographic origin of the isolates was determined.

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# МОРФОЛОШКА И МОЛЕКУЛАРНА КАРАКТЕРИЗАЦИЈА ИЗОЛАТА *COLLETOTRICHUM COCCODES* ИЗОЛОВАНИХ ИЗ ПАПРИКЕ УЗГАЈАНЕ У БУГАРСКОЈ И МАКЕДОНИЈИ

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## Резиме

*Colletotrichum coccodes* се јавља као један од узрочника антракнозе у плодовима паприке у Бугарској. Недавно је потврђено њено присуство и на плодовима паприке из Македоније. У Бугарској је ова гљивица изолована из корена прерано сазрелих биљака али у Македонији нису забележена слична запажања. Циљ овог рада је да се изврши компаративна морфолошка, културолошка и молекуларна карактеризација изолата *C. coccodes* добијених из плодова и корена паприке узгајане у Бугарској и Македонији. Даље, ова техника је примењена за утврђивање разлика између изолата *C. coccodes* добијених из корена и осталих гљивица које могу да формирају микросклероције. На ткиву домаћина, *C. coccodes* је развила ацервуларне конидиомате са плодоносним телом у облику шоље са тамно обојеним, неразгранатим, стерилним хифама са дебелим зидом које се зову сете. На хранљивој подлози, развиле су се праве, једноћелијске, вретенасте конидије састављене од слузасте хијалинске масе. За кратко време појавио се велик број ситних, тамних, глобуларних, чекињастих микросклероција почевши од центра колоније и пропорционално се шире́ћи преко агарне плоче. Два сета PCR прајмера је коришћен за секвенционирање рибозомалних интерних (ITS1 и ITS2) регија. Појединачни одзиви на ~450 bp и ~350 bp су појачани коришћењем род-специфичних (Cc1F1/Cc2R1) и врста-специфичних (Cc1NF1/Cc2NR1) прајмера, респективно. Морфолошка, културолошка и молекуларна карактеризација изолата из корена и плодова паприке је показала да је узрочник трулежа корена и антракнозе плодова један те исти патоген, *C. coccodes*. Изолати из корена су показали способност брзог мицеларног раста и стварања великог броја ситних микросклероција а конидије су производили само на SSPA. На SSPA подлози је добијен значајно већи мицеларни раст и интензитет спорулације у односу на остале испитиване подлоге и може се препоручити за производњу већих количина конидија. Није запажена никаква генетска варијација у изолатима у односу на биљни орган из којег су изоловани или географско порекло биљака.

КЉУЧНЕ РЕЧИ: ацервуле, изолати гљивице, ITS, микросклероција, паприка, биљни патогени

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# MICROMORPHOLOGICAL CHANGES ON THE EMBRYONIC MEMBRANES OF TURKEY EGGS INFECTED WITH *ASPERGILLUS FUMIGATUS* AND THEIR IMPORTANCE FOR EMBRYONIC SURVIVAL

ABSTRACT: Aspergillosis is a frequent fungal disease of young and adult poultry in our commercial flocks. Infection can occur after hatching by inhalation of *Aspergillus* conidia which can be present in contaminated hatcheries, or later, by spores from moldy litter, dust, faeces or feed. Spores from the surface of egg shell can penetrate inside of an egg. The main characteristic of aspergillosis is granulomatous inflammation of respiratory system, although generalized form is possible as well. Multiple yellow nodules can be seen as major patomorphological changes and they are usually localized in lungs, air sacs, and can also be found in spleen, brain, subcutis and eyes. Egg embryos are quite susceptible to infection by *Aspergillus fumigatus* during incubation.

In this study, the history of a case on one local farm with mortality rate of 7.2% in the turkey flock is presented. At the same time, 28 day old 30 incubated hatching turkey eggs were sampled, that were unable to hatch. The aim of the present work was to determine the cause and to identify the agent of embryonic mortality. Total of the 30 eggs were opened, and 16 of them had thickened egg membranes in the area of air sac. Membrane thickening was visible and circumscript or diffuse presence of black–grey or grey–green fungal growth was observed. Only 3 samples air sacs were filled with developed stages of fungi. To evaluate histopathological lesions, changed egg membranes were processed by standard histological technique.

Dominant microscopic finding was thickening of egg membranes as a consequence of fungal growth and many of them penetrated deep towards embryo. Most of the hyphae were growing vertically through membranes. On the outside surface of the membranes, the elements of fungi (conidial heads with phialids and spores on them), could be clearly observed. These changes were responsible for embryonic death, which on the basis of the size of dead embryos occurred between 7th and 10th day of incubation. *Aspergillus fumigatus* was isolated and identified from the content of air sacs.

KEY WORDS: *Aspergillus fumigatus,* turkey hatching eggs, egg membranes, morphological changes

## INTRODUCTION

The principal agent causing aspergillosis in poultry is *Aspergillus fumigatus.* The genus *Aspergillus* which consists of approximately 600 species belongs to the division *Deuteromycota* which is composed of anamorphs *Aspergillus fumigatus* and *Aspergillus flavus.* Both lack a sexual stage and this is why they belong to the next classification scheme: division Deuteromycota, class Deuteromycetas, order Moniliales, family Moniliacae, genus Aspergillus (S a i f et al., 2008). *Aspergillus fumigatus* is a ubiquitous, saprophytic mold that can be isolated from a variety of habitats worldwide. As a facultative pathogen, it can cause various disease manifestations, including life-threatening invasive aspergillosis in animals and humans (K u n k l e, 2003; O l i a s et al., 2010). Most frequently it occurs in turkey poults, chicks, ducklings and goslings (I v e t i ć et al., 2003; B e y t u t et al., 2004; S p a l e v i ć et al., 2010; K u r e lj u š i ć et al., 2011). Aspergillosis is frequent fungal disease of young birds, although adult birds can be infected, too. There are two forms of the disease: acute aspergillosis usually characterized by severe outbreaks in young chickens and chronic aspergillosis which occurs in adult breeder. The fungal spores are ubiquitous in nature. Exposure of poultry to fungi or spores occurs after the introduction of contaminated litter (T s i o u r i s et al., 2008), but fungi from contaminated food can be directly inhaled by chickens while consuming food. According to M i lj k o v i ć et al. (2012) *A. fumigatus* was isolated from changed parts of the skin on the foot pads in broiler chickens. In Serbia, this agent was isolated in *Coracias cyanogaster, Coraciformes* and *Fringillidae-Passeriformes* imported as free-living birds in quarantine (M i lj k o v i ć et al., 2011). K a p e t a n o v et al. (2010) reported that early infection in hatcheries is possible if fungal contamination exists. Spores from the surface of egg shell can penetrate inside of an egg (S i n g et al., 2009). The main characteristic of aspergillosis is granulomatous inflammation of respiratory system, although generalized form is possible as well. Multiple yellow nodules can be seen as major pathomorphological changes and they are usually localized in lungs, air sacs, and changes can also be found in spleen, brain, subcutis and eyes (K u r e lj u š i ć et al., 2012). The treatment of aspergillosis is very difficult, therefore preventive measures are essential. In this paper, we determined the causal agent of embryonic mortality.

## MATERIALS AND METHODS

Total of 30 samples of 28 day old incubated non-hatching eggs were obtained from one local turkey hatchery. The egg shell was pure and non-deformed. Samples were taken when turkey chicks were brought from the roller space from several boxes. The samples were collected during a period when the farm, which was dealing with fattening of turkeys which were originally from the hatchery, experienced a problem of increasing mortality rate of 7.2%.

The results of the tests were reported previously by K u r e lj u š i ć et al. (2012). After laboratory processing of the egg shell surface, the 30 non-hatching eggs were opened and 16 of them had thickened egg membranes in the area of air sac. To evaluate histopathological lesions, changed egg membranes were processed by standard histological technique. Samples were fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections about 5μm were stained with hematoxylin-eosin (HE), periodic acid Schiff (PAS) and Grocott methods. The content of air sacs and surface of egg membranes were inoculated into Sabouraud dextrose agar (SDA) with 20 IU/ml penicillin G and 40 mg/ml streptomycin sulphate, and incubated at temperature of 25°C under aerobic conditions for isolation. Primary isolation was successful after 5 days. Subcultures were made on SDA without antibiotics (5 to 5 days). Cultures were stained with Lactophenol-cotton-blue (Hi Media) drops. The identification was done according to the description in available literature (Q u i n n, 2002). No bacterial growth was observed in aerobic culture on blood agar plates for 2 days at 37°C.

## RESULTS AND DISCUSSION

After opening the turkey incubated non-hatching eggs, changes on their membranes were observed. Out of a total of 30 eggs, 16 had thickened egg membranes in the area of air sac, which were, from the outside towards the air sacs, circumscriptively or diffuse covered with black, bluish-green and white color fungal colonies (Figure 1: a, b, c).
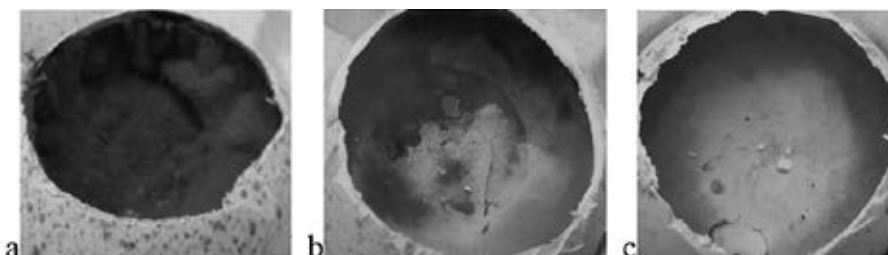


Fig. 1. – Macroscopic changes on the egg membranes

In the mycological examination on Sabouraud dextrose agar with antibiotic, fungal colony showed growth after 24 hours at 25 °C, but after four days it was white and about 2cm in diameter. By the seventh day, the colony diameter increased to 3.5 cm and there were color changes in the central part of the colonies, from bluish-green to grey-green, and by the 10th day the color of the cultures was greenish (Figure 2a). Staining of the fungus with a drop of Lactophenol-cotton-blue showed spore heads and arrangements of phialides and spores (Figure 2: a, b) *Aspergillus fumigatus.*
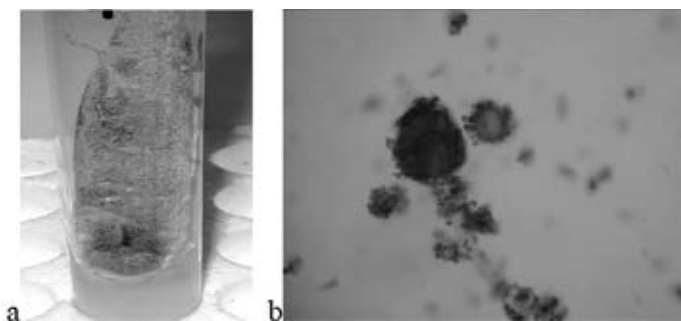
Fig. 2. – A colony of *A. fumigatus* on SDA (a)

Stained culture *A. fumigatus* with Lactophenol-cotton-blue, magnification 400x (b).

Below the inner layer of egg membrane a yellow-red yolk mass was detected which was, more or less, attached to the inner layer of the membrane in a form of pseudomembrane. Some parts of the egg membrane were black stained from penetrated elements of molds. Also, the thickness ranged up to 1 cm, and consisted of mycelium and yolk content. In our opinion, this thickened membrane formed as a result of the growth of *A. fumigatus*. As a consequence of thickened membrane the oxygen supply to the embryo was decreased, which necessarily led to its death. In our case, and based on the size of the turkey embryos (Figure 3), we assumed that they died between the seventh and tenth day of age. However, the question is whether the death of embryos was a result of suffocation or due to toxicosis.


Fig. 3. – Turkey embryos

Dominant microscopic finding was the thickening of egg membranes as a consequence of fungal growth which penetrated deeply towards the embryo (Figure 4). This was in accordance with the results presented by I v e t i ć et al. (1999).

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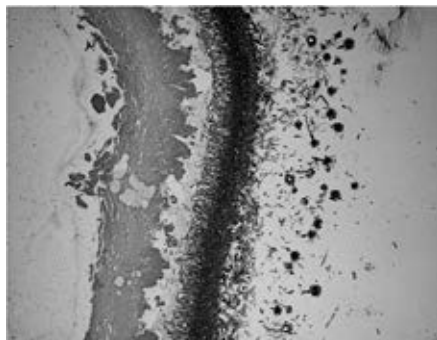
Fig. 4. – Histological section PAS-positive hyphae

*A. fumigatus* penetrating into sheath and directly towards the embryo tissue, PAS staining, magnification 200x.

Most of the hyphae grew vertically through membranes. On the outside surface of the membranes, elements of fungi were observed, and conidial heads with phialides and spores were bigger in diameter than the hyphae (Figure 5). This finding is consistent with the findings of F e m e n i a et al. (2007) for the case of artificial infection of 1-day old turkey by intra-air-sac inoculation of spore suspension of 3-day old *A. fumigatus* culture, initially isolated from a human with invasive aspergillosis (CBS 144.89) containing $10^7$ spores/animal, when similar finding was obtained.



Fig. 5. – Hyphae *A. fumugatus* growing vertically through the membrane-conidial heads with phialides and spores on them, Grocott, staining, magnification 400x

A very interesting microscopic finding was pseudogranuloma-granuloid formation of round or verrucoid shape, located on the surface egg membrane (Figure 6).
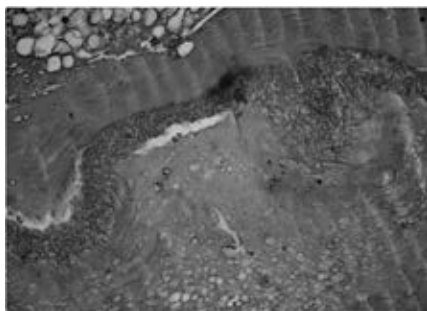
Fig. 6. – Granular formation on the egg membrane,
PAS staining, magnification 400x

Similar granular formations were described by B i o n d i ć et al. (1981), which consisted of numerous hyphae with or without protein mass. According to some authors (T o t h et al., 1979) these granuloid formations indicate the toxic effects of the molds. Immunocompromised condition is presumably induced by products of *A. fumigatus* existing in the body or environment, because this fungus can produce toxins such as gliotoxin, which has immuno-suppressive properties. The weakened bird, susceptible to subsequent diseases, ultimately died due to the acute airsacculitis and pneumonia.

The presence of *A. fumigatus* in the air of chicken hatchery is common, and it is often isolated in embryonated chicken eggs (S p a l e v i ć et al., 2008). The authors stated in their study that the control and disinfection of eggs should be conducted on the farm, as well as the control of litter, nest boxes, more frequent egg collection and careful evaluation of the quality of the eggs, with removal of all damaged and dirty eggs. Turkey hatching eggs are usually washed and sanitized before incubation because of the high percentage of soiled and stained eggs which is why this procedure is required. Eggs are sanitized by formaldehyde fumigation to eliminate pathological microorganisms that may reduce turkey performance and spread disease. Fumigation of the hatching eggs, hatchery facilities, equipment, and vehicle is an effective way of control of pathological microorganisms in the hatchery.

CONCLUSION

Hyphae of *Aspergillus fumigatus* were found in the inner layer of the egg membrane and sometimes they penetrated deeply towards the embryo. Egg membranes that suffered the previously described changes were responsible for embryonic death, which, on the basis of the size of the dead embryos occurred between the seventh and tenth day of incubation.

In this case, *Aspergillus fumigatus* was the cause of embryonal mortalities, which was confirmed by mycological and histopathological examination.

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## МИКРОМОРФОЛОШКЕ ПРОМЕНЕ НА ОПНАМА ЕМБРИОНИРАНИХ ЋУРЕЋИХ ЈАЈА ИНФИЦИРАНИМ СА *ASPERGILLUS FUMIGATUS* И ЊИХОВ ЗНАЧАЈ ЗА ЕМБРИОНАЛНО ПРЕЖИВЉАВАЊЕ

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### Резиме

Аспергилоза је често гљивично обољење младих, али могу да оболе и одрасле јединке. Инфекција може да настане већ након излегања, удисањем спора аспергилуса присутних у контаминираном инкубатору, или касније, спорама из фецеса, простирке, прашине или хране. Иначе споре могу да пенетрирају и љуску јајета и дођу у његову унутрашњост. Болест се карактерише запаљењем, пре свега респираторног система, мада понекад протиче и у генерализованој форми. Патолошке промене у виду мултипних жућкастих чворића локализоване су најчешће у плућима, ваздушним кесама, а могу бити захваћени слезина, мозак, поткожно ткиво и очи. Аспергилоза се тешко лечи због чега је неопходно да се предузму све превентивне мере којима се смањују или елиминишу услови неопходни за настанак болести.

Нашим истраживањем је обухваћено 30 ембронираних ћуређих јаја, из којих се у току инкубационог периода нису излегли ћурићи, а са циљем да се утврди узрок ембрионалног морталитета. Јаја су потицала од родитељског јата ћурака инкубираних у инкубаторској станици из околине Београда. Свих 30 јаја је отворено, а код 16 макроскопским прегледом је установљено задебљање јајчаних опни у подручју ваздушне коморе која је са спољашње стране дифузно или циркумскриптно прекривена црно-сивим или сиво-зеленим колонијама односно растом плесни. Код три случаја већи део ваздушне коморе испуњавали су развојни елементи плесни.

За микроскопско испитивање узете су промењене јајчане опне и обрађене стандардном хистолошком техником, а добијени ткивни резови обојени су НЕ, PAS и Grocott методом.

Микроскопском сликом доминирало је задебљање јајчаних опни као последица инфилтрације сплетом хифа од којих много дубље пенетрирају према ембриону. Већина њих на излазу из опни заузима вертикални положај. На спољашњој

површини опни јасно су се видели слободни елементи плесни (конидијалне главе са фијалидама и спорама на њима). Из садржаја ваздушних комора изолован је *Aspergillus fumigatus*.

Овако промењене опне јајета сигурно су одговорне за ембрионално угину-ће које се у овом случају, судећи по величини ембриона, догодило од седмог до десетог дана старости.

КЉУЧНЕ РЕЧИ: *Aspergillus fumigatus*, ембрионирана ћурећа јаја, јајчане опне, морфолошке промене

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# THE IMPACT OF *TRICHODERMA HARZIANUM*
## ON SPROUTING AND EARLY DEVELOPMENT
## OF PEPPER UNTIL THINNING

ABSTRACT: This paper presents the results of the study on the analysis of the stimulative and biopesticide effects of the fungus *Trichoderma harzianum* T-22 on germination, sprouting and early development of two pepper varieties (Amfora and Buketna 1) in organic production. The results show the significant effect of the applied T-22 strain on germination, sprouting, growth and early development of both aboveground and underground parts of plants, either used as soil or seed inoculum, in comparison to controls. It was concluded that *T. harzianum* T-22 proved its efficiency in promoting growth and development of pepper, and preventing the development of mycoses.
KEY WORDS: *Trichoderma harzianum*, biopesticide, plant growth stimulation, peppers

## INTRODUCTION

Over the last decade, organic products have become increasingly popular and economically more important. The procedures adopted in conventional production and which aim at rapid increase in productivity showed a range of adverse effects on both environment and health. Such procedures endanger any future production by destroying the long-term soil fertility. Besides the high production, protection of vegetable crops is also an important factor. Conventional production uses agro-chemical procedures against plant diseases (including intensive tillage, monoculture over large areas, use of mineral fertilizers, chemical control of weeds, pests and diseases and genetic manipulation of plants). The latter are widely used for highly-efficient, reliable and rapid operation. The chemical methods have a number of shortcomings such as environmental pollution, disruption of microbial processes in the soil, the occurrence of microbial resistance to fungicides and disruption of sanitation standards in greenhouses.

Steady increase in demand for organic products in the world suggests that this production method can be very profitable in case of proper use of natural resources, knowledge and production experience. Trichodermin, the preparation made from active ingredients of the fungus *Trichoderma spp* as well as its spore substrate, has been gaining great popularity in plant production and protection (H a r m a n et al., 2004.). When entering the soil, the preparation acts as plant protection agent (K a r et al., 2006.). Trichodermin and *Trichoderma* spore substrate do not affect either microbial processes in the soil, or the plant and its surrounding environment. To the contrary, the stimulative effect on the growth and development of various plants has been recorded.

*Trichoderma* belongs to a group of imperfect fungi and primarily develops in the soil (S a m u e l s, 2006). Its growth starts after 5-6 hours onto the culture medium (agar). Its colony achieves 90 mm in diameter in three days. Spore growth begins two days after mycelium development, gradually intensifies and reaches its maximum after 4-5 days. Fungal colony becomes green due to spore formation. The cycle of conidial stage development of pure culture ends within the course of 3-4 days (G a m s and M e y e r, 1998.).

Under different growing conditions, the structure, shape and size of the colony change, as well as mycelium color and its physical and chemical properties. The extent and nature of the change depends on genetic basis of the fungus, growth medium, and reactions to temperature and humidity. *Trichoderma spp.* are present in substantial numbers in nearly all soil types and other environments, such as the defunct wood (M o n t e, 2001). They are antagonistic to parasitic fungi; they live and grow on them and utilize their nutrients by using their own enzymes to degrade cell walls of pathogenic fungi (S a m u e l s et al., 2002.). This process (mycoparasitism) limits the growth and activity of plant pathogenic fungi. In some cases, in cooperation with mycoparasites, *Trichoderma* can produce antibiotics.

*Trichoderma* has wide temperature optimum (20-29 °C). Growth was recorded in traces at 6 °C and 34 °C, while the minimum and maximum points of its temperature and ecological valence were 5 °C and 35 °C, respectively, and no myceliar growth was recorded at those temperatures (K u b i c e k et al., 2001.). Mycelium grows faster at 25-28 °C with intensive conidial generation.

There are several mechanisms responsible for T-22 pathogen control ability in plants. As a biofungicide, it protects the root system in the rhizosphere (root zone).

Since it is a living organism, the T-22 can grow along the root system establishing barriers against pathogen attack. As long as the root system continues its active growth and development, T-22 will continue to grow along it, feeding on nutritive waste products secreted by the plant, thus removing the nutrient substances that could also attract pathogens. T-22 root colonization does not seem to interfere with the activities of nitrogen-fixing *Rhizobium* or mycorrhizae.

T-22 second defense line against root-rot fungi is the release of hydrolytic enzymes. Many pathogenic fungi contain chitin as their cell wall component.

T-22 strain contains chitinase that have proved to have the ability of breaking the cell wall of the pathogen. These enzymes function optimally in acidic environment. Although insects also contain chitin as an integral part of their exoskeleton, their pH is usually in the alkaline range. Therefore, T-22 chitinase will not damage organisms such as plants, birds, fish, humans and others (C h e t, 1987.).

This study aimed at determining *Trichoderma* stimulative effects on the growth and development of two pepper varieties widely used in commercial production in Serbia (Amfora and Buketna 1) during the period from sowing to thinning. Its effect on germination and seedling protection against pathogenic fungi in the production of seedlings in the greenhouse was also studied by completing the production cycle in an organic manner.


MATERIAL AND METHODS


As it was scheduled by the experiment, both pepper varieties were sown in three ways: 1) pure pepper seed and soil (the controls); 2) pure soil with pepper seeds inoculated with *Trichoderma* spores; and 3) pure seeds with soil inoculated with *Trichoderma* spores.

The seeds were sown in the middle of March into six containers (three for each pepper variety) that contained 66 cups. Inoculation of seeds and soils was performed seven days prior to the experiment.

Three seeds were sown into each cup (198 per container) in order to examine germination potentials for the cross-comparison and comparison with the values stated on the labels (70% for Amfora and 94% for Buketna 1 varieties). After sowing, seeds were watered and stored in a greenhouse at the temperature of 25 °C.

No other fertilizers or pesticides were used.

Sprouting and early plant development and root development were examined.


RESULTS AND DISCUSSION


No changes were observed in the containers during the first two weeks after sowing. This is typical for pepper, which takes 2-3 weeks to sprout (Table 1). The first eruption was observed 16 days after sowing, while the intense eruption began 20 days after sowing.

Table 1 shows the best results (highest number of seedlings) obtained from the cultivar Buketna 1, in samples in which either the seed or soil was inoculated with *Trichoderma* spores. By comparing the number of seedlings in control and inoculated samples, it was determined that the number was twice as high in the inoculated samples compared to the controls. The appearance of the first true leaves was noticed during the fourth week, when seedlings were re-counted and approximately the same numbers were found as in

previous counting. In Buketna 1 variety there was a slight difference between the seed and soil inoculated samples (67 and 69 plants). Amfora cultivar achieved the same results in all inoculated samples. More balanced plant growth was observed in all the inoculated samples, regardless of the variety.

Tab. 1. – Number of sprouted plants per cup 20 days after sowing

| Pepper samples | 20 days after sowing | | | Total sprouted plants |
|---|---|---|---|---|
| Plants per cup | 1 | 2 | 3 | No |
| Amfora control | 18 | 10 | 1 | 29 |
| Buketna control | 17 | 21 | 5 | 33 |
| Amfora, inoculated soil | 18 | 20 | 6 | 44 |
| Buketna, inoculated soil | 20 | 27 | 16 | 63 |
| Amfora, inoculated seed | 13 | 31 | 17 | 61 |
| Buketna, inoculated seed | 9 | 21 | 33 | 63 |

The percentage of sprouting was calculated in the fifth week after sowing. Buketna 1 variety showed lower effect of the inoculation, sprouting percentages being 79.2 % (control), 79.2 % (inoculated soil) and 84.8 % (inoculated seed). For the Amfora varieties, those percentages were 64.2 % (control), 73.7 % (inoculated soil) and 76.7 % (inoculated seed), as shown in Chart 1.
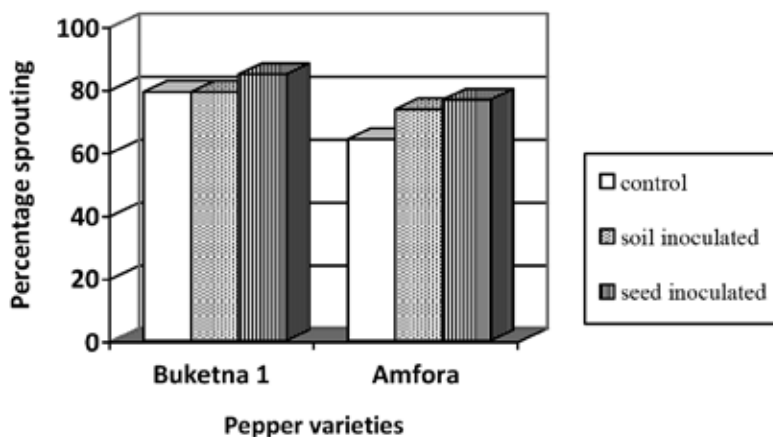


Chart 1. – Percentage sprouting five weeks after the sowing

In the inoculated Amfora samples, the percentage sprouting exceeded 70% of values stated on the label (73.7 % for soil inoculation and 76% for seed inoculation). The control Amfora samples showed lower sprouting percentage (64.3%).

In all Buketna 1 samples, percentage sprouting was below the value stated on the label (90%). The highest sprouting percentage was recorded in seed inoculated samples (84.8%), but it was still quite below the stated value.

The results of measuring of all the plants showed the average plant height in the sixth week after sowing (Table 2).

Tab. 2. – Average plant height (cm) six weeks after the sowing

| Pepper samples | Average plant height (cm) |
|---|---|
| Buketna control | 3,29 |
| Buketna, inoculated soil | 3,78 |
| Buketna, inoculated seed | 3,87 |
| Amfora control | 2,85 |
| Amfora, inoculated soil | 3,17 |
| Amfora, inoculated seed | 3,46 |

The highest sprouting percentage and greatest plant height were achieved by seed inoculated Buketna 1 variety samples.

Thinning was performed in the sixth week, allowing root development assessment. A significant difference in root development between inoculated and control plants was observed. Best developed roots in both cultivars were found in seed inoculated samples. Slightly less developed roots were observed in soil inoculated samples, while the control plants had least developed roots (Figures 1 and 2).
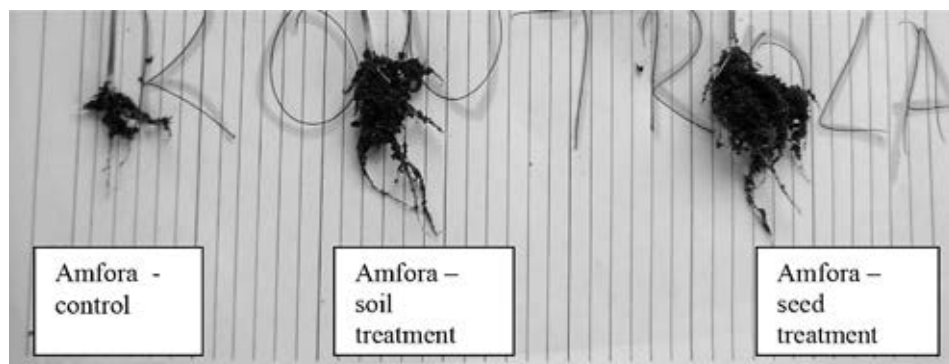


Fig. 1. – Amfora variety, root development (photo J. Đoković)

Amfora cultivar showed the greatest root development in seed inoculated samples (Figure 1). Apart from the size, the roots also showed good ramification and width which further resulted in the increase in the strength of the plant and its resistance to wind and other weather conditions, after being transferred to the field. In soil inoculated samples, root system was longer, penetrating deeper, which was also an excellent feature facilitating water and mineral matter acquisition from deeper soil layers in the open field and enabling survival during the drought.

Fig. 2. – Buketna 1 Amfora, root development (photo J. Đoković)

Buketna 1 variety (Figure 2) had similar growth and development of the root system as the Amfora variety, with somewhat less developed root system in soil inoculated. In the seed inoculated samples, plant roots spread both laterally and longitudinally, which is a very good feature because it increases the strength of plants and provides them with water and mineral nutrients from deeper soil layers, after being transferred to the open field.

## CONCLUSIONS

The results of the study suggest that the fungus *Trichoderma* has a significant effect on germination, growth and development of both aboveground and underground parts of pepper plants, both when used as a soil or seed inoculums in comparison to the controls (no inoculum). The effects are clearly variety-dependant. The effectiveness of *Trichoderma* was demonstrated in promoting growth and development of pepper, as well as in preventing the development of mycoses.

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УТИЦАЈ ГЉИВЕ *TRICHODERMA HARZIANUM* НА КЛИЈАЊЕ
И РАНИ РАЗВОЈ ПАПРИКЕ ДО ПЕРИОДА РАЗРЕЂИВАЊА

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Резиме

Рад представља резултате испитивања стимулативних и биопестицидних ефеката гљиве *Trichoderma harzianum* Т-22 на клијање, ницање и рани развој две сорте паприке (Амфора и Букетна 1) у органској производњи. Резултати сугеришу значајан ефекат примењеног Т-22 соја на све испитиване параметре, укључујући развој надземног и подземног дела биљака, без обзира на то да ли је спорама гљиве инокулисано семе или земљишни супстрат. Закључујемо да је *T. harzianum* Т-22 показала ефикасност у подстицању раста и развоја паприке, као и у превенцији развоја микоза.
КЉУЧНЕ РЕЧИ: *Trichoderma harzianum*, биопестицид, стимулација раста биљке, паприка

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# THE OCCURRENCE OF TRICHOPHYTOSIS AMONG PEOPLE AND CATTLE ON A FARM IN VOJVODINA, SERBIA

ABSTRACT: Dermatophytoses are frequent contagious fungal skin diseases that affect the skin of people and animals. Zoophile dermatophytes pose a significant problem for both human and veterinary medicine, and they are especially present among bovines. In this paper we showed a simultaneous occurrence of trichophytosis among professionally exposed people and bovines on a farm in Vojvodina, Serbia. The tested samples (skin scrapings and hair) originating from people and animals, were positive for *Trichophyton verrucosum* dermatophyte which was determined by applying a direct microscopic examination of the smears, as well as the isolation and identification of the agents.
KEY WORDS: Bovines, dermatophytosis, people, trichophythosis, *Trichophyton*

## INTRODUCTION

Dermatophytoses are frequent contagious fungal diseases that affect skin of people, animals and rarely birds (S a r g i s o n et al., 2002; C h e r m e t t e et al., 2008). These diseases are widespread around the world. The zoophile sorts of dermatophyte predominantly infect animals which become reservoirs of these agents, although sometimes the presence of geophile and anthropophile dermatophytes can be registered. Dermatophytes are represented by

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askomycetes that possess both keratophile and keratinolytic characteristics. A dominant sort of dermatophyte among bovines and other ruminants is *Trichophyton verrucosum* from the *Arthrodermataceae* (C h e r m e t t e et al., 2008). A special problem for public health is seen in the capacity of the fungi *T. verrucosum* to infect people (P a p i n i et al., 2009). Fungal infections of skin and nails are the most frequently reported among people. It is considered that one quarter of the population on this planet has superficial mycoses. The frequent dermatomycoses among people is more often noticed in the communities of lower social and economic status, i.e. among those with inadequate hygiene habits, as well as among people who have frequent and repeated direct contacts with the infected skin of animals (H a v l i c k o v a et al., 2008). Data collected in Serbia on the infections of people and animals caused by dermatophytes are scarce, although these diseases are quite frequent. P o t k o n j a k et al. have recently shown epizoological and clinical characteristics of the bovine infection with *T. verrucosum* on a bovine farm in the Autonomous Province of Vojvodina (P o t k o n j a k et al. 2011). The aim of this paper was to point to a simultaneous occurrence of the infection of people and bovines on a farm in Vojvodina, Serbia, with *T. verrucosum* being an agent.


MATERIAL AND METHODS


Total of 12 people and 566 animals were clinically examined and the sampling was performed at the same time. Out of 6 people with skin changes, which indicated a fungal infection, skin skrapings were taken from the edge of the efflorescence, previously processed with 70% alcohol. Out of the 20 bovines with clinical changes which indicated a fungal infection, hair samples and skin scrapings were collected from the edge of the efflorescence, previously processed with 70% alcohol. Regarding the laboratory testing, first a direct microscopic examination of the samples of hair and skin scrapings was done. The smears of these materials were prepared in a 10% solution of potassium hydroxide and in chlorlactophenol, and with multiple, brief exposure to open flame. The examination of the prepared smears was performed with a light microscope, magnification 400x, in order to identify the potential presence of arthroconidia. At the same time, a cultivation of bovine hair samples and bovine and human skin samples took place in the Sabouraud dextrose broth with chloramphenicol (Himedia Laboratory), at different temperatures (25° C and 37°C) during a four-week period. The identification of the grown colonies was done based on the growth rate, macroscopic and microscopic characteristics, urease test, and specific nutritive requirements for growth. A solution of lactophenol cotton blue (Himedia Laboratory) was used to examine microscopic features of the grown colonies and to stain the dermatophytes, while Trichophyton agar 1-7 (Difco^TM) was incubated at room temperature during a two-week period (R o b e r t and P i h e t, 2008).

# RESULTS

A direct microscopic examination of the samples of bovine hair and skin scraping samples taken from people and bovines, proved an overwhelming presence of arthroconidia producing ectotrix chains. After 12 days of incubation at a temperature of 37° C, substantial, round whitish cobweb-like colonies incorporated into the foundation, which corresponded to trichophyton, while similar smaller colonies grew only after the third week of cultivation at a temperature of 25° C (Fig. 1). Using the microscope to examine a smear from the culture stained with diluted lactophenol cotton blue irregular and septate hyphae were noticed (Fig. 2). The urease test was negative for the isolated trichophyton sorts. Trichophyton agars number 1 and 2 did not grow, while mild increase was detected in test tubes number 4, 5 and 7. Satisfactory increase in the tested culture was observed in the trichophyton agar number 3, with additional inositol and thiamine. The colonies that grew on the Sabouraud dextrose broth with chloramphenicol were identified as *Trichophyton verrucosum*.
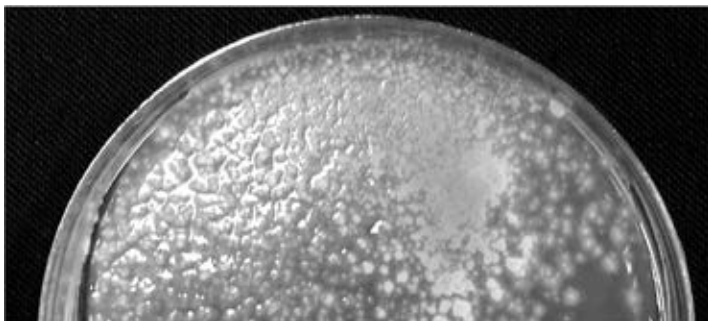


Fig. 1. – Appearance of *T. verrucosum* colonies grown on Saburo dextrose agar with chloramphenicol



Fig. 2. – Appearance of *T. verrucosum* hifa stained with lactophenol cotton blue

The analyzed material from all 6 patients proved the presence of the agent *Trichophyton verrucosum*. All the patients had characteristic efflorescences which were described as "red lumps" (Fig. 3). Material taken from bovines proved that all 20 of them were infected with *T. verrucosum*, and typical efflorescenes were: grey-white, round with a desquamate, elevated, bordered, localized predominantly on the head and the neck (Fig. 4).



Fig. 3. – Appearance of efflorescence on human arm caused by *T. verrucosum*



Fig. 4. – Appearance of efflorescence on calf head caused by *T. verrucosum*

## DISCUSSION

A g h a m i r i a n reported that zoophile dermatophytes pose a significant problem for human and veterinary medicine, and that they are especially present among bovines. In recent years, especially during winter, an increase

in the incidence of the infection with this agent has been reported among animals, and the most frequent reported agent is *Trichophyton verrucosum* in bovines, which is transferred to people via direct contact (A g h a m i r i a n and G h i a s i a n, 2011). Recently, clinical picture of dermatomycoses in people caused by zoophile dermatophytoses has been changed due to the inadequate immune response of the host, so granulomatose changes have been registered as well as invasive or deep mycoses, changes similar to mycetoma caused by *M. canis* and the ever increasing infections caused by agent *T. verrucosum* (C h e r m e t t e et al., 2008). P a p i n i reported a widespread infection caused by *T. verrucosum* in the rural area and added that it is frequently registered among cattle growers (P a p i n i et al., 2009). N é j i et al. isolated the agent *T. verrucosum* in 178 patients, which corresponded to 1.2% of all the registered dermatophytoses (N é j i et al., 2011). M a s l e n reported that agent *T. verrucosum* was in most cases isolated from the materials originating from people who were at a professional risk of infection (farm workers or cattle slaughterhouses), or from children who lived on farms and were in direct contact with the infected bovines (M a s l e n, 2000). N e n o f f said that each occurrence of infections caused by *T. verrucosum* and *T. erinacei* should be reported to the scientific and professional public (N e n o f f et al., 2012).

## CONCLUSION

In this paper, we have pointed to a simultaneous infection of people and bovines on a farm in AP Vojvodina, Serbia. Each occurrence of zoophile dermatophytes among animals can be expected to be followed by a simultaneous occurrence of the infection of directly exposed persons. Further epidemiological research, strict application of general prophylaxis, as well as vaccination of bovines are necessary in order to eradicate the source of this zoonosis.

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## ПОЈАВА ТРИХОФИТОЗЕ КОД ЉУДИ И ГОВЕДА НА ФАРМИ У ВОЈВОДИНИ, СРБИЈА

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Резиме

Дерматофитозе представљају честа, контагиозна, гљивична обољења коже људи и животиња. Зоофилне дерматофите представљају значајан проблем у хуманој и ветеринарској медицини, а посебно су присутне код говеда. У раду смо приказали истовремену појаву трихофитозе код професионално експонираних људи и говеда на фарми у Војводини, Србија. У узорцима достављеним на испитивање (скарификати коже и длака) пореклом од људи и животиња, применом директног микроскопског прегледа препарата, као и изолације и идентификације узрочника, идентификовано је присуство дерматофите *Trichophyton verrucosum*.
КЉУЧНЕ РЕЧИ: дерматофитоза, говеда, људи, *Trichophyton*, трихофитоза

*Z o r i c a   R .   L o p i č i ć*[1], *A l e k s a n d r a   S .   B o č a r o v – S t a n č i ć*[2], *M i r j a n a   D .   S t o j a n o v i ć*[1], *J e l e n a   V .   M i l o j k o v i ć*[1], *V l a d i m i r   R .   P a n t i ć*[2], *M i l a n   J .   A d a m o v i ć*[1]

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# *IN VITRO* EVALUATION OF THE EFFICACY OF PEACH STONES AS MYCOTOXIN BINDERS

ABSTRACT: The paper describes *in vitro* model for the evaluation of ability of peach shell (unmodified and modified), prepared at the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade, to adsorb different mycotoxins.

Peach stones were obtained from "Vino Župa" Company from Aleksandrovac, where they have been disposed of as by-products from their Juice Factory. After proper preparation, two sorts of peach shell particles were used: one as unmodified peach shell particles (PS) and another one obtained by acid modification, denoted as MPS.

Adsorption of six mycotoxins: aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZON), diacetoxyscirpenol (DAS) and T-2 toxin by PS and MPS was tested *in vitro*. Crude extracts of mycotoxins, produced at the Department of Microbiology of Bio-ecological Center, Zrenjanin, were used for adsorption experiments. The ability of binding mycotoxins was evaluated in the electrolyte 0.1 M $K_2HPO_4$ which pH value was adjusted to 3.0 and 7.0, respectively. Mass ratio of individual mycotoxin and peach shell samples was 1:5000. The experimental mixtures were incubated for 1 hour on a rotary shaker (185 rpm) at room temperature (22-25°C). After incubation, the extractions of non-adsorbed mycotoxins from the filtrates were performed with organic solvents, and their quantification was done by thin-layer chromatography (TLC).

KEY WORDS: peach stones, acid modification, adsorption, **in vitro,** mycotoxins

## INTRODUCTION

Biosorption has proved to be efficient, low cost and sustainable technique that uses cheap and abundant biomaterials, usually declared as waste, for removing heavy metals and organic waste mostly dyes (N a j a  et al., 2010; S u d et al., 2008; D a s et al., 2008). The authors have used one type of agricultural waste material-peach stone for *in vitro* removing of the new type of pollutants in biosorption – mycotoxins.

Among all substances that can contaminate feedstuffs, the outstanding place belongs to mycotoxins. These naturally occurring components, secondary

fungal metabolites, can have deleterious health effects on animals and humans due to the consumption of animal products. One approach in finding method(s) for prevention of toxic effects of mycotoxins is detoxification and inactivation of these fungal metabolites by using mycotoxin binders. These additives inhibit the uptake of mycotoxins by animals *in vivo*.

Agricultural waste materials mostly consist of cellulose, lignin, hemicellulose, pectin, extracts, lipids and other organic compounds that are rich in different functional groups responsible for pollutants binding. The presence of these three biological polymers (cellulose, lignin, and hemicellulose) causes richness of peach shell in hydroxyl and phenol groups which can be further chemically modified to produce adsorbent materials with improved adsorbing properties. They also have multilayer porous structure filled with openings and channels that provide large volume per sorbent surface unit, which is favorable for biosorption process (H u b b e et al., 2011). Chemical modification of cellulosic materials is often used to improve certain properties of the material or some of its components, such as material hydrophilic or hydrophobic characteristics, its elasticity, adsorptive or ion-exchange capability, thermal properties of the material or its resistance to microbiological attack, but in most cases the chemical modification serves to improve absorbing capacity of materials (S u n, 2010). Many researchers have conducted their investigations using different chemical agents (acids, bases, etc.) in order to improve absorbing properties of biomaterials toward heavy elements (W a n  N g a h  and H a n a - f i a h, 2008). Acid pretreatment serves for removal of some soluble organic impurities and it can be used to change the structure of functional cell compounds and expose binding sites to pollutants in order to improve biosorption capacity. In the case of heavy elements biosorption, acid pretreatment increased the overall negative charge of the adsorbents, which improved biosorption capacities for negative cations (E l a n g o v a n et al., 2008).

So, the aim of the presented investigation was to evaluate and compare *in vitro* the binding capacity of unmodified peach shell particles and peach shell particles treated with hydrochloric acid to six different mycotoxins: aflatoxin B1, ochratoxin A, deoxynivalenol, zearalenone, diacetoxyscirpenol and T-2 toxin.

## MATERIALS AND METHODS

### *Biosorbent preparation*

Lignocellulosis material – peach stones were obtained from "Vino Župa" Company from Aleksandrovac, where they were disposed of as by-products from their Juice Factory. The samples were manually crushed and separated from kernels, and in that way only hard stone parts were taken for further analysis. The crushed peach stones were further milled to different fractions and washed several times in tap and distilled water. The sample was sent to

chemical analysis. The content of micro and macroelements in unmodified peach stones was analyzed using standard chemical methods (Službeni Glasnik SFRJ, broj. 15/87) and the morphology of the untreated material was obtained with dried sample coated with gold and observed using JEOL JSM-6610LV SEM model.

For mycotoxins adsorption, only the fraction with diameter less than 100 μm was used. Prior to experiments, samples were dried at 60°C for 24 h, washed three times in 0.01 M HCl, and then in distilled water until negative reaction with Cl⁻ ions was reached. After drying, one part of these particles was directly used for mycotoxin adsorption as unmodified material (PS). Another sample marked as modified peach shell particles (MPS) was activated by 1M hydrochloric acid on thermostatic orbital shaker (25°C and 200 rpm). After 1 hour, flask content was filtered, particles were washed with distilled water several times, and the procedure with 1M HCl was repeated two more times. At the end, MPS was washed with distilled water until negative reaction with Cl⁻ ions was reached. All the samples were marked and stored in polypropylene bags until the experiment started.

*Production, quantification and isolation of mycotoxins*

Aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZON) were produced by solid substrate fermentations based on the methods of B o č a r o v - S t a n č i ć et al. (2009a and 2009b) and B o č a - r o v - S t a n č i ć et al. (2010), respectively. Type A trichothecenes (diacetoxy-scirpenol – DAS and T-2 toxin) were biosynthesized by submerged fermentation in liquid medium (B o č a r o v-S t a n č i ć et al., 2007). For the toxin production, the following fungal cultures were used: *Aspergillus flavus* GD-2 (leg. prof. dr G. Dimić, Technological Faculty, Novi Sad, Serbia), *A. ochraceus* CBS 108.08, *Fusarium graminearum* GZ-LES (leg. dr J. Lević, Maize Research Institute, Belgrade-Zemun, Serbia), *F. graminearum* D2 (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, Serbia), *F. semitectum* SL-B (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, Serbia), and *F. sporotrichioides* ITM-391 (leg. dr A. Bottalico, Consiglio Nazionalle delle Richerce, Istituto Tossine a Micotossine da Parassiti Vegetali, Bari, Italy).

Isolations of mycotoxins and determinations of single mycotoxin content in solid substrates were done according to standard thin-layer chromatographic method for fodder analysis (Službeni Glasnik SFRJ, br.15/87). Isolations of type A trichothecenes were done by ethyl acetate and their quantities were determined by thin-layer chromatographic (TLC) method according to R u k - m i n i and B h a t (1978). Isolated crude toxins were evaporated to dryness and dissolved in following solvents: ethanol (AFL, OTA, ZON), ethyl acetate (DAS, T-2) and methanol (DON). The final concentrations of stock mycotoxin solutions were 0.1 μg/μl (AFL) and 1 μg/μl (OTA, DON, ZON, DAS and T-2), respectively.

*Experimental procedure*

In order to perform adsorption experiments, stock solutions of mycotoxins were diluted as follows: AFL to 0.2 μg/ml, ZON to 0.8 μg/ml, and all other mycotoxins to 2.0 μg/ml with electrolyte (0.1M $K_2HPO_4$). pH value of electrolyte was adjusted with 0.1M HCl or 0.1 NaOH to 3.0 and 6.9, respectively.

The binding ability of peach stones was tested *in vitro* as follows: aliquots (50 ml) of test solutions were added to Erlenmayer flasks (250 ml) containing 500 mg of single adsorbent in the case of OTA, DON, DAS and T-2 toxin, 200 mg in the case of ZON, and 50 mg in the case of AFL. Controls were prepared by adding 50 ml of the test solutions without mineral adsorbent. The flasks were stoppered, incubated for 1 hour on rotary shaker (185 rpm) at room temperature (22-25$^o$C) and then filtered. In 25 ml aliquots of electrolyte with adsorbent (C) and without it ($C_o$), concentrations of mycotoxins were determined, after extraction with 2 x 15 ml of organic solvents: benzene (ZON), benzene-acetonitrile (AFL), and ethyl acetate (OTA, DON, DAS and T-2) respectively, by TLC methods (Službeni Glasnik SFRJ, br. 15/87; R u k - m i n i and B h a t, 1978). All analyses were performed in three replications.

The adsorption index of individual mycotoxin, in percentages, was calculated with the following formula:

$$Adsorption\,index = [\frac{C_0-C}{C_0}]\,x\,100$$

RESULTS AND DISCUSSION

The chemical analyses of unmodified peach stone particles were performed in order to elucidate the composition and content of micro and macroelements present in the materials. Peach shell consisted mostly of cellulose (58.02%), hemicellulose (16.54%), and lignin (5.02%) (B o č a r o v-S t a n č i ć et al., 2012a) (Table 1).

On the other hand, results shown in Table 2 indicate that peach stone particles contained several important minerals such as calcium (0.14%) and potassium (0.089%); toxic elements were not present in significant amounts.

Such chemical compositions of the examined peach stone samples implied that these materials could be used in animal feed as energetic materials or even as carriers of certain active substances used in agriculture and industry.

The morphology and the surface nature of the grounded PS were presented on the SEM micrograph (Figure 1) at 3000 x magnification. As it can be seen from Figure 1, the PS particles have multilayer porous surface with irregular laminated structure. The average pore diameter was less than 1 μm, which might be beneficial for mycotoxin diffusion and adsorption.

Tab. 1 – Chemical composition of unmodified peach shell (PS) in %

| Parameter (%) | PS | dPS* | JUS/ISO/ Documented methods |
|---|---|---|---|
| Dry matter | 92.23 | 100.00 | Službeni Glasnik SFRJ, br. 15/87, Method 6 |
| Moisture | 7.77 | - | Službeni Glasnik SFRJ, br. 15/87, Method 6 |
| Crude protein | 1.26 | 1.37 | Službeni Glasnik SFRJ, br. 15/87, Method 7 |
| Crude fat | 0.05 | 0.05 | Službeni Glasnik SFRJ, br. 15/87, Method 12 |
| Crude cellulose | 58.05 | 62.94 | Method VDM-111 |
| Ash | 0.42 | 0.46 | Službeni Glasnik SFRJ, br. 15/87, Method 18 |
| Nitrogen free extracts (NFE) | 32.45 | 35.18 | Službeni Glasnik SFRJ, br. 15/87, Method 20 |
| Neutral detergent fiber (NDF) | 71.12 | 77.11 | Method VDM-118 |
| Acid detergent fiber (ADF) | 66.12 | 71.69 | Method VDM-119 |
| Lignin | 16.54 | 17.93 | Method VDM-119 |

Legende: *dPS-dry basis

Tab. 2 – Micro and macroeleements present in unmodified peach shell

| Parameter | K | Na | Ca | Mg | Fe | Mn | Al |
|---|---|---|---|---|---|---|---|
| Amount (%) | 0.089 | 0.042 | 0.14 | 0.031 | 0.016 | <0.01 | 0.005 |
| Parameter | $P_2O_5$ | Pb | Ni | Cd | Zn | Cu | $SO_3$ |
| Amount (%) | 0.54 | 0.008 | 0.001 | <0.01 | 0.0015 | 0.003 | <0.02 |
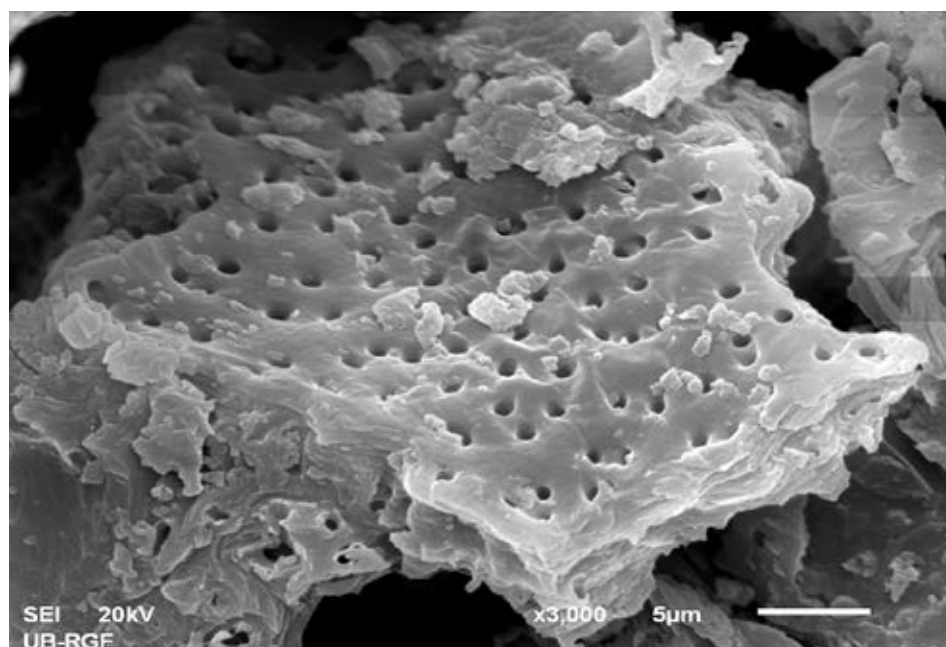


Fig. 1 – SEM micrograph of unmodified peach shell particles

Table 3 shows the adsorption indices of six mycotoxins by unmodified biosorbent – peach shell particles (PS) and peach shell particles modified by acid (MPS), calculated at two pH levels.

Tab. 3 – Adsorption indices of six mycotoxins in modified and unmodified peach shell particles at different pH values

| Adsorbent | pH | Adsorption Index (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | AFL | OTA | DON | ZON | DAS | T-2 |
| Peach shell (PS) | *3.0* | 58.82 | 42.86 | 23.08 | 50.00 | 0 | 25.00 |
| | 7.0 | 58.82 | 33.32 | 40.00 | 33.33 | 16.67 | 40.00 |
| Modified peach shell (MPS) | *3.0* | 41.18 | 42.86 | 40.00 | 33.33 | 16.67 | 50.00 |
| | 7.0 | 41.18 | 33.32 | 50.00 | 58.33 | 33.33 | 40.00 |

Data presented in Table 3 can provide better explanation by looking the graphs given in Figure 2 and Figure 3.
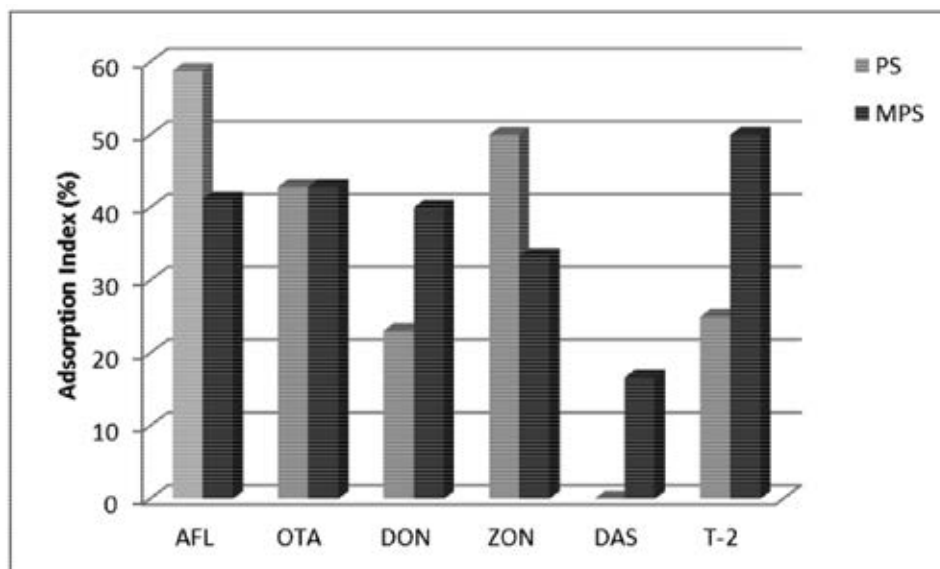


Fig. 2 – Comparison of adsorption indices (%) for modified and unmodified peach shell particles at pH 3.0

By using the TLC method, it was noted that unmodified peach shell bound more (58.82%) of applied AFL than the modified peach stones (41.18%); quantity of adsorbed AFL B1 was the same at pH 3.0 and pH 7.0 (Tab. 3). The effect of pH level on binding capacity of particular mycotoxin was quite different for other tested mycotoxins. In case of OTA, adsorption indices were the same for PS and MPS (42.86% at pH 3.0 and 33.32% at pH 7.0, respectively). Although binding of DON was observed in both analyzed samples, its adsorp-

Fig. 3 – Comparison of adsorption indices (%) for modified and unmodified peach shell particles at pH 7.0

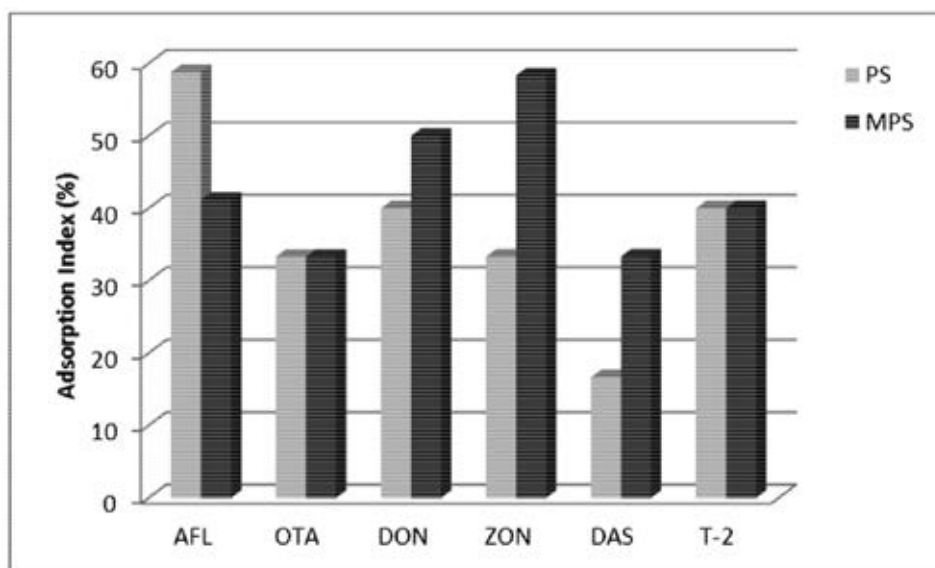tion indices were higher in modified peach shell (40.0% at pH 3.0 and 50.0% at pH 7.0, respectively). Effect of electrolyte pH value on the binding of ZON was different for different samples; PS adsorbed more ZON at pH 3.0 (50.0%) while MPS adsorbed more ZON at pH 7.0 (58.33). In case of type A tri-chothecenes (DAS and T-2 toxin), PS bound DAS only at pH 7.0 (16.67%). Higher adsorption indices for the same fusariotoxin were obtained in modi-fied PS (16.67% at pH 3.0 and 33.33% at pH 7.0, respectively). The amount of the bound T-2 toxin was the same at pH 7.0 (40.0%) but higher for MPS at pH 3.0 (50.0% compared with 25.0% in unmodified PS).

Peach stones produced from food industries as solid waste are often known as binding material in the form of ash or activated carbon, for example lead (R a s h e d, 2006) or aqueous ammonia (S o t t o-G a r r i d o et al., 2003), some textile dyes (G e r c e l et al., 2009), but not often as unmodified mate-rial that can adsorb heavy element ions (Cu) and radionuclide (uranium) (S t o - j a n o v i ć et al., 2012). Numerous data about the adsorption capacity of agricul-tural waste materials for toxic fungal metabolites are not available in Serbia (B o č a r o v-S t a n č i ć et al., 2012a; B o č a r o v-S t a n č i ć et al., 2012b, S t o j a n o v i ć et al., 2012). In case of fusariotoxins, the presented results (Tab. 3) were similar to the results obtained from our previous investigation (B o č a r o v-S t a n č i ć et al., 2012b).

The application of different lignocellulose materials such as apricot stones as one of the ingredients of mycotoxin binding additive for food and animal feed, and which has at the same time fungistatic and bacteriostatic effect, is described in patent No. 20120070516 (www.faqs.org/pattents/app/20120070516). Beside

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apricot stones, this additive also contains plant material from other prunus (e.g. prune, cherry, plum, almonds etc.) as well as plant extracts (e.g. herbal remedy, herbal extracts, powder, oil etc.). The resulting adsorbent can bind different mycotoxins, including OTA, DON, nivalenol and T-2, which are the mycotoxins difficult to be bound. According to the authors of the patent No. 20120070516, the ability of porous lignocellulosic materials to thermally collapse during melting can be used to adsorb mycotoxins irreversibly in wet system and then to entrap them after closing lignin pore structures under high temperature treatment.

As it can be seen from the presented results (Tab. 3), acid modification by 1M HCl changed the adsorption index for five out of six mycotoxins, leading in most cases to the improvement of removal, except in the case of aflatoxin B1, where decrease from 58.82 % to 41.18 % at both pH values occurred. In case of OTA, acid modification gave no changes in adsorption index at both pH values. In case of other four mycotoxins: DON, ZON, DAS and T-2, acid modification mostly improved the adsorption indices, except in the case of ZON for pH value of 7.0 where the decrease from 50.00% to 33.33% occurred.

It is not surprising that peach stones particles demonstrated ability for biosorption of mycotoxins **in vitro** conditions because of their rather high cellulose content (58.5%).

## CONCLUSION

The results presented here indicate that the peach shell particles can be used as effective biosorbents of mycotoxins. Acid modification leads to the improvement of biosorption capacity in most cases, but further investigations should be performed in order to elucidate the nature of interaction between the biosorbent and specific mycotoxin.

Similar to other *in vitro* assays, the presented assay cannot completely simulate the conditions in gastro-intestinal tract of animals, so further *in vivo* experiments are necessary to assess the efficacy of peach stones and other waste materials as mycotoxin binders.

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# *IN VITRO* ОЦЕНА КОШТИЦЕ БРЕСКВЕ КАО АДСОРБЕНСА МИКОТОКСИНА

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Резиме

Рад описује *in vitro* модел за процену способности коштице брескве (немодификоване и модификоване), припремљене у Институту за технологију нуклеарних и других минералних сировина у Београду, да адсорбује различите микотоксине.

Коштице брескве су набавњене од „Вино Жупе" из Александровца, компаније где су одложене као отпадни материјал њихове Фабрике сокова. Након одговарајуће припреме, у испитивању су коришћене две врсте честица коштице брескве: једна немодификована (PS) и друга добијена киселинском модификацијом (MPS).

*In vitro* методом је тестирана адсорпција шест микотоксина: афлатоксина Б1 (AFL), охратоксина А (OTA), деоксиниваленола (DON), зеараленона (ZON), диацетокисцирпенола (DAS) и Т-2 токсина. За експерименате адсорпције коришћени су сирови екстракти микотоксина произведени у Одељењу за микробиологију Био-еколошког центра у Зрењанину. Способност честица немодификоване и модификоване коштице брескве за везивање микотоксина је оцењивана у електролиту 0,1 М $K_2HPO_4$ чија је pH вредност подешена на 3,0 односно 7,0. Масени однос појединачних микотоксина и узорака коштице брескве је био 1:5000. Експерименталне смеше су инкубиране 1 сат на ротационој тресилици (185 о/мин) на собној температури (22-25 $^oC$). После инкубације, екстракција неадсорбованих микотоксина из филтрата експерименталних смеша су извршене органским растварачима, а њихова квантификација методом танкослојне хроматографије.

КЉУЧНЕ РЕЧИ: коштице брескве, киселинска модификација, адсорпција, *in vitro*, микотоксини

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# MONITORING OF FUNGAL SPORES IN THE INDOOR AIR OF PRESCHOOL INSTITUTION FACILITIES IN NOVI SAD

ABSTRACT: Fungal spores can cause a range of health problems in humans such as respiratory diseases and mycotoxicoses. Since children are the most vulnerable, the presence of fungal spores in the facilities of preschool and school institutions should be investigated readily. In order to estimate air contamination by fungal spores, air sampling was conducted in eight facilities of the preschool institution in Novi Sad during February and March, 2007. Sedimentation plate method was used for the detection of viable fungal spores, mostly being members of subdv. Deuteromycota (*Fungi imperfecti*).

In 32 samples a total of 148 colonies were developed, among which five genera were identified: *Penicillium*, *Cladosporium*, *Aspergillus*, *Alternaria* and *Acremonium* while non-sporulating fungal colonies were labeled as sterile mycelia. Most frequently recorded genera were *Penicillium* with 46 colonies and *Cladosporium* with 44 colonies. The genera *Aspergillus* and *Alternaria* were represented with 3 colonies each and *Acremonium* with only 1 colony. The greatest number of colonies emerged in the samples from the day care facilities "Vendi" (58 colonies) and "Panda" (49 colonies). Most diverse samples were obtained from the day care center "Zvončica", with presence of all identified genera.

These results showed notable presence of fungal spores in the indoor air of Preschool institution facilities and indicated the need for further, more complete seasonal research. Obtained information is considered useful for the evaluation of potential mycofactors that endanger health of children.

KEY WORDDS: children, day care, fungi, indoor air, Preschool institution, spores

## INTRODUCTION

Fungi are ubiquitous microorganisms that are known as important bio-contaminants of the indoor environment (A y d o g d u et al., 2005; A y d o g d u and A s a n, 2008). Their spores are easily liberated in the air and may cause diverse health problems in humans such as: irritations, infections, allergies and toxicoses (M e n e t r e z and F o a r d e, 2004; S t e t z e n b a c h et al., 2004). The most common sources of airborne fungal particles indoors are outdoor air and fungal growth encouraged by favorable environmental factors

(dampness, high temperature and the presence of organic and inorganic sub-strates) (P e s s i et al., 2000; L i a o et al., 2004).

Children spend most of their time inside the rooms of educational institutions which are common sites of air quality problems (D a i s e y et al., 2003; A y d o g d u et al., 2005). Therefore, a good indoor air quality in buildings of day care centers and schools is essential for children's safety. Various studies (F e r n g and L e e, 2002; K o s k i n e n et al., 2004; Z u r a i m i et al., 2009) showed that poor environmental conditions in day care centers can threaten the health of preschool children. Over the last decade, potential health consequences of fungal contaminants in indoor environment resulted in an increased interest for determination of potential exposure levels of these contaminants (D o t t e r u d et al., 1996; H a v e r i n e n et al., 1999; S m e d j e and N o r b a c k, 1999; S u et al., 2001; D a i s e y et al., 2003).

Aerobiology studies the release of biological particulate matter into the atmosphere, its atmospheric transport, deposition and re-suspension (S c h e i - f i n g e r et al., 2013). In Serbia, aerobiological research is focused on the studying of outdoor environment, in particular airborne pollen (S i k o p a r i j a et al., 2011). Furthermore, no aeromycological research of the indoor environment in school and preschool facilities has been conducted so far. The aim of this study was to investigate the presence of viable fungal spores in the indoor air of the day care facilities of preschool institutions in Novi Sad (Serbia).


MATERIAL AND METHODS


Airborne fungal spores were collected in February and March 2007, in eight facilities (child day care centers) of the preschool institution "Radosno detinjstvo" in Novi Sad: "Zvončica", "Plavi zec", "Vila", "Petar Pan", "Dunavski cvet", "Maslačak", "Panda" and "Vendi". Each of these facilities was visited once. The presence of airborne fungal spores was investigated in the room where children spent most of the time during their stay in the day care center. The visited child day care centers were situated in different parts of the city. They also varied according to building age and size, number of children and staff, as well as the sizes of the investigated rooms. All of the facilities were equipped with a central heating system and ventilation was provided naturally through windows. Floors were predominantly covered with carpet.

Sampling was performed before usual daily activities, between 6.30 and 8.30 am. Sedimentation plate method was used, with malt extract agar ("Torlak", Belgrade, Serbia) as the growth media. Four Petri plates were placed 1 m above the ground level in different parts of each investigated room and were exposed to air for 10 minutes by removing the cover lid. Sampled plates were incubated at 25$^{\circ}$C for 5 to 7 days. After the incubation period, grown colonies were identified and counted. Their number corresponded to the number of viable airborne spores and it was expressed as the number of colony forming units (CFU) per plate.

The use of sedimentation plate sampling method enabled the recovering of cultivable fungi which easily produce reproductive structures necessary for proper determination (mostly belonging to the group Deuteromycota or *Fungi imperfecti*). Determination of fungal genera was based on micro- and macro-morphology, as well as reverse coloration and surface coloration of colonies, according to W a t a n a b e (2002). Fungal colonies which did not sporulate after 10 days were recorded as sterile.

RESULTS

From the indoor air of eight analyzed sites, a total of 148 fungal colonies were isolated in 32 sampled Petri plates. The sterile colonies dominated with 51 CFU (34%). Sporulating fungi belonged to five genera of filamentous fungi (*Penicillium*, *Cladosporium*, *Aspergillus*, *Alternaria*, *Acremonium*). The most widespread genera, *Penicillium* and *Cladosporium*, were present in all of the investigated rooms (Tab. 1). They were also the most numerous with 46 CFU (31%) and 44 CFU (30%), respectively. *Aspergillus* and *Alternaria* were represented with 3 CFU (2%), while *Acremonium* was recorded with only one colony (1%) (Fig. 1).

Tab. 1. – Presence of isolated fungal genera in the investigated facilities

| Identified fungal genera | Number of colony forming units (CFU) | | | | | | | | Total number | Percentage (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Vendi | Panda | Masla-čak | Zvon-čica | Dunav-ski cvet | Plavi zec. | Vila | Petar Pan | | |
| Non-sporulating colonies | 27 | 14 | 2 | 2 | 3 | 3 | / | / | 51 | 34.5 |
| *Penicillium* | 26 | 7 | 6 | 1 | 1 | 1 | 3 | 1 | 46 | 31.1 |
| *Cladosporium* | 5 | 28 | 2 | 3 | 2 | 2 | 1 | 1 | 44 | 29.7 |
| *Aspergillus* | / | / | 1 | 1 | / | / | / | 1 | 3 | 2 |
| *Alternaria* | / | / | / | 1 | / | / | 1 | 1 | 3 | 2 |
| *Acremonium* | / | / | / | 1 | / | / | / | / | 1 | 0.7 |
| TOTAL | | | | | | | | | 148 | 100 |

The total counts of CFU per each examined facility are shown in Fig. 2. Day care centers "Vendi" and "Panda" stand out with the highest number of detected viable airborne fungal spores – 58 CFU and 49 CFU, respectively. The number of fungal colonies developed in the samples from other facilities was notably lower ("Maslačak" – 11 CFU, "Zvončica" – 9 CFU, "Dunavski cvet" and "Plavi zec" – 6 CFU each, "Vila" – 5 CFU). The lowest number of CFU was obtained from "Petar Pan" (4). With five isolated genera, the diversity of recorded colonies was the highest in the day care centar "Zvončica", while in "Dunavski cvet", "Panda" and "Vendi" only *Cladosporium* and *Penicillium* were identified (Tab. 1).
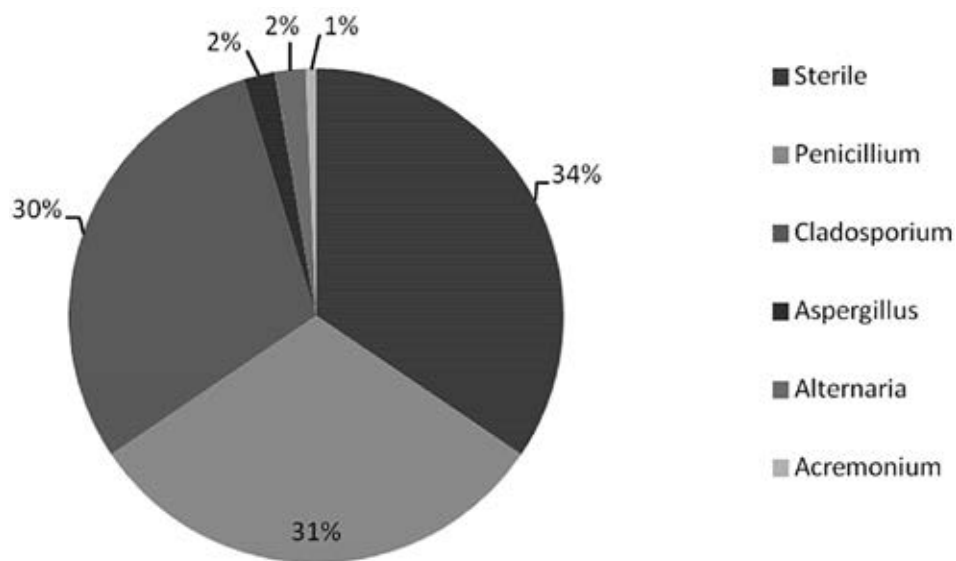
Fig. 1. – Distribution of detected fungal genera in the total number
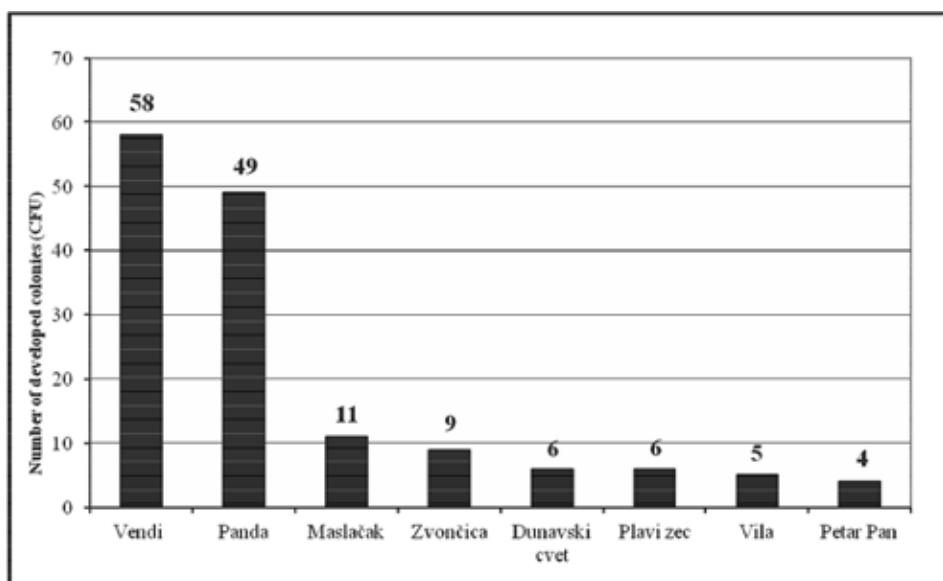of developed colonies (CFU)



Fig. 2. – Total number of fungal CFU isolated in each examined children day
care facility

## DISCUSSION

The most common airborne fungal spores in the indoor environment belong to the following genera: *Cladosporium*, *Penicillium*, *Alternaria* and *Aspergillus* (E t z e l et al., 1998; R o l k a et al., 2005; K a l y o n c u, 2008) which is in accordance with our results. Moreover, *Penicillium* and *Cladosporium* which were present in all of the investigated sites and dominated among other fungal genera detected, were found to be predominant in many other indoor aeromycological studies (H u et al., 2002; S a r i c a et al., 2002; S h e l t o n et al., 2002; L e e et al., 2006; A y d o g d y and A s a n, 2008). Similar to our study, the research conducted by A y d o g d u et al. (2005) in the indoor air of 5 schools in Turkey recognized *Alternaria* and *Aspergillus* as the most frequent, followed by *Penicillium* and *Cladosporium*. *Acremonium* was the only genus in our research that was registered in a single sample with one colony. Other investigations of indoor air, in buildings with different purpose, have also recorded presence of *Acremonium* in small percentage (S h e l t o n et al. 2002; L u g a u s k a s and K r i k s t a p o n i s, 2004; R o l k a et al., 2005) but there was an indication of possible sample contamination. The occurrence and airborne spore concentrations in indoor environment show notable seasonal variability but also greatly depend on: geographical, climatic and meteorological parameters, surrounding vegetation, specific sources of indoor contamination, as well as the sampling time and procedure (A y d o g d u et al., 2008). Many of the airborne spores recorded in the indoor environments, particularly those of *Cladosporium* and *Alternaria*, originate from outdoor sources (H i n d y and A w a d, 2000). In contrast, species of *Aspergillus* and *Penicillium* are mainly derived from indoor sources (A y d o g d u  et al., 2005).

The highest number of CFU, reported from day care centers "Vendi" and "Panda", indicated notably higher amount of viable airborne fungal spores compared to the other investigated facilities. Main contributor to the high number of CFU in samples from the day care "Vendi" was the genus *Penicillium* (45%). This might indicate that the applied ventilation methods were not adequate for the given room, resulting in promotion of fungal proliferation inside the building. As opposed to this, *Cladosporium* was the main contributor to the high number of CFU recorded in the day care center "Panda" (57%). This result might be related to the immediate vicinity of a park area (Futoški park), which could be a source of *Cladosporium* – the mold usually developed in soil, on vegetation and decaying plant material (E l l i s, 1971). However, the applied sampling method could also have affected high concentrations of *Cladosporium* and *Penicillium* in facilities "Vendi" and "Panda". With the application of culturing methods in aeromycological research, it is possible to overestimate the importance of these molds since their species grow easily in culture plates (A y d o g d u et al., 2005).

The Sedimentation Plate Method (also known as the gravity or settling plate method) was used for its practicality, low cost and the ease of use. It is a passive sampling technique that relies on particles settling out of air and is useful for the enumeration of bacteria, yeast and fungal spores (A t l a s and

B a r t h a, 1998). This method is recommended for obtaining preliminary or qualitative information (H o e k s t r a, 2002).

Airborne fungi identified in the selected facilities of the preschool institution "Radosno detinjstvo" may affect the health of children and employees. D a i s e y et al. (2003) determined that the symptoms of asthma and "sick building syndrome" are common in school children. According to P a s t u s z k a et al. (2000), *Cladosporium*, *Alternaria* and *Aspergillus* represent the main group of airborne molds to which children may be sensitized and which may cause allergic symptoms. *Alternaria* and *Cladosporium* are considered to be the most important aeroallergens in the outdoor air, as well as the cause of mycotoxicosis in humans. Frequent exposure to airborne aeroallergens of the genus *Alternaria* can result in respiratory arrest in children and adolescents with asthma (O ' H o l l a r e n et al., 1991). Spores of *Aspergillus* and *Penicillium* represent the most widespread aeroallergens in the world (A y d o g d u et al., 2005) and are recently recognized as the most important allergens of indoor air (S h e n et al., 2000; F i s h e r and D o t t, 2003; K a s z n i a – K o c o t et al., 2007). Records on the *Aspergillus* and *Penicillium* viable fungal spores could indicate the presence of molds that are known to be responsible for mycotoxin production and emission of volatile organic compounds – VOC's (K a s z n i a – K o c o t et al., 2007).

CONCLUSION

Presence of airborne fungal spores in buildings of educational facilities can affect the health of children and staff. Results of our study indicated notable presence of viable fungal spores in the air of examined preschool facilities, hence the need for further, more detailed investigations. Seasonal air monitoring of fungal spores and application of more accurate, quantitative sampling method (the use of vacuum/culture samplers) would provide a more detailed insight about indoor air quality. Information obtained by such studies is required for the assessment of health-threatening factors, medical evaluation of children and staff health condition and for the implementation of remediation procedures.

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# МОНИТОРИНГ СПОРА ГЉИВА У ОБЈЕКТИМА ПРЕДШКОЛСКЕ УСТАНОВЕ У НОВОМ САДУ

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Резиме

Споре гљива могу бити узрочници низа различитих здравствених проблема код људи, као што су респираторна обољења и микотоксикозе. С обзиром на то да деца представљају једну од најугроженијих група, било би пожељно редовно проверавати присуство спора гљива у просторијама предшколских и школских установа. У циљу процене квалитета ваздуха и евентуалног присуства спора гљива, током фебруара и марта 2007. године извршено је узорковање ваздуха у осам објеката Предшколске установе у Новом Саду. Употребљена је метода седиментационих плоча, а у центру истраживања биле су гљиве које су претежно припадници подраздела Deuteromycota (Fungi imperfecti).

У 32 узорковане петри плоче развило се укупно 148 колонија различитих родова гљива, од којих је укупно пет детерминисано: *Penicillium*, *Cladosporium*, *Aspergillus*, *Alternaria* и *Acremonium*. Гљиве чије колоније нису спорулисале, нису могле бити детерминисане и означене су као стерилне. Доминантни родови били су *Penicillium* са 46 колонија и *Cladosporium* са 44 колоније, док су родови *Aspergillus* и *Alternaria* били заступљени са по 3 колоније, а род *Acremonium* забележен је само са 1 колонијом. Највећи број колонија јавио се у узорцима из обданишта „Венди" (58 колонија) и „Панда" (49 колонија). Објекат „Звончица" био је најразноврснији на основу присуства детерминисаних родова (забележено присуство свих 5 родова).

Добијени резултати указују на значајно присуство спора гљива у ваздуху испитиваних објеката и на потребу за даљим, потпунијим испитивањима. Информације добијене оваквим истраживањима изузетно су корисне за процену потенцијалних фактора који угрожавају здравље деце.

КЉУЧНЕ РЕЧИ: деца, гљиве, ваздух, предшколске установе, просторије, споре

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# BIOINDICATIVE VALUES OF MICROFUNGI IN STARCH AND POSSIBLE DEFICIENCIES OF THE NEW SERBIAN REGULATION ON FOOD HYGIENE

ABSTRACT: The results of tests on the presence of yeasts and molds in cornstarch [AD 'IPOK' Zrenjanin, 2007–2008, made at the time when previous Regulations were valid] were analyzed in terms of bioindicative values of microfungi as indicators of quality and safety of raw material or final food products. Microbiological analysis was used to detect the presence of a number of microorganisms MMI-0001, and a questionnaire was designed at the Department of Public Health in Zrenjanin town (Republic of Serbia), where the analyses were done, regarding the microbiological tests on starch. In order to rationalize the analyses and make them more economical, several areas of product quality control (water, food, raw materials, space) were recommended either to be excluded or regarded as optional. Thus, analysis of presence of microfungi as indicators of product quality was categorized as optional. The results obtained from this research suggest a different conclusion because the bacteria in the samples indicated "microbiologically", namely bacteriologically, safe samples of food, while, on the contrary, the presence of some microfungi as distinct xerophilous or xerotolerant microorganisms, indicated that the food was mycologically non-safe. The obtained data are crucial for questioning the decision to exclude the earlier required (mycological) analysis of the samples (in the production of starch, or end products, etc.) and categorize such analyses in new Regulations as optional, depending on the manufacturer's preference. Bioindicative values of microfungi as indicators of the quality of starch, clearly point to the shortsightedness of the new Regulations on food hygiene and safety, where tests on certain microorganisms (in this case, yeasts and molds) are not legally defined as mandatory, but the Law leaves manufacturers a possibility to choose (or not to choose) the testing and frequency of testing on the presence (absence) of microorganisms, which can be risky, both in the production and marketing of the finial products.
KEY WORDS: Corn starch, Food hygiene, Microfungi, Regulations, Serbia

## INTRODUCTION

Some fungi, primarily yeasts and molds, are in various ways involved in the induction of pathological conditions in humans and animals (M a t a v u l j et al., 2005). Toxic effects of mycotoxins are manifested as different syndromes

in humans and animals, known as mycotoxicoses, and they have been manifested as cytotoxicity, hepatotoxicity, neurotoxicity, teratogenicity, mutagenicity and carcinogenicity of the target tissue, an organ or system of organs (K o c i ć – T a n a c k o v and D i m i ć, 2012). Knowledge of the role of microorganisms in food spoilage and processes that induce diseases which are transmitted through food is essential to understand the principles of effective food sanitation procedures (M a r r i o t t and G r a v a n o, 2006).

Definitions of food safety and the food itself are prescribed by the Law on Food Safety, (Službeni Glasnik RS, br. 41/2009). On the basis of this law (Article 26, Paragraph 4), the "Regulation on the general and special conditions of hygiene of food at any stage of production, processing and transportation (Službeni Glasnik RS, br. 72/2010), the so called " new Regulation" was adopted. The Law on Food Safety and Consumer Goods (Službeni Glasnik SFRJ, br. 53/1991) is no longer valid. Based on this currently invalid Law, another also presently invalid law, "the old Regulation" – Regulation on microbiological food safety in traffic (Službeni Glasnik Republike Jugoslavije, br. 26/1993, 53/1995, 46/2002) was prescribed. According to the Article 49 of this Regulation it was clearly stated that the analysis of starch on the presence of yeasts and molds is mandatory, and it also prescribed the frequency of analysis and allowable counts of microfungi in the sample. Investigations of certain microorganisms (in this case, yeasts and molds) are not exactly legally defined by the new Law, but the Law provides freedom for producers to decide on the frequency of analyses, which poses a significant health hazard.

In this study, the presence of yeasts and molds in the corn starch was analyzed. From our point of view, the frequent presence of microfungi in the analyzed raw ingredients or final food product samples implies the necessity of regular testing of all products on the presence of these microorganisms and the need to introduce this test as mandatory in the new Regulations.

MATERIAL AND METHODS

In this study the results on the presence of yeasts and molds in corn starch, as well as their bioindicative values as indicators of safety of the final food product, were analyzed for the particular company in Zrenjanin which was engaged in the production of starch during 2007–2008, when the old Regulation was still valid.

Also, a type of interview was prepared for the employees of the Department of Public Health in Zrenjanin, regarding their opinions on the former and actual microbiological tests required for starch. The interview which was in the form of survey was designed to reflect professional and competent opinions of professionals about the topic addressed.

Microbiological analysis MMI-0001 was used to detect the presence of yeasts and molds in food, as well as the presence of a number of microorganisms. The technique of preparing the food samples for testing was performed according to the procedures for determining the presence, isolation and iden-

tification of microorganisms described in the Regulations on methods of performing the analysis and superanalyses in food commodities, (Službeni Glasnik SFRJ, br. 25/80).

The smallest amount of samples to be submitted for analysis was 200 g. In case the food was suspected to have caused food poisoning or toxo-infections, the sample size was greater than 200 g, depending on the amount of the remaining foodstuff that was suspected to be the cause of food poisoning. All types of food samples for microbiological examination were prepared under aseptic conditions to prevent microbial contamination of samples from the environment.

The sample brought for microbiological control had the Sampling report and Work order, taken from the computer and sent to the laboratory for testing. Each sample had its own number, the main protocol number and the assigned laboratory number that was the same for all nutrient media until the end of the test. All samples were recorded in the Workbook according to the sampling date and under the numbers that defined them (number of sample(s), number of the main protocol and protocol number of laboratories).

Inoculated culture media were thermostated at appropriate temperatures depending on the type of the test. After 24-48 hours of incubation the reading of experimental data on culture media was done. Recorded changes on/in nutrient media were noted in the Work order form, indicating all changes related to the isolation and identification of microorganisms. Optimal analysis period was 5 to 7 days. The analysis which lasted for more than 7 days occurred in case of a repetition of some test parameters. Upon the completion of the analysis, results of microbiological quality of a sample were entered in the computer for further analysis performed by a hygiene specialist. The equipment and supplies used, examined culture media, and other contaminated material was disposed of in biohazard bags and/or metal cans.

## RESULTS AND DISCUSSION

The samples were analyzed for the presence of yeasts and molds in the plain corn starch, as an indicator of accuracy. Allowed number of yeasts and molds in 1.0 g of sample was <100, and a value of 100 is considered as permissible. The results showed that the counts in the tested samples varied greatly and ranged from 0 (which was measured in a few cases) to as many as several thousands. Tables 1, 2, 3 present the overall results on the presence of yeasts and molds in the samples from 2007 and 2008. The results showed that the counts above the allowed limit often occurred, therefore, this test should not be ignored, and in fact, it turned out to be much more needed than required.

Data obtained from the Department of Public Health in Zrenjanin suggested that quantitative composition of bacteria indicated microbiologically good quality of analyzed samples (majority of samples from 2007), while on the contrary, the quantitative composition of microfungi (number of fungal particles) indicated bad microbiological quality of analyzed samples. These

Tab. 1. – The percentage of the presence of yeasts and molds in the cornstarch in relation to the total number of samples from 2007.

| Samples | Number | Percentage (%) |
|---|---|---|
| Total number of analyzed samples | 206 | 100.00 |
| Samples with counts above the permitted | 88 | 42.72 |
| Samples with counts equal to the Permissible level | 22 | 10.68 |
| Samples with counts at permissible level | 96 | 46.60 |

Tab. 2. – The percentage of positive samples in 2007

| Samples | Number | Percentage (%) |
|---|---|---|
| A total of positive samples* | 88 | 100.00 |
| Samples positive only on yeasts and molds | 82 | 93.18 |
| Samples positive only on illicit bacteria | 4 | 4.55 |
| Samples positive both on microfungi and bacteria | 2 | 2.27 |

* the samples are considered positive if they have values above the permissible content of micro-organisms

Tab. 3. – The percentage of the presence of yeasts and molds in corn starch as compared to the total number of samples in 2008.

| Samples | Number | Percentage (%) |
|---|---|---|
| Total number of analyzed samples | 141 | 100.00 |
| Samples with counts above the permitted | 40 | 28.37 |
| Samples with counts equal to the permissible level | 10 | 7.10 |
| Samples with counts at permissible level | 91 | 64.53 |

data represent the basis for questioning the decision to exclude the mycological analysis of samples in the production of starch, or its end products, and to make the categorization analyses of microfungi optional, depending on the manufacturer's preference. The exclusion of the analyses for quantitative composition of microfungi from the list of compulsory analyses was based on earlier claims that some groups of bacteria might indicate the environmental conditions in which microfungi occur, so it would be unnecessary to perform two analyses (B o r r e g o et al., 1991). Authors of some scientific papers argue the opposite claiming that the presence of bacteria in a certain substrate does not necessarily indicate the presence of fungi, especially some toxigenic fungi of the genera *Penicillium, Aspergillus, Fusarium, Cladosporium, Alternaria, Mucor, Rhizopus*, etc., due to their xerophile nature (A r v a n t i d o u et al., 2000; S h e i n m a n et al., 2000; M a t a v u lj et al., 2005, 2006).

According to the responses of the employees who participated in the questionnaire it could be concluded that the problem of deficiencies of new Regulation existed, not only from the perspective of the problems given in

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this paper (frequency of microbiological analyses on yeasts and molds incidence), but also from other aspects which were important for food hygiene and safety. It was observed that the number of analysis on microfungal presence in food and raw material samples was considerably reduced after the introduction of new Regulations. The following conclusions were drawn based on the responses given:

- The number of analysis on microfungi counts after the adoption of new Regulation was reduced, almost completely;
- The methods for fungal presence testing, according to the new Regulation, are not better than the "old method" prescribed by the previous Regulation;
- Starch manufacturers should include in their plans for microbiological analysis more frequent performance of tests on fungal presence;
- The Guide for the implementation of microbiological criteria for food analysis does not clarify enough the new rules prescribed by new Regulation;
- The competences and responsibilities of inspection authorities are unclear;
- The number of compulsory samples analyzed on a monthly/annual basis is not clearly stated;
- Certain microorganisms and some foodstuffs are not included in the compulsory analyses;
- The requirements related to the analysis are not clear and understandable.


CONCLUSION


Bioindicative values of microfungi as indicators of the quality of starch clearly point to the shortcomings of the new rules on food hygiene, where testing on certain microorganisms (in this case, yeasts and molds) is not legally defined as mandatory, but the law gives manufacturers the possibility to choose (or not to choose) the testing and frequency of testing on the presence (absence) of microorganisms, which can be risky, both in the production and marketing of the finial products.

The simplest solution to this problem would be to prescribe clearly and accurately the defined list of microorganisms which presence would have to be analyzed in certain products, because among other things, the results of this study show that high risks exist if regular control of raw ingredients and final products on fungal presence is omitted.


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БИОИНДИКАТИВНЕ ВРЕДНОСТИ МИКРОГЉИВА У СКРОБУ
И МОГУЋЕ МАЊКАВОСТИ НОВОГ ПРАВИЛНИКА
О ХИГИЈЕНИ ХРАНЕ РЕПУБЛИКЕ СРБИЈЕ

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РЕЗИМЕ

Анализирани су резултати испитивања присуства квасаца и плесни у кукурузном скробу [АД „ИПОК" Зрењанин, 2007-2008. год., у време када је био важећи стари Правилник] у погледу биоиндикативне вредности микрогљива као индикатора квалитета и хигијенске исправности сировине или финалног производа. Микробиолошка анализа је коришћена за откривање присуства броја микроорганизама ММИ-0001, а такође дизајниран је интервју за запослене на Одељењу за јавно здравље у Зрењанину, где су урађене анализе микробиолошких тестова кукурузног скроба.

У неколико области контроле квалитета производа (вода, храна, сировине, простор), у сврху рационализације анализа и њиховог појефтињења, препоручено је избацивање неких од њих, или увођење неких од њих као необавезних. У овом случају анализирана је одлука да се учини опционом анализа присуства микрогљива као показатељ квалитета и хигијенске исправности производа. Елиминација анализе присуства гљива је објашњена ранијим тврдњама да неке групе бактерија индикују еколошке услове који погодују појављивању микрогљива, тако да би било сувишно да се анализирају обе групе микроорганизама. Наши резултати указују на другачији закључак, јер бактерије у узорцима показују „микробиолошку", односно бактериолошку исправност или неисправност узорака хране док, напротив, присуство неких микрогљива као посебне групе ксерофилних или ксеротолерантних микроорганизама, указује на миколошку, а самим тим и микробиолошку неисправност испитиваних узорака хране. Ови подаци су од кључног значаја за преиспитивање одлуке да се избаце раније обавезне миколошке анализе узорака (у производњи скроба или производа од скроба, итд.) и такве анализе у новом Правилнику категоризују као опционе, зависно од жеље произвођача.

Биоиндикативне вредности микрогљива као показатеља квалитета скроба јасно указују на кратковидости новог Правилника о хигијени хране и њеној безбедности, где тестирање на одређене микроорганизме (у овом случају квасци и плесни) није законски дефинисано као обавезно, него Закон даје произвођачима слободу да се по властитом нахођењу определе (или не определе) за тестирање или учесталост тестирања на њихово присуство (одсуство), што може да буде ризично, како у производњи тако и у пласману готових производа. Најједноставније решење овог проблема било би прописивање јасно и тачно дефинисане листе микроорганизама чије присуство би морало да се анализира код одређених производа, јер између осталог, и резултати овог рада указују колико може бити опасно ако се не врши редовна контрола испитиваног производа на микрогљиве.

КЉУЧНЕ РЕЧИ: микрогљиве, хигијена хране, кукурузни скроб, Правилник, Србија

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# MYCOTOXINS AND THEIR EFFECT ON HUMAN HEALTH

ABSTRACT: Health risks associated with the consumption of products contaminated with mycotoxins are worldwide recognized and depend on the extent to which they are consumed in diversified diet. To some extent, the presence of small amount of mycotoxins in cereals and related food products is unavoidable; this requires risk assessments which are to be carried out by regulatory bodies in several countries to help establish regulatory guidelines for the protection of public health. By assessing the levels at which these substances in food may pose a potential risk to human health, it is possible to devise appropriate risk management strategies. However, several important factors have to be taken into account in making a rational risk management decision, such as adequate toxicological data and information concerning the extent of exposure, availability of technically sound analytical procedures (including sampling), socioeconomic factors, food intake patterns and levels of mycotoxins in food commodities which may vary considerably between countries.
KEY WORDS: health risk, human health, imunosupression, legislation, mycotoxicosis, mycotoxin

## INTRODUCTION

From the aspect of human and veterinary medicine, food safety represents a significant problem. Thus, attention is directed to diseases which are closely related to different kinds of mycotoxicoses. Reports from the World Health Organization show that the presence of mycotoxins and toxic metabolites of molds in the food for human consumption is not on the decrease. Due to the obvious increase in the number of diseases caused by mycotoxins, which have been implicated as potential etiological factors, a great effort is made to identify mycotoxins present in the food and thereon to eliminate them. Besides the already known and studied mycotoxin-related diseases, World Health Organization reports also mention other diseases such as Alzheimer, multiple sclerosis and cancers.

A number of studies have documented that mycotoxins are etiological factors of a number of respiratory and neurological disorders, as well as cancerous changes, nephrotoxicity and hepatotoxicity. Recent studies have shown that mycotoxins have a biological role and direct influence on the initiation and/or recurrence of nervous system disorders. By repeated stimulation of receptors affected by mycotoxins, the relationship between mycotoxicosis and Alzheimer's disease can be explained. This can be used as the study model for MCS (*Multiple Chemical Sensitivity Syndrome*) and other syndromes.

The presence of mycotoxins in food is a problem that mankind, especially modern society, is faced with, regardless of the fact that there is a big difference between developed and underdeveloped parts of the world, primarily in the degree of food contamination with mycotoxins. In underdeveloped regions people are constantly exposed to acute or chronic mycotoxicosis. However, having due regard to the ongoing struggle for enough food in these regions, it would be unrealistic to expect a solution to the problem of mycotoxicosis. People in developed or highly developed areas of the world are less exposed to mycotoxins, primarily due to geographic and climatic conditions. In addition, there are considerable food resources, modern processing and storage of food is constantly applied, strict legislation is introduced, and there is a very strict control of mycotoxins presence in food.

### Presence of mycotoxins in animal feedstuff

Contamination with mycotoxins and molds is a world problem. According to FAO data, nowadays 25% of world wheat production is contaminated (D e v e g o w d a et al., 1998). It is evident that molds and mycotoxins represent a serious problem (SCOOP, 1996), not only in regard to successful harvest and food quality, but also in the productivity and health of animals (M i l l e r and T r e n h o l m, 1994).

### High risk foods

Since there are known limits which designate the contaminated food as safe, it is necessary to be able to recognize foods which are the most common sources of intoxication (S i n o v e c and R e s a n o v i c, 2005). Such foods on organoleptic examination usually appear safe, although mycotoxins are described as „cold blooded murderers". Mycotoxins are most commonly found in cereals, coffee, cocoa, nuts, as well as foods of animal origin in which mycotoxin residues can be found (milk, meat, offal, eggs).

*Milk and milk products.* The presence of mycotoxins in milk and dairy products is a serious food safety problem, especially for infants and children who are most susceptible to mycotoxins, and who are also most exposed to this source of poisoning. Mammals, which ingest food contaminated with aflatoxin, secrete the toxin in their milk in the form of hepatic 4 – hydroxylated

metabolite known as "milk toxin", or aflatoxin M1. AFM1 is slightly less toxic than AFB1, therefore, the International Agency for Cancer Research (IARC, 1993) classified AFM1 as potentially carcinogenic to humans. By taking into account the possibility that the OTA and other mycotoxins can be secreted into milk, and that the rumen microflora can greatly transform, it can be concluded that the content of mycotoxins in milk poses lower hazard to human health.

Powdered milk is another source of AFM1 in the human population, and is therefore subject to regulation which is extremely strict concerning this product. In the last decade of the twentieth century, AFM1 contamination in milk was reduced to a minimum in the EU because there was no legal limit on the presence of AFM1 due to the lack of certainty about the effects that very low doses of aflatoxin and its body tissue accumulation can have especially on children (F r e m y and D r a g a c c i, 1999).

*Human milk.* Mycotoxins, primarily aflatoxins, represent a serious prenatal problem as it is known that some toxins easily pass through the placental barrier and can be found in a fetus when a mother consumes contaminated food. As mycotoxins (M i r a g l i a et al., 1995) are, as a rule, excreted by milk, the highest risk of intoxication in newborns is primarily mother's milk.

*Meat and meat products.* Foodstuff, which originates from animals fed with contaminated feed, represents a potential hazard to health. Ruminant meat represents a slight danger in comparison to pork and poultry due to physiological characteristics of the rumen and specific degradation of the majority of present mycotoxins.

Aflatoxin is deposited in the tissues and organs of animals which consume contaminated feed. Deposition occurs in liver, muscles, stomach, kidneys, adipose tissue and meat, the highest deposition of aflatoxin is in the liver (R e - s a n o v i ć, 2000).

Ochratoxin is deposited primarily in kidney and liver, as well as in muscle and adipose tissue (J o n k e r and P e t t e r s s o n, 1999). Contamination of pork meat with OTA is a major problem, and that is why some European countries have introduced special monitoring in slaughterhouses where the presence of OTA is determined based on post mortem examinations (M o u s s i n g et al., 1997).

Zearalenone and its derivatives can be determined in edible portions such as liver and muscles of animals fed with contaminated feed (C i e g l e r and V e s o n d e r, 1983), as well as in the meat of clinically healthy animals (M i l i ć e v i ć et al., 2005).

Relatively rapid metabolic degradation of T-2 toxin is probably the main reason for very difficult identification of its presence in meat (P a c e, 1986).

*Eggs.* The presence of myctoxins in eggs represents a health hazard for human population. Aflatoxin B1 residues pose the highest hazard since their transformation into AFB1 in the liver of hens causes the formation of a set of hydroxylated derivatives which can pass into the egg. Besides the excretion of aflatoxin (R a d o v i ć, 1997), there is also a possibility of OTA, ZON and T-2

toxin elimination in eggs, mostly in the yolk and to a smaller extent in the egg white (F u c h s and H u l t, 1992).

*Grains.* Given that cereals are the main source of carbohydrates in the diet of people around the world, they represent the main source of mycotoxins in food. Cereals can easily be infested with fungi in various stages of production and storage. A large number of mycotoxins can be found in cereals, and many countries have enacted legislation on maximum allowed levels (S m i t h, 1997) of mycotoxins in cereals. Corn grain is considered to be the grain most highly contaminated with mycotoxins, followed by rice, barley and wheat, oilseeds and seeds of peanuts, soybeans and sunflower. The combination of more toxins is a pressing problem for the detection of mycotoxins, as well as for the monitoring of the effects that they exert in the body of humans and animals.

### Aflatoxin B1 and human health hazard

Humans are most commonly exposed to the effects of aflatoxin in three ways:
- ingestion of food of vegetable origin (mainly corn and peanuts) contaminated with aflatoxin (AFB1),
- Ingestion of contaminated milk and dairy products, including cheese and powdered milk (AFM1) and,
- Ingestion of aflatoxin residues present in meat and meat products, as well as in eggs (to a lower degree than in the previous two ways).

A number of health disorders are developed as a result of aflatoxin ingestion; they differ based on the degree, character and intensity relative to the quantity of ingested aflatoxin, length of exposure, general health status and age of the patient.

*Hepatocellular carcinoma* is one of the most common malignant diseases, and it is the fourth most common cause of death. Early epidemiological studies indicated to the close relationship between hepatocellular carcinoma and exposure to aflatoxin B1, i.e. exposure to contaminated food. The incidence of primary hepatocellular carcinoma increases logarithmically with an increase in aflatoxin ingestion which was proven by further studies. In geographic regions where hepatocellular carcinoma is rare, AFB1 contamination in food is very low.

Aflatoxins, particularly AFB1, show an emphasized carcinogenic effect (E a t o n and G r o o p m a n, 1994). The International Agency for Research on Cancer (IARC, 1993) classified the AFB1 in the group 1 carcinogens, since the risk of human primary liver cancer is very high (H e n r y et al., 2001).

*Acute toxic hepatitis* is a disease which has been described in many geographic regions, but the highest prevalence was recorded in India. The examination performed on 674 patients in 150 cities of India showed that all patients consumed moldy corn, and that aflatoxin B1 was present in most samples in quantities of 0.25-15.6 mg / kg. Considering the fact that adults eat at least 400 g of corn daily on the territory where the survey was conducted, it can be con-

cluded that exposure to aflatoxin was above 6 mg / day. Histopathological examination of the liver of patients who died of acute toxic hepatitis revealed bile duct proliferation accompanied by periductal fibrosis and cholestasis. Aflatoxin was also detected in the tested urine of affected patients.

*Kwashiorkor* is a protein deficiency which manifests as hypoalbuminemia, generalized edema, dermatosis, enlarged fatty liver, and it is common in geographic regions where seasonal occurrence of aflatoxin is present. Aflatoxin was recorded in liver samples of 36 children who deceased due to Kwashiorkor (H e n d r i c k s e, 1985). This implies that aflatoxin is one of the possible etiological factors along with the possibility that malnutrition alters aflatoxin metabolism (D e V r i e s, 1989).

*Reye's syndrome* is a form of hepatic encephalopathy in children accompanied by fatty degeneration of parenchymal organs. Although Reye's syndrome occurs in many countries around the world and geographical link with the areas with a high risk of aflatoxin ingestion has not been observed, aflatoxin is considered as one of the etiological factors. The first hypothesis of a causal connection dates back in 1963 when the presence of aflatoxin B1 and G2 in the serum of patients suffering and dying from Reye's syndrome was recorded. Also, AFB1 was detected in the liver of 27 patients suffering from Reye's syndrome, while AFM1 was detected in only 4 patients (B r y d e n, 2007). Taking into account the research carried out in conjunction with Reye's syndrome, it was concluded that the disease etiology was multifactorial, and aflatoxin played an important role in the pathogenesis.

*T4 lymphocyte deficiency* may be caused by the presence of aflatoxins in human food because it is known that aflatoxins are mitogenic factors for T4 lymphocytes and cause symptoms related to deficiency of T4 lymphocytes (G r i f f i t s h et al., 1996).

There are exact data on the dietary intake of aflatoxins, but they are based only on available data and different models for certain given values. Average daily intake of aflatoxin B1 ranged from 2-77 ng / man or 0.4-0.6 ng AFM1/man (SCOOP, 1996).

### Ochratoxin A and human health hazard

It has been believed for a long time that ochratoxin is responsible for nephropathies and urinary tract tumors in man. High exposures to OTA, high concentration in blood serum and long half-life (35 days), as well as the deposition in the kidneys foster the development of nephrotoxicity.

OTS is significant for its relationship with Balkan Endemic Nephropathy (BEN) which is a chronic kidney disorder (Krogh et al., 1977) with lethal outcome (R a d o v a n o v i ć, 1991). BEN has an endemic character and it was recorded in rural regions of the Balkans (parts of Bosnia and Herzegovinia, Croatia, Serbia, Romania, and Bulgaria) and it is more common in woman than in man. Kidneys are smaller, tubules are degenerated, and interstitial fi-

brosis and glomerule hyalinisation are present in the cortex. Tubular function is decreased and this is one of the first clinical signs.

High levels of OTA and β-2 microglobulins in the serum characterize chronic interstitial nephropathy. Previously performed studies (R a d i ć et al., 1986) confirmed that 56.6% of tested sera from patients with nephropathy from the Western Posavina region were positive for ochratoxin A. Data from Vraca (Bulgaria), where entire families were affected with BEN, showed high concentrations of OTA in the sera samples from patients. In some North African countries, especially Tunisia, epidemiological studies show a high relationship between OTA and chronic interstitial nephropathy.

On the other hand, there are contradictory data which state that there is no connection between BEN and high OTA concentrations in serum (G r o s s o et al., 2003; Abid et al., 2003). Therefore, the question on the relationship between OTA and BEN still remains unanswered. Difficulties arise due to the seasonal presence of OTA in food, allowed limits for OTA in serum and food, qualitative and quantitative laboratory procedures for OTA determination, and still incomplete knowledge about synergistic effects of different mycotoxins. In fact, citrinin stimulates the effects of OTA and citrinin concentrations in the food samples from Vrac were 200 times higher than OTA concentrations.

With high content of OTA in food (C i e g l e r and V e s o n d e r, 1983), there was a high incidence of renal adenomas and carcinomas (R a d o v a n o - v i c et al., 1991), and there is a correlation between the occurrence of BEN and urinary tract tumors. The importance of OTA induced renal carcinomas is increased due to frequent metastases in the lymph nodes and liver in humans, and multiplication of the mammary gland – fibroadenoma in women. This is why the International Agency for Research on Cancer (IARC, 1993) classified OTA as carcinogenic potential for human population (group B).

The exact data on the dietary intake of ochratoxin do not exist, and most of the information is obtained from Europe where, unlike other regions of the world, OTA is a common food contaminant. Average daily intake of OTA was estimated to be 45 mg / kg bw / week, out of which 25 ng was from grains, 10 ng from wine and meat and only 1.5 ng from pork (WHO, 1985). According to the other data and model calculations, it was believed that the daily intake of OTA was up to 92 mg / kg bw / week in countries where the consumption of grains was significant. Data from countries that had a very low incidence of nephropathy showed that daily intake of OTA ranged 1-5 mg / kg bw / day (SCOOP, 1997).

*Zearalenone and human health hazards*

Due to its estrogenic structure, it is considered that zearalenone and/or its derivatives, especially zearalanol, cause precocious puberty in children at the age of 7-8 (P a i n t e r, 1977). Epidemiological study performed in Portorico (S a e n z d e R o d r i g u e z et al., 1985) showed that areas where

children suffer from *praecox* puberty are characterized with high concentration of estradiol and its equivalent in meat, and zearalenone and its metabolites were recorded in the blood plasma of affected children. It is believed that exposure to zeralenone occurred during pregnancy of their mothers who consumed contaminated food at the time of pregnancy. A similar phenomenon was investigated in the southeastern region of Hungary, and zearalenone concentration in the blood of patients with precocious puberty ranged from 18.9-103.5 mg / ml.

F-2 toxin can cause oestrogenisation and pseudopregnancy in women and, inhibition of the normal development of testicles in men. It is also associated with the development of prostate cancer in men, and there is a hypothesis that zearalenone can be an etiological factor not only for premature puberty but for cervical cancer as well (H s i e h, 1989). However, the International Agency for Research on Cancer (IARC, 1993) believes that there has not been data reliable enough to classify F-2 toxin as potential carcinogen in the population of people (group 3). On the other hand, the use of derivatives of zearalenone was used to relieve the symptoms of menopausal disorders.

The exact data on the dietary intake of zearalenone do not exist, except for Canada and Northern Europe. Average daily intake of F-2 toxin was estimated to be from 0.03 to 00:06 mg / kg bw / day (WHO, 1985), and 1.2-1.5 ng / day, or 0:01 to 00:02 mg / kg bw / day (E r i k s e n and A l e x a n d e r, 1998). The determined values were below PTDI of 0.2 mg / kg bw / day. However, the assumption is that the mean intake of zearalenone in Europe ranges from 1-420 ng / kg body weight (EC, 2003).

*T-2 toxin and human health hazard*

Trichothecene toxicity mechanisms reside on strong inhibition of protein synthesis, and this results in a set of negative effects to human health.

Alimentary toxic aleukia (ATA) is a disorder characterized by necrotic angina, hemorrhagic diathesis and sepsis accompanied by granulocitosis as a result of bone marrow atrophy. The disease had fatal consequences in 80% of cases (J o f f e, 1978). The disease is caused by the ingestion of cereals contaminated with molds of the *Fusarium* genus (*F. poae and F. sporotrichoides*).

Nowadays, ATA is considered to be largely eradicated disease since there is a high exposure of large number of people to trichothecene mycotoxins. In addition, the International Agency for Research on Cancer (IARC, 1993) classified T-2 toxin as carcinogenic potential for the population of people (group 3).

In addition to ATA, diseases related to ingestion of food contaminated with *Fusarium* molds and / or T-2 toxin (C a n a d y et al., 2001) appeared sporadically in the second half of the twentieth century, mainly in Asia (Japan, Korea, China and India, Kashmir). The disease occurs shortly (5-30 min, max 1 hour) after the ingestion of contaminated food (0.2-0.8 mg T-2/kg) mainly wheat, and it is followed by nausea and abdominal pain (100%), irritation of

the mouth and larynx (63%), diarrhea (39%), melena (5%), vomiting (7%). Unlike ATA, none of the diseases had fatal consequences.

Based on the results of analysis performed on food for humans, the average daily intake of T-2 toxin was estimated to be 7.6 ng / kg bw (WHO, 1985). In any respect, more detailed studies need to be performed in different regions of the world, especially in European countries in order to obtain more accurate information about the overall intake of this toxin in humans.

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# МИКОТОКСИНИ И ЊИХОВ ЕФЕКАТ НА ЉУДСКО ЗДРАВЉЕ

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Резиме

Здравствени ризици повезани са конзумацијом производа контаминираних микотоксинима су препознати у целом свету. У извесној мери присуство малих количина микотоксина у житарицама и другим производима је очекивано и неминовно. То изискује процену ризика од стране регулаторних тела која морају имати улогу у успостављању регулаторних смерница за заштиту јавног здравља. Након процене нивоа микотоксина у храни потребно је осмислити одговарајуће стратегије за управљање ризиком. Неколико важних фактора треба узети у обзир при доношењу одлука о рационалном управљању ризиком, укључујући токсиколошке податке, нивое изложености, доступност аналитичких процедура, социоекономске факторе и националну легислативу.

КЉУЧНЕ РЕЧИ: болест, имуносупресија, микотоксикозе, микотоксини, законска регулатива, здравље људи

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# CORRELATION BETWEEN THE LIMIT VALUES OF LABORATORY AND CLINICAL MYCOTOXICOSIS

ABSTRACT: Analysis of feed for the presence of fungi and mycotoxins is a request necessary to meet in order to ensure a healthy and economical production in livestock. These tests are related to legal regulation which prescribes the maximum legislated content (MLC), both for the presence of mycotoxins and the total number of fungi in certain feeds. Health problems that can occur during the production of animals are sometimes caused by the presence of mycotoxins in the feed. Laboratory testing is a good practice to confirm a suspicion, and allows timely treatment of contaminated feed.

Potential problems arise under circumstances when there is a clinical outcome of mycotoxicosis and animal and laboratory findings suggest that the obtained values are below the level that is within the MLC. For these reasons, the subject of our research was to investigate the occurrence of mycotoxins and mold in feed, as well as the clinical presentation for animals that were fed with the feed with allowed values of these agents according to the recommended levels. The aim of this paper was to highlight the problems associated with clinical correlation of sick animals and laboratory findings, and suggest their overcoming.

In the period of one year, a total of 176 samples of feed (complete mixture for broilers, corn and soy products) were examined for the presence of fungi, 106 samples were examined for the presence of mycotoxins and 26 flocks of broilers and turkeys were clinically observed. Standard methods were used for isolation of molds and the ELISA test was used for the detection of mycotoxins. Clinical and pathomorphological observation of the flocks was done to determine the natural indicators of production.

Studies indicated a problem because clinical and pathomorphological findings in some cases were not correlated with laboratory findings of molds and mycotoxins in the feed, and in some cases it did not necessarily mean that the animals were healthy. Synergism and cumulative effects of mycotoxins, on the one hand, and the characteristics of each species and product category on the other hand, can create specific circumstances that can lead to disease and can increase even though the values prescribed by legislation have been met.

KEY WORDS: clinical findings, feed, molds, mycotoxins

## INTRODUCTION

Modern animal production requires the implementation of optimal zoot-echnical measures and the use of safe feed. Feeds are especially worth atten-

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tion because of their nutritional importance. As a source of essential biological and minerals, it can be a vector of transmission of various microorganisms and their metabolites. Feed can be contaminated at various stages of production and processing, and the diversity of present microorganisms depends on some of its properties, water activity, presence of oxygen, pH and nutritional potential (M a c i o r o w s k i et al., 2007). The most important factors that contribute to the presence or production of mycotoxins in feed are often related to storage and environmental conditions which, under the control of people, can be reduced (D u a r t e et al., 2011). Presence of molds and mycotoxins in foods indicates that the contamination occurred at some point in the production of feed, and that such feed is a threat to the health of animals. Mycotoxins as secondary metabolites of molds (A v a k u m o v i ć et al., 2007) require special attention when the quality of the feed is considered. Most commonly, mycotoxins enter the body through the digestive tract during the process of eating contaminated feed, but the possibility of inhalation of toxic spores and direct dermal contact should also not be ignored (Z i n e d i n e, M a ñ e s, 2009).

The legislation specified the method for determination of the presence of fungi and mycotoxins in feed and set the threshold limit value of certain toxins in animals and different product categories (Službeni Glasnik, 2010). Potential problems arise under circumstances when there is a clinical picture of mycotoxicosis in animals but laboratory findings suggest that the obtained values are below MLC. 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 of time (P e t - t e r s o n, 2004). For these reasons, the subject of our research was to investigate the occurrence of mycotoxins and mold in feed, as well as the clinical results of animals which consumed the feed with allowed values of these agents. The aim of this paper was to highlight the problems associated with clinical correlation of sick animals and laboratory findings, and to suggest their overcoming.

## MATERIAL AND METHODS

During a one year long period (autumn, 2011 – autumn, 2012) we examined a total of 176 samples of complete feeding mixtures for broilers and young turkeys used in the diet for fattening poultry and turkeys. This included a total of 103 mixtures for fattening broilers and young turkeys, 29 samples of corn and 54 soybean products (meals and cakes). Isolation of fungi was performed according to the methodology defined by international standards (EN ISO 21527-2:2011). Tests for the presence of mycotoxins in the feed used for young birds were carried out on 106 samples. It included 45 samples of mixtures for chickens and turkeys, 47 corn samples and 14 samples of corn and soybean products. Content of total aflatoxins, ochratoxin A, deoxynivalenol and T2 toxin were determined by the enzymatic immunoaffinity (ELISA) method,

using Ridascreen® test kits (R-Biopharm, Germany) (J a k š i ć et al., 2012). Methods are verified according to the European requirements (E C, 2006b).

Clinical examinations were done in 11 turkeys from fattening flocks (But Big-6) and 15 flocks of broilers (Ross, Cobb), for which standard method of clinical diagnosis, observation, pathoanatomical overview and insight into the production indicators (quantity and length of feed intake, feed conversion and mortality) was done (A v a k u m o v i ć et al., 2007; P a l i ć et al., 1994).

## RESULTS AND DISCUSSION

In Table 1 the results of feed tested for the presence of mold are summarized. During the experiment, the complete feeding mixtures for broilers and turkeys, and corn and soybean products were analyzed.

Tab. 1 – Distribution of feed samples according to mold counts and the number of faulty samples

| Feeds | No. feed samples in the given values | | | | number of faulty feed |
|---|---|---|---|---|---|
| Complete feeding mixtures | $<10^2$ | $10^2$ | $10^3$ | total | |
| Turkeys | 6 | 4 | 5 | 15 | 0 |
| Broilers | 33 | 7 | 48 | 88 | 10 |
| Total | 39 | 11 | 53 | 103 | |
| *Corn* | 0 | 5 | 24 | 29 | 0 |
| Soybean products (meals, cakes) | 31 | 11 | 12 | 54 | 0 |

Table 1 shows that a small number of feed was contaminated with mold in amounts that exceeded the MLC. *Fusarium*, *Aspergillus* and *Penicillium* were the most common species of fungi isolated in the examined feed, which is in accordance with the findings of other authors (B i n d e r, 2007), and the *Fusarium* species were the most common (over 30%). This finding corresponds to *Fusarium* results obtained from the studies conducted in different countries (Z i n e d i n e et al., 2007). Out of all tested feeds, complete mixture for fattening turkeys did not contain molds above the MLC while in the mixture for broilers 10 (11.36%) out of 88 nutrients examined were inadequate. In case of corn and soybean products, it can be seen that the tested samples of corn and soya products were within the acceptable limits for molds, according to the current Rulebook. Our findings are in agreement with the results of other researchers who have concluded that a small number of contaminated mixtures with mold is a sign of good quality feed. Satisfactory finding in soy products indicated that the heat treatment caused a significant decrease in mold (A s t o r e c a et al., 2011).

It is important to note that a significant number of samples, over 50% samples of feed for broilers and 60% samples of feed for turkeys, contained mold counts within the acceptable limits, but for the rest of the tested samples we could not claim that they were entirely free from mold because the detec-

tion threshold of the methods applied was <$10^2$. Similar observations are valid for the examined samples of corn and soybean products.

Table 2 shows the data related to the presence of mycotoxins in feed-stuffs which were studied for the presence of molds.

Table 2 also contains data for the levels of certain mycotoxins that are allowed by our legislation and regulatory legal acts of the European Union (Službeni Glasnik, 2010, E C , 2003, E C , 2006a). Legal regulations from our country set the allowed levels for the mixtures that are used for feeding of young animals, and the levels allowed according to the EU regulation are given only for aflatoxin while for other mycotoxins only maximum limits are defined.

Tab. 2 – Mycotoxin content in feed samples in comparison to legislated values
Results of the tests of complete mixtures

| Complete feeding stuffs for broilers and turkeys | | Maximum legislated mycotoxin content (μg/kg) | | | |
|---|---|---|---|---|---|
| | | Aflatoxins | Ochratoxin A | Deoxynivalenol | T2 toxin |
| Serbian regulations* | | 10 | 1000 | 300 | |
| EU directive** | | 10 | 100 | 5000 | |
| Detection limit | | <2 | <1 | <74 or <222 | |
| No. of samples | | | | | |
| Total number of samples | 45 | 44 | 37 | 19 | 34 |
| Number of faulty samples | | 1 (2 close to the limit) | 0 | 2 | 0 |
| | | | | 2 | |
| | | | | 0 | |
| Maize | | Maximum legislated mycotoxin content (μg/kg) | | | |
| | | Aflatoxins | Ochratoxin A | Deoxynivalenol | T2 toxin |
| | | 50 | | | |
| EU directive** | | 20 | 250 | 12000 | |
| No. of samples | | | | | |
| Total number of samples | 47 | 39 | 35 | 43 | 31 |
| Number of faulty samples | | 2 | 0 | 0 | 0 |
| Soybean products (meals and cakes) | | Maximum legislated mycotoxin content (μg/kg) | | | |
| | | Aflatoxins | Ochratoxin A | Deoxynivalenol | T2 toxin |
| Serbian regulations* | | 50 | | | |
| EU directive** | | 20 | 250 | 8000 | |
| No. of samples | | | | | |
| Total number of samples | 14 | 12 | 8 | 10 | 6 |
| Number of faulty samples | | 0 | 0 | 0 | 0 |

* S l. G l a s n i k  R S, br. 4/2010 Pravilnik o kvalitetu hrane za životinje, čl. 99. (Sr)
** E C ( E u r o p e a n  C o m m i s i o n ) (2003, 2006a)

Table 2 gives data on the presence of mycotoxins in the samples of complete mixtures for broilers, corn and soybean products. It can be seen that only 3 full feed mixtures for broilers meal and 2 samples of corn were inadequate regarding the allowed levels of mycotoxins, while the other samples were within the set limits.

The results of laboratory tests indicated that the number of feeds that were not appropriate according to the allowed levels was not large; however, direct production, and fattening can lead to the problem when the symptoms of mycotoxicosis exist in spite of using the feed that meets legal requirements. The significance of the obtained results can be observed from the aspect of impact of small concentrations of mycotoxins and their synergistic effects on individuals (T a m m e r a et al., 2007).

From the 15 broiler flocks monitored, and 11 flocks of fattening turkeys, clinical and pathomorphological findings revealed changes that correspond to mycotoxicosis in 4, i.e. 2 flocks, respectively. Data on clinical, pathomorphological findings and production indicators pointed to the problems caused by mycotoxins (A v a k u m o v i ć et al., 2007; P a l i ć et al., 1994). Clinical symptoms observed in the specimens included drowsiness, contamination of feathers around the cloaca, ataxia and necrotic lesions on the hard palate, tongue and mucous membrane of the turn of the horny beak. Touching of these points was reported to cause pain in the animals. Pathomorphological changes were characterized with prominent blood vessels, carotid, dark colored anticoagulated blood, atrium overfilled with blood, bleeding, subcapsular liver with rounded edges. Natural indicators have pointed to changes in mortality, prolonged period of fattening, the altered feed conversion efficiency and lower weight in the final stage of fattening. Our findings are consistent with the results reported in the works of other authors (K a p e t a n o v et al., 2012).

Necrotic lesions in the oral cavity led to a prolonged period of food and water consumption, and feed conversion was increased because more food was needed to gain 1 kg. Mortality in flocks was increased and the feeding was extended. Laboratory tests and identified health problems on the ground indicated that mycotoxins usually did not cause acute illnesses, and if it did occur it was usually due to the multiple interactions of different factors which could cause the toxicity of these substances (M o r g a v i and R i l e y, 2007). The influence of toxins can be synergic (K u b e n a et al., 1997) or depend on the amount of mycotoxins present (D ö l l and D ä n i c k e, 2011), and their cumulative effect can cause health problems.

CONCLUSION

The results obtained in the course of this research suggest that the problem of the presence of fungi and mycotoxins in the feed cannot be approached only from the aspect of their allowed limits, but the harmful effect on the health of animals and subsequent consequences should be considered as well. It is obvious that mycotoxins pose numerous threats to different types of animals

and products. Also, in addition to laboratory tests which should be practiced in order to avoid mistakes by relying only on the given threshold, clinical findings and data on pathoanatomical and production indicators are necessary steps. Results of the laboratory tests of feed point to the fact that even if the nutrient values seem to be appropriate, the health and production problems in animals can still be reported.

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## КОРЕЛАЦИЈА ГРАНИЧНИХ ВРЕДНОСТИ ЛАБОРАТОРИЈСКИХ НАЛАЗА И КЛИНИЧКЕ СЛИКЕ МИКОТОКСИКОЗА

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Резиме

Анализа хране за животиње на присуство микотоксина и гљивица је захтев који је непходно испунити како би се обезбедила здрава и економична производња у сточарству. Могући проблеми настају у околностима када се јавља клиничка слика микотоксикоза код животиња а лабораторијски налаз указује да су добијене вредности испод граничних нивоа, односно у оквиру дозвољених вредности. Из наведених разлога предмет нашег истраживања је праћење присуства микотоксина и плесни у храни за животиње, као и приказ клиничке слике које се јавља код животиња које су конзумирале храну са дозвољеним вредностима ових агенаса према правилнику. Циљ рада је да истакне проблеме везане за корелацију клиничке слике болесних животиња и лабораторијских налаза и како их превазићи.

У периоду од једне године укупно је прегледано 176 узорака хране за животиње (потпуне смеше за тов подмлатка живине, кукуруза и производа од соје) на присуство плесни, 106 узорака на присуство микотоксина и опсервирано је 26 јата бројлера и ћурића. За испитивања су коришћене ИСО методе за изолацију плесни и ELISA тест за доказивање микотоксина. Јата су прегледана клиничким и патоморфолошким методама и одређивањем натуралних показатеља производње.

Истраживања су указала на присутан проблем, да клинички и патоморфоло-шки налаз у неким случајевима не мора бити у корелацији са лабораторијским на-лазом плесни и микотоксина у хранивима као и да дозвољене вредности присуства ових материја у неким случајевима не представљају сигурност за здравље животи-ња. Синергизам и кумулативно дејство микотоксина са једне стране и карактери-стике појединих врста животиње и производних категорија са друге стране, могу створити специфичне околности које ће довести до појаве болести и онда када су задовољене вредности прописане правним регулативама.

КЉУЧНЕ РЕЧИ: клиничка слика, микотоксини, плесни, храна за животиње

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# SCREENING OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF SELECTED MACEDONIAN WILD MUSHROOMS

ABSTRACT: Regarding the development of novel safe antimicrobials of natural origin, macrofungi became attractive for the researchers in the last decade. In this study, antimicrobial potential of methanolic extracts of six wild macromycetes (*Boletus lupinus, Flammulina velutypes, Phellinus igniarius, Sarcodon imbricatus, Tricholoma aurantium, Xerocomus ichnusanus*) was evaluated. *In vitro* antimicrobial activity was investigated by the microdilution method and minimum inhibitory concentration (MIC) was determined. Testing was conducted against eleven microorganisms, including six strains of bacteria and five species of fungi. Extracts showed selective antimicrobial properties while the activities depended both on the species of microorganism and on the type and concentration of extract. The evaluated extracts demonstrated antimicrobial activity, exhibiting more potent inhibitory effects on the growth of bacteria than on fungi. The highest antibacterial and antifungal activity was observed in methanolic extract of polypore fungus *P. igniarius*.

KEY WORDS: Microdilution method, antimicrobial activity, mushrooms, Macedonia

## INTRODUCTION

Antibiotic resistance has become a global concern in recent years (W e s t h et al., 2004). Although a number of natural and synthetic antimicrobial agents have been isolated or synthesized against pathogenic microorganisms, infectious diseases remain one of the major threats to human health. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microorganisms has led to the screening of novel sources for their

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potential antibacterial and antifungal activity (C o l o m b o and B o l s i s i o, 1996; I w u et al., 1999). The fact that basidiomycetes have been insufficiently investigated, together with a variety of structural types of antibiotics which are produced by these organisms, suggests that they may be a source of new and useful bioactive compounds (A n k e, 1989). As a matter of fact, macro-fungi need antibacterial and antifungal compounds to survive in their natural environment. Therefore, antimicrobial compounds could be isolated from many mushroom species and could be of benefit for humans. Bioactive mol-ecules have been isolated not only from edible, but also from inedible species (Q u a n g et al., 2006). Reported bioactivities of mushrooms include antibac-terial, antifungal, antioxidant, antiviral, anti-tumor, cytostatic, immunosup-pressive, antiallergic, antiatherogenic, hypoglycemic, anti-inflammatory and hepatoprotective activities (W a s s e r and W e i s, 1999; L i n d e q u i s t et al., 2005). The responsible bioactive compounds belong to several chemical groups which are often polysaccharides or triterpenes (K i m et al., 2000; S u n and L i u, 2009). One macrofungi species can have various bioactive com-pounds and pharmacological effects (L i n d e q u i s t et al., 2005).

Generally, although many antimicrobial compounds have been isolated from mushrooms, the antimicrobial compounds from microscopic fungi still dominate as antibiotics on the market. Thus, the aim of this study was to ex-amine *in vitro* antimicrobial activity of methanolic extracts from selected macromycetes: *Boletus lupinus, Flammulina velutypes, Phellinus igniarius, Sarcodon imbricatus, Tricholoma aurantium, Xerocomus ichnusanus.*


## MATERIAL AND METHODS

### *Fruiting body selection*

Samples of the wild macromycetes *Boletus lupinus, Flammulina velu-types, Phellinus igniarius, Sarcodon imbricatus, Tricholoma aurantium, Xe-rocomus ichnusanus* were collected from different locations in Macedonia, in autumn 2011. Geographical location and natural habitat of the mushroom specimens are shown in Table 1. Taxonomic identification was done in Myco-logical Laboratory at the Institute of Biology, Faculty of Natural Sciences and Mathematics  in Skopje, by implementing standard methods of microscopic and chemical techniques (coloring of fruit bodies and spores), and appropriate literature (A l e s s i o, 1985; B e r n i c c h i a, 1990; B r e i t e n b a c h and K r ä n z l i n, 1986, 1991, 1995; D a n k e, 2004; F e r n á n d e z, 1997; H o r a k, 2005; J a h n, 1990; J ü l i c h, 1984; K n u d s e n and V e s t e r h o l t, 2012; K r i e g l s t e i n e r, 2000a, 2000b, 2001; R y v a r d e n, 1991;

R y v a r d e n and G i l b e r t s o n, 1994). The representative voucher specimens were deposited in Macedonian Collection of Fungi (MCF) at the Institute of Biology.

Tab. 1. – Geographical Location and Natural Habitat of the Mushroom Species Studied for Antimicrobial Potential

| mushroom species | natural habitat | geographical location |
|---|---|---|
| *Boletus lupinus* | mycorrhizal (on ground in open oak forest) | Galichica Mt. (Ohrid side) |
| *Flammulina velutypes* | saprotrophic (on stump of deciduous trees) | Botanical garden, Skopje |
| *Phellinus igniarius* | parasitic (on living willow trunks) | Vicinity of the town of Kumanovo |
| *Sarcodon imbricatus* | saprotrophic (on soil in conifer forest) | Suva Gora Mt. |
| *Tricholoma aurantium* | mycorrhizal (on ground in pine forest) | Suva Gora Mt. |
| *Xerocomus ichnusanus* | mycorrhizal (on ground in open oak forest) | Galichica Mt. (Prespa side) |

## Preparation of methanolic extracts of mushrooms

The fruiting bodies were cleaned to remove any residual compost/soil and subsequently air-dried in the oven at 40°C. Dried specimens were ground to fine powder and extracted by stirring with 80% (v/v) methanol in ultrasonic bath for 30 min at 4°C, and then centrifuged at 12000 rpm for 15 min. Supernatants were used for the evaluation of antimicrobial potential of the samples. The organic solvent in the extracts was evaporated until dry, under vacuum. The yields of methanolic extracts of the fruiting bodies are presented in Table 2.

Tab. 2. – Yield of Mushroom Methanolic Extracts

| sample | mushroom species | yield of extracts[a] (g/100 g of dry mushroom) |
|---|---|---|
| 1 | Boletus lupinus | 37.67 ± 4.04 |
| 2 | Flammulina velutypes | 30.00 ± 0.00 |
| 3 | Phellinus igniarius | 2.20 ± 0.17 |
| 4 | Sarcodon imbricatus | 39.00 ± 1.15 |
| 5 | Tricholoma aurantium | 36.67 ± 1.15 |
| 6 | Xerocomus ichnusanus | 38.50 ± 2.12 |

[a] Each value is the mean of three replicate determinations ± standard deviation.

## In vitro *antimicrobial assay*

### Test microorganisms

Antimicrobial activities of methanol extracts were tested against 11 microorganisms, including 6 strains of bacteria: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus pumilus* NCTC 8241, *Sarcina lutea* ATCC 9341 and *Bacillus subtilis* ATCC 6633, and 5 species of fungi: *Saccharomyces cerevisiae* ATCC 16404, *Candida albicans* ATCC 10231, *Aspergillus niger* I.N. 1110, *Aspergillus sojae* CCF and *Penicillium* spp. FNS FCC 266.

The microorganisms were provided from the collection held in the Microbiology Laboratory, Faculty of Natural Sciences and Mathematics in Skopje.

### Suspension preparation

Microbial suspensions were prepared in accordance with the direct colony method. The turbidity of initial suspension was adjusted by comparison with 0.5 McFarland's standard (A n d r e w s, 2005). The initial suspension contained about $10^8$ colony forming units (CFU)/mL. Additionally, 1:100 dilutions of initial suspension were prepared in sterile 0.9% saline.

### Microdilution method

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) using the microdilution method with resazurin, an indicator of microbial growth (Sarker et al., 2007). The 96-well plates were prepared by dispensing 50 µL of nutrient broth, Mueller-Hinton broth for bacteria and Sabouraud dextrose broth for fungi in each well. A volume of 50 µL from the stock solution of tested extracts (concentration: 100 mg/mL for mushroom specimens *Boletus lupinus, Flammulina velutypes, Sarcodon imbricatus, Tricholoma aurantium, Xerocomus ichnusanus* and 20 mg/ml for polypore fungus *Phellinus igniarius*) was added into the first row of the plate and then two-fold serial dilutions of extracts were performed. The obtained concentration range was from 50 mg/mL to 0.025 mg/mL for extracts of *Boletus lupinus, Flammulina velutypes, Sarcodon imbricatus, Tricholoma aurantium, Xerocomus ichnusanus*, and from 10 to 0.0049 mg/mL for *Phellinus igniarius*. MIC was defined as the lowest concentration of the tested extracts that prevented a resazurin color change from blue to pink.

The tested extracts were dissolved in 10% (v/v) DMSO in sterile water. A solvent control test was performed to study the effect of DMSO on the growth of microorganisms. The test proved that DMSO had no inhibitory effect on the tested organisms.

Each test plate included growth control and sterility control. All tests were performed in duplicate and MIC values were constant.


RESULTS AND DISCUSSION

*In vitro* antimicrobial activity was investigated by the microdilution method and MIC was determined. The data relating to the antimicrobial activities of fruit body samples is summarized in Table 3. Antimicrobial activity was observed in all species included in the study. It was detected that all of them had antimicrobial activity against at least four of the test microorganisms employed. Most of these activities were antibacterial.

Tab. 3. – Minimum inhibitory concentration (MIC)a of methanolic extracts from mushroom samples.

| Test organisms | B. lupinus | F. velutypes | P. igniarius | S. imbricatus | T. aurantium | X. ichnusanus |
|---|---|---|---|---|---|---|
| | | | Samples | | | |
| B. subtilis | 6.25 | 12.5 | 2.5 | 6.25 | 3.125 | 6.25 |
| B. pumilus | 25 | 3.125 | 5 | 25 | 12.5 | 12.5 |
| S. aureus | 50 | 50 | 5 | 25 | 25 | 12.5 |
| S. lutea | 6.25 | – | 2.5 | 12.5 | 12.5 | 6.25 |
| P. aeruginosa | 25 | 50 | 5 | 25 | 12.5 | 25 |
| E. coli | – | – | 10 | 50 | 50 | 50 |
| A. niger | – | – | – | – | – | – |
| A. sojae | – | – | – | 50 | – | – |
| Penicilium spp. | – | – | 10 | – | 50 | 50 |
| S. cerevisiae | – | – | – | – | – | – |
| C. albicans | – | – | – | – | – | – |

[a] Minimum inhibitory concentration (MIC); values given in mg/mL.
(–) No inhibition

Results from this study showed that for all examined extracts no antimicrobial activity was recorded against the tested yeasts, *S. cerevisiae* and *C. albicans,* and the mold *A. niger.* The highest antibacterial activity was obtained in the extract from polypore fungus *P. igniarius* against *B. subtilis* and *S. lutea* (MIC=2.5 mg/mL). According to our results *P. igniarius* exhibited inhibitory effect against all tested bacterial strains with MIC values ranging from 2.5 to 10.0 mg/mL. Also, the highest antifungal activity observed in this study was in methanolic extract of *P. igniarius* (MIC=10.0 mg/mL) against *Penicillium* spp. Those results are in accordance with earlier reported data which confirmed the antimicrobial activity of this *Phellinus* species (S i t t i - w e t and P u a n g p r o n p i t a g, 2008; B a l a k u m a r et al., 2011).

It is also evident from these results that the Gram-positive bacteria (*Bacillus subtilis*, *Bacillus pumilus*, *Sarcina lutea* and *Staphylococcus aureus*) were more sensitive to the examined macrofungi extracts. The results from our study are in agreement with literature data which showed that the bacteria were more sensitive to the antimicrobial agents compared to fungi (H u g o and R a s s e l l, 1983). This observation may be explained by the differences in the cell wall structure that can produce differences in antibiotic susceptibility of the cells (W a l s h and A m y e s, 2004). It is well known that Gram-negative bacteria possess an outer membrane and a periplasmic space, both of which are absent from Gram-positive bacteria (B a s i l e et al., 1998).

The fungal cell wall structure might also be the reason for relatively high resistance towards antimicrobial agents, which was observed in microfungal species tested in this study. The results from this study show that, apart from *P. igniarius* extract, mushroom extracts inhibited the tested fungi in relatively high concentration of 50 mg/ml. The explanation might be found in protein–polysaccharides and amorphous homo- and heteropolysaccharides

which are identified as constituents of the fungal wall matrix that, among other functions, cement the skeletal portion of the wall, render the elasticity, hydrophilic properties, and determine the antigenicity of the cells (F a r k a š, 2003).

## CONCLUSION

The current study was undertaken to measure the antimicrobial potential of methanolic extracts from fruiting bodies of six wild macromycetes (*Boletus lupinus, Flammulina velutypes, Phellinus igniarius, Sarcodon imbricatus, Tricholoma aurantium, Xerocomus ichnusanus*). Results from this study showed that all analyzed mushroom extracts exhibit antimicrobial activity. With an increasing number of bacteria that have developed resistance to commercial antibiotics, extracts and derivatives from mushrooms hold great promise for novel medicines. Regarding the development of natural antimicrobials from macrofungi, further research could be focused on the identification and quantification of individual antimicrobial compounds in the selected mushroom extracts.

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# СКРИНИНГ АНТИБАКТЕРИЈСКЕ И АНТИФУНГАЛНЕ АКТИВНОСТИ СЕЛЕКТИРАНИХ ГЉИВА ИЗ МАКЕДОНИЈЕ

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## Резиме

Тренд развоја нових безбедних антибиотика природног порекла у последњој деценији је разлог да макромицете постану атрактивне за истраживаче. У овој студији спроведена је евалуација антимикробног потенцијала метанолних екстраката добијених из шест врста макромицета (*Boletus lupinus, Flammulina velutypes, Phellinus igniarius, Sarcodon imbricatus, Tricholoma aurantium, Xerocomus ichnusanus*). *In vitro* антимикробна активност испитана је микродилуционом методом и одређена је минимална инхибиторна концентрација (МIC) примерка. Тестирање је спроведено на једанаест микроорганизама, укључујући шест сојева бактерија и пет врста гљива. Екстракти су показали селективне антимикробне карактеристике. Антимикробна активност је зависила од врсте микроорганизма и од типа и концентрације екстракта. Анализовани екстракти показали су моћније инхибиторне ефекте на раст бактерија него на гљиве. Најизраженија антибактеријска и антифунгална активност је утврђена у метанолним екстрактима врсте *P. igniarius*.
КЉУЧНЕ РЕЧИ: микродилуциона метода, антимикробна активност, гљиве, Македонија

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# INVASIVE ZOOPATHOGENIC *MASTIGOMYCOTINA* IN REPUBLIKA SRPSKA, SERBIA AND NEIGHBORING COUNTRIES WITH SPECIAL REFERENCE TO *APHANOMYCES ASTACI*

ABSTRACT: Non-native alien zoopathogens have had a profound impact on the health of aquatic zoobiota in Republika Srpska and the Republic of Serbia as well as around the region and the world as a whole. The movement of disease-causing microfungi around the world has taken its toll on many different organisms, and continues to intensify with an increase in global transport of cargo, people, and animals. Those who study fungi have little information about the characteristics of invasive, zopathogenic fungi because of the fact that a particular fungus can inhabit many different organisms, and may be pathogenic to some of these hosts but not to the others. Crayfish plague (*Aphanomyces astaci*) broke out through the Danube River to the territory of Bačka and Banat (today A.P. Vojvodina, the Republic of Serbia) in 1879. Until 1880, the disease spread through the Sava River to the northern Bosnia (today Republika Srpska). From 1955 until 1970, almost all territories of Republika Srpska and Republic of Serbia were afflicted with the crayfish plague disease. Despite the fact that the problems of the spread of crayfish plague in the territory of the Republika Srpska and the Republic of Serbia have been of great significance, there is little data available for analyzing this issue. According to IUCN criteria, and mostly due to the *Aphanomyces astaci* invasion, the degree of endangerment of noble crayfish in Serbia was evaluated as "Endangered", which was a higher degree than the international level determined for this species, i.e., "Vulnerable".
KEY WORDS: fungi, invasive, *Aphanomyces astaci*, Republika Srpska, the Republic of Serbia

# INTRODUCTION

The commonly used definition of biological invasions, in scientific communities, implies negative ecological impact. The term 'invasive' covers any non-indigenous species that has spread and become abundant in a new geographical location regardless of the actual ecological impact (P e t i t et al., 2004; D e s p r e z-L o u s t a u et al., 2007). By using this definition, it can be concluded that fungi with economic, but unknown ecological impact, should be regarded as invaders (Tab. 1).

Successful invaders can be defined as species able to pass through a series of filters corresponding to the successive steps of the invasion: transport, establishment and spread (K o l a r and L o d g e, 2001; R i c h a r d s o n et al., 2000; S a k a i et al., 2001). Unlike plant and animal invasions, deliberate introductions have probably had a minor role in fungal introductions. Desirable fungi that were introduced in new habitats mostly included pathogenic fungi used for classical biological control (CBC). Few examples of fungal invasions, resulting from the escape of purposefully introduced pathogenic fungi, were reported (S e l o s s e et al., 1998).

Tab. 1. – Potential or reported ecological interactions among zoopathogenic *Mastigomycotina*

| Interaction | Result | Examples | References |
|---|---|---|---|
| Specialist pathogen (relatively) in natural ecosystem; host–pathogen not coevolved (host jump) | Emerging disease | Crayfish plague *Aphanomyces astaci* (*Saprolegniaceae, Oomycota*) (Europe), carried by introduced North American crayfish, with chronic infections, transmitted to native European crayfish | D a s z a k et al., 2000; A l d e r m a n, 1996, |
| Generalist pathogen in natural ecosystem; host–pathogen not coevolved (host jump) | Emerging disease | Frog chytrid *Batrachochytrium dendrobatidis* (*Chytridiomycota, Chytridiomycetes, Rhizophydiales*) (North America and Europe) | D a s z a k et al., 2000; B r i g g s et al., 2010; R a c h o w i c z et al., 2005; F i s h e r et al., 2009; M o r e l, 1999 |
| Animals that interact with native and invasive fungal parasites | Vectors of exotic and native parasites; increase in population of native parasites | *Saprolegnia ferax*, a pathogen of amphibians, is greater in toad population exposed to hatchery-reared trout; Exotic reptiles can serve as vectors of *Basidiobolus ranarum* (causes basidiobolomycosis in humans, horses and other vertebrates) | R i z z o, 2005; W e b b e r, 1990 |

With the exception of a few well known examples of invasive animal pathogens, microfungi are generally poorly represented in date bases of invasive or alien species. Emerging infectious diseases can be defined as diseases caused by pathogens showing new or increased geographical range, new host range or caused by new or recently evolved pathogens. Fungi is the important

Tab. 2. – Fungi in available databases of invasive species (D e s p r e z-L o u s t a u et al., 2007)

| Region | List | Fungi | Source |
|--------|------|-------|--------|
| Austria | Neobiota (invasive) | 83 | E s s l and R a b i t s c h (2002) |
| England | Non-native species | 198 | H i l l, M. et al. (2005) |
| Latvia | Alien species | 7 | http://lv.invasive.info/ |
| Lithuania | Invasive species | 98 | http://www.ku.lt/lisd/ |
| Nordic–Baltic states | Alien species | 98 | http://sns.dk/nobanis/ |
| Poland | Alien species | 81 | http://www.iop.krakow.pl/ias/ |
| Switzerland | Alien species (invasive) | 6 | Wittenberg R (2005) |
| World | Global invasive species (worst) | 9 | http://www.issg.org/database/welcome/ |

taxonomic group, responsible for emerging infectious diseases in general, and introduced pathogens have also been recognized as an important cause of emerging diseases in animals, although fungi are less present among animal pathogens (D a s z a k et al., 2000; D o b s o n and F o u f o p o u l o s, 2001). (Tab. 2). The most obvious impacts of fungal invasions were epidemics (zoonoses) caused by exotic pathogenic fungi. It was estimated that 65–85% of pathogens worldwide were alien at the location where they were recorded (P i m e n t e l et al., 2001). Such invasions can have significant ecological, economic and social consequences.

Despite of the fact that these species exist in neighboring countries (Table 3) or in more remote European countries (Table 4), in Republika Srpska and Bosnia and Herzegovina, just a few species were registered as invasive, such as ascomycete *Cryphonectria parasitica* (Murrill) Barr., nominated as one of "World's worst" invaders (http://www.issg.org/database/species/ecology.asp?si=124&fr=1&sts=sss &lang=EN). Even though "fungal" species, belonging to *Mastigomycotina* group, represents a significant cause of serious diseases on various groups of organisms, there are no evidence on this group in any of the databases, neither in Bosnia and Herzegovina, in First national Report for the Convention on Biodiversity, (Federal Ministry of Environment and Tourism of Bosnia and Herzegovina, 2008), nor in the Republic of Serbia (Table 4). However, P a v l o v i ć et al. (2006) reported about the first finding of *Orconectes (Faxonius) limosus* in the Serbian part of the River Danube, what could lead to the indirect conclusion on the replacement of almost extinct *Astacus astacus*.


## RESULTS OF ANALYSED AVAILABLE DATA

The list of aquatic animal diseases/pathogens and susceptible species that are considered for monitoring purposes with regard to the zoning and approval of an area/farm in Bosnia and Herzegovina listed *Aphanomyces astaci,* causing the well known disease crayfish plague, with no further comments or explanations (A l a g i ć, 2009).

Tab. 3 – Non-indigenous invasive species *Aphanomyces astaci* (*Oomycetes, Mastigomycotina*) in Republika Srpska and the Republic of Serbia, in neighboring and regional countries

| Country | Recent finding | Source |
|---|---|---|
| Albania | | http://www.cbd.int/invasive/doc/legislation/Greece.pdf<br>http://www.iucnredlist.org/details/2431/0 |
| Austria | | F r a n k e 1894<br>A l d e r m a n, 1996 |
| Bosnia and Herzegovina | | http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail<br>http://www.iucnredlist.org/details/153745/0<br>A l a g i ć 2009<br>A l d e r m a n, 1996 |
| Bulgaria | 2010 | H u b e n o v a et al. 2010<br>A l d e r m a n, 1996 |
| Croatia | 2012 | http://www.iucnredlist.org/details/2430/0<br>P â r v u l e s c u et al. 2012<br>A l d e r m a n, 1996<br>http://www.iucnredlist.org/details/2191/0 |
| Czech Republic | 2009<br>2011<br>2012 | K o z u b í k o v á et al. 2006, 2009<br>http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail<br>A l d e r m a n, 1996 |
| Greece | | T h e o c h a r i s 1986<br>http://www.cbd.int/invasive/doc/legislation/Greece.pdf |
| Hungary | 2010 | K o z u b í k o v á et al. 2010<br>A l d e r m a n, 1996 |
| Italy | 2009<br>2010<br>2011 | C o r n a l i a 1860<br>C a m m à et al. 2010<br>A q u i l o n i et al. 2011<br>http://www.iucnredlist.org/details/2430/0<br>http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail |
| Macedonia FYR | | Not reported till 2000<br>http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail |
| Montenegro | | No available data<br>http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail |
| Romania | 2012 | P â r v u l e s c u et al. 2012<br>S c h r i m p f et al. 2012<br>A l d e r m a n, 1996 |
| Slovakia | | http://www.iucnredlist.org/details/2191/0<br>J a n s k ý and K a u t m a n 2007<br>A l d e r m a n, 1996 |
| Slovenia | | A l d e r m a n, 1996 |
| Turkey | 2008<br>2012 | H a r l i o ğ l u 2008<br>K o k k o et al. 2012<br>B a r a n and S o y l u 1989 |

According to the OIE (2012), *Aphanomyces astaci* is a member of the group of organisms commonly known as the water molds. Although regarded to be fungi for a long time, this group, the *Oomycetes*, is now considered protists and are classified with diatoms and brown algae in a group called the *Stramenopiles* or *Chromista*. Four groups (A–D) of *A. astaci* were described based on random amplification of polymorphic DNA polymerase chain reaction (D i e g u e z - U r i b e o n d o  et al., 2009): Group A (the so called *Astacus* strains) comprises a number of strains that were isolated from *Astacus astacus* and *Astacus leptodactylus*; these strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus* strains I) includes isolates from both *A. astacus* in Sweden and *Pacifastacus leniusculus* from Lake Tahoe, the USA. Imported *P. leniusculus* have probably introduced *A. astaci* and infected the native *A. astacus* in Europe. Group C (*Pacifastacus* strains II) consists of a strain isolated from *P. leniusculus* from Pitt Lake, Canada. Another strain (Pc), isolated from *Procambarus clarkii* in Spain, falls into group D (*Procambarus* strain). This strain shows temperature/growth curves with higher optimum temperatures compared with the isolates from northern Europe (D i e g u e z - U r i b e o n d o  et al., 1995). *Aphanomyces astaci* strains that have been present in Europe for many years (group A strains) appear to be less pathogenic than strains which have been introduced more recently with crayfish imports from North America since the 1960s.

The crayfish plague is a well documented example of fungal zoonoses (Table 3, Table 4). The fungus *Aphanomyces astaci* (*Saprolegniaceae, Oomycota*), which causes the well known disease crayfish plague, was introduced to Italy, probably through ballast water discharge from North American ship in 1860 (C o r n a l i a, 1860). It was also spread to other countries: to Sweden in 1907, Spain in 1958, Norway in 1971, the United Kingdom in 1981, and Turkey in 1987 (D i é g u e z-U r i b e o n d o, 1997; O i d t m a n n et al., 1997). The plague wiped out native populations of the noble European crayfish (*Astacus astacus*) almost to the point of extinction in some countries. In the Greece's Actions on IAS report (2010), *Aphanomyces astaci* was listed as the major invasive alien species, (http://www.cbd.int/invasive/doc/legislation/Greece.pdf), and L o w e  et al., (2004) considered this fungus as one of the 100 of the world's worst invasive alien species.

The most comprehensive review on the spread of crayfish plague was presented by A l d e r m a n (1996). Importantly, crayfish plague continued to spread in Europe because American crayfish species continued to spread on the continent by natural movements or due to stocking by humans (e.g. the American signal crayfish and red swamp crayfish). The signal crayfish (*Pacifastacus leniusculus*) was introduced as a replacement in waters where *Astacus astacus* had been, or had almost been extinct, what caused new problems since signal crayfish, being immune to the disease and as a carrier of infection, increased the spread of the plague to previously unaffected populations of noble crayfish (H o l d i c h, 1988). *Aphanomyces astaci* can be transferred from one water body to the next also through fishing gear, contaminated traps, by the transfer of infected animals, and probably even by predators

Tab. 4 – Non-indigenous invasive species *Aphanomyces astaci* (*Oomycetes, Mastigomycotina*) in Republika Srpska and the Republic of Serbia, and in more remote European countries

| Country | Source |
|---------|--------|
| Belarus | A l d e r m a n, 1996 |
| Finland | N y l u n d and W e s t m a n 1992<br>W e s t m a n and N y l a n d 1979 |
| France | A l d e r m a n, 1996 |
| Germany | O i d t ma n n et al. 1997<br>S c h ä p e r c l a u s 1927<br>S u r b e c k 1903<br>A l d e r m a n, 1996 |
| Ireland | A l d e r m a n, 1996<br>R e y n o l d s 1988<br>M a t t h e w s and R e y n o l d s 1990 |
| Italy | http://www.iucnredlist.org/details/2430/0 |
| Latvia | M a n n sf i e l d 1942<br>A l d e r m a n, 1996 |
| Lithuania | T z u k e r z i s 1964<br>M a z y l i s and G r i g e l i s 1979<br>A l d e r m a n, 1996 |
| Norway | V r å l s t a d, et al. 2011<br>T a u g b ø l et al. 1993<br>A l d e r m a n, 1996 |
| Spain | D i é g u e z-U r i b e o n d o 2006<br>http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail |
| Sweden | A l m 1929<br>B o h m a n et al. 2006<br>A l d e r m a n, 1996 |
| Russia | A r n o l d 1900<br>A l d e r m a n, 1996<br>H o f e r 1900 |
| Ukraine | A l de r m a n, 1996 |
| EUK | O i d t m a n n et al. 2005<br>A l d e r m a n et al. 1984<br>H o g g e r 1984<br>http://www.iucnredlist.org/details/2430/0 |

preying on crayfish (N y l u n d & W e s t m a n 1992, O i d t m a n n et al., 2005).

Beside the crayfish plague, most fungal diseases affecting freshwater crayfish are associated with poor water quality which has to be achieved by environmental management. Fungicides or general disinfectants, such as formalin, may be used for identification of causes of fungal infections. There are no cures for crayfish plague infection. Normally, 100% of susceptible crayfish species in a population will be killed after the fungus is introduced. (http://www.crayfishmates.com/crayfish_diseases/fungi.html).

The recent worldwide outbreaks of amphibian chytridiomycosis, which contributed to the population decline, might also be caused by anthropogenic

introduction of the pathogen, *Batrachochytrium dendrobatidis,* outside its endemic range (R a c h o w i c z et al., 2005).

## DISCUSSION

The first pioneering attempt in the Republic of Serbia to make provisional list of invasive fungal species was done within the Project on "Invasive alien species in Vojvodina", which lasted from 2005 to 2010, and was supported by the Republic Fond for the Environmental Protection, coordinated with the Provincial Secretariat for the Environmental Protection and Technological Development of Autonomous Province of Vojvodina. Seven fungal species were listed in this provisional list and two of them belong to *Mastigomycotina* group, but none of zoopathogenic fungi were listed in this first proposal. (http://iasv.dbe.pmf.uns.ac.rs/index.php?strana=pocetak).

Only limited reports examine the aquatic fungi in Serbia. The aquatic fungal community recorded in five large reservoirs of Serbia comprised mostly autochthonous species: *Achlya americana, A. diffusa, A. racemosa, Dictyuchus sterile, Isoachlya toruloides, Leptomitus lacteus, Pythium ultimum, Saprolegnia ferax, S. hypogyna*, *S. monica* (R a n k o v i ć, 2005). Most of them were zoopathogenic. *Leptomitus lacteus* was reported to be pathogenic for the fish spawn (K i z i e w i c z, 2004), and *Achlya americana* and *Saprolegnia ferax,* were proven to be pathogenic for some fish species (C h a u h a n et al., 2012), while *Achlya racemosa, Dictyuchus sterile,* and *Saprolegnia ferax,* had 100% frequency of occurrence amongst the infected fish (O g b o n n a and A l a b i, 1991).

Crayfish plague outbreaks were characterized by mass mortalities of noble European crayfish without any apparent effects on other aquatic organisms. Crayfish trade and fishing activities spread the plague throughout Europe covering most of the European countries (E d e r, 2004; K o z u b í k o v á et al., 2006; Š o u t y-G r o s s e t et al., 2006; V r å l s t a d et al., 2011b). According to A l d e r m a n (1996), crayfish plague broke out through the Danube River to the territory of Bačka and Banat (today A.P. Vojvodina, the Republic of Serbia) in 1879, and spread to the Drava River basin in 1881 (today Croatia). Until 1880, the disease spread through the Sava River to the northern Bosnia (today Republika Srpska), all to the Slovenian territory (1885). From 1955 until1970, almost all territories of Republika Srpska and Republic of Serbia were afflicted with the crayfish plague disease (Table 5).

Mostly due to the spread of crayfish plague, significant declines occurred in the range of *Astacus astacus* species: approximately 52% decline was recorded in England for 10 years, 52% decline in France between 1995 and 2003, and 99.5% decline was estimated for a ten year period in the South Tyrol region of Italy. These countries were once greatly abundant with this species. Since there is no information on the rate of decline for all the countries in this species range, the situation in Republika Srpska and Serbia is likely to be similar to that seen in France and Italy as the main threats, alien crayfish species

and *Aphanomyces astaci*, are present throughout much of this species range. This species is estimated to have undergone a 50-80% decline over a 10 year period over its global range. Sibley (2002) has suggested that in case that the current trend in the decline of this species continues, it will face possible extinction in Britain within the period of 30 years (http://www.iucnredlist.org/details/2430/0). According to IUCN criteria (Version 3.1), the degree of endangerment of *A. astacus* in Serbia was evaluated as "Endangered" ("EN"), which was a higher degree than the international level determined for this species, i.e., "Vulnerable" ("VU"). (S i m i ć et al., 2008).

Tab. 5. – Available data on alien invasive species *Aphanomyces astaci* in Republika Srpska (Bosnia and Herzegovina) and in the Republic of Serbia

| Country | Recent finding | Source |
|---|---|---|
| Bosnia and Herzegovina | – | http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail<br>http://www.iucnredlist.org/details/153745/0<br>A l a g i ć 2009<br>A l d e r m a n, 1996 |
| Republika Srpska | | http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail A l d e r m a n, 1996 |
| Republic of Serbia | | http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail<br>http://www.iucnredlist.org/details/153745/0<br>A l d e r m a n, 1996 |

CONCLUSION

Non-native alien zoopathogens have had a profound impact on the health of aquatic zoobiota in Republika Srpska and Republic of Serbia as well as around the region and the world as a whole. Crayfish plague (*Aphanomyces astaci*) broke out through the Danube River to the territory of Bačka and Banat (today A.P. Vojvodina, the Republic of Serbia) in 1879. Until 1880, the disease spread through the Sava River to the northern Bosnia (today Republika Srpska). From 1955 until 1970, almost all territories of Republika Srpska and Republic of Serbia were afflicted with the crayfish plague disease. Despite of the fact that the problems of the spread of crayfish plague in the territory of the Republika Srpska and Republic of Serbia have been of great significance, there is little data available for analyzing this issue. According to IUCN criteria, and mostly due to the *Aphanomyces astaci* invasion, the degree of endangerment of noble crayfish in Serbia was evaluated as "Endangered", which was a higher degree than the international level determined for this species, i.e., "Vulnerable".

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# ИНВАЗИВНЕ ЗООПАТОГЕНЕ *MASTIGOMICOTINA* У РЕПУБЛИЦИ СРПСКОЈ, СРБИЈИ И СУСЕДНИМ ЗЕМЉАМА СА ПОСЕБНИМ ОСВРТОМ НА *APHANOMICES ASTACI*

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### Резиме

Алохтоне зоопатогене микрогљиве имају дубок утицај на здравље акватичних зообиота у Републици Српској и Републици Србији, као и широм региона и света у целини. Зоонозе које изазивају микрогљиве широм света угрожавају различите организме, и та претња се интензивира са повећањем глобалног транспорта робе, људи и животиња. Миколози и они који се на овај или онај начин баве гљивама располажу са релативно мало информација о карактеристикама инвазивних, зоопатогених гљива, јер једна паразитска врста гљива може да насељава различите организме, и може бити патогена за неке од ових домаћина, али не и за друге. Узрочник куге нашег племенитог рака (акватична плесан *Aphanomyces astaci*) доспео је 1879. године Дунавом на територију Бачке и Баната (данас АП Војводина, Република Србија). Реком Савом болест се већ до 1880. године проширила територијом северне Босна (данас Република Српска, БиХ). Од 1955. до 1970. год. готово целокупну територију Републике Српске и Републике Србије освојила је ова зооноза. Упркос великом значају проблема ширења ракове куге на територији Републике Српске и Републике Србије, мало је доступних података који се баве овим питањем. Према IUCN критеријумима, углавном због инвазије *Aphanomices astaci* степен угрожености врсте племенитог рака у Србији се оцењује као „угрожена", што је за степен више од међународног нивоа утврђеног за ову врсту, тј. „рањива".

КЉУЧНЕ РЕЧИ: микрогљиве, инвазивне, *Aphanomices astaci*, Република Српска, Република Србија

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# LICHENS IN SERBIAN LEGISLATION

ABSTRACT: In this paper an overview of official regulations on the protection of lichens in the Republic of Serbia is presented, and provisions of individual regulations are analyzed. Also, the effects of adopted regulations on the protection of endangered species of lichens are discussed and general measures to improve the protection of lichens in the future are proposed. Finally, the need for further studies on lichens is suggested as a basis for their effective protection and conservation.
KEY WORDS: Lichens, conservation, protection, law regulations, Serbia

## INTRODUCTION

Lichens are currently considered to be fungi that live in symbiosis with a photobiont, an autotrophic green alga (phycobiont), or cyanobacterium (cyanobiont) or, in some cases, both. The fungal partner (mycobiont) in most lichens (98%) belongs to ascomycets, and zygomycetes and rare basidiomycets make up the remainder. The symbiotic relationship is often characterized as mutualistic, that is, both partners benefit. However, recent evidence suggests that, while the fungus is dependent on its autotrophic partner, the photobiont is often fully content to live alone (W e d i n et al., 2004; K a r a m a n et al., 2012).

Fungi take more dominant role and cultivate photosynthesizing algae for food and in return provide a shady, moist, vitamin-rich environment, so scientists classify lichens based on their associated fungal species. Whether the fungi were harvesting algae or cyanobacteria, the symbiotic modus operandi of the lichens proved to be the same. Perhaps Trevor Goward, the lichen curator at the University of British Columbia Herbarium, describes them best: "Lichens are fungi that have discovered agriculture" (G r i c e, 2010).

Apart from the other reasons for protection and conservation of fungi, one could mention that lichens comprise a vast and yet largely untapped source of potentially powerful new pharmaceutical products. In particular, and most

importantly for modern medicine, they represent an unlimited source of poly-saccharides with anti-tumor and immunostimulatory properties. Most of them, if not all, contain biologically active polysaccharides that differ in chemical compositions, most of them belonging to the group of β-glucans which have specific chemical linkages that are needed for their anti-tumor action (S h i b a t a et al., 1968; N i s h i k a w a, 1969, O l a f s d o t t i r and I n g ó l f s d o t t i r, 2001).

Unfortunately, the notion that lichen species, just like other organisms on our planet, can become endangered has been neglected so far. Moreover, awareness of the risk of lichen species reduction and the fact that some species have already disappeared has come too late and many anthropogenic lichen deserts have already been reported worldwide (G i l b e r t, 1971; H e n d e r s o n, 1987; D a s, 2013), as well as in Serbia (K u y u n d h i c y et al., 1998 a,b; M a t a v u l j et al., 1998; C v i j a n et al., 2008).

Generally, it is considered that the main reasons for categorization of lichens as threatened organisms are disappearance and contamination of their habitats primarily caused by humans, due to pollution of the atmosphere, industrialized agriculture, unfavorable forestry practices and anthropogenic alterations of large habitat areas. All of these issues have led to the degradation of lichen habitats.


## LICHENS AS POLLUTION MONITORS


In 1859 L.H. Grindon attributed the declining lichen flora of South Lancashire to increasing air pollution and, in 1866, W. Nylander concluded from the studies conducted in Paris that lichens might serve as practical indicators of air quality. Subsequent studies from all over the world, which were published in over 500 scientific publications, vindicated Nylander's views (H a w k s w o r t h and R o s e, 1970).

In 1912 it was appreciated that the lichen vegetation on trees in urban areas could be divided into zones easily recognizable in the field. Three or four zones can be most commonly distinguished: (1) an inner "lichen desert" with no lichens, or at least no foliose and fruticose species, (2) an intermediate "struggle" or "transition" zone where foliose and fruticose species begin to appear but are poorly developed (often divided into an ''inner'' and "outer" zone where foliose and fruticose species, respectively, first appear), and (3) an outer "normal" zone with lichen vegetation unaffected by pollution.

The lichen growth can be affected by different gaseous air pollutants such as: high sulfur dioxide concentration, carbon compounds in smoke, fluorides, car fumes (carbon monoxide, oxides of nitrogen, lead containing compounds, hydrocarbons) and dust, photo-chemical smog (ozone, peroxyacetyl nitrate, nitrogen oxides etc.), heavy metals (iron, lead, zinc, and copper), radioactive isotopes of metals (radionuclides), agricultural chemicals (pesticides, especially fungicides, fertilizers) (H a w k s w o r t h, 1971, L o p p i and C o r s i n i, 1995).

Lichenoflora in Vojvodina, including bank region of the River Tamiš and the Danube-Tisza-Danube Canal hydrosystem has been explored randomly (K u y u n j i c y et al., 1998a,b; K u j u n d ž i ć and M a t a v u l j, 1998; M a t - a v u l y et al., 1998; M a t i j a š e v i ć, 1988), and more systematically within the study of former Yugoslavia lichen flora (K u š a n, 1953; M u r a t i, 1992, 1993; M a t a v u lj and Đ u r đ e v i ć, 2005).

After collection of a substantial body of knowledge on the vulnerability of lichens in the last decade of the twentieth century, lichens slowly began to be incorporated in nature protection programs, and the frameworks of actions that address their conservation became more formal and recognized (to a greater or lesser extent) by some states. At that time, the need to introduce some kind of control was also recognized in the Republic of Serbia. The present paper provides an overview of legislation in Serbia regarding the protection of lichens in nature. The main objective of this paper was to present a chronological review of regulations on the protection of lichens, analyze the effects of enacted regulations on the population of lichens, and propose the ways to improve their conservation and protection in the future.

Regulations that apply only indirectly to macromycetes, including lichens, such as laws related to forestry, national parks and similar (e.g. laws regarding nature conservation but not explicitly mentioning lichens) were not considered nor were the laws governing other areas related to lichens, such as regulations focused on pharmaceutical or food industry, or the protection of materials, medical and related aspects.

The basic condition for the preservation of lichens (or any other organism) is increased awareness of existing problems which requires in depth study, rapid and satisfactory taxonomic inventories, and extensive ecological and chorological studies. Although mycological and lichenological research data have been collected in the Republic of Serbia for nearly a century, these studies were done randomly and non-systematically: driven by individual enthusiasm rather than as a part of a systematic research project; thus, these data are not sufficient to guide the decisions and regulations for the protection and preservation of lichens (I v a n č e v i ć, 1995).

Adequate protection of lichens can only be established based on solid and reliable scientific data collected through systematic and long-term scientific studies. It is therefore necessary to make a substantial investment in basic lichenological research. Another necessary condition for determining the state of endangered lichens is careful monitoring of population sizes, abundance, diversity and distribution over a long period, using standardized methodologies. Then, based on the collected data a Red List of endangered lichens can be created, preferably using generally accepted IUCN classifications (IUCN, 2001). However, it may be inappropriate to delay protective measures until the expected optimum level of knowledge about the population of lichens and other macromycets is reached (M a t a v u l j et al., 1998; M a t a v u l j and K a r a m a n, 2004).

The material used in this paper consists of legal provisions of the Republic of Serbia (laws and other regulations) relating to environmental protection:

*Закон о заштити природе. Службени гласник Социјалистичке Републике Србије бр. 29, 1988* – [Nature conservation law, 1988]; *Одлука о стављању под заштиту биљних врста као природних реткости. Службени гласник Социјалистичке Републике Србије 11, 17. 03. 1990* – [Decision on putting plant species under protection as natural rarities, 1990]; *Одлука о изменама и допунама одлуке о стављању под заштиту биљних врста као природних реткости. Службени гласник СРС 49, 15. 08. 1991* – [Decision on amending the decision on putting plant species under protection as natural rarities, 1991] – *Закон о заштити животне средине. Службени гласник Републике Србије 66/1991, 83/1992, 53/1993, 67/1993, 48/1994 и 53/1995* – [Environmental protection law, 1991]; *Уредба о заштити природних реткости, Службени гласник Републике Србије 50, 09. 07. 1993* – [Regulation on the protection of natural rarities, 1993]; *Наредба о контроли коришћења и промета дивљих биљних и животињских врста. Службени гласник Републике Србије 50, 09. 07. 1993. и 36/1994* – [Directive on control of use and trade of wild plant and animal species, 1993]; *Наредба о стављању под контролу коришћења и промета дивљих биљних и животињских врста. Службени гласник Републике Србије 16, 05. 04. 1996. и 44/1996* – [Directive on control of use and trade of wild plant and animal species, 1996]; *Наредба о стављању под контролу коришћења и промета дивљих биљних и животињских врста. Службени гласник Републике Србије 17, 07. 04. 1999* – [Directive on control of use and trade of wild plant and animal species, 1999]; *Закон о заштити животне средине. Службени гласник Републике Србије 135/2004 и 36/2009* – [Environmental protection law, 2004]; *Уредба о стављању под контролу коришћења и промета дивље флоре и фауне. Службени гласник Републике Србије 31/2005, 45/2005-испр., 22/2007, 38/2008, 9/2010* – [Regulation on putting the use and trade of wildlife under control, 2005]; Convention on the conservation of European wildlife and natural habitats – the Bern convention (the Republic of Serbia signed and ratified this convention on 9 January 2008 and implementation began on May 1, 2008); *Закон о заштити природе. Службени гласник Републике Србије 36, 12.05.2009 и 88/2010* – [Nature conservation law, 2009]; *Правилник о проглашењу и заштити строго заштићених и заштићених дивљих врста биљака, животиња и гљива. Службени гласник Републике Србије 5, 05. 02. 2010* – [Regulation on the proclamation and protection of strictly protected and protected wild species of plants, animals and fungi, 2010]. Listed legal provisions of the Republic of Serbia (laws and other regulations) were reviewed and analyzed in this paper.

## OVERVIEW OF REGULATIONS ON THE PROTECTION OF LICHENS IN REPUBLIC OF SERBIA

- Nature Conservation Law (Službeni Glasnik RS, br. 29/88), Decision on putting plant species under the protection as natural rarities (Službeni Glasnik br. 11/90, March 17, 1990), and Decision on amending the decision on

putting under the protection of plant species as natural rarities (Službeni Glasnik RS, br. 49/91, August 15, 1991), by alteration (amendments) of the text of the Decision of 1990, as well as The Environmental protection law (Službeni Glasnik RS, br. 66/91, 83/92, 53/93, 67/93, 48/94 and 53/95), does not consider mushrooms or lichens. Regulation on the Protection of natural rarities (Službeni Glasnik RS br. 50/93, June 9, 1993) and the Directive on control of the use and trade of wild plant and animal species (Službeni Glasnik RS, br. 50/93, June 9, 1993 and 36/94), placed no lichen species under control. The Directive on control of the use and trade of wild plant and animal species (Službeni Glasnik RS, br. 16/96, April 5, 1996 and 44/96) and the Directive on control of the use and trade of wild plant and animal species (Službeni Glasnik RS, br. 17/99, April 7, 1999) placed none of the lichen species under protection, too.

The Republic of Serbia signed and ratified the Convention on the conservation of European wildlife and natural habitats – Bern convention, on January 9, 2008 and provisions began to be implemented on May 1, 2008, without any special reference to the lichens. Also, Environmental protection law, (Službeni Glasnik RS, br. 135/04 and 36/09), under the latest amendments to this Regulation (from 2010), put none of the lichen species under protection. Nature conservation law (Službeni Glasnik RS, br. 36/09, May 12, 2009, and 88/10), and Regulation on proclamation of putting the use and trade of wildlife under control (Službeni Glasnik RS, br. 31/05, 45/05, 22/07, 38/08, 9/10), provided a basis for lichen protection because the latest amendments of this Regulation (from 2010) included a list of the following species of lichens (Tab. 1), or strictly protected lichen species (Tab. 2).

Tab. 1. – List of protected lichen species according to the Regulation on the declaration and protection of protected and strictly protected wild species of plants, animals and fungi. Službeni Glasnik RS, br 5/10.

| Latin name | Authors | English name* | Serbian name |
|---|---|---|---|
| *Cetraria islandica* | (L.) Ach., 1803 | True Iceland lichen, Iceland cetraria lichen | прави исландски лишај |
| *Evernia prunastri* | (L.) Ach., 1810 | Oakmoss lichen, Ring lichen | шљивин лишај, храстов лишај |
| *Pseudevernia furfuracea* | (L.) Zopf, 1903 | Purper geweimos, Treemoss, Tree lichen | пурпурни лишај |
| *Usnea* spp. (Excluded Strictly protected *Usnea* species) | Dill. ex Adans., 1763 | Old Man's Beard, Beard Lichen | дедина брада |
| *English and Serbian names and authors, added in the Table for this edition, are missing in the original List in the text of Law Regulation | | | |

Tab. 2. – List of strictly protected lichen species according to the Regulation on the declaration and protection of protected and strictly protected wild species of plants, animals and fungi. Službeni Glasnik RS, br. 5/10.

| Latin name | Authors | English name* | **Serbian name** |
|---|---|---|---|
| *Alectoria sarmentosa* | (Ach.) Ach. , 1810 | Witch's hair | вештичја коса |
| *Anaptychia crinalis* | (Schleich.) Vězda, 1977 | Fringed eyed centipede lichen | ресасти лишај |
| *Cetrelia cetrarioides* | (Duby) W.L.Culb. & C.F.Culb. | Giant shield lichen | штитасти лишај |
| *Collema fragrans* | (Sm.) Ach. , 1814 | Clustered mini-jelly lichen | згрудвани мали пихтијасти лишај |
| *Collema nigrescens* | (Hudson) DC. | Blistered jelly lichen | мехурасти црни пихтијасти лишај |
| *Evernia divaricata* | (L.) Ach., 1810 | Ring lichen | прстенасти лишај |
| *Fuscopannaria saubinetii* Syn. *Vahliella saubinetii* | (Mont.) P.M. Jørg., 2008 | Pink-eyed shingle lichen | црополики ружичасти лишај |
| *Graphis elegans* | (Borrer ex Sm.) Ach., 1814 | Elegant script lichen | исписани лишај |
| *Heterodermia speciosa* | (Wulfen) Trevis., 1868 | Powdered shield fringe lichen, Powdered centipede lichen | ресасто-штитасти стоноги лишај |
| *Hypogymnia vittata* | (Ach.) Parrique | Vitt tube lichen | увијено-тракасти лишај |
| *Lempholemma polyanthes* | (Bernh.) Malme, 1924 | Bubbly skin lichen | мехурасто-кожасти лишај |
| *Leprocaulon microscopicum* | (Vill.) Gams ex D. Hawksw. | Mealy lichen | брашњави лишај |
| *Leptogium hildenbrandii* Syn. *Leptogium papillosum* | (Garov.) Nyl. 1856 (de Lesd.) C.W. Dodge, 1933 | Hildenbrand's skin lichen | хилденбрандов кожасти лишај |
| *Leptogium saturninum* | (Dicks.) Nyl., 1856 | Saturn skin lichen, Bearded jellyskin | длакави кожасти лишај |
| *Leptogium teretiusculum* | (Wallr.) Arnold | Terete skin lichen | ресасто-кожасти лишај |
| *Letharia vulpina* | (L.) Hue, 1899 | Wolf lichen, Timber wolf | вучји лишај |
| *Lobaria amplissima* | (Scop.) Forss., 1883 | Lungwort, Lung moss | лишај плућњак велики |
| *Lobaria scrobiculata* | (Scop.) DC., 1805 | Textured lungwort, Textured lung lichen | лишај плућњак мрежасти |
| *Menegazzia terebrata* | (Hoffm.) A.Massal., 1854 | Treeflute, Honeycombed lichen | чешљолики лишај |
| *Moelleropsis nebulosa* | (Hoffm.) Gyeln., 1940 | Blue-gray grainy crust lichen | облаколики зрнасти лишај |
| *Nephroma bellum* | (Sprengel) Tuck., 1841 | Kidney lichen | бубреголики лишај |
| *Normandina pulchella* | (Borrer) Nyl. | Clam lichen | шкољкасти лишај |
| *Pannaria rubiginosa* | (Ach.) Bory, 1828 | Brown-eyed shingle lichen, Matted lichen | смеђеоки плишани црополики лишај |

| | | | |
|---|---|---|---|
| *Parmotrema chinense* | (Osbeck) Hale & Ahti, 1986 | Powdered ruffle lichen, Chinese parmotrema lichen | таласасти кинески прашкасти лишај |
| *Peltigera collina* | (Ach.) Schrader, 1801 | Tree pelt, Felt lichen | филцани кожасти лишај |
| *Peltigera malacea* | (Ach.) Funck, 1827 | Veinless pelt, Felt lichen | глатки филцани лишај |
| *Physcia biziana* | (A. Massal.) Zahlbr., 1901 | Frosted rosette lichen, Rosette lichen | розетасти смрзнути лишај |
| *Physcia leptalea* | (Ach.) DC. | Fringed rosette lichen | розетасти ресасти лишај |
| *Physcia tribacia* | (Ach.) Nyl., 1874 | Edge-granulated rosette lichen | розетасти рубно--зрнасти лишај |
| *Sclerophora peronella* | (Ach.) Tibell | Pin-like lichen | чиодасти лишај |
| *Solorina spongiosa* | (Ach.) Anzi, 1862 | Fringed chocolate chip lichen | сунђерасто-иверасти лишај |
| *Sphaerophorus globo-sus* | (Huds.) Vain., 1903 | Coral lichen, Globe ball lichen | коралолики вршно--кугласти лишај |
| *Thelotrema lepadinum* | (Ach.) Ach., 1803 | Bark barnacles, Barnacle lichen | богињави, приштолики лишај |
| *Trapeliopsis wallrothii* | (Flörke in Spreng.) Hertel & Gotth. Schneider, 1979 | Scaly mottled-disk lichen, Wallroth's trapeliopsis lichen | громуљичави Валротов лишај |
| *Tuckneraria laureri* | (Kremp.) Randlane & A.Thell, 1994 | Laurer's edged lichen | Лауреров ињем оперважени лишај |
| *Usnea longissima* | Ach., 1810 | Methuselah's beard lichen, Beard lichen | метузалемова дугачка брада |
| *Usnea scabrata* | Nyl., 1873 | Straw beard lichen, Beard lichen | сламната дедина брада |
| *English and Serbian names and authors, added in the Table for this edition, are missing in the original List in the text of Law Regulation | | | |

Regulation on the declaration of protected and strictly protected wild species of plants, animals and fungi (Službeni Glasnik RS, br. 5/10, February 5, 2010) did not put any of the lichen species under protection. This review of existing laws and regulations provides insight into basic trends that appear to be determining the approach toward protection of lichens in Serbia.

After the Nature conservation law of 1988, the Serbian Government adopted the Environmental protection law of 1991. This Act then took over the protection of endangered species that were still designated as "natural rarities", which was an inadequate definition subjected to sharp criticism by environmentalist experts. Based on this Law from 1991, the Regulation on the protection of natural rarities was adopted in 1993 (Službeni Glasnik, br. 50/93) but, unfortunately, endangered species of lichens were not included and their protection was omitted. Regrettably, lichens, together with other macromycets were at the time still perceived by the public as a less important part of the plant kingdom, and their unique and important role in nature was not understood.

At that time, the largest gap existed between inadequate legal protection and enormous pressure on nature and lichen habitats, which had become seriously endangered. Due to the alarming situation related to the protection of lichens and fungi in Serbia (which was similar to that in some other countries of Southeast Europe) the European Council for the Conservation of the Fungi expressed its concern at their 1997 meeting in Vipiten, Italy (B o h l i n, 2006). Numerous signals indicated a worsening situation for threatened lichen species in our country. Thus, by late 1998, Serbia started work on new documents that were supposed to provide adequate protection for endangered lichens. A directive on control of the use and trade of wild species was issued in April 1999 (Službeni Glasnik, br. 17/99). In this document, fungi were listed separately from plants for the first time, and species of lichens were mentioned. In addition, the nomenclature of species' names was corrected. Finally, some provisions on how to protect endangered species were listed. Unfortunately, this directive was not written in the form proposed by the expert lichenologists.

A new Law on environmental protection was adopted in 2004, and on the basis of this Law a new Regulation on control of the trade of wild fauna and flora was passed in 2005 (Službeni Glasnik, br.31/05). This Regulation has been applied to this day without any significant alterations.

New Nature conservation law (the first since 1988) was adopted in 2009 (Službeni Glasnik, br. 36/09) and it introduced new solutions, as a result of the tendency to be harmonized with EU regulations. Article 59 stated which parts of this law (which is currently inactive) would be applied upon accession of the Republic of Serbia to the European Union. In Article 27, the protected species were listed as protected natural goods which could have the status of "protected" or "strictly protected" species. Moreover, a large number of Articles of the Law was concerned with the protection of lichen species' habitats, providing the necessary protection of these species. This allowed the prescription of new, more effective conservation measures. In accordance with this Law, the Regulation on the proclamation and protection of protected and strictly protected species of plants, animals, lichens and fungi was adopted (Službeni Glasnik, br. 05/10).

The Environmental protection law (2004), which was used for preparation of the Regulation on putting the use and trade of wildlife under control (2005) does not recognize the new Nature conservation law (2009), since it was accepted much earlier. The Nature conservation law (2009) does not include ordinances from the Regulation on putting the use and trade of wildlife under control (2005), which was prepared according to the older Environmental protection law (2004), so one subset of species protection is regulated according to the old Environmental protection law (2004) and another by the new Nature conservation law (2009). In this way, both laws are broken by the same activity, but the supervising inspection services do not have any evidence.

In addition to domestic legislation, there are also obligations from international conventions signed by Serbia that have obligatory character. Regula-

tions of the Bern convention, which protects the flora, fauna and habitat of species in Europe, came into force in Serbia in mid 2008. Lichens have not yet been officially included into the lists of species covered by the Bern convention, primarily for administrative and political reasons, and their protection under the provisions of the Bern convention is not mandatory in Serbia. The list of species of lichens that have been proposed for inclusion in the Bern convention is now in the form of an official proposal confirmed by the Standing Committee of the Bern convention.

Unfortunately, a project for making a revised version of the Red list for lichens and other fungi was proposed to the state authorities in 2007, including a detailed evaluation of vulnerability factors (I v a n č e v i ć et al., 2007), but its implementation has not been approved yet. However, Article 36 of the Nature conservation law (2009) provides that: "…species that are or may become endangered shall be protected as strictly protected wildlife, or protected wildlife. The species protected under this law shall be determined on the basis of national and international Red lists or Red books, professional findings and scientific knowledge." Moreover, the same Article provides that the Red book or Red list may be adopted by the Ministry of environmental protection. Consistent application of these legal provisions, once they are enforced, should provide a scientific basis for protection measures and help align Serbian legislation with the legislation of other countries that have made more progress in this field.

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## ЛИШАЈИ У ЗАКОНОДАВСТВУ РЕПУБЛИКЕ СРБИЈЕ

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Резиме

У овом раду је дат преглед званичних прописа о заштити лишаја у Републици Србији уз анализу одредаба појединих прописа. Такође се разматрају ефекти усвојених прописа о заштити угрожених врста лишаја и предлажу опште мере за побољшање заштите лишаја у будућности. Коначно, указује се на потребу интензивирања студија о лишајима што би обезбедило основу за њихову ефикасну заштиту и конзервацију.

КЉУЧНЕ РЕЧИ: лишаји, очување, заштита, законска регулатива, Република Србија

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# *SCHIZOPHYLLUM COMMUNE* – THE MAIN CAUSE OF DYING TREES OF THE BANJA LUKA ARBORED WALKS AND PARKS

ABSTRACT: In the frame of investigation of the main cause of dying trees of the main arbored walks (Mladena Stojanovića Aley and Park), the investigation of the presence and diversity of macrofungi in Banja Luka City were undertaken in the period 2006-2011. Relatively poor generic diversity of lignicolous (pathogenic or potentially pathogenic and saprotrophic) macrofungi with only 16 species representing this group (13 basidiomycets: *Schizophyllum commune, Fomes fomentarius, Stereum hirsutum, Coriolus versicolor, Flammulina velutipes, Pseudotrametes gibbosa, Ganoderma applanatum, G. lucidum, G. adspersum, Polyporus squamosus, Meripilus giganteus, Laetiporus sulphureus, Auricularia auricula-judae*, and 3 ascomycets*: Nectria cinnabarina, Xylaria hypoxylon, X. polymorpha*) were recorded. Such a poor qualitative composition of this very important fungal group could be explained by the reduction in the number of plant species in arbored walks and alleys, as well as the reduction in the number of fungi resistant to heavy air pollution caused by nearby (1-5m) fuel combustion in engines.

Although only  preliminary, our results pointed to the necessity of conservation and protection of the most beautiful features of Banja Luka and its alleys and arbored walks, by undertaking the measures of curing damaged trees and treating them with fungicides in order to wipe out the epiphytia caused in more than 95% of cases (dated May 2011) by Split-gill (*Schizophyllum commune*), present on dead wood but also on damaged trees of *Aesculus hyppocastaneum* (127 trees), *Tilia cordata* (124 trees), *Tilia platyphyllos* (36 trees), *Tilia argentea* (40 trees), *Acer negundo* (20 trees), *Platanus acerifolia* (2 trees), *Robinia pseudoacacia* (3 trees), *Fraxinus ornus* (1 tree), *Betula pendula* (1 tree), *Catalpa* sp. (2 trees), etc. Altogether, during the last decade, around 200 trees collapsed or were sanitary cut in Banja Luka arbored walk from the Malta site to the Green bridge, a total length around 5 km. The reason for this was primarily due to Split-gill fungus and the restoration of arbored walks in the streets extremely polluted by engine fuel consumption in the zone of Mladena Stojanovića street. By analyzing the trees along the City of Banja Luka main street it could be concluded that, besides the appearance of suffocation of plants, due to wide asphalt surfaces that are located immediately next to the tree-trunks and heavy air pollution, fungi caused illnesses are the most important cause of the decline of trees. With its great adaptation to arid climate and ability to resist to the air pollution, *Schizophyllum commune* turned out to be the most aggressive

and successful universal fungal invader of trees from old alleys, even threatening immuno-compromised human individuals. However, man and his direct or indirect impacts contribute to the dying of trees much faster than the fungal pathogens in the busiest and most polluted Mladena Stojanovića street.

# INTRODUCTION

Split-gill fungus, *Schizophyllum commune* Fries, is probably the most widespread existing macroscopic fungus that is found on every continent except Antarctica, where there is no wood to be used as a substrate. Besides the most common worldwide species, there are a few less common species of *Schizophyllum*. It does not appear to be very closely related to the other gilled mushrooms, and most researchers categorize this species in its own order: *Schizophyllales* (Family Schizophyllaceae, Phylum Basidiomycota). As a result of its omnipresence, it is also one of the most studied mushrooms on earth.

Its fruiting body could be 1-6 cm wide, fan-shaped when attached to the side of the log, irregular to shell-shaped when attached above or below, upper surface is covered with small hairs, dry, white to grayish or tan, under surface composed of gill-like folds that are split down the middle, whitish to grayish, without a stem, flesh tough, leathery, pallid.

The gills function to produce basidiospores on their surface. Spore print of this fungus is white, and microscopic features of spores are: 3-4 x 1-1.5 µm, cylindrical to elliptical and smooth. Fruiting bodies are inedible due to small size and leathery consistence.

The fruit body can be described as a compound structure consisting of compressed radiate cupules. What appear to be gills are the margins of two adjacent cupules with proliferating marginal tissue which makes it look like the "gill" is split. They appear to be split because they can dry out and rehydrate, and thus open and close many times over the course of a growing season. The fruiting bodies to the right are probably a year old or more. This is a great adaptation for an arid climate, with sporadic rains. Unlike other mushroom species, the mycelium only has to produce one set of fruiting bodies per year, which can then almost totally dry out and rehydrate and keep functioning. It is a great strategy for fungal reproduction. One can go out in the dead of winter and find sporulating fruiting bodies of this fungus. It is a very successful wood-decaying fungus that causes white rot.

Owing to the work of John Raper and his colleagues at Harvard University in the 1950s-1970s, it is well known that it is a single widespread species. They collected worldwide samples of this fungus. After collecting and germinating the spores into mycelium, they were able to get individuals from all over the world to mate with one another. During that time they were also able to divide the species in mating types (sexes) based on their mating reactions. As long as two strains are of different mating types they are able to mate and form fertile offspring (R a p e r and K r o n g e l b, 1958; R a p e r and H o f f m a n, 1974).

*S. commune* is the best-known agent of human infection among the *Basidiomycotina*. Besides being considered (conditionally) as a more and more aggressive plant patogen causing white rot, this fungus has been reported recently to cause allergic bronchopulmonary mycosis (K a m e i et al., 1994) in an otherwise healthy female, and it was repeatedly isolated from the sputum of a patient with chronic lung disease (C i f e r r i et al., 1956). Other reports of *S. commune* infection include cases of meningitis (C h a v e z – B a t i s t a et al., 1955), sinusitis (C a t a l a n o et al., 1990, K e r n and U e c k e r, 1986; R o s e n t h a l, 1992), ulcerative lesions of the hard palate (R e s t r e p o, 1973), and possible onychomycosis (K l i g m a n, 1950) in both immunocompetent and immunosuppressed hosts. Although it is sometimes difficult to evaluate the significance of isolation of *S. commune* from clinical specimens (G r e e r, 1977, 1994; K l i g m a n, 1950), there have been a number of well-documented reports especially involving the nasal mucosa, hard palate, and lung (C a t a l a n o et al., 1990; K e r n and U e c k e r 1986; R o s e n t h a l, 1992). However, K a m e i et al. (1994), suggested that diagnosis of infection caused by *S. commune* may be difficult because of the failure to observe clamp connections on hyphae, the absence of fruiting body formation in the dark, and also the possibility of identification only when the fungus is a dikaryon capable of producing basidiocarps. All confirmed cases of *S. commune* infection have been based on isolates which form characteristic fruiting bodies in culture. Additional features which allow the identification of an isolate as *S. commune* include narrow hyphal pegs or spicules (C a t a l a n o et al., 1990; C h a v e z – B a t i s t a et al., 1955) present on some hyphae. G r e e r (1977) reported that the pegs could also be observed on hyphae growing in tissue.

Tab. 1. – Phytopathogenic fungi on trees of avenues and parks in Banja Luka City (A n o n i m, 2001)

| Tree species | Pathogenic fungi on leaves and leaf and twig shoots | Pathogenic fungi on tree trunks, branches and root system |
|---|---|---|
| *Tilia* spp. | *Gnomonia tiliae, Cercospora microsora Phyllosticta tiliae, Septoria* sp. | *Nectria* sp., *Exosporium tiliae, Discella desmazierii, Diaporthe eres, Hercospora tiliae, Pyrenochaeta pubescens, Ganoderma applanatum, Coriolus versicolor, Stereum* sp., *Armillariella mellea, Pholiota* sp., *Phellinus dryadeus, Inonotus hispidus, Leptoporus adustus, Schizophyllum commune* |
| *Aesculus hippocastanum* | *Guignardia aesculi, Mycosphaerella hippocastani* | *Inonotus hispidus, Coriolus hirsutus, Coriolus versicolor, Phellinus* sp., *Fomes fomentarius, Pholiota* sp., *Ganoderma applanatum, Leptoporus adustus, Stereum* sp., *Polyporus squamosus, Armillariella mellea* |
| *Acer* spp. | *Phyllosticta* sp. *Phleospora* sp. *Leptothyrium platanoides* | *Coriolus versicolor, Nectria cinnabarina, Polyporus squamosus, Inonotus hispidus, Stereum* sp., *Coriolus hirsutus, Leptoporus adustus, Verticillium* sp., *Diaporthe* sp., *Cryptodiaporthe* sp., *Armillariella mellea* |
| *Platanus* sp. | *Gnomonia veneta* | *Phellinus* sp., *Erwinia* sp., *Verticillium* sp. *Diaporthe* sp |

## MATERIAL AND METHODS

Systematic mycological survey of trees of Banja Luka arbored walk of the most frequent street Mladena Stojanovića, as well as nearby located Mladena Stojanovića park and wider region of the Park edge of the Banja Luka City of the Republic of Srpska (Bosnia) were done during the 2006-2011 period of time.

Fungi were identified on the basis of both morphological and anatomical properties of fruit bodies and according to specific chemical reactions using modern keys (A i n s w o r t h et al., 1973; C e t t o, 1979; H e r m a n n, 1990; P h i l l i p s, 1983; Uzelac, 2009; W e b s t e r, 1980).

## RESULTS AND DISCUSSION

Results of earlier recording of phytopathogenic fungal species in the investigated avenues and parks in Banja Luka City are shown in Table 1 containing the list of 10 species inhabiting leaves and leaf and twig shoots, as well as around 30 lignicolous pathogenic species (A n o n i m, 2001). Most of them can be found during the whole year, regardless of the season, except *Flammulina velutipes* which appears usually during late winter and early spring seasons.

According to data from the quoted Tab. 1, the presence of "epixylous" fungi that cause wood rot in the reviewed alleys was considerable. Attacks of these organisms lead to the decomposition of wood elements. With some exceptions (*Armillariella mellea* rot, for example) these diseases do not cause dying of trees, but if left uncontrolled, they become dangerous, because in the final stages they lead to the drying of trees or their fractures and falling. These diseases may remain unnoticed until the appearance of fruiting bodies or cavities, fractures or drying of trees. By analyzing the trees in Banja Luka streets it could be concluded that besides the appearance of suffocation of plants, due to wide asphalt surfaces that are located very next to tree-trunks (10-50cm), fungi caused illnesses are the most important cause of the decline of trees. Faster than fungal pathogens, man and his direct or indirect impacts have contributed to the dying of trees along the busiest and most polluted Mladena Stojanovića street (A n o n i m, 2001).

Out of 16 lignicolous macrofungi, recorded during the period of our investigations which was from 2006-2011, 13 species belonged to Basidiomycotina, and 3 to Ascomycotina Phylum. Sic species belonged to the family Polyporaceae, followed by 3 species belonging to Ganodermataceae, 2 to Xylariaceae, and 1 species the families Schizophyllaceae, Auriculariaceae, Hypocreaceae (Nectriaceae), Meripilaceae, and Tricholomataceae (Tab. 2).

Considering the poor qualitative composition of host plants (the substrates consisted mainly of seven tree species: *Aesculus hyppocastaneum*, *Tilia platyphyllos, T. cordata, T. tomentosa, Acer negundo, Platanus acerifolia*, *Robinia pseudoaccacia*, and scarce individual trees of *Acer campestre*,

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Tab. 2. – Generic composition of lignicolous macrofungi on investigated trees of arbored walks along Mladena Stojanovića Alley and park in Banja Luka City

| Species | Order and Familia | Substrate | Locality |
|---|---|---|---|
| *Schizophyllum commune* Fries | *Agaricales (Schizophyllales) Schizophyllaceae* | On various substrates (tree trunks and stumps, tree branches) of surveyed trees and bushes | Alley, Park, Park outskirts |
| *Coriolus versicolor* (L.) Quél. 1886 *Trametes vrsicolor* (L.) Pilát. | *Aphyllophorales Coriolaceae (Polyporaceae)* | White willow tree trunk; Lime fallen branch; Hornbeam stumps | Alley, Park |
| *Fomes fomentarius* (L.) J.J. Kickx 1867 | *Aphyllophorales (Polyporales) Polyporaceae* | Old trunk of *Populus* sp.; Living White willow tree trunk; Living Lime tree trunk. | Alley, Park |
| *Ganoderma adspersum* (Schulzer) Donk | *Polyporales Ganodermataceae* | Living Lime tree trunk. Boxelder tree trunk, | Park |
| *Ganoderma applanatum* (Pers.) Pat. | *Polyporales Ganodermataceae* | Unidentified tree trunk, White willow tree trunk | Park |
| *Ganoderma lucidum* (Curtis) P. Karst | *Polyporales Ganodermataceae* | Unidentified tree trunk | Park |
| *Laetiporus sulphureus* (Bull.: Fr.) Murrill 1920 | *Agaricales Polyporaceae* | Living tree of White willow | Park |
| *Meripilus giganteus* (Pers.) P. Karst. 1882 | *Agaricales Meripilaceae* | Horse-chestnut living tree trunk | Alley |
| *Polyporus squamosus* (Huds.) Fr. 1821 | *Polyporales Polyporaceae* | Horse-chestnut stump Boxelder tree trunk, | Park |
| *Trametes gibbosa* (Pers.) Fr. 1838; *Pseudotrametes gibbosa* (Pers.) Bond. and Singer 1944 | *Agaricales Aphyllophorales Polyporaceae* | Boxelder tree trunk, Horse-chestnut stump Unidentified tree stump, | Park |
| *Nectria cinnabarina* (Tode: Fr.) Fr. 1849 | *Ascomycotina Hypocreales, Hypocreaceae (Nectriaceae)* | Dry fallen branch of *Tilia* sp.; Dry fallen branch of Black locust Boxelder fallen branches, | Aley, Park |
| *Xylaria hypoxylon* (L.) Grev.(1824) | *Ascomycotina Xylariales, Xylariaceae* | Horse-chestnut stump Unidentified tree stump, | Aley |
| *Xylaria polymorpha* (Pers.: Fr.) Greville | *Ascomycotina Xylariales, Xylariaceae* | Horse-chestnut stump | Aley |
| *Stereum hirsutum* (Willd.) Pers. 1800 | *Russulales Polyporaceae (Stereaceae)* | Dry fallen branch of *Tilia* sp.; White willow stump | Park, Park edge |
| *Auricularia auricula-judae* (Bull.) J.Schröt. | *Auriculariales Auriculariaceae* | Elder bush dry fallen branch Black locust fallen dry branch | Park, Park edge |
| *Flammulina velutipes* (M.A. Curtis) Singer, 1951 | *Agaricales (Marasmiaceae) Tricholomataceae* | on *Juglans regia* tree trunk (backyard of the house in Bože Varićaka street 1) Unidentified tree stump | Park edge |

*Gleditsia triacantos, Morus nigra* and *Sorbus torminalis)* it was not surprising that relatively poor generic composition of lignicolous macrofungi of the investigated region was found. This diversity would be even poorer if tree substrates (*Sambucus nigra; Salix alba, Juglans regia, Populus* sp.), from Mladena Stojanovića Park outskirts and surrounding area were excluded from the analysis.

Altogether, during the last decade, around 200 trees collapsed or were sanitary cut in the Banja Luka arbored walk, from the Malta site to the Green bridge, a total length of around 5 km. This was due to the damage caused primarily by Split-gill fungus and the restoration of arbored walks in the nearby streets characterized by highly polluted air of Mladena Stojanovića alley, caused by the engine fuel combustion. Similar reports have been recently written in Lithuania (Snieskiene, 2011), where deterioration of state of *Aesculus hyppocastaneum* from larger cities was recorded, caused by infection with *Schizophyllum commune* together with other fungal pathogens.

By analyzing the trees along Banja Luka main street it could be concluded that, besides the appearance of suffocation of plants, due to wide asphalt surfaces that are located next to tree-trunks and heavy air pollution, fungi caused illnesses are the most important cause of the decline of trees. With its great adaptation to arid climate and high resistance to air pollution, *Schizophyllum commune* turned out to be the most aggressive and successful universal fungal invader of various species of plants from old alleys and parks (Tab. 3), threatening the immunocompromised and even healthy human individuals (Tab. 4). However, faster than fungal pathogens, man and his direct or indirect impacts have contributed to the dying of trees along the busiest and the most polluted Mladena Stojanovića street.

Tab. 3. – *Schizophyllum commune* incidence on investigated trees of arbored walks along Mladena Stojanovića Alley and in Mladena Stojanovića Park and park surrounding area in Banja Luka

| Tree species | English name | Fungal substrate | Locality |
|---|---|---|---|
| *Aesculus hyppocastaneum* | Horse-chestnut tree, Conker tree | Living tree trunk, Tree stumps | Alley, Park |
| *Tilia platyphyllos* | Large-leaved lime, Large-leaved lime | Living tree trunk, Tree stumps and fallen branches | Alley, Park |
| *Tilia cordata* | Small-leaved lime, Small-leaved lime | Living tree trunk, Tree stumps | Park |
| *Tilia tomentosa* | Silver lime, Silver linden | Living tree trunk, Tree stumps | Park |
| *Acer negundo* | Box elder, Boxelder maple, Maple ash | Living tree trunk Tree stumps | Park |
| *Platanus acerifolia* | London plane, London planetree, Hybrid plane | Tree stump, Fallen branch | Alley, Park |
| *Sorbus torminalis* | Wild service tree, Chequers tree, Checker tree | Living tree trunk, Fallen branch | Park |

| *Morus nigra* | Black mulberry | Living tree trunk | Park |
|---|---|---|---|
| *Gleditsia triacanthos* | Honey locust | Living tree trunk, Fallen branch | Alley, Park |
| *Robinia pseudoaccacia* | Black Locust, False acacia | Dry fallen branch Living tree trunk | Aley, Park |
| *Ailanthus altissima* | Tree of heaven, Ailanthus | Living tree trunk | Park edge |
| *Catalpa bignonioides* | Cigar tree, Catalpa, Indian bean tree Catawba | Living tree trunk | Park edge |
| *Juglans regia* | Persian walnut, Common walnut | Tree trunk (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Betula pendula* | Silver birch | Tree trunk (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Sambucus nigra* | Elder, Elderberry, Black elder, Elder bush | Bush branch (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Salix alba* | White willow Golden willow | Living tree trunk and branches, Tree stump | Park edge |
| *Fraxinus ornus* | Manna ash, South European flowering ash | Living tree trunk | Park edge |
| *Acer campestre* | Field maple | Bush branch (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Ligustrum vulgare* | Wild privet, European privet | Bush branch (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Cydonia oblonga* | Quince | Tree trunk (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Malus domestica* | Apple | Tree trunk (backyard of the house in Bože Varićaka street, no 1. | Park edge |
| *Prunus cerasus* | Sour cherry | Living tree trunk | Park edge |
| *Prunus domestica* | Plum | Living tree trunk | Park edge |
| *Hibiscus syriacus* | Rose of Sharon, Rose mallow, St Joseph's rod | Bush branch (backyard of the house in Bože Varićaka street, no 1. | Park edge |

Extremely high incidence of Split-gill fungus in Banja Luka statistically increases the possibility of allergic reactions or other forms of mycosis, already registered in similar climatic conditions (K a m e i  et al., 1994; R o s e n t h a l, 1992; ), especially with the growing number of immunocompromised individuals. Xerophilic, or at least xerotolerant fungal species (Z a k  and W i l d - m a n, 2004) have found optimal conditions for expansion, due to our more and more arid climate conditions.

Tab. 4. – Recent reports on *Schizophyllum commune* infections on humans in the Region

| Case | Illness | Treatment | source |
|---|---|---|---|
| – | *Sch. commune* in sinunasal mucus | – | Buzina et al., 2002, 2003 |
| 56-year-old male | inflamed bronchogenous cyst of previously immunocompetent patient | surgery | Bulajic et al., 2006 |
| 44-year-old immuno-competent male, Belgrade | sinusitis associated with headache, transient diplopia, and dizziness | treated with systemic antimycotics (liposomal amphotericin B, 2 weeks before and 1 week after surgery) and systemic and topical corticosteroids, followed by itraconazole for 2 months) | Pekic et al., 2010 |
| Immuno-competent male | allergic fungal rhinosinus-itis with sellar propagation and hyperprolactinemia | – | Arsic Arse-nijevic et al., 2010 |
| 32-year-old female | rhinosinusitis with nasal polyps | combination of oral itraconazole and topical corticosteroid therapy combined with surgery | Perić et al., 2011 |

## CONCLUSION

Although preliminary, our results point to the necessity of conservation and protection of the most beautiful features of Banja Luka and its alleys and arbored walks, by means of undertaking the measures of curing damaged trees and treating them by fungicides in order to eliminate the epiphytia caused, in more than 95% of cases (dated May 2011), by Split-gill (*Schizophyllum commune*) present on dead wood as well as on damaged trees of *Aesculus hyppocastaneum* (127 trees), *Tilia cordata* (124 trees), *Tilia platyphyllos* (36 trees), *Tilia argentea* (40 trees), *Acer negundo* (20 trees), etc.

During the last decade, around 200 trees collapsed or were sanitary cut in Banja Luka arbored walk from the Malta to the Green bridge, a total length of around 5 km. This was due to the damage caused primarily by Split-gill fungus and the restoration of arbored walks near the streets characterized by much polluted air, along Mladena Stojanovića street, caused by the engine fuel combustion. By analyzing the trees along the main street of Banja Luka it could be concluded that, besides of appearance of suffocation of plants due to wide asphalt surfaces, located next to tree-trunks, and heavy air pollution, by fungi caused illnesses are the most important cause of the decline of trees.

With its great adaptation to arid climate, and resistance to the air pollution, *Schizophyllum commune* turned out to be the most aggressive and successful universal fungal invader of trees from old alleys, threatening immunocompromised and even healthy human individuals. However, faster than fungal pathogens, man and his direct or indirect impacts contribute to the dying of trees along the busiest and most polluted Mladena Stojanovića street.

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## *SCHIZOPHYLLUM COMMUNE* – ГЛАВНИ УЗРОЧНИК ОДУМИРАЊА ДРВЕЋА ДРВОРЕДА И ПАРКОВА ГРАДА БАЊЕ ЛУКЕ

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Резиме

У оквиру истраживања главног узрока одумирања стабала бањалучких дрвореда (Алеја у улици Младена Стојановића и Парку Младена Стојановића), истраживани су присуство и разноврсност лигниколних макрогљива у периоду 2006-2011. године. Релативно сиромашна генеричка разноврсност лигниколних (патогених или потенцијално патогених и сапротрофних) макрогљива са само 16 врста које представљају ову групу (забележено је 13 базидиомицета: *Schizophyllum commune, Fomes fomentarius, Stereum hirsutum, Coriolus versicolor, Flammulina velutipes, Pseudotrametes gibbosa, Ganoderma applanatum, G. lucidum, G. adspersum, Polyporus*

*squamosus, Meripilus giganteus, Laetiporus sulphureus, Auricularia auricula-judae* и 3 аскомицете: *Nectria cinnabarina, Xylaria hipoxylon, X. polimorpha*). Тако сиромашан квалитативни састав ове веома важне групе гљива може се објаснити редуковањем дрвореда и алеја на мали број биљних врста, али и смањењем броја гљива отпорних на велика загађења ваздуха изазвана веома блиским издувним гасовима (1-5 m) који настају сагоревањем горива у моторима.

Иако прелиминарни, наши резултати указују на неопходност очувања и заштите најлепших карактеристика Бање Луке, њених алеја и дрвореда, на неопходност предузимања мера лечења оштећених стабала и третирања фунгицидима у циљу елиминације епифитије, у више од 95% случајева (од маја 2009), изазване лигниколном фитопатогеном гљивом *Schizophyllum commune*, констатованом на мртвом дрвету, али и на оштећеним стаблима *Aesculus hippocastaneum* (127 стабала), *Tilia cordata* (124 стабала), *Tilia platiphillos* (36 стабала), *Tilia argentea* (40 стабала), *Acer negundo* (20 стабала), *Platanus acerifolia* (2 стабла), *Robinia pseudoacacia* (3 стабла), *Fraxnus ornus* (1 стабло), *Betula pendula* (1 цај), *Catalpa sp.* (2 стабла), итд. Све у свему, током последње деценије, око 300 стабала је угинуло или уклоњено санитарном сечом у бањалучкој алеји Младена Стојановића од Малте до Зеленог моста, укупне дужине око 4 km, услед оштећења изазваних првенствено гљивом *Schizophyllum commune*, тако да рестаурација дрвореда дуж саобраћајнице која се одликује веома загађеним ваздухом, изазваним унутрашњим сагоревањем моторних горива постаје готово немогућа мисија. Анализирајући дрвеће дуж главне улице града Бање Луке могло би се закључити да је, поред појаве „гушења" биљака, због великих површина асфалта које се налазе непосредно поред стабала дрвећа и тешких загађења ваздуха, болести проузроковане гљивама су најважнији узрок пропадања дрвећа. Са својим израженим моћима адаптације на сушне климатске услове, и изванредно отпоран на загађења ваздуха, испоставља се да је гљива *Schizophyllum commune* најагресивнији и најуспешнији универзални инвадор стабала старих бањалучких алеја и паркова, чак претећи имунокомпромитованим особама људске популације. Нажалост, човек својим директним или индиректним утицајима брже и успешније од патогених гљива доприноси умирању дрвећа дуж најпрометније и најзагађеније бањалучке улице.

КЉУЧНЕ РЕЧИ: Град Бања Лука, алеја, парк, лигниколне гљиве, *Schizophyllum commune*

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# METHODOLOGICAL APPROACHES FOR UNRAVELING ILL-NATURED MOMENTS OF GENERALLY GOOD-NATURED *SACCHAROMYCES CEREVISIAE*

ABSTRACT: The yeast *Saccharomyces cerevisiae* is a two-sided coin; it has a beneficial, biotechnological value with a strong connotation of reliable safety, but also a negative, potentially pathogenic nature on the other hand. This review is discussing the methodological approaches that were used for unraveling putative virulence traits that should separate pathogenic and non-pathogenic strains. These methods can be sorted in four main groups regarding physiology/morphology, genetics, proteomics and host-pathogen interactions. They can be used to enrich the knowledge regarding e.g. invasive growth, rapid molecular identification, cell-wall adhesions and virulence of yeast pathogens, respectively. Most of these methods have been adapted mainly to the leading yeast pathogen *Candida albicans* or bacterial pathogens. *S. cerevisiae,* on the other hand, can act as a weak opportunistic pathogen in certain conditions and its pathogenicity is not comparable to the pathogenicity of *C. albicans*, therefore it is welcomed that new, modified methods are introduced. New approaches already showed a strong induction of invasive growth under nutrient starvation and at temperatures typical for human fever (37-39 °C), revealing the potential of *S. cerevisiae* strains to cause a disease in humans. A repressive effect on the other hand was observed in the presence of salts, anoxia and some preservatives, which implies the importance of food safety measures. The role of cell-wall proteins, which are involved in the adhesion to plastic material like catheters and other cell wall components that bind to the epithelial surface in the host, should be subjected to more detailed studies. The mechanisms for antimycotic resistance and rapid molecular identification techniques that would identify potentially pathogenic strains should be the focus of research, as well.
KEY WORDS: *Saccharomyces cerevisiae*, virulence, adhesion, cell-wall proteins, invasive growth, azole resistance

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Historically, *Saccharomyces cerevisiae* is one of the oldest and best studied domesticated microorganisms. There are indicationa that wine and beer were produced even 7000 years b.c. (D e m a i n, et al., 1998). Today, *S. cerevisiae* is used in many biotechnological applications among which the production of beverages, yeast biomass (leaven and yeast biomass used for feed), and the production of vitamins and enzymes for food industry are most spread and socio-economically important (D e m a i n, et al., 1998). Yet, this species still has some characteristics which we do not understand and this causes serious problems to immunocompromised patients. Despite its GRAS status, *S. cerevisiae* was placed in the "biosafety level one" because it can cause superficial and mild systemic infections (d e H o o g, 1996; M u r p h y and K a v a n a g h, 1999). There are three characteristics which, when combined, challenge physicians at the treatment decision: i) the ability to grow at elevated temperatures (i.e. 39 °C); ii) resistance to azole antimycotics (e.g. fluconazole, itraconazole), and iii) symptoms highly similar to those of *Candida* infection (K o n t o y i a n - n i s and R u p p, 2000; S a l o n e n et al., 2000; S a v i n i et al., 2008; S o b e l et al., 1993). Why challenge? Because in the case of immunocompromised patients there is often no time for the identification of pathogen and antimycotics are administered immediately. Unfortunately, there are not many antimycotics to choose from. Amphotericin B is a golden standard but has many severe side effects; therefore, physicians try to avoid it (N a v a r r o – G a r c i a et al., 2001). Azole antimycotics are often better choice, but if the causative agent is an azole-resistant pathogen (e.g. *S. cerevisiae*, *Candida glabrata*), the treatment will be a failure and the condition of a patient can get worse.

Regarding the spectrum of virulence factors, fungal pathogens are inferior to bacterial pathogens, but due to their eukaryotic origin, the spectrum of targets to which antimycotics can act without damaging host cells, is much narrower. Main fungal virulence factors include; the ability to grow at elevated temperatures (i.e. above 37 °C), adherence to cell tissues (e.g. epithelium), extracellular enzymes (e.g. proteinases), immunomodulation of the host immune response (e.g. by cell-wall components like mannan), dimorphism (i.e. pseudohyphae, true hyphae) and invasive growth. Yeast *Candida albicans* covers all these virulence factors and that is why it is so efficient pathogen. Fungal pathogenesis includes these four main steps: i) entrance into host; ii) adherence to surface epithelium, which is covered with thick layer of dynamic host microbiota in concentration of $10^{12}$ celic/g (G u a r n e r and M a l - a g e l a d a, 2003; Z a n e l l o et al., 2009) and therefore represents a crucial and most challenging step for yeast; iii) colonization and the expression of virulence factors listed above, which actually separate pathogenic from non-pathogenic microorganisms (H o s t e t t e r, 1994; M a d i g a n et al., 1997); and iv) spreading through host (i.e. invasion, which is a second meaning of the word, besides the invasive growth, which describes the penetration into solid surface). Summing this up, the study of pathogenic fungi should cover physiological and morphological studies, as well as main -omics to improve the understanding of pathogenesis, what can then offer solutions for combating mycoses. Figure 1 represents this approach.
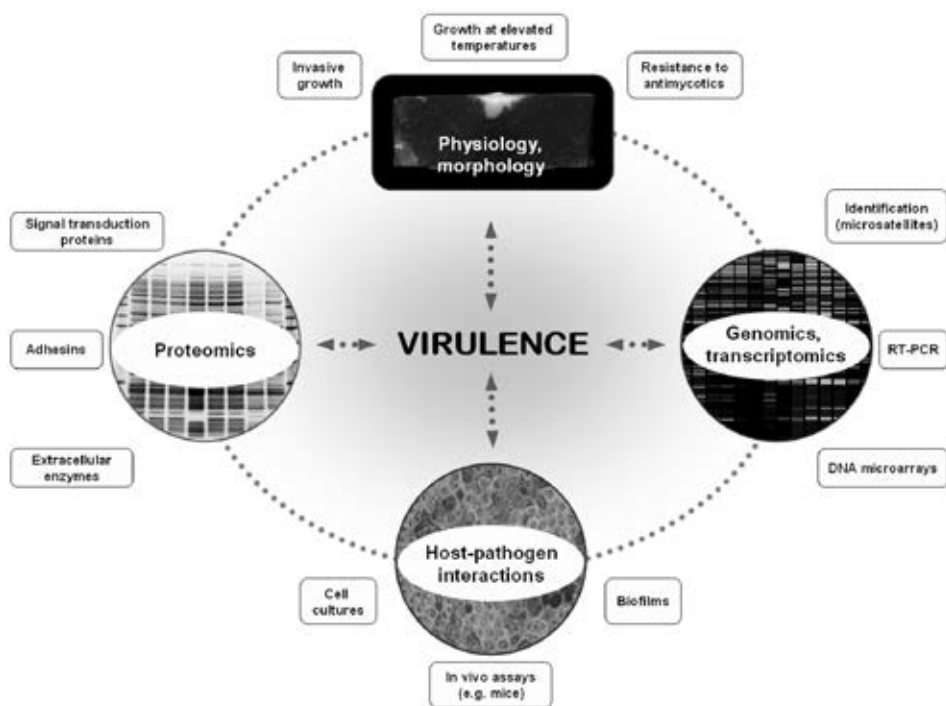
Fig. 1 – The concept of studying yeast virulence.

Before 1990, *Cryptococcus neoformans* and *C. albicans* were the main threats among yeast pathogens, but with progress in medicine, increase of immunocompromised patients (because of AIDS, leukemia, neutropenia etc.), prophylaxis, prolonged hospitalizations and the increased use of immunosuppressive agents, antimycotics and broad spectrum antibiotics, other non-*albicans* yeast species emerged, like *C. glabrata* and *S. cerevisiae* (D o n o w i t z et al., 2001; H u r l e y et al., 1987; K r c m e r y et al., 1999; M a l g o i r e et al., 2005). In the reports found, the latter was responsible for about 4% of fungal infections (C a s a l o n e et al., 2005; H e n r y et al., 2004; M u r p h y and K a v a n a g h, 1999; P i a r r o u x et al., 1999; S m i t h et al., 2002). The first reported case is was from 1976, when a 68-old man got fever and it was discovered that the cause was a systemic infection with *S. cerevisiae* (J e n s e n and S m i t h, 1976). The source of infection was leaven, which he was taking as a food additive. The number of reports increased rapidly in the 90s (E n a c h e – A n g o u l v a n t and H e n n e q u i n, 2005; H e n r y et al., 2004; S m i t h et al., 2002). The figure 2 shows the increase in publications which refer to *Saccharomyces* and "invasive growth". The interest in this area is obviously still not ceasing.
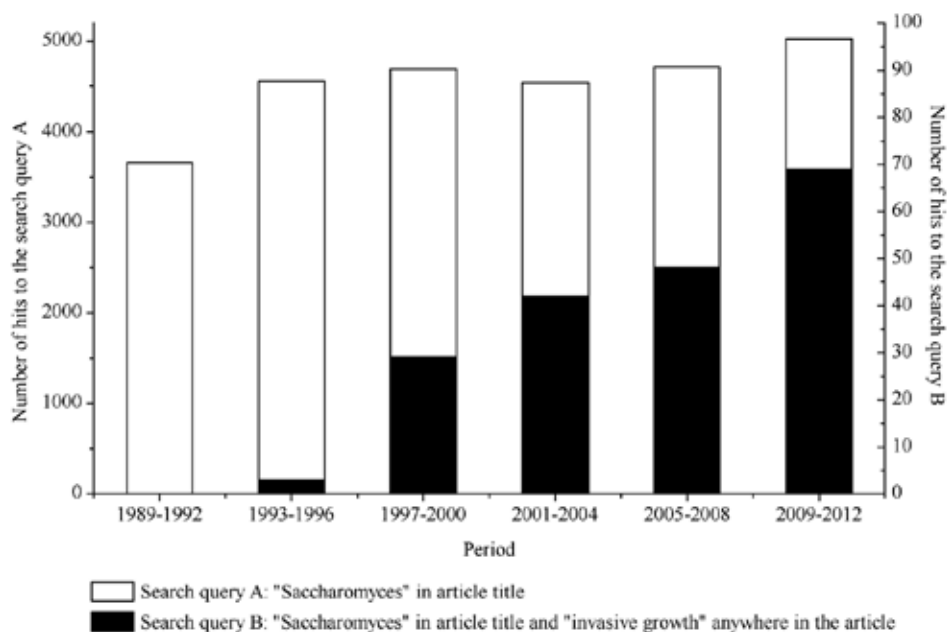
Fig. 2 – The increase in publications referring to *S. cerevisiae* invasiveness during the last two decades. The data was obtained from the webpage Scirus (Elsevier, 2013).

## Clinical facts

*Saccharomyces* infections do not choose specific age group or sex of a patient, since the study of 92 documented cases of *Saccharomyces* invasive infection reported the average age $44.3 \pm 26.9$ years old and women/men ratio was 45:35. Symptoms are clinically indistinguishable from an invasive candidiasis and include elevated temperature, chorioretinitis (fluffy yellow exudates), esophagitis (yellow-white plaques on an erythematous background) and other nonspecific symptoms that could be related to the underlying disease, which makes the diagnosis of *Saccharomyces* infection often unexpected and difficult (E n a c h e – A n g o u l v a n t and H e n n e q u i n, 2005; M u r p h y and K a v a n a g h, 1999). Moreover, in the case of misdiagnosed infections which are treated with azoles, the treatment itself represses other sensible species and improves *S. cerevisiae* competitiveness.

Transmission occurs mostly through food and dirty hands; however, what is interesting is that according to one report the source for 40% of *Saccharomyces* infection was a probiotic variety *Saccharomyces boulardii* (E n a c h e – A n g o u l v a n t and H e n n e q u i n, 2005; H e n r y et al., 2004; J e n s e n and S m i t h, 1976; S m i t h et al., 2002). The probiotic is sold under different names all over the world (DiarSafe® and Travla® in the UK, Ultra-Levure® in France, Perenterol® in USA and Florastor® in Canada) and it is supposed to help against digestive problems (E l m e r et al., 1996; K l e i n et al., 1993;

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Z a n e l l o et al., 2009). In addition to direct effects of *S. cerevisiae* virulence factors, the yeast is also indirectly connected to numerous diseases including Crohn's disease, antiphospholipid syndrome, systemic lupus erythematosus, type 1 diabetes mellitus, and rheumatoid arthritis, as these disorders were found to be correlated to high levels of anti-*S. cerevisiae* autoantibodies (R i n a l d i et al., 2013).

## *Virulent traits of S. cerevisiae*

The main virulent trait of *S. cerevisiae* is its ability to grow at elevated temperatures – some strains can grow up to 45 °C (B a r n e t t et al., 2000). This is an important virulent trait because if the patient's fever and immune system do not repress the growth of this pathogen, only few solutions remain. Another virulent trait which is important to point out is adhesion. It has great adhesiveness to polystyrene, polypropylene and polyvinylchloride, which is important for the patients with catheters (B o w e n et al., 2001; D o u g l a s et al., 2007; R e y n o l d s and F i n k, 2001). Adhesion to epithelium and pathogenesis of this species is, in general, poorly examined. Two other virulent traits are frequently mentioned with *S. cerevisiae* virulence. One is pseudohyphae, which are formed as a consequence of bipolar budding in which buds do not separate from the mother cell (d e  L l a n o s et al., 2006b). Although pseudohyphae stimulate flocculation and adhesion, the role of pseudohyphae remains unclear. The second is invasive growth (R o b e r t s and F i n k, 1994), the ability to grow into the solid matrices.

*S. cerevisiae* strains are highly diverse regarding its virulence. The tests in murine system (B y r o n et al., 1995; M c C u s k e r et al., 1994b) showed that the strain YJM311 was very efficient pathogen, since it reached 100% mortality, while the strain YJM222 reached 0% at the same infectious dose. This raised the question about what makes the *S. cerevisiae* pathogen and which putative virulent traits above correlates with pathogenicity described in murine system.

## *Agar invasion assay*

*S. cerevisiae*, as a chemoheterotroph, obtains energy and biomass material from the nutrients found in the contact environment. During the evolution, it has developed mechanisms of detecting the scarcity of environmental nutrients (G a g i a n o et al., 2002). Moreover, some strains developed also the mean of growing, with which cells penetrate solid medium to colonize nutrient-rich areas. This ability was soon related to virulence but it has never been proved. The idea to use agar plates for determination of *S. cerevisiae* invasiveness was first applied in the agar invasion assay (R o b e r t s and F i n k, 1994). The assay compared the growth of yeast colonies before and after the washing of colonies off the agar surface. The invasive colonies, which remained in the agar, confirmed the invasive growth. With the use of the assay the first mechanisms of *S. cerevisiae* dimorphism and invasive growth were

revealed (R o b e r t s and F i n k, 1994). It was soon discovered that it is nutrient depletion, especially nitrogen depletion, that triggers molecular mechanisms, especially MAP (*m*itogen-*a*ctivated *p*rotein) transduction kinase pathway, which results in morphological changes and invasive growth (B r a u s et al., 2003; C u l l e n and S p r a g u e 2000; G a g i a n o et al., 2002; G i m e n o and F i n k, 1994; G i m e n o et al., 1992; G o g n i e s et al., 2006; G u o et al., 2000; K l i n g b e r g et al., 2008; L o and D r a n g i n i s, 1998; P a l e c e k et al., 2002; P a n et al., 2000; R o b e r t s and F i n k, 1994; S c h e r z et al., 2001; S t a n h i l l et al., 1999). In most publications, the biggest role for adhesion and invasion was assigned to a cell-wall adhesin/flocculin Flo11 (B r a u s et al., 2003; C u l l e n and S p r a g u e 2000; G i m e n o et al., 1992; G o v e n d e r et al., 2008; G u o et al., 2000; L o and D r a n g i n i s, 1996; L o and D r a n g i n i s, 1998; M o s c h et al., 1999; N a v a r r o – G a r c i a et al., 2001; P a n et al., 2000; R e y n o l d s and F i n k, 2001; R o b e r t s and F i n k, 1994; R u p p et al., 1999). However, as it seemed that this 196 kDa mannoprotein (D o u g l a s et al., 2007) could be the key for explaining the invasive nature of invasive strains, the correlation has never been proved completely. Moreover, wide-genome microarray analyses could not confirm the correlation (B r e i t k r e u t z et al., 2003). It was also suggested that other Flo11-independent mechanisms are involved (B r a u s et al., 2003; B r e i t-k r e u t z et al., 2003; C u l l e n and S p r a g u e, 2002; G u l d e n e r et al., 2004; G u o et al., 2000; K i m and K i m, 2002; L i u et al., 1993; L o e b et al., 1999; M a d h a n i and F i n k, 1997; M a o et al., 2009; N i et al., 2004; P a l e c e k et al., 2000; P a l e c e k et al., 2002a; P a n et al., 2000; R u a et al., 2001; T r a c h t u l c o v a et al., 2004).

Since the agar-invasion assay was qualitative, the need for quantitative assay was welcomed. First steps in this direction were made by Drees and co-workers (D r e e s et al., 2005), while the quantitative agar-invasion assay was first introduced 14 years after the original assay (Z u p a n and R a s p o r, 2008). It joined the ideas to use agar plates as solid media and modern densitometry technique, which enabled the determination of the level of strains' invasiveness (Figure 3). In addition, new parameter was introduced, namely relative invasion, which describes the strain's preference for growing invasively when compared to superficial growth. This parameter has, therefore, the potential to make predictions about the invasive mechanisms; on the basis of all possible combinations of superficial growth, invasive growth, and relative invasion, we have proposed 8 models of invasive growth that are a) stimulated, b) repressed, or c) not specifically altered by specific environmental stimuli (Z u p a n and R a s p o r, 2010). The assay was used to study various environmental conditions that could have an influence on invasive growth of *S. cerevisiae*. Besides the confirmation that the nitrogen depletion stimulates invasive growth, it was also shown that the relative invasion in these nitrogen depleted media was up to 100%, meaning that the strains grew exclusively in the medium matrix. Strong stimulation of invasive growth was observed also in the cases of glucose depletion and at elevated temperatures (above 37 °C), revealing the potential of *S. cerevisiae* strains to cause a disease in humans.
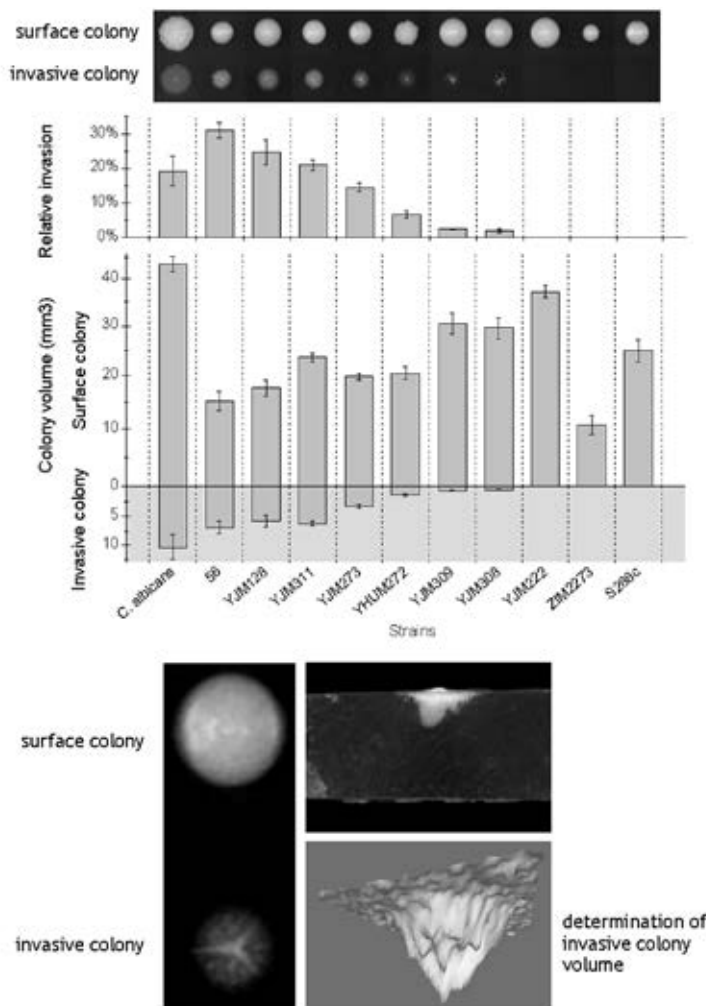
Fig. 3 – Quantitative agar-invasion assay. For more details see the original publication (Z u p a n  and R a s p o r, 2008).

On the other hand, a repressive effect was observed in the presence of salts, anoxia and some preservatives. Interestingly, strain YJM222, which was reported as non-virulent in murine system (B y r o n et al., 1995) did not show invasive growth at any growth condition, while the most virulent strain in the test, YJM311 (B y r o n et al., 1995), was among the most invasive on the test, especially at 42 °C (Z u p a n and R a s p o r, 2010). The experiments raised questions again on what distinguishes invasive and non-invasive strains and if the characteristic responsible for invasive growth determines strain's virulence. Therefore, the assay was soon coupled to the newly introduced method of analyzing cell-wall proteome (Z u p a n et al., 2009).

*Cell-wall proteins*

Yeast cell-wall determinants define strain's adhesion, flocculation, and the ability to form biofilms (B a y l y et al., 2005; B o j s e n et al., 2012; H o s t e t - t e r, 1994; L o and D r a n g i n i s, 1998; P e n d r a k and K l o t z, 1995; V a n M u l d e r s et al., 2009; W h e e l e r et al., 2003). They mainly include cell-wall proteins, abundantly modified by mannose branches, thus extending over a wide range of molecular masses, mainly from 60 to 200 kDa and some even above that range (C h a f f i n et al., 1998; M o u k a d i r i et al., 1997; M r s a et al., 1997; v a n  d e r  V a a r t et al., 1995; Z l o t n i k et al., 1984). Genes that are coding main mannoproteins isolated in yeast pathogens are orthologs; *ALS1* (*C. albicans*), *EPA1* (*C. glabrata*), *FLO11* (*S. cerevisiae*), *MPF3* (*C. neoformans*), *BAD1* (*B. dermatitidis*) etc. (L i and P a l e c e k, 2003; N a v a r r o – G a r c i a  et al., 2001). Gene studies forecast 38 potential cell-wall proteins, while 20 of them has been identified (C a r o et al., 1997; C h a f f i n et al., 1998; K l i s et al., 2006; K l i s et al., 2002; K o l l a r et al., 1995; M r s a et al., 1997; N g u y e n et al., 1998; V e r s t r e p e n and K l i s, 2006; Y i n et al., 2007; Z l o t n i k et al., 1984). However, the function is still unknown for many of them. The methods for isolation of glycoproteins include reducing agents (e.g. DTT, b-mercaptoethanol), detergents (SDS), leach (NaOH, KOH), and hydrolitic enzymes (e.g. glucanase, chitinase) (B o n y et al., 1997; d e G r o o t et al., 2004; K l l a r et al., 1995; M a g n e l l i et al., 2002; N g u y e n et al., 1998; S h i b a t a et al., 1983; Z l o t n i k et al., 1984). One of the most specific methods for cell-wall protein detection is immunohistochemical staining; glycoproteins are biotinylated before the cell is disrupted, therefore high specificity is assured (M o n t e a g u d o et al., 2004; M r s a et al., 1997). However, with the exception of enzymatic approach, these methods are highly aggressive to molecular structures, therefore extensive purification and/or great amounts of cell biomass are needed for efficient isolation. With the intention to analyze very small amounts of biomass, like in the case of invasive colonies, which is 10-3000 smaller amount of cell biomass than the amount used in other publications (d e  G r o o t et al., 2004; K o l l a r  et al., 1995; M a g n e l l i et al., 2002; N g u y e n et al., 1998; S h i b a t a et al., 1983; Z l o t n i k et al., 1984), the advantages of enzymatic approach were used in a quantitative cell-wall protein profiling of invasive and non-invasive *S. cerevisiae* strains (Z u p a n et al., 2009). Cell-wall protein isolation was performed enzymatically on viable cells by using mild, isosmotic reaction conditions and pure, proteinase free glucanase. The results of quantitative SDS-PAGE analysis revealed the presence of up to 20 protein bands with molecular masses in the range 60–220 kDa. In addition, comparative analysis of cell-wall protein profiles resulted in significant changes in the protein profile expression relevant to different cultivation temperature, cell morphology (invasive vs. non-invasive growth) and yeast strain. The differences were most significant among different strains; again, proteins were most abundant in strain YJM311, which was the most virulent strain used in the experiment (B y r o n et al., 1995), while the protein profile of strain YJM222, which was the least virulent,

showed the lowest amounts of cell-wall proteins (20× less if compared with YJM311). The results may indicate that adhesiveness which is not conditioned solely by specific adhesins, but more likely by the amount of adhesions expressed on the surface of cells.

*Host-pathogen interactions – in vitro testing of strain pathogenicity*

The criticism of agar-invasion assays and similar tests that simulate the environment in which the examining phenomenon occurs is that the results are not extrapolatable *in vivo*. Therefore, *in vitro* assays are good solutions for avoiding experiments on animals. In this aspect, cell cultures offer valuable working tool. As they can simulate human gut epithelium, they can be used for assessing beneficial or negative aspects on human health regarding probiotic/ prebiotics and pathogenic microorganisms (A l o i s i o et al., 2012; B o t i c et al., 2007; C e n c i c et al., 2002; P o g a c a r et al., 2010), nutraceuticals/functional foods/food supplements (C e n c i č and C h i n g w a r u, 2010b; L a n g e r-h o l c et al., 2011), new drugs (C e n c i č and L a n g e r h o l c, 2010), beneficial plant extracts (C e n c i č and C h i n g w a r u, 2010a), etc. The knowledge about yeast pathogenicity is mostly based on experiments with *C. albicans* and consecutively, cell culture assays have been adapted to this yeast pathogen, e.g. adhesion assays (A r i e et al., 1998; B a i l e y et al., 1995; J o n g et al., 2001; K l i n g b e r g et al., 2005; Z i n k et al., 1996), invasion assays (D i e t e r i c h et al., 2002; H o w a r d, 2001; J o n g et al., 2001; O d d s et al., 2001), and citotoxicity assays (B a t i s t a, 2005; B e r g a m i n i et al., 1992; F r e s h n e y, 2000; M o s m a n n, 1983). Actually, the assays are very similar to the assays used with bacterial pathogens (B a d g e r et al., 1999; B u r k-h o l d e r and B h u n i a, 2009; H u a n g et al., 1995; R a m a r a o and L e r e-c l u s, 2006; v a n P u t t e n and P a u l, 1995). One of the obvious problems with assays based on bacteria is that yeasts are eukaryotes and methods that are based on the detection of eukaryotic cell-culture response e.g. metabolic activity (test MTT) and release of $H_2O_2$, NO, or $O_2^-$ etc. could not be used. This is one of the reasons why is the pathogenicity of *S. cerevisiae* so poorly understood. *S. cerevisiae* is not an aggressive pathogen and its pathogenesis is most probably very different from the pathogenesis of *C. albicans*. Namely, *C. albicans* is only distantly related to *S. cerevisiae* and among pathogenic yeasts represent a unique organism with unique characteristics like germ-tubes, true hyphae and "reversed dimorphism" (yeast form in the host and hyphae outside the host). Even *C. glabrata* is more related to *S. cerevisiae* than to *C. albicans*. Therefore, the methods used to study *C. albicans* or bacterial pathogenicity are hardly suitable for studying pathogenicity of *S. cerevisiae* and other weaker yeast pathogens, because they will always show zero effect as it was already showed (K l i n g b e r g et al., 2008). This is also the reason why there are no cell-culture publications which would show the difference among strains of *S. cerevisiae* regarding its virulence. Differences surely exist and they were shown in *in vivo* experiments in murine system (B y r o n et al., 1995; M c  C u s k e r

et al., 1994b), therefore the assays should be modified to see this difference properly. In this case, agar-invasion assays can help to select appropriate test strains. The steps in this direction were made in the doctorial dissertation, in which new solution regarding modifications of existing cell-culture assays were proposed and preliminary results were documented (Zupan, 2010).

## Conclusion and future perspectives

Besides the topics covered in this review many other approaches, presented in Figure 1 are still waiting to be explored. A development of new methods on cell cultures for the study of *S. cerevisiae* virulence is surely an important perspective as stressed above. When a reliable phenotypic virulent trait is established, new genetic markers for rapid detection of virulent strains should be established. Analysis of microsatellites seems to be most promising in this area (H e n n e q u i n et al., 2001; M a l g o i r e et al., 2005), but till now little success with selection of primers has been shown. Another important aspect is also yeast biofilms, which are interesting from the view of adhesion and antimicrobial resistance. The latter is important also because unlike *C. albicans*, *S. cerevisiae* is resistant to azole antimycotics (S o b e l et al., 1993) and presents a problem when amphotericin B can not be administered. When looking deeper into the problem, signal transduction pathways and its genetic background responsible for the expression of virulent traits can be examined using modern proteomic and genetic approaches (C u l l e n and S p r a g u e, 2000; L i u et al., 1993; M a d h a n i and F i n k, 1997; P a l e c e k et al., 2002a). Nevertheless, the main obstacle lies perhaps in the perception of *S. cerevisiae* as a pathogen. It is used in many biotechnological applications and even sold as a probiotic. From this perspective it represents no threat for healthy individuals and attracts little attention. However, it is also one of the best studied organisms on the planet and a perfect model for many human illnesses and why would it not be a perfect model for studying new emerging yeast pathogens, which are winning the battle against antimicrobials?

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МЕТОДОЛОШКИ ПРИСТУПИ ЗА РАЗОТКРИВАЊЕ НЕПОВОЉНИХ
ОСОБИНА ГЕНЕРАЛНО БЕЗБЕДНОГ *SACCHAROMYCES CEREVISIAE*

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Резиме

Квасац *Saccharomyces cerevisiae* показује две особине; има корисну, биотех-
нолошку вредност са јаком конотацијом поуздане безбедности, као и негатив-
ну, потенцијално је патогене природе с друге стране. Овај преглед расправља ме-
тодолошке приступе који су коришћени за разоткривање вирулентних особина
које треба да одвоје патогене и непатогене сојеве. Ове методе могу бити сортиране

у четири главне групе у погледу физиологије / морфологија, генетика, протеомика и интеракција између патогена и домаћина. Оне се могу користити да обогате знање о нпр. инвазивном расту, брзој молекуларној идентификацији, адхезинима ћелијског зида и вируленцији квасца, респективно. Већина ових метода је углавном прилагођена за водећи патогени квасац *Candida albicans* или бактеријске патогене. *S. cerevisiae*, с друге стране, може да делује као слаб опортунистички патоген у одређеним условима и његова патогеност није упоредива са патогеношћу *C. albicans*; стога су уведене нове, измењене методе. Нови приступи су већ показали јаку индукцију инвазивног раста услед недостатка хране и на температурама типичним за људску грозницу (37-39 °C), откривајући потенцијал *S. cerevisiae* сојева да изазову болест код људи. Репресивни ефекат с друге стране је примећен у присуству соли, аноксија и неких конзерванса, који повезује значај мера безбедности хране. Улогу протеина ћелијског зида, који су укључени у адхезију на пластичне материјале као што су катетери и друге компоненте ћелијог зида које се везују за епителне површине домаћина, треба подвргнути детаљнијим студијама. Механизми резистенције на антимикотике и технике брзе молекуларне идентификације које идентификују потенцијално патогене сојеве треба да привуку оштрију пажњу истраживања.

КЉУЧНЕ РЕЧИ: *Saccharomyces cerevisiae*, вируленција, адхезија, протеини ћелијског зида, инвазивни раст, резистентност на азоле

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# THE CONTRIBUTION OF BIOETHANOL TO SUSTAINABLE DEVELOPMENT IN SERBIA

ABSTRACT: The pollution caused by the use of fossil fuels for the production of mechanical or electrical energy is one of the most important environmental issues nowadays. In this respect, biofuels represent a viable source of energy. Bioethanol as a renewable energy source is derived from organic material of plant origin, so-called biomass, thus reducing environmental pollution. The aim of this study was to analyze the potential of bioethanol in meeting future energy demands in the Republic of Serbia.
KEY WORDS: bioethanol, bioprocess, renewable energy, Serbia, sustainable development

## INTRODUCTION

The national reserves of oil and gas in Serbia are limited and the country is heavily dependent on the import of oil. The oil import bill is a serious strain on the country's economy and has been deteriorating the balance of payment. The country has become increasingly dependent on fossil fuels and its energy security builds upon the fragile supply of imported oil that is subject to disruptions and price volatility (G o l u š i n and M u n i t l a k - I v a n o v i ć, 2009). Contribution of renewable energy sources to total energy consumption of Serbia contemporary amounts to less than 1%, apropos of 280 GWh/year. By combining the methods of introducing new and renewable sources, systematic application of methods for increasing energy efficacy, as well as methods for introducing new technologies, the percentage of contribution of non-conventional energy sources in Serbia could be increased by as much as 20% (D o d i ć et al., 2009 a, b).

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In Serbia, bioethanol for fuel has still not been produced, although there are indications of preparation for the construction of plants for such purposes. That is why, it is necessary for the competent bodies of the Serbian Government to introduce corresponding programs and plans that should define and regulate the production of bioethanol as a fuel in Serbia as soon as possible. Having in mind that we are situated in Europe, and that we are oriented to joining the EU, it is probable that regulatory as well as stimulative measures ought to be similar to the already existing ones in the EU in that region.

Taking into account our agricultural production, it can be concluded that the raw materials for bioethanol production in Serbia can primarily be sugar- (sugar beet) and starch-containing raw materials (corn, wheat surpluses, potato surpluses or waste potato), followed by specifically intended raw materials planted on the unused soils (hybrid sorghum, Jerusalem artichoke – topinambour, triticale) ( D o d i ć et al., 2010). According to the estimations, the agricultural production in Serbia has already realized annual surpluses of cereals in the past couple of years, amounting to about 1 million of tons (mainly corn, and after it, wheat) that could be used for the production of bioethanol as a fuel. Besides this, it is estimated that there is some 1.000 km$^2$ of non cultivated soils in Serbia that offer possibilities for planting cultures intended for bioethanol production (sorghum, Jerusalem artichoke).

Unavoidable prerequisite for the realization of economical production of bioethanol from husbandry cultures is the complete utilization of all by-products, primarily spent grains and slopes, with their basic application as feed. Also, the applied process technology, as well as microbial species and their adaptation to the process conditions have a significant effect on the process economy. In that respect, modern processes, that are much more efficient and energy saving, are favorable for substrate pretreatment, fermentation and especially, distillation and dehydratation stages of ethanol production.

In pursuance of the experiences and norms that are established in the EU, bioethanol that is to be applied as a mixture with motor gasoline has to fulfill the requirements regarding its maximal allowable water content of 3.000 ppm (B r i d g w a t e r et al., 2002; R a m a c h a n d r a et al., 2000). Besides this, bioethanol intended for fuel must have high ethanol content and must be refined and denatured. Apart from defining bioethanol that is to be used in Serbia as a fuel, it is also necessary to adjust the existing rules to the corresponding European standards.

Demands for bioethanol as an addition to motor fuel, which amounted about 5.75% in the year 2010 in Serbia, would be about 80,000 tons. The needs for bioethanol in pharmaceutical and chemical industries, as well as for the production of strong liquors are to be added to this quantity of bioethanol.

This paper presents the potential for the development of bioethanol production in Serbia.

Production of bioethanol on small farms can be successfully applied for processing of only 30 kg of corn per day, with obtaining crude ethanol in the so-called "brandy ladle" and the use of lygnocellulosic agricultural wastes as an energy source. Figure 1 shows basic material balance of such a production of ethanol (V u č u r o v i ć et al., 2011).
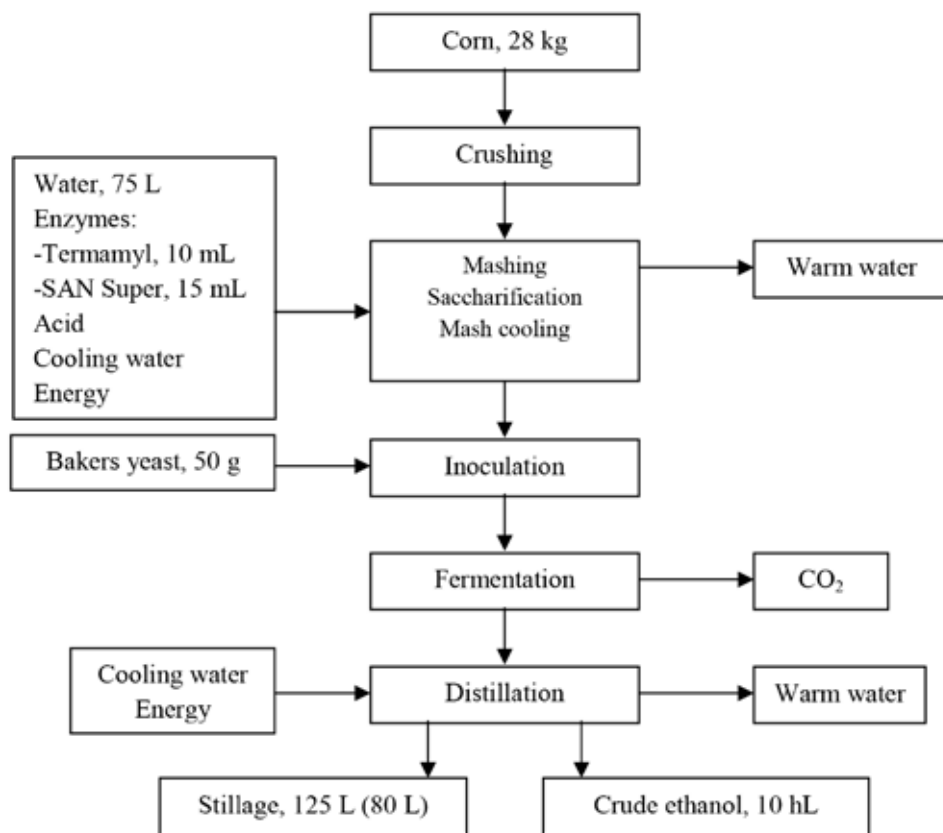


Fig. 1. – Schematic presentation of material balance for bioethanol production on small farms

If one of the strategic decisions for processing starchy raw materials on small farms is to be adopted, consumption of slopes or spent grains in so-called wet feeding of livestock appears as an imperative. In such a case, for example defined in Fig. 1, the minimal number of animals that should be permanently fed in the stockyard is 3–4 cattle, or about 20–30 pigs. In case of larger capacity, such as processing of about 300 kg of corn per day and production of about 1.000 liters of stillage per day, it is necessary to have crib for fattening of 30

young bulls all around the year, with animals of different ages (V u č u r o v i ć et al., 2011).

In case there is a need for the construction of a larger number of such plants, the only possible solution lies in the construction principle of the so-called "satellite plants", which will enable the production of crude ethanol on small farms, thus obtaining and consuming stillage for animal feeding, and consuming agricultural wastes as energetic fuels. Such obtained crude bioethanol would be transported to a central rafination plant, distillation-rectification unit which has modern equipment (Fig. 2).
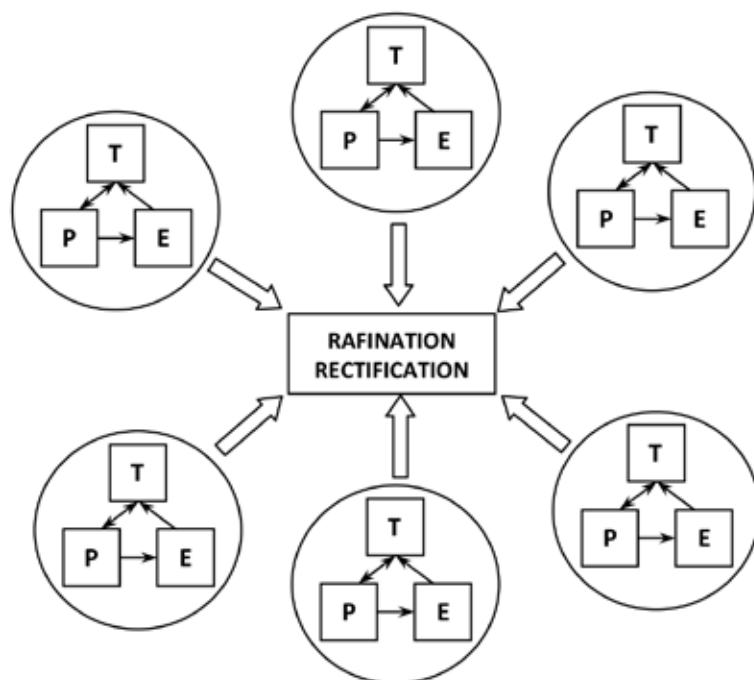


Fig. 2. – Conceptual scheme of bioethanol production on small farms in Serbia, with rectification in the common central processing unit (E – production of crude bioethanol; T – animal feeding; P – agricultural production)

*Bioethanol production on large farms*

If stillage is to be used as feed in wet feeding, it is estimated that, due to the restrictions imposed by the size of animal farm, the upper capacity limit for such plants is some 10 – 15 tons of corn per day, that is, the production of 3.000 – 3.500 hL of absolute ethanol per day. Basic material balance of such production is outlined in Fig. 3. In such case, for animal feeding it necessary to have a herd with 1.300 – 1.700 milking cows or 5.000 – 25.000 heads of sheep and/or pigs (B a r a s et al., 1996).

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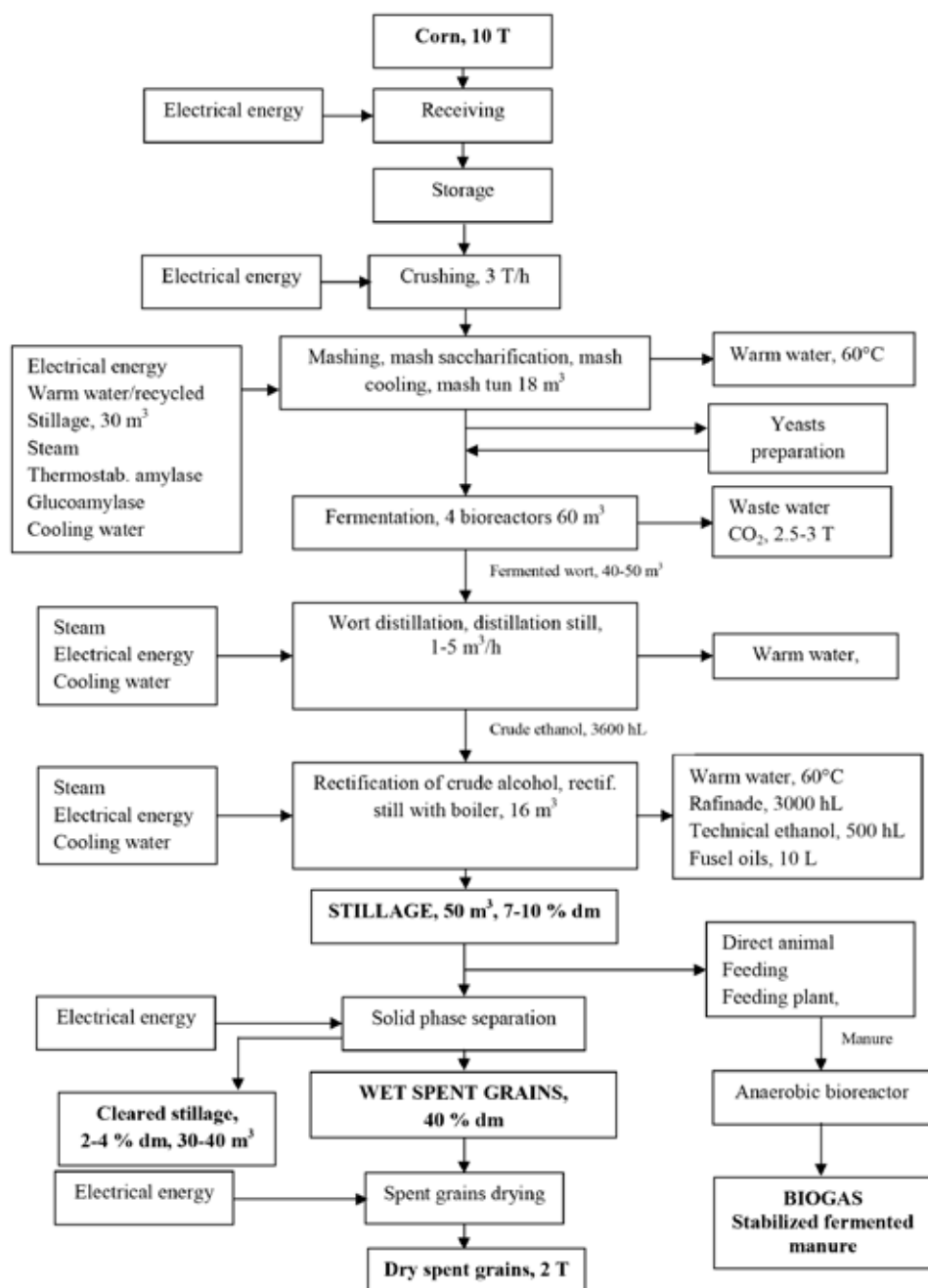
Fig. 3. – Block scheme of the technological process for processing 10 tons of corn per day

In the case of working at small farms (Figs. 1 and 2), the obtained excrements supposedly should not cause any problems, because by using the primitive process they could be used as manure. In spite of that, more convenient solution and unavoidable solution for larger enterprises (Fig. 4) is their conversion by anaerobic biotechnological conversion into the stabilized manure (bio-fertilizer) and biogas (energent). So, the obtained biogas could be used for supplementing the energetic needs of ethanol factory, as well as of the farm itself (G o l u š i n et al., 2011).
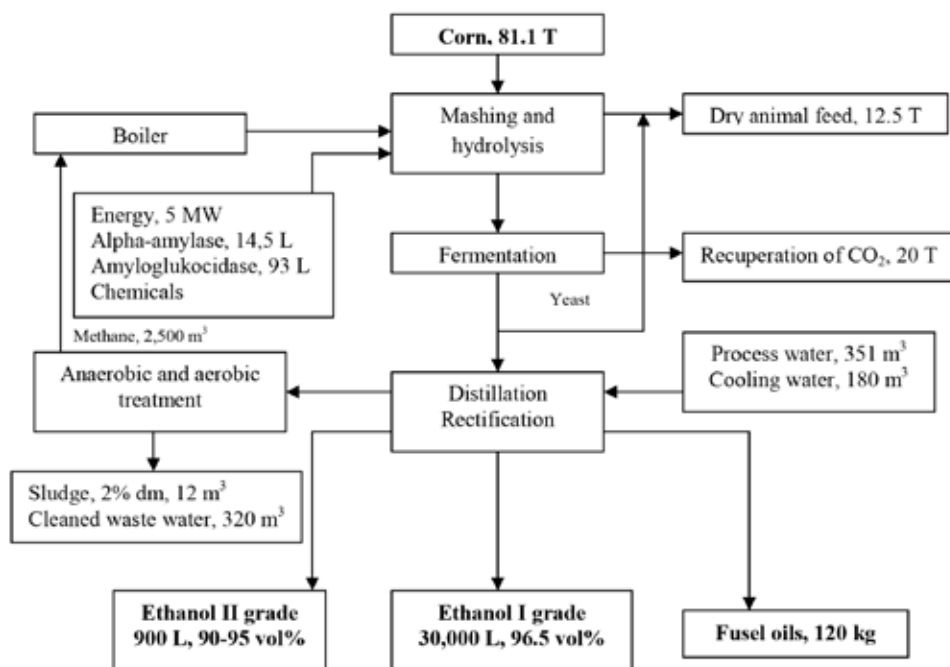


Fig. 4. – Scheme of the conventional technological model for ethanol production from cereals with its material balance for a capacity of 30,000 hL/day

Such an approach has considerable effect on the environment because it solves the questions of waste water streams from the ethanol factory, as well as the questions of waste waters and unpleasant odors from animal production, especially in the case of pig feeding. With no need to perform broader analysis, it is clear that the construction of a plant for bioethanol production on larger farms represents serious investment actions. The principal disadvantage of these actions lies in the fact that, due to relatively small capacity, it is almost impossible to apply contemporary knowledge in domains of distillation and rectification. Therefore, energetically extensive solutions, which started at the beginning of the last century, have to be applied.

Considering the facts that the construction of such enterprises with relatively large capacities requires possessing or constructing the farms for stillage

consumption by wet feeding process, and that problems of excrements must be solved, the value of the necessary investments significantly increases. Because of that, such a solution is recommended only under exceptional circumstances when favorable conditions permit it. However, preference is given to the abovementioned system of small plants with central rafination, or to the construction of large, and even of huge (industrial) plants for processing of starchy raw materials into bioethanol.

## CONCLUSION

By analyzing the existing capacities and equipment for the production of bioethanol in Serbia, it can be concluded that the existing equipment and capacities cannot satisfy the needs for the production of bioethanol for fuel. With the reconstruction and modernization of the existing plants, especially in domains of raw materials processing and bioethanol dehydratation, they could be reconditioned for the production of a part of bioethanol for fuel. Nevertheless, the capacities would be insufficient for fulfilling the needs of ethanol as a supplement to motor fuels, so the construction of new capacities is required. Pursuant to that, several concepts could be discussed; one of them is the construction of larger plants, or the construction of so-called "satellite" plants for the production of crude ethanol on small farms, with obtaining and consumption of used grains (for livestock feeding), using at the same time agricultural waste as supplies of energy for these plants. Such obtained crude bioethanol is supposed to be transferred on raffination into central, contemporary equipped distillation-rectification installation.

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## ДОПРИНОС БИОЕТАНОЛА ОДРЖИВОМ РАЗВОЈУ У СРБИЈИ

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### Резиме

Загађење изазвано употребом фосилних горива за производњу механичке или електричне енергије представља једно од најважнијих еколошких питања данашњице. У том погледу биогорива представљају одрживо решење за производњу енергије. Биоетанол се као обновљиви енергетски извор добија из органских материја биљног порекла, тзв. биомасе, чиме се смањује загађење животне средине. Циљ овог рада је анализа потенцијала биоетанола у задовољавању будућих енергетских потреба у Републици Србији.

КЉУЧНЕ РЕЧИ: биоетанол, биопроцес, обновљива енергија, Србија, одрживи развој

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# THE EFFECT OF PRECONDITIONING CELLS UNDER OSMOTIC STRESS ON HIGH ALCOHOL PRODUCTION

ABSTRACT: This paper focuses on the research into the influence of salt on physiology of the yeast, *Saccharomyces cerevisiae*. Specifically, the work focused on how NaCl affected the growth, viability and fermentation performance of this yeast in laboratory-scale experiments. One of the main findings of the research presented involved the influence of salt "preconditioning" of yeasts which represents a method of pre-culturing of cells in the presence of salt in an attempt to improve subsequent fermentation performance. Such an approach resulted in preconditioned yeasts having an improved capability to ferment high-sugar containing media (up to 60% w/v of glucose) with increased cell viability and with increased levels of produced ethanol (higher than 20% in vol.). Salt-preconditioning was most likely influencing the stress-tolerance of yeasts by inducing the synthesis of key metabolites such as trehalose and glycerol which act to improve cells' ability to withstand osmostress and ethanol toxicity. The industrial-scale trials using salt-preconditioned yeasts verified the benefit of the physiological engineering approach to practical fermentations. Overall, this research has demonstrated that a relatively simple method designed to adapt yeast cells physiologically – by salt-preconditioning – can have distinct advantages for alcohol fermentation processes.
KEY WORDS: alcohol production, osmotic stress, preconditioning, viability, yeast

## 1. INTRODUCTION

During alcoholic fermentation, yeasts are subjected to several physic-chemical stresses such as: initially, high sugar concentrations and low temperature; and latterly, increasing ethanol concentrations. Such conditions trigger a series of biological responses in an effort to maintain yeast cell viability and cell cycle progress, but very few studies of yeast stress responses have been reported in wine strains. In laboratory strains of *Saccharomyces cerevisiae*, many studies have focused on transcriptional activation and gene expression.

Such responses can be distinguished by different stages: cellular changes that occur immediately as direct consequences of the physic-chemical forces; activation of the primary defensive processes and changes in cell homeostasis. Concerning the osmostress, a number of physiological changes take place, including: efflux of intracellular water, with associated rapid reduction in total cell volume, including the vacuole (A l b e r t y n et al., 1994); transient increases in glycolytic intermediates (A l e x a n d r e and C h a r p e n t i e r, 1995); accumulation of cytosolic glycerol; and triggering of the HOG (Hyper Osmotic Glycerol) signaling pathway (A l e x a n d r e H. et al., 2001).

With salt stress, microorganisms such as the yeast *S. cerevisiae* develop systems to counteract the specific effects of sodium chloride. For example, salt-induced stress results in both ion toxicity and osmotic stress and cellular defense responses are based on sodium exclusion and osmolyte synthesis, respectively. The latter includes polyols, specifically glycerol that are accumulated intracellularly (A l l i s o n S. D. et al., 1999, A r n e b o r g N. et al. 1995 A t t f i e l d P. V., 1987). Other products synthesized by yeast under osmostress conditions are trehalose and glycogen that may collectively represent 25% of the dry cell mass depending on the environmental conditions. The disaccharide trehalose accumulates not only during salt adaptation (A t t - f i e l d P. V., 1997, A t t f i e l d P. V. and B e l l P.J.L., 2003), but also in response to a number of other stress conditions including protection against high temperature where it acts by stabilizing proteins and maintaining membrane integrity (B a c k h u s L. E., 2001, B e l l i n g e r Y. and L a r h e r F., 1987, B e n e y L., et al., 2001).

Exposing yeast cells to a hyper osmotic environment leads to a rapid initial efflux of cellular water into the medium, effectively dehydrating the cell. Intracellular water can also be recruited from the vacuole into the cytoplasm, thus partially compensating for sudden increases in water loss. Additionally, the cytoskeleton collapses leading to depolarization of actin patches. Cell dehydration leads to growth arrest, and cellular accumulation of compatible solutes to balance intracellular osmotic pressure represents a major compensatory or adaptation mechanism. Depending on the osmotic stressor, the compatible solutes can be glycerol, trehalose, amino acids, and fatty acids in cell membranes. Hyperosmotic stress caused by sodium chloride leads to the increases in intracellular glycerol concentrations due to elevated biosynthesis, increased retention by cytoplasmic membranes, or decreased dissimilation or uptake of glycerol from the medium. Glycerol is produced during glycolysis by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (GPD) (B e t t K. E. and C a p p i J. B., 1965, B l o m b e r g A., and A d l e r L., 1989, B l o m b e r g A., and A d l e r L., 1992, B r e w s t e r J. L. et al., 1993). Under osmotic stress, GPD activity is enhanced and this requires an equimolar amount of cytoplasmic NADH resulting in decreased reduction of acetaldehyde to ethanol and increased oxidation to acetate. The observed decrease in the synthesis of alcohol dehydrogenase as well as the increase of the aldehyde dehydrogenase could account for this alteration in flux.

The present work was based on the hypothesis that osmotic stress caused by NaCl would improve wine yeast viability due to accumulation of cellular protecting molecules and pre-adapted yeast cells under osmotic stress conditions can be used as inoculums to run high gravity fermentations and to produce high alcohol content.

Recent research (L o g o t h e t i s S. et al., 2006, 2007, 2012) has shown the benefits of applying a mild osmotic stress to yeasts physiology and fermentation performance. For example, preconditioning yeast cells with salt imparts an ability to tolerate subsequent fermentation stresses due to high alcohol content, high sugar concentrations, low pH and fluctuating temperatures.

## 2. MATERIALS AND METHODS

### 2.1 Yeast cultures

Three different yeast strains of *S. cerevisiae* were used for laboratory experiments. Strains designated as *DV 10, Fermol* and *Syrah,* which were produced by Lalvin kindly gifted by EnoChimiki S.A. Athens Greece, Speedal S.A. Thessaloniki Greece and Martin Vialatte, were kindly gifted by Ampeloiniki S.A. Thessaloniki Greece.

### 2.2 Inoculum preparation (cell preconditioning)

Yeast cells were grown in defined medium containing (per liter deionised water): 100 g D-glucose, 1 g $K_2HPO_4$, 1 g $K_2H_2PO_4$, 0.2 g $ZnSO_4$, 0.2 g $MgSO_4$, 2 g yeast extract and 2 g $NH_4SO_4$. All the media components were purchased from Sigma Chemical Company. Medium contained NaCl (commercial NaCl was used) 6% w/v for 24 hours were used for pre conditioning under osmotic stress experiments. The total volume of the medium for each fermentation medium was 250 ml.

### 2.3 Fermentation media preparation

The medium for experimental laboratory fermentations consisted of the following: 550 g/L glucose, 1 g/L $K_2HPO_4$, 1 g/L $K_2H_2PO_4$, 0.2 g/L $ZnSO_4$, 0.2 g/L $MgSO_4$, 2 g/L yeast extract and 2 g/L $NH_4SO_4$. Mineral components and the glucose were sterilized separately at 120ºC, and 2 Atm pressure for 20min. Batch fermentations were carried out in 300 ml volume of glass flasks containing 250 ml of growth medium without shaking at 25°C. After inoculation, 1 mL was periodically taken directly from each flask in order to monitor the differences between stressed and unstressed yeast cells with respect to yeast population growth and cell viability.

## 2.4 Yeast growth and viability determination

Yeast cell number was determined by using the Thoma haemocytometer and yeast cell viability by using the methylene blue method of Lee et al., (1981).

Cell viability was determined using the Thoma haemocytometer as follows: 1 mL of sample medium was taken and diluted in 9 mL of deionised water. The sample of 1 mL of this solution was dissolved with 1 mL of 10% v/v methylene blue solution and left for 10 minutes. Aliquots of 1μL were placed on the haemocytometer by using a Pasteur pipette. The haemocytometer was then microscopically observed by an optical microscope (Olympus model CHK2-F-GS microscope). Yeast cell viability was calculated and expressed as follow: Viability (%) = a/n x 100.

Where a: number of metabolically active cells; n: total cell number. Since cellular viability needed to be determined immediately after hyperosmotic treatments, vital staining with methylene blue, which is rapid and accurate, was used.

## 2.5 Fermentation analysis

The analysis of glucose, alcohol and glycerol was made by ELITE LaChrom HPLC system comprised of a VWR  HITATCH L-2130 pump, a VWR  HITATCH L-2200 autosampler fitted with a 20 μL sample loop, and a VWR  HITATCH L-2455 Diode Array detector. Peak data were collected with Agilent EZChrom Elite Client/Server Enterprise Data System Aminex HPX-87X.

## 2.6 Statistical analyses

All experiments were conducted in triplicate. Results were analyzed with the statistical program called BioStat Plus 2008 (version 5.3.0.6 by AnalySoft Comp) using the Basic Describe Statistics package.

## 3. RESULTS AND DISCUSSION

### 3.1 The effect of preconditioning under osmotic stress conditions caused by NaCl on growth and viability of the industrial yeast strains

It is known that when NaCl is added to a growth medium containing yeast cells the intracellular concentration of $Na^+$ increases, and this has a negative effect on yeast growth and viability (R o d r i g u e z - N a v a r r o and O r t e g a, 1981). Similarly, Fuping et al. (2005) have shown that treatment of *S. cerevisiae* cells with KCl of up to 6% causes a great loss of viability. A gradual in-

crease of sodium chloride in a growth medium containing yeast cells can cause a cell growth arrest depending on the sodium chloride concentration (N o r b e r g and B l o m b e r g, 1997). Experiments have shown that the leavening ability of baker yeasts decreases dramatically during cultivation in media containing sodium chloride concentrations between 0 and 3% (O d a and T o n o m u r a, 1992). Previous research (M o r r i s et al., 1986) showed that the greatest loss in cell viability was caused under hyper osmotic conditions between 0 and 1 OSM, (OSM=Osmolarity=5.85%NaCl w/v). Interestingly, between 1 and 4 OSM, the loss of viability was smaller (M o r r i s et al., 1986). Comparative studies on the effect of osmotic stress on *S. cerevisiae* and non-*Saccharomyces* strains showed that non-*Saccharomyces* strains displayed higher salt tolerance (G a r - s i a et al., 1997). In general, previous studies have revealed that osmotic stress and especially sodium chloride-induced stress cause growth arrest and have negative effect on the viability of yeast cells.

In contrast, latest research works have shown that long adaptation to saline stress (up to 10% of NaCl w/v) have positive effect on yeast cell viability (L o g o t h e t i s S. et al., 2006).

In the following laboratory-scale series of experiments (Figures 1 to 2), the effects of preconditioning to saline stress and saline stress (6% NaCl w/v) on industrial wine yeast cells growth and viability were investigated. The findings indicate that the preconditioning caused a faster growth and the difference between untreated cells and the cells which were pretreated under osmotic stress was significantly high. The results shown in Fig 1 indicate the difference between the three species. If we compare Figures 1 and 2 which show yeast growth and yeast viability, it can be seen that yeast cells exposed to sodium chloride have the highest growth in comparison to the cells which were untreated. Especially, Figure 2 shows the highest difference regarding cell growth for a time period of 88 hours. Previous research supports the concept that when *S. cerevisiae* cells are exposed to high concentrations of NaCl, they show reduced viability resulting in cells acquiring tolerance against a severe salt shock (up to 1.4 M NaCl= 8.19 % NaCl w/v) following the previous treatment with 0.7 M NaCl (4.09 % NaCl w/v) (Varela et al., 1992). Overall, previous reports describe the osmotic stress of yeast cells for no more than a few hours, but in the current research, cells are treated for 120 hours (Fig. 1) during the fermentation process.

### 3.2 High Gravity Fermentation

Previous studies have reported that some agents, like sodium chloride, play an important role in minimizing or inhibiting the fermentation process, specifically with regard to glucose utilization for the production of yeast biomass (E l-S a m a r g y and Z a l l, 1988). Much earlier studies regarding this subject had been performed. For example, in the 1920s, studies concerned with the fermentability of yeasts during cereal wort fermentation under different sodium chloride concentrations showed that the fermentative ability of
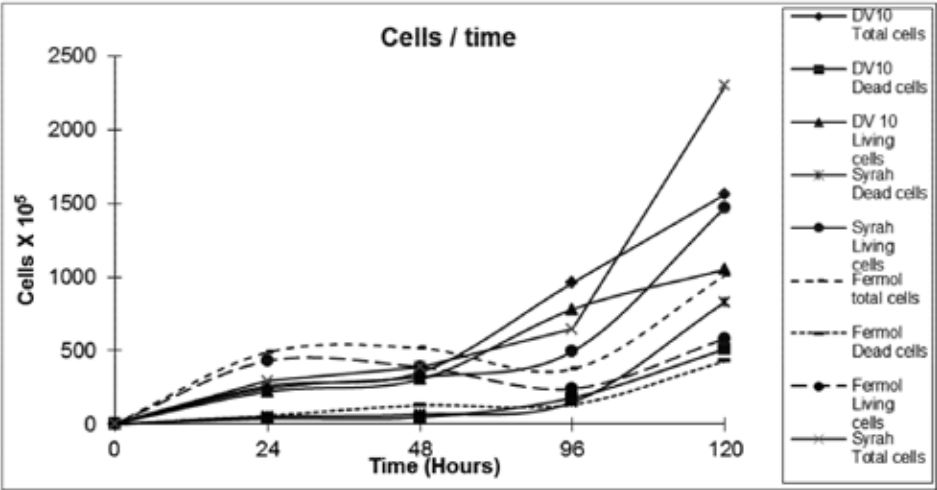
Fig. 1. – Influence of salt-induced osmotic stress (6% w/v NaCl) on yeast cell growth. *Saccharomyces cerevisiae* (strains DV10, Syrah and Fermol) was grown on a glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined by methylene blue staining using a haemocytometer at the intervals shown and the standard error was between 2.75 and 4.28%.
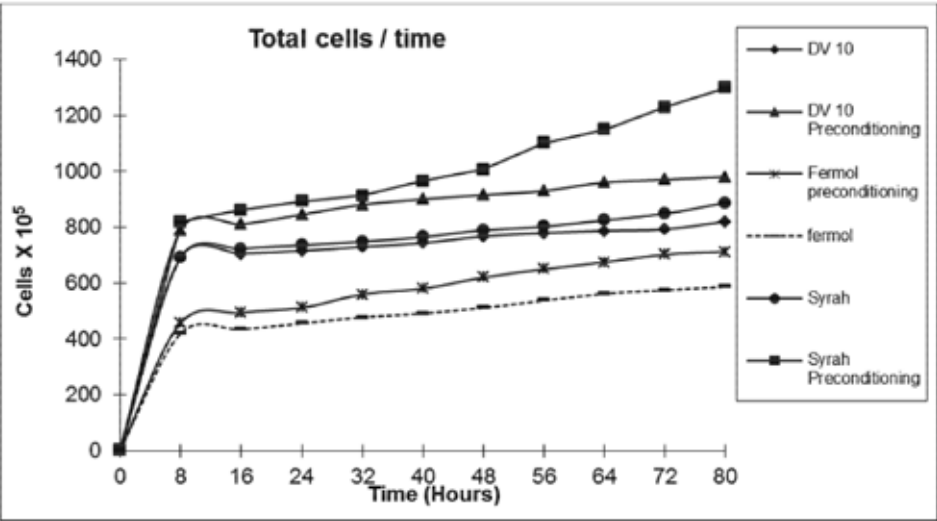


Fig. 2. – Influence of preconditioning under osmotic stress (6% w/v NaCl) on yeast cell growth. *Saccharomyces cerevisiae* (strains DV10, Syrah and Fermol) was pretreated under osmotic stress conditions caused by 6% w/v NaCl for 24 hours and grown on a glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined by methylene blue staining using a haemocytometer at the intervals shown and the standard error was between 2.03 and 3.88%.

yeast increased when pre-conditioned with 5% w/v NaCl, but for higher concentrations, a gradual reduction of fermentative efficiency was reported (S p e a k m a n et al.,1928). In the second part of the presented work, it is clearly shown that preconditioning of the yeast cells under 6% NaCl for 24 hours had a good glucose utilization considering that the starting concentration of glucose was 550 g/L Fig 4.

The main hypothesis of this section was that the preconditioned yeast cells had acquired an increased alcohol tolerance and ethanol production capability. Additionally, osmotic pre-stress due to NaCl-preconditioning enabled yeast cells to ferment sugars at high concentrations and produce high yields of alcohol. We hypothesize that the likely mechanism for this phenomenon lies at the level of elevated stress gene expression, especially linked to the increases in the intracellular concentration of stress-tolerance metabolites such as glycerol and trehalose.

Figure 3 shows that the level of alcohol after 288 hours of fermentation was 23.5% in volume since the highest level that Syrah strain can rich, as specified byMartin Viallate, is 16.5 % in volume.
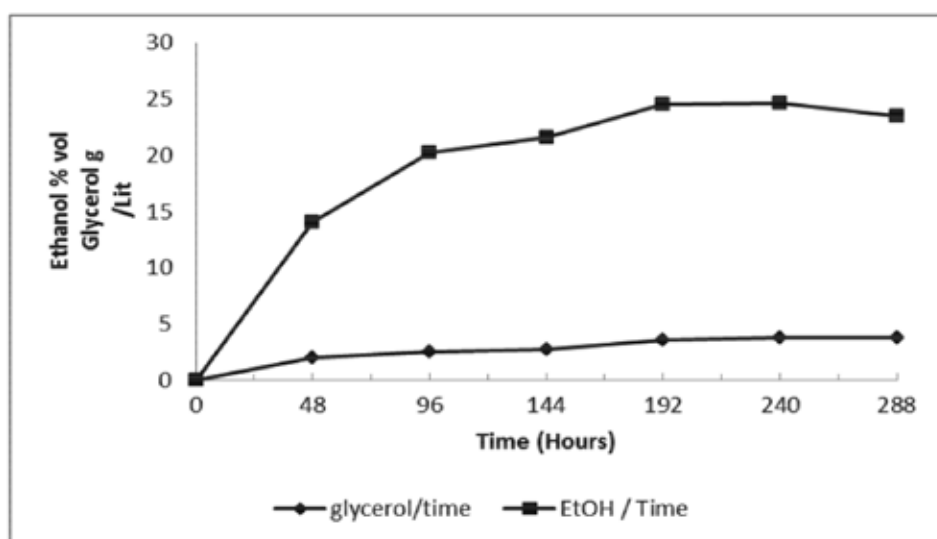


Fig. 3. – Alcohol and glycerol production by salt-preconditioned wine yeasts during fermentation. Fermentations were conducted with preconditioned yeasts (strain *Syrah*) using 6% NaCl and growing cells for 24 hours prior to inoculation. Standard error was from 3.4 to 4.95%.
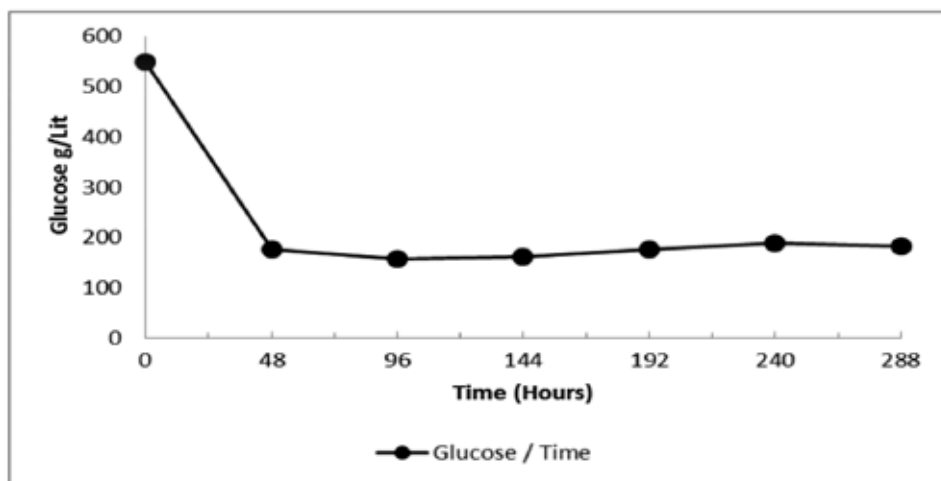
Fig. 4. – Sugar consumption by salt-preconditioned wine yeast during high gravity fermentation. Fermentations were conducted with preconditioned yeasts (strain *Syrah*) using 6% NaCl and growing cells for 24 hours prior to inoculation. Standard error was between 3.7 and 4.6%.

## 4. CONCLUSIONS

To summarize, this research work has revealed that osmotic pre-stress due to NaCl-preconditioning enabled yeast cells to ferment sugars at high concentrations and achieve high yields of alcohol. We hypothesize that the likely mechanism for this phenomenon lies at the level of elevated stress gene expression, especially linked to the increases in the intracellular concentration of stress-tolerance metabolites such as glycerol and trehalose. Additional research that has already been made (L o g o t h e t i s S., et.al., 2012) can verify the applicability of NaCl-conditioning in other yeast biotechnologies like brewing industry or distillery industry.

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## УТИЦАЈ ПРЕКОНДИЦИОНИРАЊА ЋЕЛИЈА У УСЛОВИМА ОСМОТСКОГ СТРЕСА НА ПОВИШЕЊЕ ПРИНОСА АЛКОХОЛА

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Резиме

Овај рад се односи на испитивање утицаја соли на физиологију квасца *Saccharomyces cerevisiae*. Тачније, у раду се испитује утицај NaCl на раст, вијабилност и ферментациону активност овог квасца у лабораторијским условима. Најважније откриће овог истраживања се односи на утицај прекондиционирања ћелија квасца натријум-хлоридом као начина припремне култивације ћелија у присуству соли, на повећање ферментационог учинка овог квасца. Показало се да прекондиционирани квасци имају побољшану способност ферментације у медијумима са високом концентрацијом шећера (до 60% m/v глукозе) уз повећану вијабилност ћелија и повишен ниво продукције етанола (преко 20% vol). Прекондиционирање у условима повишене концентрације соли је највероватније имало утицаја на повећање толеранције стреса код ћелија квасца изазивајући синтезу кључних метаболита као што су трехалоза и глицерол који делују у правцу повећања способности ћелије да се одупре осмотском стресу и токсичном деловању етанола. Пробе у погонским условима у којима је коришћено прекондиционирање у описаним условима су потврдиле да приступ физиолошког инжењеринга може да допринесе унапређивању практичних резултата. Ово истраживање је показало да релативно једноставан поступак прекондиционирања ћелија квасца у условима повишене концентрације соли може значајно да побољша учинак процеса алкохолне ферментације.

КЉУЧНЕ РЕЧИ: производња алкохола, осмотски стрес, прекондиционирање, вијабилност, квасац

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# USE OF NON-SACCHAROMYCES *TORULASPORA DELBRUECKII* YEAST STRAINS IN WINEMAKING AND BREWING

ABSTRACT: Selected *Saccharomyces* yeast strains have been used for more than 150 years in brewing and for several decades in winemaking. They are necessary in brewing because of the boiling of the wort, which results in the death of all yeast cells, with the exception of some Belgian style beers (ex. Lambic), where the wort is left to be colonized by indigenous yeast and bacteria from the environment and ferment naturally. In winemaking their use is also pertinent because they provide regular and timely fermentations, inhibit the growth of indigenous spoilage microorganisms and contribute to the desired sensory characters.

Even though the use of selected *Saccharomyces* strains provides better quality assurance in winemaking in comparison to the unknown microbial consortia in the must, it has been debated for a long time now whether the use of selected industrial *Saccharomyces* strains results in wines with less sensory complexity and "terroir" character.

In previous decades, non-*Saccharomyces* yeasts were mainly considered as spoilage/problematic yeast, since they exhibited low fermentation ability and other negative traits. In the last decades experiments have shown that there are some non-*Saccharomyces* strains (*Candida, Pichia, Kluyveromyces, Torulaspora*, etc) which, even though they are not able to complete the fermentation they can still be used in sequential inoculation-fermentation with *Saccharomyces* to increase sensory complexity of the wines.

Through fermentation in a laboratory scale, we have observed that the overall effects of selected *Torulaspora delbrueckii* yeast strains, is highly positive, leading to products with pronounced sensory complexity and floral/fruity aroma in winemaking and brewing.

KEY WORDS: wine, beer, fermentation, yeast, *Saccharomyces cerevisiae, Torulaspora delbrueckii*

## INTRODUCTION

Most of the non-*Saccharomyces* yeast strains are considered as spoilage yeast due to low ethanol tolerance, low fermentation ability and other negative

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sensory traits, but some strains have been isolated from a variety of species (*Candida, Pichia, Kluyveromyces, Torulaspora*, etc.) that even though they are not able to complete the fermentation, they can still be used in sequential inoculation-fermentation with *Saccharomyces* to increase sensory complexity of the wines (J o l y et al., 2003a,b; 2006, C i a n i et al., 2010). N i s s e n et al. (2003) found that early death of *Torulaspora delbrueckii* during mixed fermentations with *S. cerevisiae* was not due to the presence of ethanol or any other toxic compound but cell–cell contact-mediated mechanism. These non-*Saccharomyces* strains have been commercialized and at least 3 *T. delbrueckii s*trains are now available to the winemaking industry. *T. delbrueckii* strains have also been used traditionally in the production of German style wheat beers (Hefeweizen) for their banana, bubblegum and clove-like flavors. During wine fermentation, *T. delbrueckii* yeast strains produce noticeably higher concentration of higher alcohols, esters, terpenes and phenolic aldehydes as well as other molecules like 2-phenylethanol, linalool, methylvanillin (F a g a n et al., 1981; H e r r a i z et al., 1990; L e m a et al., 1996; K i n g et al., 2000; P l a t a et al., 2003; R e y n a l et al., 2011), which impart a distinct floral and fruity aroma and add to the sensory complexity giving a "wild/natural" fermentation effect. *T. delbrueckii* strains, when compared to *S. cerevisiae* strains, generally exhibit osmotolerance (A l v e s – A r a u j o et al., 2007; B e l y et al., 2008), higher demand for nitrogen and oxygen (V i s s e r et al., 1991; M a u r i c i o et al., 1998; H o l m H a n s e n et al., 2001; H a n l et al., 2005), lower production of volatile acidity, acetaldehyde and acetoin (especially in high gravity fermentations) and depending on the strain, low/medium glycerol, succinic acid, polysaccharides production, volatile thiols like 3-sulfanylhexan-1-ol and other compounds (B e l y et al., 2008; R e y n a l et al., 2001; J o l y et al., 2003a,b; 2006; R e n a u l t et al., 2009; C i a n i et al., 2010; Z o t t et al., 2011).


## MATERIALS AND METHODS


Yeast strains: One strain of *T. delbrueckii* (Td28), two strains of *S. cerevisiae*, (Sc12 and Sc31) isolated from fermenting musts in Greece and 3 commercial *T. delbrueckii* strains, Level 2® (Lallemand), Zymaflore® Alpha (Laffort), Viniflora® Prelude™ (Hansen), as well as a commercial brewing yeast WB-06 by Fermentis, were used in this experiment.

Isolation and conservation media were: YEPD agar consisting of 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar-agar; Lysine agar consisting of 11.7 g/L yeast carbon base, 0.9 g/L L-Lysine, 20 g/L agar-agar; YM agar consisting of 3g/L malt extract, 5g/L peptone, 10 g/L glucose, 20 g/L agar-agar. The media were supplemented with 0.1 g/L chloramphenicol. Sterilization occurred at 121 ºC for 15 minutes.

Fermentation media for wine: for inoculums and fermentations, a synthetic must simulating the grape must composition (N a g a t a n i et al., 1968; S t r e h a i a n o et al., 1984) was used with the following composition: 1 g/L

yeast extract, 2 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4.7H_2O$, 5 g/L $KH_2PO_4$, 50 g/L glucose (for inoculums) and 195 g/L glucose (for fermentations), 3 g/L tartaric acid, 3 g/L L-malic acid, 0.2 g/L citric acid, 2 g/L, pH was adjusted at 3.5 with 1N KOH. For sensory analysis a natural wine produced from fermented grape must of the Greek *Vitis vinifera* var. *Assyrtiko* was used, with the following composition: 204 g/L sugars, total acidity 6.1 g/L expressed as tartaric acid, yeast-assimilable nitrogen 243 mg/L, pH 3.24 and total sulfur dioxide of 35 mg/L. Inoculum $3*10^6$ cells/L, with viability over 96%.

Fermentation medium for brewing: the medium was reconstituted from malt extracts made from wheat and barley malts (dry unhopped extract "Spraymalt Wheat" and liquid "Connoisseurs Range" hopped extract for "Wheat Beer" from Muntons plc) with bottled chlorine-free water to an original gravity of 1.044, and fermentation was followed through weight loss. The inoculum ratio was 7g dry yeast/10 L, with viability over 96%.

Sterilization occurred at 121 ºC for 15 minutes. Media components were purchased as following: yeast extract from bioMerieux, Yeast carbon base, peptone and L-Lysine from Difco, and all other from Sigma-Aldrich.

Analyses for sugars, organic acids, ethanol and glycerol were performed with an ELITE LaChrom HPLC system comprised of a VWR HITATCH L-2130 pump, VWR HITATCH L-2200 autosampler fitted with 20 µL sample loop, and VWR HITATCH L-2455 Diode Array detector and RI detector. Peaks data were collected with Agilent EZChrom Elite Client/Server Enterprise Data System. The column was an Aminex HPX-87X from Biorad, the mobile phase was $H_2SO_4$ 0,05N at 0.4 mL/min, with a column temperature of 40 ºC. Samples were treated for protein removal by mixing 8 parts of sample with 1 part $Ba(OH)_2$ 0.3N and 1 part 5 % $ZnSO_4$ solutions, left for 10 min at room temperature, centrifuged and sterile filtrated through 0.45 mm cellulose acetate filters (Sartorius).

Volatile substances were measured using 8500 Perkin Elmer Gas Chromatographer, with a Head Space Perkin Elmer 8500µ, with a Shimadzu integrator C-R3A using a silica SGE 25 AQ3/BP 20, 25m x0.33 mm column with 0.5 µm film thickness (T a t a r i d i s et al., 1998; T a t a r i d i s, 2001).

Yeast cell number was determined using a Thoma type haemocytometer and yeast cell viability using the methylene blue method by L a n g e et al., (1993). Yeast biomass was measured by dry weight and correlated with optical density measures (O.D.) at 620 nm.

Sensory analysis was conducted with a panel of 10 expert enologists and brewers. All experiments were conducted in triplicate with 1.8 L for synthetic must, 5L for grape must, and 3.5L for wort. Samples were taken and analyzed at regular intervals.

Statistical analysis for the percentage of error, standard error, standard deviation, variation coefficient and curve fitting (smoothing by spline functions) was conducted using Microsoft Excel (N e u i l l y and C e t a m a, 1998; R e i n c h, 1967).

## RESULTS AND DISCUSSION

In synthetic must fermentation at 20 ºC (Figures 1, 2 and 3) there was a clear difference between the fermentation kinetics of *S. cerevisiae* strains Sc12, Sc31 and *T. delbrueckii* Td28. Sc12 was a rapid fermenting strain, Sc31 was a slow fermenting strain. Td28 was even slower fermenting strain than expected for *T. delbrueckii* strain, but it was able to complete the fermentation leaving no sugars, despite the popular belief that due to low alcohol tolerance *T. delbrueckii* strains are not capable of doing so. No lag phase was observed for the three strains.

Td28 cells were significantly smaller than *S. cerevisiae* cells, however the total dry biomass was higher for Td28 when compared to Sc31, but lower when compared to Sc12 (Table 1).
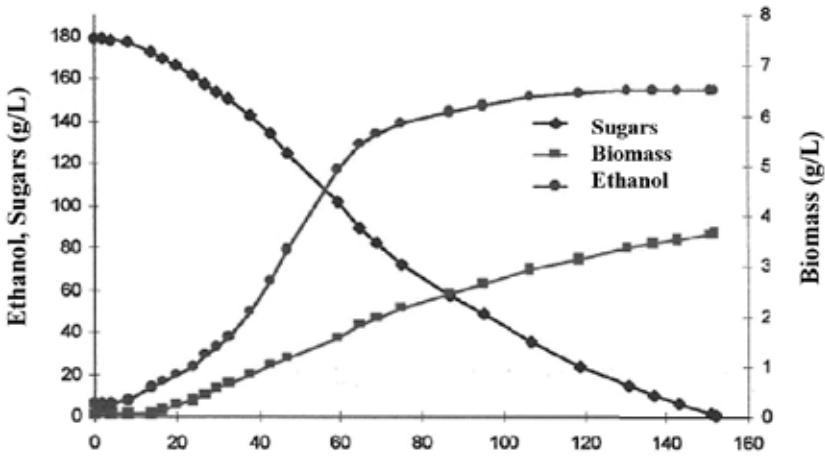


Fig. 1. – Fermentation kinetics of Sc12 at 20 ºC.
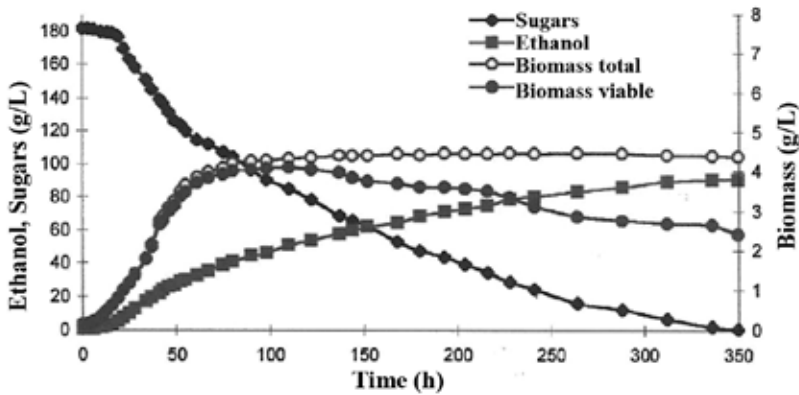


Fig. 2. – Fermentation kinetics of Sc31 at 20 ºC.
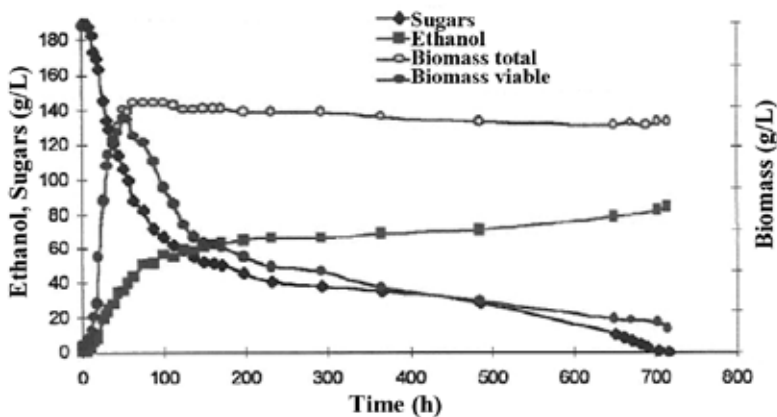
Fig. 3. – Fermentation kinetics of Td28 at 20 ºC.

Correlation between optical density (O.D.) at 620 nm and dry weight was calculated for each strain, from exponentially growing cells, after appropriate dilutions:

Sc12    dry biomass g/L=2.0045*(O.D.)+ 0.1962    ($R^2$=0.9883)
Sc31    dry biomass g/L=1.9739*(O.D.)+0.0942    ($R^2$=0.9928)
Td28    dry biomass g/L=1.4678*(O.D.)+0.1511    ($R^2$=0.9948)

Maximum total cell population was $118*10^6$ cells/mL for Sc12, $110*10^6$ cells/mL for Sc31, and $277*10^6$ cells/mL for Td28. At the end of the fermentation, dry biomass yield was higher for Sc12; however, at maximum biomass Td28 exhibited higher yield than the *S. cerevisiae* strains. Further experiment (data not shown) revealed that oxygen additions in the first stages of fermentation can improve *T. delbrueckii* growth, biomass yield and survival rate/viability. Ethanol production, final sugar concentration and final pH were similar for all strains, without significant differences. Ethanol yields were similar for the three strains, with *S. cerevisiae* strains having slightly higher values. Ethanol productivity, as well as sugar consumption rate, for Sc12 was significantly higher than those of Sc31 and Td28. Biomass productivity was higher for Td28, lower for Sc12 and even lower for Sc31. Maximum specific growth rate was higher for Td28 and lower for Sc12 and Sc31. Cell viability for Sc12 was over 96% throughout the fermentation, for Sc31 after the growth phase (maximum biomass) viability declined gradually to 60% at the end of the fermentation, and for Td28 the loss of viability was rapid, down to 10% at the end of the fermentation.

Tab. 1. – Kinetic characteristics (mean values) of fermentations at 20 ºC.

| | Yeast strains | | |
|---|---|---|---|
| | Sc12 | Sc31 | Td28 |
| Initial sugars (g/L) | 179±8.95 | 182.2±8.23 | 189.2±8.79 |
| Final sugars (g/L) | 1±0.45 | 0.25±0.49 | 0 |
| Initial ethanol (g/L) | 1±0.07 | 0.36±0.09 | 0.22±0.03 |
| Final ethanol (g/L) | 87.23±4.32 | 90.04±5.3 | 89.43±4.6 |
| Ethanol (% vol) | 11.05±0.32 | 11.41±0.45 | 11.33±0.51 |
| Initial biomass (g/L) | 0.25±0.013 | 0.14±0.017 | 0.17±0.02 |
| Initial biomass (g/L) | 6.51±0,035 | 4.38±0.041 | 5.69±0.09 |
| Maximum population (cell/mL) | 118*$10^6$±6.3% | 110*$10^6$±6.5% | 277*$10^6$±7.3% |
| Initial pH | 3.5±0.05 | 3.43±0.05 | 3.45±0.05 |
| Final pH | 3±0.05 | 3.16±0.05 | 3.03±0.05 |
| Biomass yield (g/g) | 0.035 (end) | 0.023 (end) | 0.029 (end) |
| Biomass yield (g/g) at max biomass | 0.048 (at 87 h) | 0.052 (at 80 h) | 0.058 (at 63 h) |
| Ethanol yield (g/g) | 0.484 | 0.492 | 0.4715 |
| Max specific growth rate (h$^{-1}$) | 0.065 | 0.1048 | 0.1435 |
| Sugar consumption rate (g/L/h) | 1.171 | 0.520 | 0.264 |
| Ethanol productivity (g/L/h) | 0.567 | 0.256 | 0.124 |
| Biomass productivity (g/L/h) | 0.067 (at 63 h) | 0.051 (at 80 h) | 0.093 (at 63 h) |
| Fermentation time (h) | 152±6.3% | 350±8.4% | 717±9% |

Means of triplicate fermentations ±SD or % of error

Glycerol production was higher for Sc31, followed by Sc12, with Td28 having lower concentration (Table 2). Volatile acidity (acetic acid) was slightly higher for Td28 than Sc31 and Sc12, just as the lactic acid concentration. However, Td28 had much higher (almost twice as much) production of succinic acid than the two *S. cerevisiae* strains (Table 2).

Acetaldehyde production was low for all strains, with Sc31 having less than the other two. Propanol-1 production was higher for Td28, isobutanol production was the same for Td28 and Sc12 and 25% higher for Sc31. Ethyl acetate concentrations for the three strains had no significant differences, and the sum of amyl alcohols was lower for Td28. However, the concentration of 2-phenyl ethanol with its distinctive rose-like aroma was significantly higher for Td28 than Sc31 (almost half) and Sc12 (almost a third) (Table 2). As *T. delbrueckii* strains are used in consecutive fermentation in winemaking, followed by inoculation by *S. cerevisiae,* in order to achieve fast fermentation completion, the fermentation with Td28 was also analyzed during the mid fermentation point in order to see which metabolite concentrations would be found. According to the data shown below, in the middle of the fermentation by Td28, acetic acid and ethyl acetate production was low, but organic acids and other metabolite production was high, even higher than that of the *S. cerevisiae* at the end of their fermentations (Table 2).

Tab. 2. – Fermentation products (mean values) at 20 ºC.

| Concentration | Yeast strains | | | |
| | Sc12 | Sc31 | Td28 | Td28 mid fermentation |
| --- | --- | --- | --- | --- |
| Glycerol (g/L) | 5.49±0.33 | 5.98±0.41 | 6.03±0.09 | 4.87±0.07 |
| Acetic acid (g/L) | 0.15±0.01 | 0.21±0.02 | 0.16±0.01 | 0.08±0.01 |
| Lactic acid (g/L) | 0.13±0.01 | 0.08±0.01 | 0.2±0.02 | 0.05±0.01 |
| Succinic acid (g/L) | 0.34±0.02 | 0.44±0.02 | 0.7±0.02 | 0.405±0.02 |
| Acetaldehyde (mg/L) | 9±0.71 | 5.3±0.84 | 10±0.89 | 7±0.63 |
| Propanol-1 (mg/L) | 32.5±4.01 | 35.4±3.72 | 55±4.15 | 76±3.89 |
| Isobutanol (mg/L) | 31±3.53 | 41.4±3.72 | 31±4.23 | 22.2±3.51 |
| Ethyle acetate (mg/L) | 45±4.32 | 43.5±3.91 | 41.5±4.00 | 29.2±3.67 |
| Sum of Amyl alcohols (mg/L) | 82.6±5.06 | 100±0.01±5.21 | 63±4.31 | 82.5±6.34 |
| Phenylethanol* (mg/L) | 3.6±0.27 | 5.9±0.8 | 9±0.04 | not analyzed |

* determined by direct injection
Means of triplicate fermentations ±SD

With regard to the sensory analysis of wines produced from *Assyrtiko* grapes, ten experienced enologists were asked to assess comparatively the wines produced with the three strains, using a scale from 1 (worst) to 10 (best) for 16 attributes in 4 major groups: Sight (color, viscosity, brilliance, depth), Nose (aroma, faults, variety, intensity), Palate (complexity, concentration, fruit, length), Finish (aftertaste, balance, tannin / phenolics, acid). As it is shown below (Figure 4) the wine produced with Td28 scored significantly higher averages for aroma, variety, intensity, complexity, fruit and acid. The panel concluded that the Td28 wine was more crisp and fresh, with higher flower/fruit aromas (Figure 4). *Assyrtiko* grape musts were also fermented by consecutive fermentation with each commercial *T. delbrueckii*, followed by *S. cerevisiae*, according to the manufacturer's recommendations, with results similar to Td28.
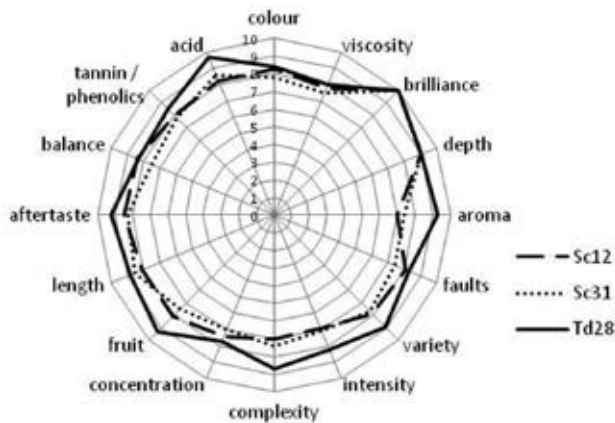


Fig. 4. – Average sensory notes for the wine produced by three strains Sc12, Sc31, Td28.

In brewing, almost all yeast strains that are used belong either to *S. cerevisiae* species (in case of ales-top fermenting yeast) or *S.pastorianus* (ex *uvarum*, ex *carlsbergenesis*) species (in case of pils/lagers-bottom fermenting yeast). There are some exceptions, the case of Lambics, were a consortium of indigenous yeast and bacteria from the air populate/contaminate the wort and are left to ferment naturally (or inoculated with commercial mixed cultures). Recently, some brewers have started using non-*Saccharomyces* (like Brettanomuces/ Dekkera strains), in order to obtain specific sensory characteristics. Even though *T. delbrueckii* is frequently mentioned on several web pages on the internet as a typical yeast used in the production of Bavarian style "weiss" (wheat) beers, we have not been able to find scientific references on their use in beer. The only references that could be found on them are related to them as spoilage yeast. Thus, we have undertaken the task of conducting some preliminary experiments on their use in brewing "wheat" style beers. Wheat beers are produced from wort that has been obtained using barley malts and a percentage of wheat, malted or unmalted.

Brewing was conducted at 20 ºC with wort reconstituted from liquid and dry malt extract specific for this beer type. Fermentation of 3.5 L batches with either Td28 *T. delbrueckii* strain or commercial WB-06 *S. cerevisiae* strain revealed that Td28 was able to ferment maltose (A l v e s – A r a u j o et al., 2007), but at a rate of 30% slower than with the WB-06 strain. Final gravity was high for both strains (Table 3). Maturation was conducted after the primary fermentation in capped beer bottles with the same yeast, at room temperature (25 ºC) for 7 days, followed by 14 days at 10 ºC.

Tab. 3. – Final gravity and primary fermentation duration at 20 ºC.

|  | Yeast strains | |
| --- | --- | --- |
|  | **WB-06** | **Td28** |
| Initial gravity | 1.044 | |
| Final gravity | 1.009 | 1.012 |
| Primary fermentation (h) | 157.2 | 204.4 |

Td28 also exhibited slightly less sedimentation (giving beers with more typical wheat haze appearance) in comparison to WB-06 which was also considered as a low sedimentation strain. Still, further experiments are necessary for validation. Sensory analysis performed by expert brewers found (on a scale 1 to 10) that WB-06 strain was not a very fast strain (compared to other yeasts recommended for wheat beers previously used in our laboratory), but exhibited a subtle estery character and phenol flavor which were typical of wheat beers and also mentioned by the manufacturer. Td28 showed higher estery notes than WB-06 with rose, bubblegum and banana aromas, but lower phenol flavors. Buttery notes (diacetyl) varied considerably between the bottles, and the differences between the two yeasts were also high and they could not be properly quantified. The overall average note of the brewer's panel was

higher for the Td28 strain, thus demonstrating a potential for brewing wheat beers (Figure 5).
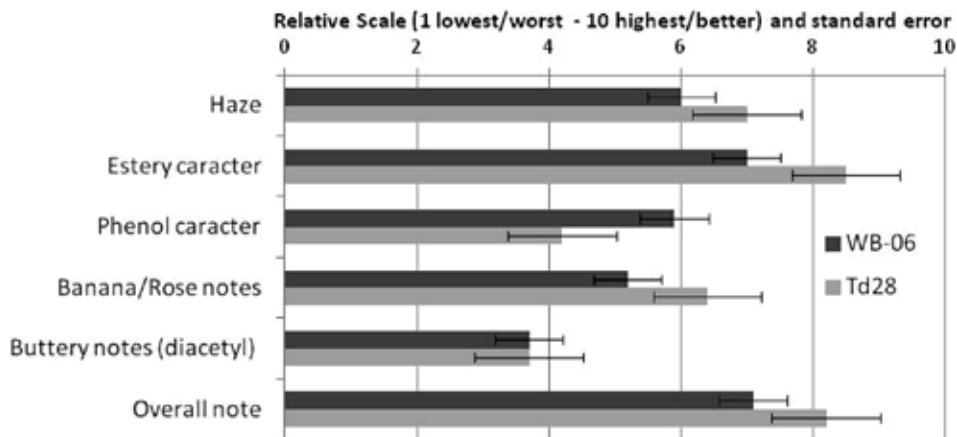


Fig. 5. – Comparison between wheat beers produced with WB-06 and Td28 strains at 20 ºC.

CONCLUSIONS

With respect to the wine production, results from laboratory scale fermentation showed that the *T. delbrueckii* Td28 strain was a slow fermenting strain. However, it has the ability to complete the process of wine fermentation twice or three times needed for *S. cerevisiae*. In addition, it is capable of higher production of organic acids, as well as 2-phenyl ethanol, acceptable production of acetic acid and glycerol. From a sensory point of view, the wines produced with Td28 retain high acidity and fresh character, while also having significantly higher sensory notes regarding the overall complexity and fresh flower and fruity aromas.

With regards to brewing, Td28 was able to consume maltose, which is the major sugar in wort, more slowly than the commercial *S. cerevisiae* strain WB-06. Td28 exhibited more pronounced ester character, complexity and intensity, but lower phenol character.

Although further experiments with more strains are necessary, the overall effects of selected *Torulaspora delbrueckii* yeast strains are highly positive, leading to pronounced sensory complexity and floral/fruity aroma in winemaking and brewing.

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# КОРИШЋЕЊЕ NE-SACCHAROMYCES СОЈЕВА КВАСЦА *TORULASPORA DELBRUECKII* У ПРОИЗВОДЊИ ВИНА И СЛАДА

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Резиме

Одабрани сојеви квасца *Saccharomyces* се више од 150 година користе у производњи слада а у винарству неколико деценија. Ови сојеви су неопходни у производњи пива и слада због кувања сладовине, током које долази до уништавања свих ћелија квасаца са изузетком неких врста пива у белгијском стилу (нпр. Ламбиц) код којих се сладовина оставља да се колонизује нативним квасима и бактеријама из околине и на тај начин природно ферментише. У производњи вина, примена *Saccharomyces* сојева је стална јер обезбеђује правилну и уредну ферментацију, спречава раст нативних (дивљих) микроорганизама изазивача кварења и доприноси жељеним сензорским карактеристикама вина.

Иако коришћење одабраних *Saccharomyces* сојева обезбеђује сигурније очување квалитета у производњи вина у односу на непознату, дивљу микрофлору у шири, већ дуже време се расправља о томе да ли коришћење индустријских *Saccharomyces* сојева има за последицу вина слабије сензорске комплексности и карактера који мање зависи од климатских услова, локалитета и земљишта (тероар).

У претходним деценијама, ne-Saccharomyces квасци су сматрани проблематичнима и изазивачима кварења јер су показивали слабију ферментациону способност и друге нежељене особине. Током последњих деценија, показано је да неки ne-Saccharomyces родови попут *Candida, Pichia, Kluyveromyces, Torulaspora*, иако не могу да комплетирају ферментацију, могу да се користе у поступку секвенционалне инокулације-ферментације заједно са *Saccharomyces* квасцем и доприносе сензорској комплексности вина.

Током ферментације у лабораторијским условима, уочили смо да је укупан ефекат одабраних квасних сојева *Torulaspora delbrueckii* веома задовољавајући и доприноси добијању вина и пива изражене сензорске комплексности и цветно/воћне ароме.

КЉУЧНЕ РЕЧИ: вино, пиво, ферментација, квасац, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*

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# OPTIMIZATION OF MEDIUM FOR ANTIMYCOTIC PRODUCTION BY *STREPTOMYCES* SPP.

ABSTRACT: Numerous species of the genus *Streptomyces*, on the appropriate cultivation medium in the process of submerged biosynthesis, as a product of the secondary metabolism, and under aerobic conditions synthesize pharmacologically active compounds. The aim of presented study was optimization of different nitrogen sources in the cultivation medium for the production of antimycotics using a strain of *Streptomyces* spp. isolated from the environment. Experiments were carried out in accordance with Box-Behnken design with three factors at three levels (peptone: 3.0 g/l, 7.0 g/l and 11.0 g/l; yeast extract: 1.0 g/l, 3.0 g/l and 5.0 g/l; soybean meal: 5.0 g/l, 15.0 g/l and 25.0 g/l) and three repetitions in the central point. Cultivation mediums were analyzed for determination of residual sugar, residual nitrogen, pellet diameter and RNA. Also, antimycotic activity of the obtained cultivation mediums was determined using diffusion disc method on the *Aspergillus* spp. as the test microorganism. For the optimization of selected parameters, a Response Surface Methodology was used and the obtained data were analyzed using the software package DESIGN EXPERT 8.1. Achieved model with a coefficient of determination ($R$) of 0.952 predicted that the maximum inhibition zone diameter (24.0 mm) against microorganism *Aspergillus* spp. and the minimum amount of residual sugar (0.551528 g/l) under applied experimental conditions was produced when the contents of varied nitrogen sources were: peptone 11.0 g/l, yeast extract 4.32 g/l and soybean meal 25.00 g/l.

KEY WORDS: Antimycotic activity, Nitrogen sources, Medium optimization, *Streptomyces* spp.

## INTRODUCTION

*Streptomycetes* and related actinomycetes continue to be prolific sources of novel secondary metabolites with a range of biological activities that may ultimately find application as pharmaceutically useful compounds (B i b b,

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2005). Antibiotic biosynthesis is a specific property of microorganisms and depends greatly on culture conditions. It is necessary to develop optimum conditions to make the production of an antibiotic feasible. The ability of *Streptomyces* cultures to form antibiotics is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and culturing, hence the medium constitution together with the metabolic capacity of the producing organism greatly affect antibiotic biosynthesis (W a k s m a n, 1961).

Fungal phytopathogens cause serious problems worldwide in agriculture and food industry and many of them produce mycotoxins which are harmful to humans and livestock (O s k a y, 2009). Fungal infections are caused by eukaryotic organisms and for that reason they generally present more difficult therapeutic problems than bacterial infections do. In fact, there are relatively few agents that can be used to treat fungal infections. New fungicides are needed in agriculture, food protection, and medicine due to the increase in resistant pathogens, occurrence of new infectious diseases and the toxicity of currently used compounds (V a l a n a r a s u et al., 2010). According to the reviewed literature, many strains of *Streptomyces* isolated from different environments, showed strong antifungal activity against different fungal pathogens with broad spectrum of antibacterial activity (S i n g h and R a i 2012; V a l a n a r a s u et al., 2010; A t t a, 2012; A l – A s k a r, 2011).

Response Surface Methodology (RSM) is a collection of statistically designed experiments and analyses that directs the investigation of numerous factors and their interactions. This approach minimizes the number of probes required to identify critical factors and possible synergism between factors. In this research, an antifungal antibiotic was produced by *Streptomyces* spp. isolated from the environment. The aim of this paper was to optimize the contents of peptone, yeast extract and soybean meal as nitrogen sources, by applying RSM in the cultivation medium for the antimycotic biosynethesis.


## MATERIAL AND METHODS

As the production microorganism, strain of *Streptomyces* spp. isolated from the environment, was used in this experiment. The medium for the growth of microorganisms had the following characteristics (g/l): glucose (15.0), soybean meal (10.0), $CaCO_3$, (3.0), NaCl, (3.0), $MgSO_4$, (0.5), $(NH_4)_2HPO_4$, (0.5), $K_2HPO_4$, (1.0) (Ilic et al., 2010). In accordance with the defined aim and experimental plan of the study the mediums for the biosynthesis of antimycotic had different contents of peptone (3.0 g/l; 7.0 g/l and 11.0 g/l), yeast extract (1.0 g/l; 3.0 g/l and 5.0 g/l) and soybean meal (5.0 g/l; 15.0 g/l and 25.0 g/l). Mediums also contained (g/l): glucose (15.0), $(NH_4)_2SO_4$ (5.0), $CaCO_3$ (3.0), NaCl (3.0), $MgSO_4$ (0.5), $K_2HPO_4$ (1.0). The pH value of the mediums was adjusted to $7.2 \pm 0.1$ prior to autoclaving. The biosynthesis of antimycotic was carried out in an Erlenmeyer shake flasks containing one third of the cultivation

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medium. The inoculation was performed by adding 10 % (v/v) of inoculums prepared under aerobic conditions, on growth medium, at 27°C for 48 h and agitation rate of 150 rpm. Cultivation on mediums for antimycotic biosynthesis was carried out under aerobic conditions with external mixing (laboratory shaker, 150 rpm) at a temperature of 27°C for 7 days.

At the end of the cultivation, depending on the requirements of applied analytical method, the sample was or was not processed before measuring. The separation of solid from liquid phase in the cultivation medium was carried out by centrifugation at 10.000·G for 10 minutes (Eppendorf Centrifuge 5804, Germany). Diameters of pellets were determined by microphotography analysis using the Leica QWin software. Microphotographs were taken using an optical microscope (Carl Zeiss, Germany) with 10 x enlargement. Since the pellets were approximately elliptical, diameter of the pellets was calculated as equivalent to the diameter of a circle which area was identical to the area of an ellipse that represented a two-dimensional projection of the measured pellet. The RNA content was determined from the cultivation sediment by a modified method of M u n r o and F l e c k (1966). The content of reduced sugars was determined in the liquid phase of cultivation fluid, using the method with dinitrosalicylic acid (DNS) (M i l l e r, 1959). Total nitrogen content, in the supernatant of cultivation medium, was determined by the Kjeldahl method (C o h e n, 1926). After centrifugation, liquid phase was evaporated with rotary vacuum evaporators (Ika-werke, Staufen), so the final sample weight was 1/10 of the initial mass of the supernatant. The antimycotic activity of obtained cultivation medium supernatants against *Aspergillus* spp. was tested by the diffusion – disc method using sterile discs (HiMedia, India) and volume of analyzed samples was 10 µl (Bauer, 1966). Sabouraud agar (Torlak®) was used for the growth of test microorganism. After incubation at 30° C for 48 hours, the inhibition zones were measured by a special ruler (HiAntibiotic ZoneScale, Himedia ®).

When applying the RSM, the selection of a corresponding experimental plan is a key step (F e r r e i r a et al., 2007). The Box-Behnken experimental design with three factors at three levels and three repetitions in the central point was used to test the effects of different contents of nitrogen sources in antimycotic production. Table 1 represents Box-Behnken experimental plan and the range and levels of the variables investigated in this study.

Statistical analysis of the obtained experimental data was performed using STATISTICA 9.0 software. The significance of the impact of each of the factors and their interactions were determined by comparing the t-value of each coefficient in the regression equation. Response surfaces were drawn with a constant value of one of the parameters at the center of the plan, while remaining two parameters were varied. For optimization of parameters, method of desired (required) function was used and obtained data was analyzed using the software package DESIGN EXPERT 8.1.

Tab. 1. – Box-Behnken experimental plan and varied values of factors

| Run | Coded values | | | Real values [g/l] | | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Peptone | Yeast extract | Soybean meal |
| 1 | -1 | -1 | 0 | 3 | 1 | 15 |
| 2 | 1 | -1 | 0 | 11 | 1 | 15 |
| 3 | -1 | 1 | 0 | 3 | 5 | 15 |
| 4 | 1 | 1 | 0 | 11 | 5 | 15 |
| 5 | -1 | 0 | -1 | 3 | 3 | 5 |
| 6 | 1 | 0 | -1 | 11 | 3 | 5 |
| 7 | -1 | 0 | 1 | 3 | 3 | 25 |
| 8 | 1 | 0 | 1 | 11 | 3 | 25 |
| 9 | 0 | -1 | -1 | 7 | 1 | 5 |
| 10 | 0 | 1 | -1 | 7 | 5 | 5 |
| 11 | 0 | -1 | 1 | 7 | 1 | 25 |
| 12 | 0 | 1 | 1 | 7 | 5 | 25 |
| 13 | 0 | 0 | 0 | 7 | 3 | 15 |
| 14 | 0 | 0 | 0 | 7 | 3 | 15 |
| 15 | 0 | 0 | 0 | 7 | 3 | 15 |

## RESULTS AND DISCUSSION

Antimycotic biosynthesis media were formulated by varying content of different nitrogen sources, peptone, yeast extract and soybean meal, in accordance with the aim of this paper and applied experimental plan. The results of 15 experiments based on the Box-Behnken design with three factors at three levels and three repetitions in the central point, presented in Table 1, represent an average value of three repeated samplings. Values of varying independent variables ($X_1$, $X_2$ and $X_3$) and their responses values of dependent variables for residual sugars, residual nitrogen, pellet diameter, RNA and diameters of inhibition zones ($Y_1$, $Y_2$, $Y_3$, $Y_4$ and $Y_5$) are shown in the Table 2.

In addition to the results of determination required by the experiment plan, levels of sugar and nitrogen conversions are also shown. These values were calculated based on the initial contents of sugars in the medium (15.0 g/l) and the residual sugar content, as well as the estimated initial content of nitrogen in the medium and the residual nitrogen content. Calculation of initial nitrogen content was based on nitrogen amounts in the used nitrogen sources determined by Kjeldahl method (peptone, 129.5 mg/g; yeast extract, 89.32 mg/g; soybean meal, 75.46 mg/g).

The level of sugar conversion (Table 2) was high (over 90 %) for all observed combinations of organic nitrogen sources, so it could be concluded with high probability that sugar was the limiting nutrient in this process. The highest value of sugar conversion (98.82 g/l) was achieved in the medium which contained the lowest amount of soybean meal (5.0 g/l). The level of

Tab. 2. – Residual sugar, residual nitrogen, pellet diameter, RNA and inhibition zone diameter in cultivation mediums based on the Box-Behnken experimental plan

| Run | Residual sugar [g/l] $Y_1$ | Sugar conversion [%] | Residual nitrogen [g/l] $Y_2$ | Nitrogen conversion [%] | Pellet diameter [μm] $Y_3$ | RNA [mg/100g] $Y_4$ | Inhibition zone diameter [mm] $Y_5$ |
|---|---|---|---|---|---|---|---|
| 1 | 2.4699 | 83.53 | 1.8032 | 32.67 | 159.90 | 14.5782 | 10 |
| 2 | 3.2931 | 78.05 | 2.7703 | 25.24 | 152.91 | 16.5906 | 16 |
| 3 | 4.2341 | 71.77 | 2.0606 | 31.93 | 152.36 | 22.4217 | 20 |
| 4 | 1.0585 | 92.94 | 2.0848 | 38.75 | 129.01 | 17.4430 | 19 |
| 5 | 1.4114 | 90.59 | 1.2845 | 19.55 | 147.12 | 15.9277 | 20 |
| 6 | 0.1764 | 98.82 | 2.1988 | 29.74 | 111.47 | 26.3034 | 17 |
| 7 | 1.2937 | 91.38 | 2.0344 | 43.54 | 162.23 | 22.8903 | 17 |
| 8 | 0.6469 | 95.69 | 2.5472 | 45.09 | 117.42 | 17.7755 | 24 |
| 9 | 1.4114 | 90.59 | 1.6100 | 33.83 | 178.67 | 14.6494 | 16 |
| 10 | 0.1764 | 98.82 | 1.7010 | 39.04 | 135.40 | 21.6774 | 17 |
| 11 | 1.4702 | 90.20 | 2.3841 | 39.53 | 153.79 | 15.3051 | 15 |
| 12 | 1.4114 | 90.59 | 2.4769 | 42.12 | 123.42 | 13.4942 | 16 |
| 13 | 1.4114 | 90.59 | 2.6374 | 21.65 | 162.36 | 17.5082 | 11 |
| 14 | 1.1173 | 92.55 | 2.2606 | 32.85 | 163.83 | 18.1639 | 10 |
| 15 | 0.1764 | 98.82 | 2.5106 | 43.24 | 138.35 | 19.0123 | 10 |

sugar conversion was the highest in mediums in which the sum of all added nitrogen sources was average.

The level of nitrogen conversion in all observed combinations of organic nitrogen source contents was less than 50%. This indicated that the amount of organic nitrogen source was too high, which is unjustifiable from the aspect of losses due to raw material costs and their preparation, complicated separation and purification processes and organic load on effluents.

The results of experiments based on the Box-Behnken plan were fitted into a second degree polynomial. Results of the fitted selected responses (regression coefficients), their significance and coefficients of determination are shown in Table 3. A *p*-value is used to assess the statistical significance for each coefficient of the regression equations. Regression coefficients, with a significance of 95 %, are significant if the value of their significance coefficients (*p*-value) is less than 0.05 and they are bolded in the table.

High values (over 0.900) of the coefficients of determination (*R*) indicate good fitting of experimental results on pellet diameter, residual sugars, residual nitrogen, RNA and inhibition zone diameters for test microorganism *Aspergillus* spp.

In the residual sugar model all quadratic regression coefficients were significant, as well as peptone and yeast extract coefficients of interaction. Linear regression coefficients of the initial contents of peptone and yeast extract, as well as peptone and yeast extract coefficients of interaction were

Tab. 3. – Regression coefficients, their significance and coefficients of determination of models for residual sugar, residual nitrogen, pellet diameter, RNA and inhibition zone diameter for test microorganism *Aspergillus* spp.

| Responses Effects | $Y_1$ Coefficient | $Y_1$ p-value | $Y_2$ Coefficient | $Y_2$ p-value | $Y_3$ Coefficient | $Y_3$ p-value | $Y_4$ Coefficient | $Y_4$ p-value | $Y_5$ Coefficient | $Y_5$ p-value |
|---|---|---|---|---|---|---|---|---|---|---|
| *Intercept* | | | | | | | | | | |
| $b_0$ | 3.2496 | 0.0706 | -3.4422 | **0.0175** | 142.7388 | **0.0037** | -1.1993 | 0.8344 | 34.3150 | **0.0027** |
| Linear | | | | | | | | | | |
| $b_1$ | -0.5243 | 0.0857 | 0.7627 | **0.0066** | 19.0312 | **0.0109** | 0.4412 | 0.6594 | -4.1198 | **0.0124** |
| $b_2$ | -1.0291 | 0.0723 | 1.2332 | **0.0112** | -23.6037 | **0.0456** | 7.7405 | **0.0067** | 0.6564 | 0.7552 |
| $b_3$ | 0.2007 | 0.0777 | 0.0715 | 0.3079 | 0.2761 | 0.8830 | 0.7426 | 0.0859 | -1.7500 | **0.0070** |
| Quadratic | | | | | | | | | | |
| $b_{11}$ | 0.0508 | **0.0192** | -0.0239 | 0.0691 | -1.4358 | **0.0045** | 0.1242 | 0.0828 | 0.2943 | **0.0065** |
| $b_{22}$ | 0.2622 | **0.0071** | -0.0892 | 0.0846 | 1.6672 | 0.2155 | -0.6141 | **0.0440** | 0.3021 | 0.3026 |
| $b_{33}$ | -0.0083 | **0.0176** | -0.0007 | 0.6928 | -0.0731 | 0.1807 | 0.0051 | 0.6027 | 0.0446 | **0.0082** |
| Interaction | | | | | | | | | | |
| $b_{12}$ | -0.1249 | **0.0074** | -0.0858 | **0.0077** | -0.5111 | 0.4071 | -0.2185 | 0.1043 | -0.2187 | 0.1438 |
| $b_{13}$ | 0.0037 | 0.5503 | -0.0025 | 0.5573 | -0.0573 | 0.6338 | -0.0968 | **0.0071** | 0.0625 | 0.0562 |
| $b_{23}$ | 0.0147 | 0.2567 | 0.00002 | 0.9979 | 0.9803 | **0.0074** | -0.1105 | 0.0541 | -0.0000 | 0.9999 |
| $R^2$ | 0.941 | | 0.925 | | 0.936 | | 0.915 | | 0.916 | |

significant for the residual nitrogen model. In the pellet diameter model coefficients for the initial contents of peptone and yeast extract were significant, as well as quadratic coefficient for peptone, yeast extract and soybean meal coefficient of interaction. Linear regression coefficients of yeast extract and their quadratic coefficient, as well as peptone and soybean meal coefficient of interaction were significant for the RNA model. Linear regression coefficients of yeast extract and its quadratic coefficient, as well as all coefficients of interaction were not statistically significant in the model of inhibition zone diameter of *Aspergillus* spp.

## Optimization of production medium for antimycotics effective against Aspergillus *spp.*

The final goal of the application of the response area procedure was the optimization of the observed process so that the developed models could be used for simulations and optimizations. Observed responses were residual sugar and inhibition zone diameter for test microorganism. Residual sugar was minimized, while the inhibition zone diameter was maximized. A model with a coefficient of determination (*R*) of 0.952 predicted that the maximum inhibition zone diameter (24.0 mm) against microorganism *Aspergillus* spp. and the

minimum amount of residual sugar (0.551528 g/l) in applied experimental conditions was produced with the following contents: peptone 11.0 g/l, yeast extract 4.32 g/l and soybean meal 25.0 g/l.

Effects of independent variables (peptone and yeast extract contents) on the inhibition zone diameter for test microorganism are displayed on contour diagrams for the optimal value of soybean meal (25.0 g/l), Figure 1.
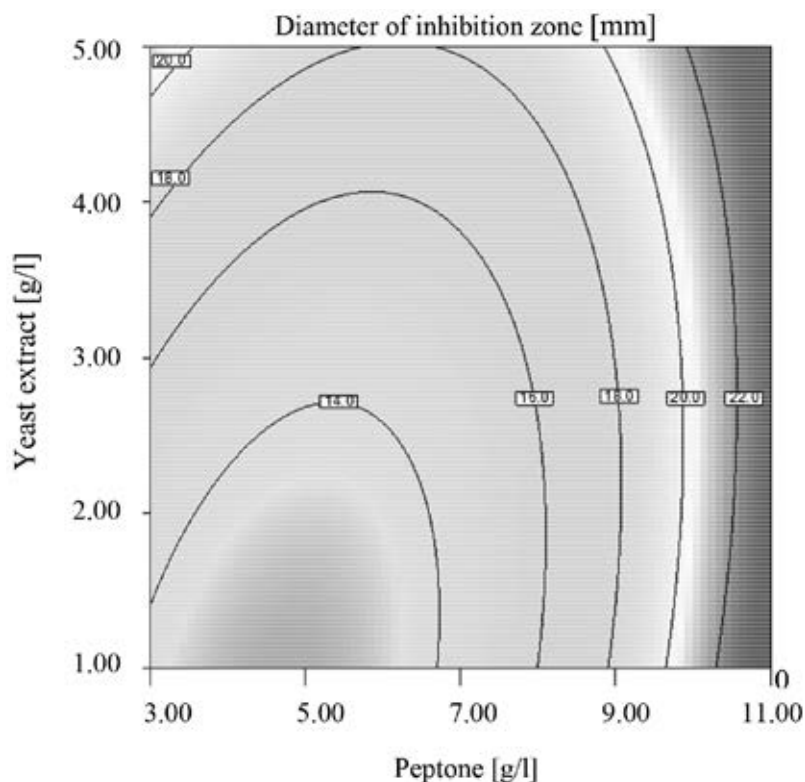


Fig. 1. – Contour diagram showing relations of inhibition zone diameter for *Aspergillus* spp. and content of peptone and yeast extract at constant level of soybean meal (25.0 g/l)

Diameter of inhibition zone for *Aspergillus* spp., with a constant content of soybean meal (25.0 g/l), as presented in Figure 1, decreased with the decrease of peptone content in the medium. With constant content of peptone the response did not change significantly when changing the yeast extract content, and it resulted in small values of peptone content. By changing the peptone content, with constant content of yeast extract, the response value was affected with small concentrations of yeast extract. For the biosynthesis of antimycotics effective against *Aspergillus* spp., when the content of soybean meal in the medium was optimal, the highest concentration of peptone was needed (about 11.0 g/l) while the yeast extract content had no effect.

*Estimation of response value based on optimal values*
*of independent variables*

Based on the optimal values of independent variables (contents of peptone, yeast extract and soybean meal) estimated by optimization and values derived by using the second degree polynomials defined by statistical analysis of experimental data, the values of dependent variables (residual sugar and nitrogen, pellet diameter, RNA and diameter of inhibition zone) were calculated. The results are shown in Table 4.

Tab. 4. – Estimation of response value based on optimal values of contents of peptone, yeast extract and soybean meal

| Test micro-organism | Pep-tone [g/l] | Yeast extract [g/l] | Soybean meal [g/l] | Residual sugar [g/l] | Residual nitrogen [g/l] | Pellet diameter [μm] | RNA [mg/100g] | Inhibition zone diameter [mm] | D |
|---|---|---|---|---|---|---|---|---|---|
| *Aspergillus* ssp. | 11.0 | 4.32 | 25.0 | 0.5512 | 2.2949 | 134.52 | 13.4678 | 24.0 | 0.952 |

Results in Table 4 show that the optimum content of peptone in the medium for the biosynthesis of antimycotics that affect *Aspergillus* ssp., under the applied experimental conditions was 11.0 g/l. Optimum contents of yeast extract and soybean meal were 4.32 g/l and 25.0 g/l, respectively.

Tab. 5. – Estimated values gained by optimization of residual sugar and inhibition zone diameter for test microorganism

| Test microorganism | Estimated values | | RSM | |
|---|---|---|---|---|
| | Residual sugar [g/l] | Inhibition zone diameter [mm] | Residual sugar [g/l] | Inhibition zone diameter [mm] |
| *Aspergillus* **ssp.** | 0.5512 | 24.0 | 0.551528 | 24.0 |

Estimated values of residual sugar and diameter of inhibition zone for the applied test microorganism were very close to the values predicted by optimization (Table 5), which indicated that the model developed by applying the response surface function was adequate.

CONCLUSION

Based on the results of the present study, a model with coefficient of determination 0.952 estimated that the lowest amount of residual sugar 0.551528 g/l and the largest possible diameter of inhibition zone of 24.0 mm, with its antimycotic activity against test microorganism *Aspergillus* spp. and under the applied experimental conditions yielded a cultivation medium containing 11.0 g/l peptone, 4.32 g/l yeast extract and 25.0 g/l soybean meal. Based on the obtained

results, a model developed using the response surface methodology is valid since the estimated values of residual sugar 0.5512 g/l and inhibition zone diameter of 24.0 mm were very close to the predicted values (0.551528 g/l and 24.0 mm, respectively).

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# ОПТИМИЗАЦИЈА ХРАНЉИВЕ ПОДЛОГЕ ЗА ПРОИЗВОДЊУ АНТИМИКОТИКА ПРИМЕНОМ *STREPTOMYCES* SPP.

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## Резиме

На одговарајућој хранљивој подлози многе врсте рода *Streptomyces*, процесом аеробне субмерзне биосинтезе, као производ секундарног метаболизма, синтетишу фармаколошки активне компоненте. Циљ овог рада је оптимизација различитих извора азота у култивационом медијуму за производњу антимикотика применом одабраног соја *Streptomyces* spp. изолованог из природног окружења. Експерименти су вршени у складу са Box-Behnken-овим планом варирањем три фактора на три нивоа (пептон: 3,0 g/l, 7,0 g/l и 11,0 g/l; екстракт квасца: 1,0 g/l, 3,0 g/l и 5,0 g/l; сојино обезмашћено брашно: 5,0 g/l, 15,0 g/l и 25,0 g/l) и са три понављања у централној тачки. У култивационим течностима је одређен резидуални шећер, резидуални азот, пречник пелета и RNA. Такође, применом дифузионе-диск методе са тест микроорганизмом *Aspergillus* spp., одређена је антимикотичка активност добијених култивационих медијума. За оптимизацију одабраних параметара примењена је метода одзивних површина и добијени резултати су анализирани софтверским пакетом DESIGN EXPERT 8.1. Добијени модел са коефицијентом детерминације ($R$) од 0,952 предвиђа да се максимални пречник зоне просветљења (24,0 mm) према микроорганизму *Aspergillus* spp., и минимална количина резидуалног шећера (0,551528 g/l) у примењеним експерименталним условима, добија када садржаји пептона, екстракта квасца и сојиног обезмашћеног брашна у хранљивој подлози износе 11,00 g/l, 4,32 g/l и 25,00 g/l, редоследом.

КЉУЧНЕ РЕЧИ: антимикотичка активност, извори азота, оптимизација подлоге, *Streptomyces* spp.

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# *TRAMETES SUAVEOLENS* AS LIGNINOLYTIC ENZYME PRODUCER

ABSTRACT: Species of the genus *Trametes* represent one of the most efficient lignin-degraders which can be attributed to a well developed ligninolytic enzyme system. Current trends are screening of ability of new species to produce these enzymes, as well as the optimization of conditions for their overproduction. Therefore, the aim of the study was to evaluate the potential of *T. suaveolens* to synthesize laccase and Mn-oxidizing peroxidases during fermentation of the selected plant raw materials. Level of enzyme activities was measured on 7, 10 and 14th day of submersion, as well as the solid-state fermentation of wheat straw and oak sawdust in the presence of $NH_4NO_3$ in previously determined optimal nitrogen concentration of 25 mM. The enzyme activity was determined spectrophotometrically using ABTS and phenol red as the substrates. The highest level of laccase activity (1087.1 U/L) was noted after 7 days of wheat straw solid-state fermentation, while during the submerged cultivation the production of the enzyme was not noted. Submerged cultivation in oak sawdust-enriched medium was the optimal for activity of Mn-dependent peroxidase (1767.7 U/L on day 14) and Mn-independent peroxidase (1113.7 U/L on day 7). Introduction of *T. suaveolens* to produce ligninolytic enzyme represented the base for further study, as well as the determination of relation between enzyme activity and rate of lignin degradation. It could lead to greater possibility of fungal species selection with high delignification capacity, which could take participation in sustainable production of food, feed, fibres, and energy, environmentally friendly pollution prevention, and bioremediation.
KEY WORDS: Laccase, Mn-oxidizing peroxidases, Plant residues, *Trametes suaveolens*

## INTRODUCTION

White-rot fungi are well known for the unique set of extracellular oxidases and peroxidases that enable efficient degradation of lignin, the second (after cellulose) most abundant natural branched polymer and aromatic material on the Earth (H a m m e l, 1997; L e o n o w i c z et al., 1999). Well developed ligninolytic enzyme system in the species of the genus *Trametes*, composed from laccase and Mn-dependent peroxidase, is responsible for significant level of lignin mineralization (Ć i l e r d ž i ć et al., 2011). Regarding the fact

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that annual production of various plant raw materials is enormous (approximately 170 – 200 billion tons), the interest in their environmentally friendly transformation into valuable products such as food, feed, fibers, and biofuels where fungal ligninolytic enzymes have the main role, has increased (K i m  and D a l e, 2004). Thus, wheat straw and oak sawdust represent abundant plant residues, which could be prospective substrates for the bioconversion into fungal biomass and lignocellulolytic enzymes due to their appropriate chemical composition (M c k e a n and J a c o b s, 1997; R a k i c et al., 2006). However, since efficiency of the lignin degradation depends on the potential of the organism-degrader, oxidative mechanisms, and culture conditions (W a n et al., 2010), screening and selection of new species with tremendous synthesis of the ligninolytic enzymes, as well as the defining of optimum conditions for their overproduction, has currently become a trend.

The aim of this study was assessment of the potential of *T. suaveolens* to produce ligninolytic enzymes during solid-state and submerged fermentation of selected plant raw materials.

## MATERIALS AND METHODS

### *Organism and growth conditions*

Culture of *T. suaveolens* HAI 300 was obtained from the Institute of Evolution, University of Haifa, Israel (HAI) and kept in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

The inoculum was prepared by inoculation of 100 mL synthetic medium (glucose, 10.0 g/L; $NH_4NO_3$, 2.0 g/L; $K_2HPO_4$, 1.0 g/L; $NaH_2PO_4$ x $H_2O$, 0.4 g/L; $MgSO_4$ x $7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH 6.5) with 25 mycelial disks (Ø 0.5 cm, from 7 day-old culture from malt agar), and incubation on a rotary shaker at 100 rpm, at room temperature (22 ± 2 °C) for 7 days. The obtained biomass was washed and homogenized with 100 mL of sterile distilled water in laboratory blender.

Activities of laccase and Mn-oxidizing peroxidases were studied after both solid-state and submerged fermentation of wheat straw and oak sawdust, residues that were previously dried at 50 °C and grounded to 0.5-1.0 cm.

Solid-state cultivation was carried out at 25 °C in 100-mL flasks containing 2 g of wheat straw and 5 g of oak sawdust, respectively, as carbon sources, and 10 mL of modified synthetic medium (without glucose, with $NH_4NO_3$ and previously determined optimal nitrogen concentration of 25 mM, and pH 6.5). Prepared flasks were inoculated with 3 mL of homogenized inoculum. Extraction of the synthetized laccase (EC 1.10.3.2), Mn-dependent peroxidase (MnP, EC 1.11.1.13), and Mn-independent peroxidase (MnIP, EC 1.11.1.16) was performed after 7, 10, and 14 days of cultivation by stirring the samples with 50 mL of distilled water on magnetic stirrer for 10 min at the temperature of 4 °C and centrifugation of 3000 rpm.

Fifty mL of modified synthetic medium was enriched with the same carbon sources and amounts, and it was inoculated with 5 mL of inoculum (in 250-mL flasks) and incubated at room temperature on a rotary shaker (at 100 rpm) for 7, 10, and 14 days during the submerged cultivation. The obtained biomasses were separated by centrifugation and supernatants were used to estimate the enzymatic activity.

Five replicates for each carbon source and measurement points were done.

*Enzyme activity assays*

Activity of the ligninolytic enzymes was determined spectrophotometrically. Laccase 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) ($\varepsilon_{436} = 29300$ $M^{-1}$ $cm^{-1}$) in 0.1 M phosphate buffer (pH 6.0), at 35 ºC. Mn-oxidizing peroxidases activities were determined with 3 mM phenol red ($\varepsilon_{610} = 22000$ $M^{-1}cm^{-1}$) in succinic acid disodium salt/albumin from bovine serum/DL-lactic acid sodium salt buffer (pH 4.5). The reaction mixture ($V_{tot} = $ 1 mL) consisted of buffer, sample, 2 mM $H_2O_2$, and phenol red, with or without 2 mM $MnSO_4$ (for MnP and MnIP, respectively), and reaction was stopped by 2M NaOH. Enzymatic activity of 1 U was defined as the amount of enzyme that transformed 1 μM of substrate per min.

Specific enzyme activity was defined by determination of total protein amount (mg/mL) using the method of S i l v a et al. (2005).

RESULTS

The activities of Lac and Mn-oxidizing peroxidases in crude extracts of *T. suaveolens* varied depending on the selected plant raw materials, type of fermentation, and cultivation period.

The highest MnP activity level (1767.7 ± 175.7 U/L) was recorded after 14 days of oak sawdust submerged fermentation, while the maximum MnIP activity (1113.7 ± 94.7 U/L) was obtained under the same cultivation conditions but on the 7th day (Fig. 1). Contrary to the mentioned results, the obtained values of Mn-oxidizing peroxidases activity were significantly lower in wheat straw submerged fermentation. During solid-state fermentation, wheat straw proved to be a better carbon source for MnP, and oak sawdust for MnIP production (Fig. 1).

Laccase synthesis was reported only during the solid-state cultivation in wheat straw-enriched medium, while under other studied conditions no activity of the enzyme was noted. Activity of the enzyme decreased with respect to the period of cultivation; the highest value was obtained after 7 days of cultivation (1087.1 ± 143.9 U/L), while on the 10th and 14th day the values were twice as low (495.5 ± 73.8 U/L) and even 5-fold (216.2 ± 46.5 U/L), respectivelly.
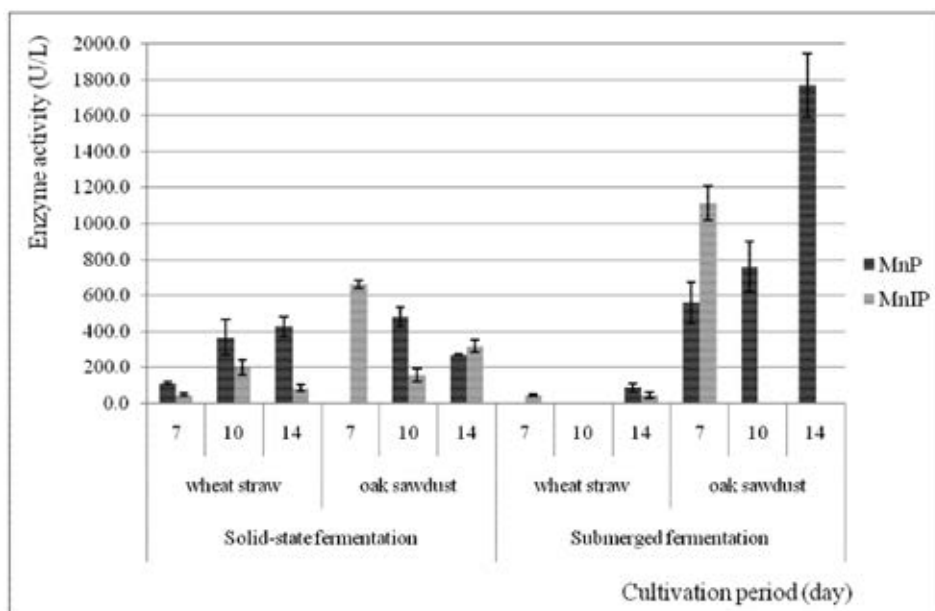
Fig. 1. – Mn-dependant peroxidase (■) and Mn-independent peroxidase (■) activity of *Trametes suaveolens* on selected plant raw materials during solid-state and submerged fermentation. The values in the figures correspond to mean values ± S.E of five replicates.

The laccase/MnP ratio changed from approximately 10:1, on the 7th day of cultivation, to 1:2, after 14 days of solid-state fermentation of wheat straw. The ratio laccase and MnIP ranged from 23:1, on the 7th day, to approximately 2.5:1 at other measurement points.

The total protein production was the highest on the 14th day of solid-state fermentation of wheat straw (147.3 ± 8.4 mg/mL) and the lowest after 14 days of solid-state cultivation in oak sawdust-enriched medium (5.7 ± 2.5 mg/mL), which was reflected on the specific enzyme activities.

DISCUSSION

The importance of this study lies in the facts the one more good ligninolytic enzyme producer, *T. suaveolens*, was discovered, the list of well lignin degraders was broadened, and cultivation conditions for enzyme overproduction were created. E l i s a s h v i l i et al. (2008; 2009) emphasized that the enzyme synthetic potential significantly depended on the cultivation type. Namely, contrary to the results obtained for *T. suaveolens* HAI 300, results of numerous studies showed that conditions of submerged cultivation were favorable for laccase production, while solid-state cultivation was appropriate for MnP synthesis (S u n et al., 2001; J a s z e k et al., 2006; D i n i s et al.,

2009; E l i s a s h v i l i et al., 2009). Thus, S u n et al. (2001) obtained significantly higher values of MnP activity in *T. gallica*, *T. pubescens*, and *T. versicolor* when stationary submerged fermentation of wheat straw, banana and apple peelings was substituted with a solid-state one. J a s z e k et al. (2006) and D i n i s et al. (2009) also reported that MnP was the predominant ligninolytic enzyme with maximum activity being even 10 times higher than the maximum laccase level, during solid-state fermentation of wheat straw.

Contrary to the data of D i n i s et al. (2009) and A s g h e r at al. (2010), the results obtained for *T. suaveolens* HAI 300 showed that laccase and Mn-oxidizing peroxidases were not expressed constitutively and did not show synergistic action. Likewise, the maximum values of laccase and Mn-oxidizing peroxidase activity, noted on the 7th and 14th day of cultivation, respectively, were not in accordance with previously described mechanism of lignin biodegradation (G ó m e z – T o r i b i o et al., 2001). Namely, according to these authors, Mn-oxidizing peroxidases that oxidizes $Mn^{2+}$ to $Mn^{3+}$, which then directly oxidizes lignin, are involved in initial attack on lignocellulose because of the fact that laccase is too large molecule to penetrate into non-modified plant cell walls.

Numerous studies have also demonstrated that lignocellulosic waste composition (concentrations of soluble carbohydrates and inducers), as well as species and strain physiological diversity caused by genetic variability, also affect enzyme synthesis (E r d e n e t al., 2009). E l i s a s h v i l i et al. (2008) noted significantly lower laccase/MnP ratio after wheat straw submerged fermentation by *T. versicolor* in comparison to the ratio obtained during solid-state fermentation of the same residue by *T. suaveolens* HAI 300, which could be explained by intergenetic diversity, different conditions, and availability of compounds that enhance enzyme synthesis in substrates (E l i s a s h v i l i et al., 2009).

In comparison to the other species of the genus *Trametes*, *T. suaveolens* HAI 300 belongs to a group of white-rot fungi which ligninolytic enzymes are characterized with significant activities. Thus, level of laccase activity in *T. suaveolens* HAI 300 was higher for approximately 8-fold and even 200-fold than in *T. versicolor* and *T. hirsute*, respectively, while differences in MnP activity were lower, from insignificant 2-fold to 56-fold in comparison with *T. trogii*, *T. versicolor*, and *T. hirsute*, respectively (E r d e n et al., 2009; L e v i n et al., 2010). These results could be explained by intergenetic diversity caused by evolution and adaptation to various environmental conditions.

Despite the presented data on *T. suaveolens* HAI 300 ligninolytic enzyme activities, additional research is needed to complete the knowledge about their efficiency. Namely, finding a direct correlation between enzyme activity and lignin degradation is not easy due to the complexity and variety of lignocellulose degradation mechanisms.

# CONCLUSION

The obtained results showed that cultivation type and period, as well as the type of plant residue played a significant role in the regulation of ligninolytic enzyme activities in the studied species. The special contribution of the study is introduction to the *T. suaveolens* enzyme system, which has not been evaluated until now. However, despite the fact that the species represented a good enzyme producer, the synthetisis mechanism was not common when compared to the previous results; solid-state cultivation was the optimum for laccase activity while Mn-oxidizing peroxidases reached the peak of activity during submerged cultivation. A special feature of the species is the period of appearance of enzyme activity peak, which was the 7th day of cultivation for laccase and 14th day of cultivation for MnP.

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## *TRAMETES SUAVEOLENS* ПРОДУЦЕНТ ЛИГНИНОЛИТИЧКИХ ЕНЗИМА

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РЕЗИМЕ

Врсте рода *Trametes* представљају најефикасније лигнин-деградере због добро развијеног лигнинолитичког ензимског система који чине лаказа и Mn-зависна пероксидаза. Садашњи трендови су скрининг способности нових врста да продукују ове ензиме као и оптимизација услова за њихову обимну синтезу. Због тога, циљ овог истраживања је био проучавање потенцијала *T. suaveolens* да синтетише лаказе и Mn-оксидујуће пероксидазе током ферментације одабраног биљног материјала. Ниво ензимске активности је мерен 7-ог, 10-ог и 14-ог дана течне и чврсте ферментације пшеничне сламе и пиљевине храста у присуству $NH_4NO_3$, у оптималној концентрацији азота од 25 mM. Ензимска активност је одређивана спектрофотометријски коришћењем ABTS и фенол црвеног као супстрата. Највиши ниво активности лаказе (1087.1 U/L) забележен је након 7 дана чврсте фер-

ментације пшеничне сламе, док у условима течне ферментације овај ензим није продукован. Течна култивација у медијуму са пиљевином храста је била оптимал-на за активност Mn-зависне пероксидазе (1767.7 U/L 14-ог дана) и Mn-независне пероксидазе (1113.7 U/L 7-ог дана). Познавање потенцијала *T. suaveolens* да про-дукује лигнинолитичке ензиме представља основу за даља истраживања, одре-ђивање односа између ензимске активности и степена разградње лигнина. То ће водити већој могућности селекције врста са високим капацитетом делигнифи-кације, које се могу користити у производњи хрне, хранива, папира и енергије, као и у заштити животне средине и биоремедијацији.

КЉУЧНЕ РЕЧИ: лаказа, Mn-оксидујућа пероксидаза, биљни преостаци, *Trametes suaveolens*

РИБАРСКО ГАЗДИНСТВО

ЕЧКА

АКЦИОНАРСКО ДРУШТВО

ЛУКИНО СЕЛО — ЗРЕЊАНИН

# Mirotin-Energo

## Kratak profile naše kompanije

Kompanija Mirotin je osnovala firmu Mirotin-Energo d.o.o. čija osnovna misija je realizacija projekata iz obnovljivih izvora energije na bazi resursa sa kojim raspolažu kompanije Mirotin-a, pre svega PD "Sava Kovačević" A.D. koja sa svojih više od 4000 ha kvalitetne zemlje pod navodnjavanjem i ima organizovanu proizvodnju mleka na govedarskoj farmi od 2200 grla i mlekaru "Dana" kapaciteta od 50.000 litara obrade mleka dnevno.

Osnova za realizaciju biogasnih postrojenja je stajnjak koji se planira godišnje u količini od 27.000 t. Takođe je planirano za proizvodnju električne i toplotne energije koriste otpaci od ratarske i povrtlarske proizvodnje u sklopu PD "Save Kovaćević". Nakon fermentacije dobija se kvalitetno i stabilno đubrivo koje je planirano za korišćenje na zemljištu imanja PD „Sava Kovaćević" kao i deo za doradu stajnjaka u vidu komposta.

Istraživanja i procene su da postoje sirovine za izgradnju 3 MW biogasnog postrojenja što će biti realizovano u narednom periodu.

## Opšte informacije o investiciji

Investitor: Mirotin-Energo d.o.o.
Početak radova: 15.8.2011.
Vrednost projekta: 5,8 miliona €
Finansiranje: 75% kroz investicioni kredit ERSTE banke i 25% sopstvenog učešća
Instalisana snaga: 1 MW električne i 1 MW toplotne energije
Količina proizvodnje biogasa: 4.000.000 m3 (50% CH4)
Dozvola za gradnju dobijena 4.11.2011.
Upotrebna dozvola: 16.08.2012.
Prvo puštanje CHP u rad: 29.09.2012.

## Projekte izvode:

- Biogest Energie und Wasser Technik GmbH (www.biogest.at)
- Projektant BOR-ING d.o.o. Kula

Građevinski deo projekta: GIK Inženjering d.o.o. Kanjiža sa podizvođačima
Tehničko tehnološki nadzor: PRO-ING d.o.o. Novi Sad
Finansijski nadzor: SGS Beograd Ltd. (www.sgs.com)
Studija izvodljivosti: prof. dr Attila Kovács
Laboratorijska ispitivanja: Insitut za biogas Segedin

Objekat se sastoji od dva fermentora prečnika 40m i 24m koji se nalaze u jednom objektu po sistemu PowerRing koje je zaštićeno rešenje od Biogest-a. Posle fermentacije i nakon separacije odvaja se čvrsta faza na pisti koja je dimenzija 81mx100m a tečna faza koje ima 70% se lageruje u postfermentoru kapaciteta 5.500 m3 na kome se nalazi balon za rezervoar gasa od 3100 m3. Proizvedeni gas se koristi CHP jedinice koja je smeštena u pogonskoj zgradi (Jenbacher 416). Za napajanje objekta i isporuku električne enrgije izgrađena je trafo stanica 20/0,4 kV snage 1MW. U pogonskoj zgradi je smeštena oprema za upravljanje i kontrolu celokupnog pogona. Planirana je proizvodnja oko 8.200.000 kWh električne i isto toliko toplotne energije. Cena za ovako dobijenu električnu energiju prema važećoj Uredbi i feed in tarifi je 14.2 €c/kWh (za ovu snagu). Planiran je rad na godišnjem nivou od minimalno 8.200h (od raspoloživih 8.760h). Za stabilizaciju rada sistema, instaliran je prikjučak sa gasnom rampom za prirodni gas koji u skladu sa Uredbom može na godišnjem nivou da meša do 10% prirodnog gasa. Toplotna energija se planira da se plasira za lokalne potrošače u Vrbasu (bolnica i ostali potrošači) i za sopstvene potrebe PD "Sava Kovačević" za sušenje semenske i merkantilne proizvodnje. Trenutno su ispitivanja i studije kojim treba da se potvrde stvarne mogućnosti sirovinske osnove biljne proizvodnje i ostalih otpadaka "Save Kovačeviča". Razmatra se mogućnost da se promenom strukture sirovina poveća proizvodnja gasa i na bazi toga i povećanje proizvodnje struje i toplote.

U sklopu ovog objekta isporučen je sistem za automatsko čišćenje štala koje je isporučila firma GEA. Sav tečni stajnjak će se čistiti kontinualno u 4 velika prihvatna šahta odakle će se nakon mlevenja transportovati cevovodom direktno u predskladište fermentora. Takođe, sa piste gde će biti deponovana silaža i ostali materijali obezbeđeno je priključenje svih tečnih faza, a čvrsti stajnjak i silaža će se ubacivati kroz dva usipna koša kapaciteta 2x80 m3 koji su opremljeni sa mlinovima čekićarima.

Za potrebe kontrolisanje količine ulaznih sirovina instalirana je kolska automatska vaga za kontrolu ulaska sirovina sa strane i eventualno merenje postfermentorske mase ako se bude izvozila iz pogona. Ostala merenja su obezbeđena kroz usipne koševe gde takođe postoji merenje protoka čvrste mase kroz ova dva usipna koša.

Za bezbedan rad pogona primljena je potrebna radna snaga za zadatak vođenja i kontrole procesa koja je obučavana u sličnim objektima u Češkoj.

Zbog značaja i složenosti objekta i opreme održavanje Jenbacher sistema je povereno konzorcijumu Habo-Cummins Serbomonte. Sistem je opremljen sa sistemom daljinske kontrole, a praćenje sistema je omogućeno kroz jedinstveni SCADA sistem gde se permanentno prikupljaju i obrađuju podaci sa objekta.

# NAUČNA OPREMA ZA MIKROBIOLOGIJU



Prodajni program kompanije VICOR d.o.o. namenjen mikrobiološkim laboratorijama obuhvata opremu renomiranih svetskih proizvođača potrebnu za stvaranje i održavanje sterilnih uslova, inokulaciju, kultivaciju, inkubaciju i identifikaciju mikroorganizama, kao i potrošni materijal potreban svakoj laboratoriji: automatske pipete; testove za hemijsku i biološku kontrolu sterilizacije; pribor za uzorkovanje: sigurnosne kese, uzorkivače, eze i držače za eze; ELISA testove i PCR kitove, kompletan Micro program vodećeg svetskog proizvođača BD, Becton&Dickinson sa brendovima BD Difco[tm], BD BBL[tm] itd, koji obuhvataju dehidratisane i gotove hranljive podloge, različite brze testove i antibiogram diskove.

По рецептури Сверуско научно-истраживачког института за пољопривреду микробиологију из Санкт Петербурга

БиоГенесис
Лек за
повртарске леје

# ЕКСТРАСОЛ

## Микробиолошки препарат
## Стимулативног и фунгицидног
## дејства

**Обезбеђује:**

- ревитализацију (оздрављење) земље и земљишта при сталном узгајању биљака на истом месту

- заштита биљака од широког спектра патогених микроорганизама

- антистресни ефекат при расађивању гомоља, луковица, расада у земљиште, пресађивања садница

- смањење 20-30% потребе биљака за хранљивим елементима захваљујући азотофиксирајућим и фосфатомобилизирајућим особинама

- регулацију свих основних физиолошких функција биљака што има за последицу стимулацију раста, постојаност према земљишно-климатским условима, повећање карактеристика квалитета и приноса 20-30%

- неоштећеност кромпира и гомоља при прихрани

- стимулацију и заштиту ослабљених собних и биљака у стакленичкој производњи

Препарат је добио сертификат - пасош Руске Академије пољопривредних наука

Апсолутно је безбедан за човека и животиње

Ограничења: нема
Рок трајања: 24 месеца
Температура чувања од 5-25 ºC

Произведено по рецептури
Др. Владимира Ћеботара

Произвођач:
БИОГЕНЕСИС доо Бачка Топола

Представништво Београд, Дубровачка 3/7
Тел/факс: 011/3033-565, 062/18617-01
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