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2007


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Зборник Матице српске за природне науке издаје Матица српска
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INTRODUCTION

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

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

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

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

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

The second scientific meeting MYCOLOGY, MYCOTOXICOLOGY AND MYCOSES with international participation is organized within Department of Sciences of Matica srpska, and held from 18—20 April 2007 in Matica srpska, Novi Sad.

President of the Organizing Committee
Professor Marija Skrinjar, Ph.D.

Зборник Матице српске за природне науке / Proc. Nat. Sci., Matica Srpska Novi Sad, № 113, 9—16, 2007

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THE CONTENT OF DEOXYNIVALENOL AND ZEARALENONE IN CERTAIN PARTS OF *FUSARIUM* INFECTED WHEAT HEADS

ABSTRACT: During the year 2006, climatic conditions were favourable for the appearance of head blight in the majority of localities in which wheat was grown in our country. In the locality of Apatin, in certain plots, the amount of detected infection was up to 25 infected heads per m^2. During the harvest, heads with distinct disease symptoms and sporulation of *Fusarium graminearum* fungi were gathered. Grains from the parts of heads with manifested disease symptoms were separated into separate samples, together with the grains above and below the infested head part. Apart from ocular evaluation, the percentage of grain infestation by *Fusarium* genus fungi was determined in all three sample categories, using wet chamber method. Deoxynivalenol (DON) was determined in the samples after extraction, using acetonitrile-water (84:16, v/v) solution. Quantitative amount of DON was determined using liquid chromatography with DAD detector at 220 nm. The content of DON in the samples was as follows: grains with manifested disease symptoms 353,4 ppm (μg/g), grains above the infested head part 0,225 ppm (μg/g), grains below the infested part 0,125 ppm (μg/g). The content of zearalenone in the samples was determined using thin layer cromatography method. This toxic agent was determined only in the samples taken from the head part in which disease symptoms were clearly manifested in the amount of 2,1 ppm (μg/g).

KEY WORDS: deoxynivalenol, *Fusarium* head blight, *Fusarium graminearum*, zearalenone

INTRODUCTION

Wheat heads, in our agroecological conditions, are often exposed to the *Fusarium* genus fungi infection which causes *Fusarium* head blight. Apart from inevitable decrease in yield, mycotoxins, that are almost always produced by these fungi in the infested grains, represent the greatest danger for humans and animals. Apart from *Fusarium* genus, also developed on the grain, there

are numerous saprophytic fungi from the genera of *Alternaria, Mucor, Bipolaris, Epicoccum, Cladosporium, Penicillium, Stemphylium* etc., which, by their enzymatic activity, destroy proteins and in great degree decrease the technological quality of the product (Clear and Patrick, 1993; Šarić et al., 1997; Korona et al., 1995; Csosz, 2002; Bagi et al., 2004; Bagi et al., 2005; Balaž et al., 2006).

Among fungi which are regularly isolated from wheat seeds, it is well known that toxins are produced by species belonging to genera of *Fusarium, Aspergillus, Penicillium, Alternaria, Mucor, Rhizopus, Streptomyces* etc. Among toxins produced by *Fusarium* genus species, the most frequent and the most important ones, in a great number of agricultural products, are deoxynivalenol (DON) and zearalenone (ZEA) toxins, which are known to cause serious health problems to people and animals (Peraica et al., 1999; Schnerr et al., 2002; Sundstol Eriksen, 2003).

The aim of this work was to determine the presence and concentration of toxins in the infested grains taken from the head part with clearly manifested *Fusarium* head blight symptoms, as well as in grains from the head parts above and below the manifested symptoms. In the part of an intensively infested head, to a greater or lesser extent, shrunk grains were formed which are, together with chaff, partially removed during the harvest. However, from the point of view of food safety, it is extremely important whether DON or ZEA are accumulated in other parts of the head, where grains are formed, and which are then combine harvested and transferred into food.

MATERIAL AND METHODS

The intensity of *Fusarium* head blight infestation, during the vegetative period of 2005/06, was monitored in the locality of Apatin on Renesansa variety. Meteorological factors are represented in Table 1, according to decades, in the period from March to July, when they could affect the intensity of the occurrence of this disease. Crops preceding wheat on the plot were: sugar beet in 2002, wheat in 2003, corn in 2004, sunflower in 2005.

Tab. 1 — Meteorological factors in the locality of Sombor (from April to June 2006)

Month	Decade	Temperature (°C)	The amount of precipitation (mm)
March	I	2.0	6.2
	II	2.5	22.4
	III	10.6	22.5
Average		5.2	51.1
April	I	10.4	32.5
	II	12.0	74.5
	III	15.9	0.0
Average		12.8	107
May	I	13.2	10.9
	II	17.5	7.8
	III	18.0	35.4

Average		16.2	54.1
June	I	13.1	73.2
	II	21.7	13.4
	III	25.3	14.0
Average		20.0	100.6
July	I	22.0	22.8
	II	23.2	2.8
	III	25.7	24.9
Average		23.7	50.5

The evaluation of the intensity of head infestation was done in June 20, 2006, in the pheno-phase of early waxy ripeness (scale BBCH 7.77), when the average number of infested heads was determined on the sample of 600 heads/m^2, at four randomly chosen places on the plot.

Heads with symptoms of *Fusarium* head blight were gathered during the wheat harvest (July 10, 2006). Determination of *Fusarium* head blight causal agent was conducted using method by Burgess et al. (1988). In the greatest number of cases, infection by the fungus was determined in the mid-plant parts of the head. By separating the infested part from the non-infested upper and lower head parts, three grain categories were formed in order to investigate the presence and content of mycotoxins. Apart from ocular separation, seed infestation was also determined using the method of incubation in moist chamber (Pitt and Hocking, 1985).

The content of deoxynivalenol (DON) in grains from different parts of ear, was determined by method of liquid chromatography (HPLC). The samples were ground, homogenized, and 25.0 g of the sample were extracted with 100 cm^3 of CAN-water (84:16, v/v). six cm^3 of crude extract was cleaned up on CACC column (activated charcoal — alumina — Celite — cation exchange resin). The cleaned up extract was evaporated to dryness, dissolved in 3 cm^3 of ethyl acetate, and quantitatively transferred to an evaporation vessel by triple washing with 1.5 cm^3 the ethyl acetate (Jajić, 2004). The eluate was evaporated to dryness only. The purified, evaporated residue was redissolved in 300 µl of methanol, and a 15 µl of aliquot solution was injected into the LC system under the following chromatographic conditions: mobile phase, a mixture of solvents ACN-water (16:84, v/v), λ = 220 nm, flowrate 0.6 cm^3/min. Calibration curves used for quantitative determination were constructed on the basis of the area under DON chromatographic peaks, using standard working solutions.

The content of zearalenone was determined by thin layer chromatography (TLC) method. Extraction and purification were performed according to Balzer et al. (1978) method. Evaporated residue was redissolved in 100 µl of chloroform. 10, 25 and 50 µl of extract were applied to the plate using a micropipette, along with 10, 15, 25 and 50 µl of standard zearalenone solution (C = 5 µg/cm^3). Quantitative determination was based on the comparison of fluorescence intensity of sample spots and standard solution spots.

RESULTS

The average intensity of *Fusarium* head blight intestation in the locality of Apatin was 24,25% (Table 2), which is considerably more than the intensity of infection in average years (B a g i, 1999).

Tab. 2 — The intensity of *Fusarium* head blight infestation in the locality of Apatin in 2006

	Number of infested heads/m^2 Replication				
	I	II	III	IV	X
Infestation intensity	25	26	27	19	24,25

Based on morphological and breeding characteristics of isolates from the infested heads on PDA and CLA substrates, *Fusarium graminearum* fungus was determined. Grain infestation, according to the investigated categories, was determined using the wet chamber method (Table 3).

Tab. 3 — The infestation of grains taken from different head parts

Sample	The percentage of *F. graminearum* infestation (%)
Grains from the head part with manifested disease symptoms	100
Grains from the head part above the infested spot	5
Grains from the head part below the infested spot	2

The content of deoxynivalenol in the samples was as follows: grains with manifested disease symptoms 353,4 ppm (μg/g), grains above the infested head part 0,225 ppm (μg/g), grains below the infested head part 0,125 ppm (μg/g). The presence of zearalenone was determined in the grain samples from the head part in which symptoms were clearly manifested in the amount of 2,1 ppm (μg/g).

DISCUSSION

Mycotoxins in groceries represent serious threat to human and animal health (H a r i s et al., 1999; Š k r i n j a r et al., 2005). The types and quantities of toxins formed in the cereal grains infested by *Fusarium* genus fungi depend on the fungus type and isolate, the time when the infestation appeared, grain crops genotype, as well as on the environmental conditions, above all temperature and humidity (P e r k o w s k i et al., 1995; B o č a r o v - S t a n č i ć, A. 1996; B a g i et al., 2000; P a u l et al., 2005). Among numerous toxins, extremely important are the ones belonging to the group of trichothecene, which include DON as well as zearalenone. Correlation was determined between the formation of DON toxin and the degree of pathogenicity of *F. graminearum* isolate, which indicates the role of DON in the isolate virulence (H a r r i s et al., 1999; G o s w a m i and K i s t l e r, 2005). It is considered

that zearalenone is an estrogenic toxin which affects the formation of fruiting stadium of *F. graminearum* (H o m d o r k et al., 2000).

The length of the period over which *Fusarium* genus fungi could infect heads, depends predominantly on climatic conditions during sensitive wheat phenophase, which lasts for about ten days, starting from the beginning of blooming to the grain formation (B a l a ž, 1987). In spikelets infected at the moment of blooming, grains are not formed, and in spikelets in which at the moment of infestation grain was developed to a certain degree, shrunk, poorly filled grains appear. Shrunk grains also appear in case that fungus infests head spindle, in the head part above the manifested spot. Most of the shrunk grains are removed, together with chaff and grain clippings during combine harvest, which also depends on the combine type and adjustment i.e. on the strength of air current that separates lighter head parts from the wheat grains.

To sum up, toxins transferred into groceries on the one hand, originate from those head parts in which poorly filled grains, are formed and which are infected by *Fusarium* genus fungi, not removed during combine harvest, and on the other, the quantity of toxins depends on the conditions of wheat storage, since in inadequate conditions toxic fungi are spread to noninfested grains, i.e. fungi activity in already infested grains produces mycotoxins. According to the obtained results, the quantity of DON and ZEA toxins is the greatest in grains from the head part, with manifested symptoms of *Fusarium* head blight, and in which 100% grain infestation was determined, which matches the results of other authors according to whom great positive correlation was determined between fungus biomass in the infested grains and the quantity of DON (S n i j d e r s and P e r k o w s k i, 1990). DON toxin in non-infested parts (above and below the infested head part) is not created, i.e. it does not move with the movement of plant assimilates. Minimal quantities of DON determined in these grains are the result of the presence of a few percents of infested grains, which could be determined only by incubation on moist blotter. The obtained data lead to the conclusion that mycotoxins are not transferrable from the head parts containing high quantity of fungi and toxin biomass into other head parts, i.e. into grains. They also point to the liability of conclusions made by Z h o u et al. (2002), who recommend the choice of favourable type of resistence against the spread of parasite within plant tissue, with the aim of preventing DON accumulation in grain crops. By preventing the growth of hyphae within the plant, the creation of toxins in the infested tissue is localised.

Based on these results, it can be concluded that in the measures with the aim of preventing toxins from appearing in groceries, apart from the measures for preventing the head infestation to develop (soil cultivation, fertilisation, crop rotation, chemical protection, resistant genotypes), and optimal storage conditions (humidity, temperature, the presence of insects etc.), measures of combine adjustments could also be included, which enable the removal of substantial part of infested grains, together with chaff which could drastically reduce the danger of mycotoxins to become part of nutrition chain. More precise role of the appropriate combine adjustment in removing infested grains should be invastigated more thoroughly in the future, and included as an important measure in avoiding mycotoxicosis in humans and animals.

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САДРЖАЈ ДЕОКСИНИВАЛЕНОЛА И ЗЕАРАЛЕНОНА У ПОЈЕДИНИМ ДЕЛОВИМА ФУЗАРИОЗНИХ КЛАСОВА ПШЕНИЦЕ

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Резиме

Током 2006. године климатски услови су били повољни за појаву фузариозе класа пшенице у већини локалитета гајења ове културе у нашој земљи. У локалитету Апатин на појединим парцелама констатована је зараза и до 25 заражених класова по m^2. За време жетве прикупљени су класови са јасним симтомима бо-

лести и спорулације гљиве *Fusarium graminearum*. Из класова су у посебне узорке одвојена зрна из дела са симптомима обољења, као и зрна изнад и испод захваћеног дела класа. Поред окуларне оцене, проценат захваћености зрна гљивама из рода *Fusarium* је одређен у све три категорије узорака, методом на филтер папиру. У узорцима одређен је деоксиниваленол (ДОН) након екстракције са смешом ацетонитрил-вода (84:16, v/v). Квантитативни садржај ДОН-а је одређен течном хроматографијом са ДАД детектором на 220 nm. Садржај ДОН-а је у узорцима био следећи: зрна са испољеним симптомима обољења 353,4 ppm (μg/g), зрна изнад захваћеног дела класа 0,225 ppm (μg/g), зрна испод захваћеног дела 0,125 ppm (μg/g). Садржај зеараленона у узорцима одређен је методом танкослојне хроматографије, при чему је овај токсин утврђен само у узорцима из дела класа на којем су се јасно испољавали симптоми и то у количини од 2,1 ppm (μg/g).

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WHEAT SAFETY IN RELATION TO PRESENCE AND CONTENT OF DEOXYNIVALENOL

ABSTRACT: HACCP (***H**azard **A**nalysis and **C**ritical **C**ontrol **P**oint*) is a concept which identifies, evaluates, and controls hazards, significant for food safety. It is applicable to the entire food chain, "from farm to table". Prevention of a foodborne mycotoxin contamination of commodities is the most rational and cost-effective method for preventing adverse effects of fungal metabolites on human and animal health. Deoxynivalenol (DON) belongs to the group of mycotoxins produced by certain Fusarium species, which can damage several vital organs, or demonstrate immunotoxic effect, when ingested in small amounts for a longer period of time. Of particular concern is exposure of children to this mycotoxin through cereals, which are believed to lead to reduced weight gain and decreased liver weights. For that reason, we tried to present HACCP concept for preventing wheat contamination with deoxynivalenol. To be able to apply this system, hazards must be identified, and risks assessed, and for that purpose a real picture of area (region) in which preventive measures shall be applied, need to be established. According to the results of the study conducted in the laboratory at our department, DON contaminated wheat samples in the region of Vojvodina accounted for 41,6% in 2004 and 2005, whereas amounts of deoxynivalenol ranged from 57 to 1840 μg/kg.

KEY WORDS: HACCP, deoxynivalenol, wheat

INTRODUCTION

HACCP system itself consists of two fundamental components: HA and CCP. HA is a risk analysis, that is, identification of potential hazards in each cycle of food production, and assessment of their effects on human health. CCPs (critical control points) are actions introduced throughout the production process by which food safety risks can be prevented, eliminated or reduced to acceptable levels.

Application of HACCP system is compatible with the quality management system, being in compliance with ISO 9000 series of standards, and it

implies adoption of appropriate measures required for food safety management. Prior to introduction of HACCP system in any organizational part of the food manufacturing establishment, the relevant organizational part must function in compliance with the principles of GAP (Good Agricultural Practice), GMP (Good Manufacturing Practice), GHP (Good Hygiene Practice) and relevant food safety legislation. For the purpose of avoiding any food-related risks, HACCP method is to be used as an effective way of managing and controlling food safety hazards in the preparation and handling of food and food products.

HACCP INTRODUCTION PROCEDURE

— Assemble of HACCP team
— Description of the product
— Identification of the intended product use

Since HACCP system involves multidisciplinary approach, multidisciplinary team should be assembled, consisting of specialists with a thorough knowledge of the product, production process, management and quality assurance principles. This team could include personnel from the production/sanitation, quality control, laboratory, engineering and independent outside experts to advise about the identified issues, or problematic areas. The task of the team is to ensure that all conditions for the application of the HACCP principles have been met and necessary documentation prepared, and the team members, therefore, must have the knowledge and expertise to develop an HACCP plan.

The product and production process description should include the name of the product, ingredients and composition, packaging, shelf life, storage conditions, and distribution control measures, if applicable.

The intended use of the product refers to its normal use by end-users, or consumers. The HACCP team must specify where the product will be sold, as well as the target group and labeling instructions which should be followed.

In our case, control is aimed at deoxynivalenol (DON), a frequent contaminant of cereals. The global frequency of mycotoxin-contamination of food appear to be increasing in the recent years. Mycotoxins may occur during the different stages of grains production. *Fusarium* species can be formed in the field preharvest, and are usually referred to as "field mycotoxins", and may continue to be formed in storage if conditions are favorable.

Fusarium is one of the most widespread genera of plant pathogenic fungi responsible for destructive and economically very important diseases of cereal crops. *Fusarium* strains are responsible for wilts, rots and the like in legumes, coffee, pines, wheat, corn, carnation and grasses.

Disease of wheat known as Fusarium head blight (FHB) is caused primarily by *Fusarium graminearum* species. This disease reduces yield and seed quality grade and spreads easily, particularly in regions with humid climatic conditions (B o w d e n et al., 2003).

Symptoms: FHB is detected immediately after blooming. Infected heads become whitish, whereas leaves and stalks remain unchanged. A portion of

stalk immediately below the head is sometimes of chocolate brown colour. Infected wheat seeds are shrunken and typically chalky white or pinkish. Seeds above the infected spikelet will shrunk if the mould enters the glume and disrupts food supply. Apart from reducing yield and grade, this disease may also contaminate the grain with fungal toxins (mycotoxins). FHB is favoured by wet conditions during the flowering and early stages of kernel development. FHB is recognized in the field by premature bleaching of infected spikelet, and the production of orange, spore-bearing structures (S e r a f i n c h o n, 2003). During wet weather, there may be whitish, occasionally pinkish, fluffy fungal growth on infected heads in the field. Diseased spikelet can contain visibly affected kernels. The grading term given to visibly affected wheat seeds is fusarium damaged kernel (FDK).

The last line of defense occurs when *F. graminearum* infections are identified in cereal fields. Such occurrence would trigger an eradication strategy where the crop is immediately ensiled or harvested, and the residue carefully managed and cultivated. The field would be taken out of cereal production for three years to allow the crop residue to degrade, resulting in the eradication of the pathogen. *F. graminearum* does not survive in fields where the host material has degraded and is no longer present. During the growing season, growers should check the fields for the presence of this disease. They should check for symptoms on cereal heads, about three weeks after anthesis, and in the grain at harvest. If *F. graminearum* is found, the planting of varieties least susceptible to the disease should be considered.

Mycotoxins produced by *Fusarium* species significantly differ in their structure, and the toxin production is primarily due to genetic factors, but is also affected by the conditions in which fungi grow, such as: the amount of available water in substrate, for fungi growth (a_w), ambient temperature, presence of aerobic medium, relative air moisture, type of medium or substrate, pH environment, level of sustained damage on kernels, total number of fungi and share of toxicogenic species, as well as the presence of competitive microflora etc. For moulds growth, humidity over 12%, and water activity above (a_w) 0,7, is needed. Temperature favourable for toxin production ranges from –5 to +60°C, and pH from 5—7.

Fusarium is a large and complex species containing strains adapted to different environmental conditions all over the world.

Different strains from the same species may behave differently in mycotoxin production. There is also a regional difference in mycotoxins produced by Fusarium species, as a result of genetic variation and climatic factors. Generally speaking, it is not possible to determine series of conditions or factors having critical effect on the moulds growth and mycotoxin production. It is a distinctive feature of biosynthesis of secondary metabolites; the produced amount is conditioned not only by the environmental and nutritional parameters, prevailing during the production, but is also affected by the previous crop residues and the incidence of mould occurrence. Mycotoxin type and concentration is always determined by the mould, substrate and external factors. External factors affecting mould production can be physical, chemical and biological, but almost always in interaction with each other. The sheer fact that

mycotoxins are able to synthesize in a few days under favourable ambient conditions, suggests that mycotoxin hazards are permanently present (Narech, 2006).

Trichothecenes are secondary toxic products produced by several moulds, including species of the genera *Fusarium, Myrothecium, Trichodederma, Trichothecium, Stachybotrys* and *Verticimonosporium.* More than 170 trichothecenes have been isolated and chemically characterized up to now. Trichothecenes are tetracyclic, sesquiterpenoid compounds with a 12,13-epoxy-group. Epoxide group C12,13 of all trichothecenes is believed to be responsible for their toxicity. Based on the presence of characteristic functional groups, they are divided into type A (T-2 toxin, diacetoxyscirpenol), type B (deoxynivalenol, nivalenol), type C and D. Trichothecenes from groups A and B are probably the most widely distributed mycotoxins, and according to the Report of the EU Commission, trichothecene toxins from group B are more prevalent in grains than those from group A. The most common trichothecene from group B is deoxynivalenol (DON) (57%), followed by 15-acetyledeoxynivalenol (20%), nivalenol (16%), fusarenon X (10%), and 3-acetyledeoxynivalenol (8%). Out of trichothecenes from group A, the most common is T-2 toxin (20% of analyzed samples), followed, to a lesser extent, by other toxins, namely, HT-2 toxin (14%), T-2 triol (6%), DAS (4%), MAS (1%), and verrucarol, but only in one out of 121 analyzed samples (SCOOP, 20).

F. graminearum and *F. culmorum.* are fungi most often associated with deoxynivalenol production have been Presence of this moulds is prevalent in grains, wheat and corn, in particular, causing their rotting. They can also occur on barley, oats and rye, as well as grain products-malt, beer and bread (Scientific Committee on Food, 1999).

Hot and moist climate favours wheat infection with these moulds, taking place throughout the entire blooming phase. The optimum temperature for *F. graminearum* growth is found to be 25°C, annuall % of humidity, and for *F. culmorum* 21°C, and 87% of humidity. According to Martins and Martins (2002) the most favourable conditions for DON production are 22°C (6,0 mg/kg) and 28°C (5,5 mg/kg), after 35 days of incubation. According to the same authors, there is no DON production at temperature of 37°C.

The presence of DON and other *Fusarium* mycotoxins in Europe is of a particular importance, primarily due to climatic conditions prevailing in that region. The Study of scientific corporation for food issues — sub-commission for trichothecene (SCOOP, 2003) included 11 EU states. Of 11.022 analyzed samples, DON presence was found in 57%. In a huge number of wheat and wheat flour analyzed for DON presence (6358), 61% of the samples were found to be positive, in the detection range from 2 μg/kg (Sweden) to 50 000 μg/kg (France).

The EU Scientific Committee for Food (Commission Regulation No 856/2005) has established maximum tolerable levels for DON in cereals and their products: unprocessed cereals other than durum wheat, oats and maize (1250 μg/kg), unprocessed durum wheat and oats (1750 μg/kg), unprocessed maize (1750 μg/kg), cereal flour, including maize flour, maize grits and maize meal (750 μg/kg), bread, pastries, biscuits, cereal snacks and breakfast cereals (500

μg/kg), pasta (dry, 750 μg/kg), processed cereal based food for infants and young children, and baby food (200 μg/kg).

Tab. 1 — Deoxynivalenol content in wheat (μg/kg) (SCOOP, 2003)

Country	Year	Total no. of samples	% of positive samples	Max. DON content	Average DON content
Austria	1999	68	57	1250	310
Austria	2000	62	61	6090	1203
Austria	2001	36	50	1230	334
Belgium	2001	33	15	504	343
Germany	1999	26	80	764	285
Germany	2000	27	66	402	159
Denmark	1999	16	87	527	198
Denmark	2000	28	92	330	63
Denmark	2001	30	63	204	56
Finland	1999	37	67	264	88
Finland	2000	35	71	1026	234
Finland	2001	39	35	376	90
France	2001	53	100	2125	190
France	2002	3	33	120	95
Norway	2001	147	34	464	84
Netherlands	1998	215	84	3280	546
Netherlands	1999	273	53	1946	245
Netherlands	2000	1111	76	5000	354
Netherlands	2001	863	27	2300	437

P i e t e r s et al. (2001) calculated a provisional TDI of 1,1 μg/kg of body according to the results of the study conducted in the Netherlands. Based on this limit, they proposed a concentration limit of 129 μg DON/kg for wheat, a level designed to protect the health of children who are heavy consumers of wheat based products. This team of scientists concluded that in the 1998—2000 period (high amounts of DON were established in wheat) DON intake exceeded provisional TDI, particularly in children, which might have adverse effects on some children. In part because of the concerns raised in their studies, even stricter limits on DON exposures in bread have been established by the Dutch government, namely, 120 DON μg/kg BW for processed wheat and wheat-based products with wheat content of 33%, and 60 μg/kg.

Results of wheat samples analyzed for the presence and content of DON in the laboratory at the Department for Livestock Breeding at the Faculty of Agriculture in Novi Sad are shown in Table 2.

Tab. 2 — Content of deoxinivalenol in samples of wheat collected in Vojvodina (μg/kg)

Year	Average no. of samples	% of positive Samples	Average	Range
2004	4	50	1230	620—18400
2005	12	33,3	182	57—423

Trichothecenes are strong inhibitors of protein synthesis, targeting and damaging, in particular, cells that are actively dividing (thymus, lymph gland, te-

stes, small intestine, spleen). These toxins can, either alone or in combination with each other, cause acute or chronic trichothecene-toxicoses, illnesses characterized by dermatitis, vomiting, food refusal, mucous and skin bleeding, hemorrhagic diarrhoea and sterility. Clinical toxicity picture includes food refusal, vomiting, tachycardia, hemorrhagic diarrhea, edema, skin and mucous necrosis, destruction of hematopoietic tissue, leukocyte and thrombocyte, leucopenia and nervous disorders. Symptoms depend on the type of toxin involved, concentration and exposure time.

Decreased food intake and vomiting are associated with increased activity of central serotonin system in brain. After lower, and especially after higher levels of exposure to deoxynivalenol, reduced growth and development have been reported in most animal species. This has been confirmed by a great number of studies as the most obvious effect of deoxynivalenol toxicity. Two year study conducted on laboratory animals showed that quantity leading to growth and body weight reduction was 0,1 mg/kg of body weight a day. There is no evidence of more significant accumulation of this mycotoxin in the body and body fluid, and detoxification is done through deepoxidation, resulting in partial conversion of this mycotoxin in deepoxy DON, and its excretion after conjugation with glucuronic acid.

Deoxynivalenol inhibits DNA and RNA synthesis at a cell level, and protein synthesis at ribosomal level. The toxin has a haemolytic effect on erythrocytes. Higher doses of DON have adverse effect on heart, liver, spleen and thymus.

An issue of great concern is a heat stability of DON, since it is a very stable compound, both during storage and processing, and as such enters the food chain (EFSA, 2004).

GENERAL PRINCIPLES OF HACCP CONCEPT (CAC, 1997)

Principle 1 Identify the potential hazards

In this stage, HACCP team identifies potential hazards associated with food production at all stages, from primary production, processing, manufacture, and distribution until the point of consumption. After that, the likelihood of occurrence of each hazard needs to be assessed and necessary preventive actions and measures taken to prevent, eliminate or reduce the identified hazard to acceptable levels.

Principle 2 Determine critical control points (CCP)

Critical control points (CCP) are points, steps, or operational procedures in the cycle of production at which potential hazards may occur, and be eliminated, or reduced to acceptable levels by preventive actions and measures.

Mycotoxins synthesize in the field, and their synthesis continues in bins, and thereafter in storages of finished products. On such a long path, and under various conditions, a number of CCPs can be identified. Some of them are: type and manner of tillage, crop rotation, type of seed (hybrids), weather conditions, presence of moulds and insects, harvest season, drying efficiency, microclimatic conditions in storage facilities.

Principle 3 Establish the critical limits

A critical limit is a criterion that has to be fulfilled. It separates acceptability from unacceptability. All parameters for different CCPs need to be measured for as short time as possible, to allow timely decisions and corrective actions. The critical limits must be supported by scientifically based data, must be verifiable, measurable and applicable for all CCPs. Sources of information for establishment of critical limits are: laws and regulations, internal requirements, actual practices and experts' recommendations.

Principle 4 Establish a monitoring system for each CCP

Monitoring is a scheduled measurement or observation of a CCP relative within its critical limits.

Critical control points are monitored in order to:

— establish when critical limit is exceeded, and human health hazards increased,

— identify the problem before it occurs,

— verify HACCP plan,

— assure and confirm the product quality.

Apart from control, operating limits are also established for the parameters based on which the process is run. They are usually more conservative than control limits, need not be part of HACCP documentation, and are used as a buffer, to adjust the process and prevent critical limit from exceeding.

Principle 5 Establish corrective actions

Corrective action is any action to be taken when monitoring indicates that critical control points in CCP depart from the critical limits. There are two types of corrective actions: intervention (intermediate) and prevention.

Principle 6 Establish verification procedures

Verification procedures need to be established to verify whether the HACCP system is working correctly, and whether the identified hazards are within control limits. Verification activities include:

— HACCP system validation

— review of the CCP monitoring results,

— product testing/analysis,

— HACCP system internal audits.

DON level checks must be carried out in regular, prescribed intervals in the field and in the final product to assess whether it is kept within acceptable levels. When there is a deviation from the critical limit, it is considered as a loss of control, and requires adequate actions to be taken. Critical limits may be adjusted, or new control measure must be introduced.

Principle 7 Establish documentation and record keeping

Efficient and accurate record keeping is essential to the application of an HACCP system, to confirm that the system is working effectively. Records are also used for tracking trends and identifying causes of deviations, for introduction of corrective and preventive actions and measures.

CONCLUSION

Mycotoxins are inevitable food contaminants which enter the food chain at one point or another. In the developed world, advances in food handling and safety procedures have largely eliminated the consumption of food with high levels of mycotoxins, and very few people suffer health problems from dietary exposures to mycotoxins.

Cereals (or wheat) are on the top of the hypothetical warning list regarding the mycotoxin contamination, particularly due to possible health effects on children.

HACCP system is widely recognized as the most effective approach for producing safe food. It is almost a universally accepted method of present and future times. Knowledge and understanding of factors favourable for fungi growth are of crucial importance for successful prevention, since they can reduce exposure of humans and animals to mycotoxins to minimum possible levels. It is still not possible to eliminate completely mycotoxins from food products. Therefore, comprehensive efforts must be made to reduce health risks attributable to mycotoxins to acceptable levels and respond to food safety issues and concerns.

Advantages of HACCP system application are that it reduces foodborne diseases, ensures safety of food, adheres to legislation, inspections and surveillance requirements, enhances competitiveness, removes barriers in international trade, and increases profit.

Implementation of HACCP system is not to be considered as the end of the process. Current maintaining of HACCP plan is to provide real benefits.

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БЕЗБЕДНОСТИ ПШЕНИЦЕ У ОДНОСУ НА ПРИСУСТВО И САДРЖАЈ ДЕОКСИНИВАЛЕНОЛА

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Резиме

Превенција контаминације хране токсогеним плеснима је најрационалнија и економски најоправданија метода за спречавање могућих последица деловања њихових метаболита на здравље људи и животиња. Деоксиниваленол (ДОН) припада групи фузаријумских микотоксина, оштећује поједине унутрашње органе, док у малим количинама кроз дужи период делује имунотоксично. Посебно је наглашена изложеност деце овом токсину преко хлеба, а претпоставља се да код њих доводи до смањења телесне тежине и смањене тежине јетре. У овом раду приказан је HACCP концепт у превенцији контаминације пшенице деоксиниваленолом. Да би се применио овај систем мора се прво идентификовати опасност и проценити ризик, а за то је неопходна реална слика за подручје (регион) у којем се желе спроводити мере превенције. Према резултатма испитивања која се спроводе у лабораторији нашег департмана на пшеници са подручја Војводине % контаминираних узорака у 2004. и 2005. години износио је 41,6, а количине деоксиниваленола кретале су се од 57 до 1840 μg/kg.

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ZEARALENONE PRODUCTION DURING MICRO-MALTING OF BARLEY

ABSTRACT: The zearalenone (ZEA) content was determined during a micro-malting process (after steeping, germination, kilning and degermination, as well as in barley samples before micro-malting process) of two winter two-rowed barley samples, grown at Kragujevac location. In all phases of micro-malting, isolation and determination of *Fusarium* spp. were performed.

It was established that barley samples, before malting, were contaminated with zearalenone (barley sample 1—9.7 μg/kg, barley sample 2—9.2 μg/kg).

The following *Fusarium* spp. were isolated: *F. avenaceoum*, *F. culmorum, F. poae, F. sporotrichioides, F. tricinctum* and *F. verticillioides*.

In both barley samples zearalenone content increased during steeping (86.5 μg/kg and 37.4 μg/kg), decreased during germination (12.5 μg/kg and 26.8 μg/kg), and increased after kilning (62.9 μg/kg and 71.2 μg/kg). In the finished malt, the zearalenone content in sample 1 was 35.7 μg/kg dry matter, and in sample 2 was 17.8 μg/kg dry matter.

KEY WORDS: Barley, *Fusarium* species, micromalting, zearalenone

INTRODUCTION

Zearalenone (F-2 toxin), 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone, is a nonsteroidal mycotoxin with low acute toxicity, strong estrogenic, and anabolic properties to humans and animals (European Commission, 2000; Hussein and Brasel, 2001; Logrieco et al., 2003; Rhyn and Zöller, 2003; Yiannikouris et al., 2004). This is due to its ability to couple with the estrogenic receptor, resulting in severe effects on the reproductive system (Zöllner et al., 2000).

As a secondary metabolite of *Fusarium* species, ZEA is the most frequent cereal contaminant (wheat, barley and oats) (Zöllner et al., 2000; Yiannikouris et al., 2004; Galvano et al., 2005). Tolerance levels in grains and grain products have been set in severeal countries with a concentration

range from 30 to 1000 μg/kg (Zöllner et al., 2000). The most common *Fusarium* mycotoxins in European barley grains are zearalenone and deoxynivalenol (Legzduna and Buerstmayr, 2004).

ZEA may be transmitted from contaminated grains into beer during the brewing process (Scott, 1996). A study of Nigerian traditional corn beer brewing showed a 51% carry over of ZEA from malt homogenate to a finished product. Resulting from that, ZEA was found at high incidence and concentration in African beers, which is in distinct contrast to the surveys of European, Canadian and Korean beers, where only one beer sample was determined to contain ZEA at a level of 100 μg/kg (Zöllner et al., 2000).

The malting process is one of the critical points during beer production, regarding the formation of factors that can affect human health, particularly as malting conditions favour the development of many fungi, mostly *Fusarium* species which can produce zearalenone, fumonisins, trichothecenes and moniliformin. *Fusarium* species enter the malting process through barley grains, water for steeping or malting vessels, and the growth of these during malting, especially steeping and germination, is favoured by high water activity and lower temperatures (Noots et al., 1998).

Fusarium species, during malting, besides production of ZEA and other fusariotoxins may affect barley germination (Rabie et al., 1997), decrease the grain size (Schwarz et al., 2001) (which decreases the yield and therefore results in significant economic losses (Schaafsnma, 2002)), lower α-amylase activity (Kleemola et al., 2001), synthesize alkaline proteases which hydrolyze barley proteins (Pekkarinen et al., 2003), and even affect the subsequent fermentation process by having an adverse effect on the yeast (Rabie et al., 1997), gushing of beer (Haikara, 1983; Schwarz et al., 1996; Kleemola et al., 2001; Vanne and Haikara, 2001, Sarlin, 2005) and the resultant beer by causing off-flavours and colours (Vaag, 1985).

Due to all above mentioned, the objective of the present study was to investigate zearalenone production during micro-malting, and the occurrence of *Fusarium* species in brewer's barley samples during micro-malting.

MATERIALS AND METHODS

Brewer's barley samples. Two varieties of two-rowed winter barley (sample 1 and 2), crop 2004, were collected in storage rooms at Kragujevac location. A total of 3 kg of each variety were sampled. Micro-maltings of 1kg barley samples were performed with a micromalting unit "Seeger", Germany. A standard micro-malting process, described by Schuster et al. (1999), was used as shown in Table 1. Chlorinated tap water (drinking quality) was used during the micromalting process.

Tab. 1. — Standard micromalting procedure

Process phase	Day	Duration, h	Description
Steeping	1	6	Steeping in 1st water 14°C
		18	Aeration 14°C; weighing
	2	4	Steeping in 2nd water 14°C
		20	Aeration 14°C; weighing
	3	2	Steeping in 3rd water 14°C
Germination		22	Aeration 14°C; weighing; correction of moisture content to 44.5% by spraying with water
	4	24	Aeration 14°C; turning
	5	24	Aeration 14°C; turning
	6	24	Aeration 14°C; turning
	7	28	Aeration 14°C; weighing; turning and transfer into kilning compartment
Kilning	8	20	Kilning (12 h/40°C — 3 h/60°C — 5 h/82°C); weighing; degermination, weighing

Zearalenone analysis. Zearalenone was determined in both barley samples before micromalting, after steeping, after germination (green malt), after kilning, and after degermination (malt). The zearalenone extraction from the barley samples was done according to the Vicam instruction sheet, by mixing 20 g of milled sample with 2 g of NaCl and 50 mL of the mixture of 90:10 acetonitrile-water in the high-speed glass blender for 2 minutes. The obtained extract was filtrated through the filter paper, and then 10 mL of the filtrate were mixed with 40 mL of washing buffer (PBS/0.1% Tween-20, VICAM). The homogenized mixture was filtrated through the 1.0 μg microfibre filter. The obtained filtrate (10 mL) was passed through Zearalenone Test Immunoaffinity column (1—2 drops/second). Ten microlitres of washing buffer and 10 mL of distilled water (1—2 drops/second) were passed through the column. Zearalenone was deabsorbed out from the column with 1.0 mL of methanol (HPLC purity) into a glass test tube at approximately 1 drop/second. Then, 1 mL of the Zearala Test developer (VICAM) was added to the tube and the content was well homogenized. The test tube was placed into the graduated fluorometer (VICAM series 4), in which the zearalenone concentration was read in μg/kg (V i c a m, 1997).

Isolation and identification of *Fusarium* spp. Samples for mycological analyses were taken before the micromalting process, after the first, second and third day of steeping, after the first day and at the end of germination, after the kilning, and after malt degermination. The dilution plate technique was used for isolating fungi from the samples and for determining their total count per 1 g, or 1 mL.

Twenty grams of the sample were diloted in 180 mL of sodium chloride solution and homogenized for 10 min in a homogenizer. Petri dishes (in triplicate) were inocoluted with 1 mL of each dilution (10^{-1}—10^{-6}). Incubation was carried out at 25°C for 5 and 7 days. Sabouraud maltose agar (SMA) with chloramphenicol (1%) and oxytetracyclin (1%) was used as an isolation medium.

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

Determination of the isolated pure fungal cultures was done according to the taxonomic properties described by Nelson et al. (1983).

RESULTS AND DISCUSSION

The obtained results indicate that both barley samples were naturally contaminated with zearalenone (sample 1—9.7 μg/kg and sample 2—9.2 μg/kg). Species of *Fusarium* genus and ZEA production varied during barley micromalting.

In barley sample 1 three species of genus *Fusarium* were identified during micromalting: *F. poae*, *F. tricinctum* and *F. sporotrichioides* (Fig. 1).

The most intensive growth had *F. poae*, during the second day of micromalting. An increase in the level of *F. tricinctum* was found during the third day of micromalting. *Fusarium sporotrichioides* reached a maximum level during the fourth day of micromalting.

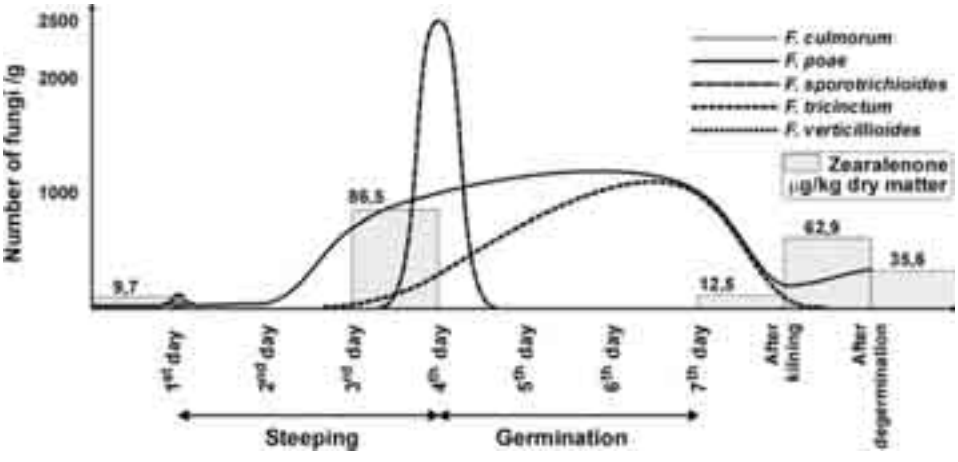

Fig. 1. — *Fusarium* species development and changes in zearalenone content during micromalting in barley sample 1

Four species of *Fusarium* genus were identified in barley sample 2: *F. poae*, *F. verticillioides*, *F. avenaceum* and *F. equiseti*. *Fusarium avenaceum* was identified only on the first day, and *F. equiseti* only on the second day of micromalting (Fig. 2). Two dominant species, *F. poae* and *F. verticillioides*, were also identified in water for steeping. *Fusarium sporotrichioides* was also

identified in steeping water, but not during the micromalitng of barley sample 2. On the first day of micromalting, intensive growth of *F. poae* was observed. Intensive growth of *F. poae* continued until the seventh day of micromalting. *Fusarium verticillioides* was identified on the second day of steeping. Growth of *F. verticillioides* decreased between the second and the third day of steeping. After the third day of steeping, growth of *F. verticillioides* was very intensive until the seventh day of micromalting. *F. verticillioides* became the most dominant species on the fourth day of micromalting (first day of germination), until the kilning (Fig. 2).

This study shows that the growth of *Fusarium* spp. is most intense during germination of barley in both samples. In contrast, A c k e r m a n n (1998) noted that the growth of *Fusarium* spp. takes place particulary during steeping of barley.

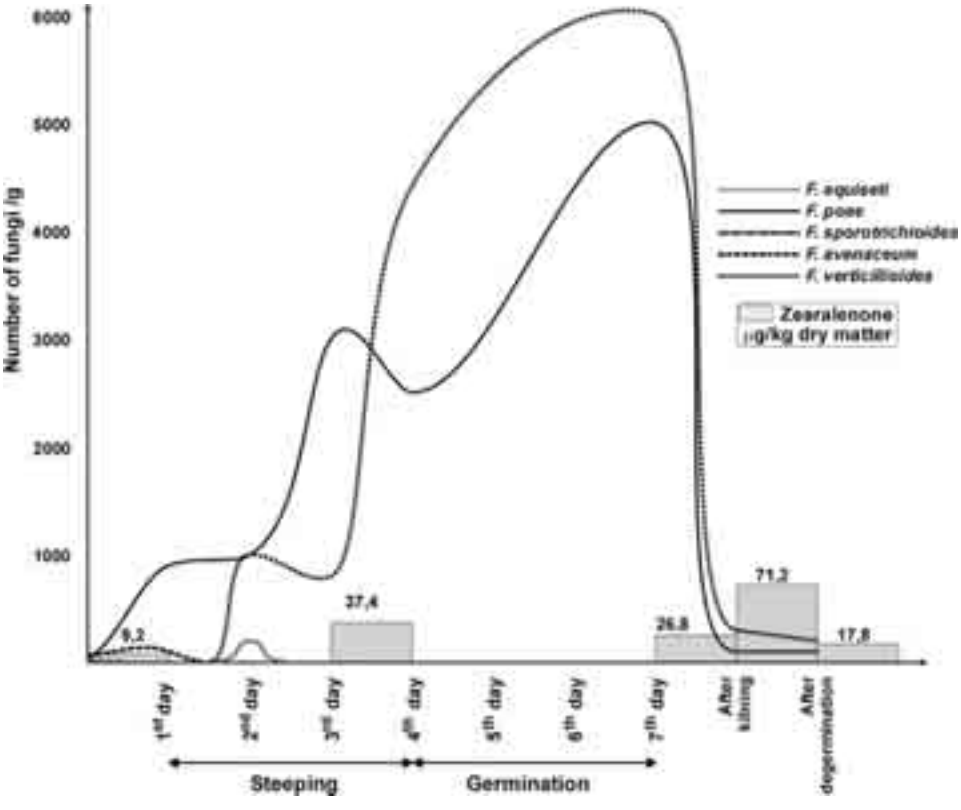

Fig. 2. — Development of *Fusarium* species and zearalenone content during micromalting in barley sample 2

During the micromalting, determined zearalenone contents varied from 9.7 to 86.5 µg/kg dry matter in barley sample 1, and from 9.2 to 71.2 µg/kg dry matter in barley sample 2 (Figs. 1 and 2). The zearalenone content increased during steeping in both samples, which is in contrast with the results of

Schwarz et al. (1995). Germination reduced the zearalenone content in both barley samples. At the end of germination the zearalenone content in sample 2 (26.8 μg/kg) was two times higher than that in sample 1 (12.5 μg/kg).

Decrease in zearalenone content can be attributed to reaction with barley components, metabolism of barley enzymes, or influence of other barley micropopulation (Dupire, 2003; Schwarz et al., 1995).

Zearalenone content increased during kilning of both samples (62.9 μg/kg dry matter in 1, and 71.2 μg/kg dry matter in 2) and was higher than in green malt. Schwarz et al. (1995) obtained similar results during kilning. After kilning, zearalenone content increased most probably because of unfavourable conditions for fungal growth. Under unfavourable growth conditions *Fusarium* species synthesize zearalenone (Smith and Moss, 1985).

After degermination, ZEN content decreased as it was party removed with the germ. In the malt, zearalenone content was 35.7 μg/kg dry matter in sample 1 and 17.8 μg/kg dry matter in sample 2.

The obtained results indicate that during micro-malting zearalenone content increased. Zearalenone content determined in finished malt was higher than in barley. Beer produced from this malt can be potentially harmful for human health.

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САДРЖАЈ ЗЕАРАЛЕНОНА ТОКОМ МИКРОСЛАДОВАЊА ЈЕЧМА

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Резиме

Зеараленон (син. Т-2 токсин) је нестероидни микотоксин, са ниском акутном токсичношћу и израженим естрогеним и анаболичким ефектима на људе и животиње. Као секундарни метаболит *Fusarium* врста најчешћи је природни контаминент житарица (кукуруз, пшеница, јечам, овас). Процес сладовања, којим се производи слад, који је основна сировина за производњу пива, погодује развоју *Fusarium* врста, па је стога циљ рада био да се одреди садржај зеараленона и учесталост *Fusarium* врста током микросладовања јечма.

За испитивање су одабрана два узорка озимог дворедог јечма, са локалитета Крагујевац. Одређивање садржаја зеараленона вршено је у јечму непосредно пре почетка микросладовања, након močења (намочени јечам), након клијања (зелени слад), након сушења (суви слад) и након отклицавања (слад). Изоловање и идентификација *Fusarium* врста су такође вршена у наведеним фазама микросладовања.

У узорцима јечма пре почетка микросладовања садржај зеараленона је био 9,7 μg/kg и 9,2 μg/kg. Синтеза зеараленона и састав *Fusarium* врста су варирали током микросладовања јечма. У зависности од фазе микросладовања изоловано је и идентификовано различито присуство *F. avenaceum, F. culmorum, F. sporotrichioides, F. tricinctum, F. verticilioides* и *F. poae*. И у једном и у другом узорку измерен је повећан садржај зеараленона након močења (86,5 μg/kg и 37,4 μg/kg), смањење након клијања (12,5 μg/kg и 26,8 μg/kg), повећање након сушења (62,9 μg/kg и 71,2 μg/kg) и смањење након отклицавања (35,7 μg/kg и 17,8 μg/kg). Промене садржаја зеараленона током микросладовања јечма може се приписати метаболизму ензима јечма, реакцији микотоксина са састојцима зрна као и одстрањивањем дела микотоксина са сладном клицом.

Добијени резултати показују да се поступком микросладовања садржај зеараленона повећао у односу на почетни узорак јечма, тако да пиво добијено од оваквог слада може представљати потенцијални ризик по здравље људи.

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OPTIMIZATION OF LABORATORY CONDITIONS FOR BYOSINTHESIS OF TYPE A TRICHOTHECENES

ABSTRACT: Type A trichothecenes, T-2 toxin and diacetoxyscirpenol — DAS, belong to one of the most toxic groups of fusariotoxins. Although larger quantities of them can be found more often in cooler parts of Europe, regarding their methabolic characteristics and the types of illnesses they provoke, it is obvious that even smaller quantities of these toxins can cause serious health disturbances of humans and animals in climatic conditions of Serbia.

Having in mind the importance of these substances, the aim of this study was to carry out the optimization of laboratory conditions under which screening of *Fusarium* spp. isolates from Serbia, regarding T-2 toxin and DAS production, should be done.

Four cultures of *Fusarium sporotrichioides*, originating from different regions throughout the world, were under present investigation: ITM-391 (Italy), KF-38/1 (Poland), M-1-1 (Japan) and R-2301 (Germany). According to the previous literature data, all of these isolates were T-2 toxin producers, and some of them were also DAS producers. The influence of medium composition (different C and N atoms sources, microelements etc.), as well as aeration (in liquid media), on byosinthesis process of these mycotoxins, *in vitro* conditions was investigated. In the case of most *Fusarium sporotrichioides* isolates, highest yields of T-2 toxin and DAS were achieved under the conditions of more intense aeration, and with the use of glucose (5 or 20%) as a C atom source. Fermentation in semi-synthetic liquid medium, using a rotary sheaker, was more suitable for screening the toxicity of the fungal isolates in pure culture because of: shorter period of incubation, more simpler sample preparation, obtaining less interfering materials in crude toxin extracts, and possibility for more precise definition of factors influencing the yield of trichothecenes.

KEY WORDS: biosynthesis, DAS, *Fusarium sporotrichioides*, T-2 toxin

INTRODUCTION

Representatives of fungal genus *Fusarium* are primarily plant pathogens, and occur mostly in association with plants and cultivated soils. Unlike most *Aspergillus* and *Penicillium* species, fusaria grow on crops before harvest, and only at high water activity (aw) levels in the substrate. Therefore, their toxic metabolites are produced before, or immediately after the harvest. Kimura et al. (2001) demonstrated that there is a causal role of fusaria mycotoxins in plant disease development. In case of some host-pathogen interactions, it was proved, by the use of contemporary molecular genetic techniques, that fusariotoxins are highly associated with disease initiation or enhanced virulence.

Several *Fusarium* species present on cereals worldwide, causing "head blight" (scrab) of small grain cereals, or "ear rot" of corn, are capable to accumulate, in infested kernels, few mycotoxins, some of which have the relevant impact on human and animal health. One of the main groups of *Fusarium* toxins are trichothecenes, with T-2 toxin, HT-2 toxin, neosolaniol, fusarenon-X etc. (Marasas et al., 1984). The primary mechanism of action of these strongly toxic sesquiterpenes, with tricyclic nucleus and an epoxide at C-12 and C-13, is the inhibition of protein synthesis at the level of a ribosome. 60S-ribosomal protein L3 is the major site of this inhibitory activity (Desjardins, 2003). The main simptoms of illnesses caused by these compounds, are vomiting, inflammation, diarrhoea, cellular damage of the bone marrow, thymus, spleen and mucous membrane of the intestines, and depression of circulating white blood cells (Marasas et al., 1984).

The presence of type A trichothecenes (T-2 toxin and diacetoxyscirpenole — DAS) in central and northeastern Europe is conecteded with *Fusarium* species *F. poae* and *F. sporotrichioides* (Bottalico, 1998). In mid-70s of the last decade proved that these fungi and their trichotecenes were associated with the death of at least 100,000 Russian people in the period 1942—1948. This exceptionally unpleasant disease, now called Alimentary Toxic Aleukia (ATA), is characterized by fever, haemorraghic rash, necrotic angina, extreme leucopenia, agranulocytosis, sepsis and exhaustion of bone marrow (Joffe, 1978). These symptoms resemble more closely those of radiation sickness, than bacterial or fungal toxicoses.

Besides T-2 toxin, *F. sporotrichioides* can biosynthesize other mycotoxins too, of which the most important are DAS, butenolide, fusarenon-X, neosolaniol and nivalenol primarily (Marasas et al., 1984). Although DAS is less toxic than T-2 toxin, in general it shows similar effects on animals and humans. Fortunately, *F. sporotrichioides* is not so commonly occurring species. It can be found mainly in temperate regions with cereals as main crops, although it has been also isolated from peanuts and soybean (Pitt and Hocking, 1985).

The production of trichothecenes proceeds from farnesyl pyrophosphate via hydrocarbon trichodiene. A sequence of oxygenations, isomerisations, cyclizations, and esterifications leads from trichodiene to more complex trichothecenes such as T-2 toxin, DON and NIV. Until now, eleven genes (*TRI3* — *TRI13*) coding trichothecene biosynthesis have been located, and their function

has been established by the target gene disruption in *F. graminearum* and *F. sporotrichioides* (D e s j a r d i n s, 2003).

Y u (2001) tried to give the answer on the following questions: how biosynthesis of various mycotoxins is controlled, and whether there are global controlling mechanisms for both sporulation and mycotoxin production. Investigations of this author revealed the existence of correlation with the pivotal role of signal transduction in cellular regulatory, communicatory, and responsive process, that led him to the hypothesis that heterometric G-protein signalling components and RGS proteins are upstream determinants in controlling processes of growth and development of *F. sporotrichioides.* From three *Fusarium* species (*F. graminearum, F. sporotrichioides* and *F. verticillioides*) important RGS protein was identified, as well as EST sequences that identify 9 out of 15 target signaling components in *F. sporotrichioides.*

Having in mind the importance of type A trichothecenes and toxin-producing *Fusarium* species, the aim of this study was to carry out the optimization of laboratory conditions for toxin biosynthesis of type A trichochecenes (T-2 toxin and DAS) in pure culture, in order to obtain faster and simpler procedure for screening of toxigenic potential of *Fusarium* spp. isolates.

MATERIAL AND METHODS

Microorganisms. *F. sporotrichioides* isolates, known as T-2 toxin producers, and some of them as DAS producers too, were under present investigation: 1) ITM-391, leg. dr A. Bottalico, Consiglio Nazionale delle Richerce, Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy; 2) KF-38/1 from barley, leg. dr Y. Chelkowski, Department of Plant Pathology, The Agricultural Faculty, Warsaw, Poland; 3) M-1-1 from soybeans, leg. dr Y. Ueno, Faculty of Pharmaceutical Sciences, Tokyo, Japan and 4) R-2301, leg. dr D. Latus, Germany (B o č a r o v - S t a n č i ć and M u n t a ñ o l a - C v e t k o v i ć, 1988; N a g a y a m a et al., 1988; M a š i ć et al., 1997). Stock cultures of the fungi were maintained on potato-dextrose agar at 4—6°C.

Inoculation of fermentation media was performed with 5 pieces (5 x 5 mm) of fungal material originated from Petri dish, sowed with tested culture, and subcultivated for 7 days on potato-dextrose agar (PDA) at 27°C.

Cultivation conditions. 1) Inoculated Erlenmayer flasks (500 ml), containing 100 or 250 ml of different semisynthetic liquid media, were cultivated on rotary sheaker (150 or 180 rpm) at room temperature (21—26°C); 2) inoculated Roux bottles, containing 50 g of wet sterilized corn kernels (initial moisture 58.4%), were cultivated at 30°C for 4 weaks. All cultivations were performed in duplicate.

Liquid fermentation media: 1) ***GPY*** (5% of glucose + 0.1% of peptone + 0.1% of yeast extract) pH 5,8; 2) ***GPY^{Zn}*** (5% of glucose + 0.1% of peptone + 0.1% of yeast extract + 0.0009% of $ZnSO_4$ x 7 H_2O) pH 5,9; 3) ***SPY*** (5% of saccharose + 0.1% of peptone + 0.1% of yeast extract) pH 6,5 and 4) ***GPYM*** (20% of glucose + 0.4% of peptone + 0.1% of yeast extract + 0.05% of

K_2HPO_4 x $3H_2O$ + 0.025% of $MgSO_4$ x 7 H_2O + 0.025% of KCl + 0.0009% of $ZnSO_4$ x 7 H_2O) pH 5,8.

Analysis of fusariotoxins: 1) After cultivation on rotary sheaker, liquid cultures were filtered. Crude extracts of type A trichothecenes (DAS i T-2 toxin) were obtained by the use of ethyl acetate. Further purification was done by the method of Romer et al. (1978), while thin layer chromatography was performed according to Pepeljnjak and Babić (1991). 2) The samples obtained after cultivation on corn grain, were dried during 24 h or more, at 60°C, until constant weight was obtained. After pulverization of dried samples, type A trichothecenes were extracted and purified from them, by the method of Romer et al. (1978). Thin layer chromatography was done in the same way as previously.

RESULTS AND DISCUSSION

During present investigations in liquid semi synthetic media, the influence of aeration, C atom source and concentration, as well as mineral supplements on *F. sporotrichioides* toxigenicity *in vitro* conditions was investigated (Table 1). In the case of temperature conditions, cultivation of fusaria was not performed when temperature stress conditions, according to Joffe (1983), but at relatively constant room temperature (21—26°C), having in mind the observation of Richardson et al. (1985), and the one of Rabie et al. (1986), what in warmer parts of the world, such as North Carolina or South Africa, toxigenic strains of *Fusarium* spp., associated with fusatiotoxicoses of domestic animals, can be found.

Toxigenic potential of *F. sporotrichioides* isolates. Having in mind the results of our previous investigations (Bočarov-Stančić et al., 1986; Muntañola-Cvetković et al., 1991), which proved that *F. sporotrichioides* cultures originated from Serbian cereals had toxigenic potential, this species was chosen for optimisation of laboratory conditions for type A trichothecene production.

All tested *F. sporotrichioides* cultures biosynthesized T-2 toxin independently of cultivation conditions, although the best results were achieved in liquid glucose media (***GPYM*** and ***GPY***). The best producer of this type A trichothecene was ITM-391 isolate, in which yields ranging from 0.16—120.0 mg/l were recorded (Table 1). Lower concentrations of T-2 toxin obtained in the present investigation from strain: KF-38/1, M-1-1 and R-2301, than those obtained in the investigation of Mašić et al. (1997), can be explained by the fact that under laboratory conditions, important deteriation of toxigenic potential can be observed regardless of their preservation conditions (Bočarov-Stančić et al., 1989). Although in some cases, as proved by Joffe and Yagen (1977), isolates can even after 30 years of cultivation on artificial media, retain the original high biosynthetic capacity, if isolations of strains were done in other ecological conditions.

DAS was recorded in 75% of tested isolates, but exclusively in higher aeration conditions (180 rpm) in liquid media with ***GPYM*** and/or ***GPY***. The best

producer of this fusariotoxin was the isolate from Polish barley KF-38/1, which biosynthesized from 1.6—12.0 mg/l. During the cultivation of the same culture on moist, sterile natural substrate (Table 1), not only that duration of the cultivation was much longer (28 days in comparison to 3 days for submerged cultivation), but also purification procedure was more complicated, because of the presence of more interfering substances. Besides that, much lower yields of T-2 toxin and DAS were obtained (Table 1). Although other authors, such as Pereira and Kemmelmeier (2000), use similar cultivation conditions for investigation of toxigenicity of fusaria (natural substrate, 21 days at 25°C), better results can be achieved in liquid media because of more precise definition of the factors influencing trichothecene production, and obtaining cleaner extracts, in which simpler purification is necessary, so that loss of toxin is less outstanding. The results of the present investigation confirm our previous conclusions (Bočarov-Stančić and Muntañola-Cvetković, 1988).

The obtained results point out the weak toxigenic potential for DAS production, but not inadequate cultivation conditions in liquid semi-synthetic media, because high yields of the same fusariotoxin (64.0 mg/l) can be achieved by other fusaria, such as *F. semitectum* under the similar cultivation conditions (Bočarov-Stančić et al., 2005).

Aeration. During submerged cultivation on rotary sheaker with higher aeration (180 rpm), the highest yields of type A trichothecenes were achieved in ***GPY*** enriched with mineral supplements (***GPYM***), and with smaller volume of liquid medium in cultivation flasks (100/500 ml). According to data presented in Table 1. yields of T-2 toxin ranged from 12.0 to 120.0 mg/l, and DAS from quantities below detection limits up to 12.0 mg/l, respectively. Under the same conditions, a little bit weaker, but rather high yields were of type A trichothecenes, obtained in ***GPY*** (250/500 ml): T-2 toxin from 8.0 to 120.0 mg/l, and DAS from quantities below detection limits up to 8.0 mg/l. Although the same concentration of microelement Zn was present in ***GPYM*** and ***GPY^{Zn}***, poorer results were obtained: T-2 toxin from 0.08—0.16 mg/l (values near detection limits of applied TLC method), while DAS was not detected under the same conditions (Table 1).

In the case of submerged cultivation with lower aeration (150 rpm), the best results were achieved in ***GPY*** medium (250/500 ml). Although DAS was not detected in this case, the yields of T-2 toxin ranged from 8.0 to 64.0 mg/l. Data shown in Table 1 point out that higher aeration had positive effect on type A trichothecene biosynthesis, although the influence of media composition could not be neglected.

C atom source. The best results were achieved in ***GPYM*** medium (T-2 toxin from 12.0—120.0 mg/l, and DAS max. 12.0 mg/l, respectively). In the case of the same media composition, except that different sugars were used as C atom sources, the obtained results are presented in Table 1. ***GPY*** medium resulted in higher yields of T-2, in 75% of tested samples, and DAS in 100% of the samples during cultivation under higher aeration conditions (180 rpm). In the case of ***SPY*** medium, concentration of DAS was below detection limit,

Tab. 1. — Yield of type A trichothecenes (DAS, T-2 toxin) under different cultivation conditions of *F. sporotrichioides*

Isolate design.	Cult. condit. / Fusariot.	Fusariotoxin yield (mg/l or mg/kg)									
		250/500 ml						100/500 ml			
		GPY		*GPY^{Zn}*	*SPY*		*GPYM*	*GPY*	*SPY*	Corn grain	RANGE (mg/l or mg/kg)
		180 rpm	***150 rpm***	**180 rpm**	**180 rpm**	***150 rpm***	**180 rpm**	**180 rpm**	**180 rpm**		
ITM-391	DAS	3.2	*n.d.*	n.d.	n.d.	*n.d.*	4.0	—	—	—	**n.d. — 4.0**
	T-2	120.0	*64.0*	0.16	48.0	*64.0*	120.0	—	—	—	**0.16—120.0**
KF-38/1	DAS	8.0	*n.d.*	n.d.	n.d.	*n.d.*	12.0	0.24	n.d.	1.6	**n.d. — 12.0**
	T-2	64.0	*32.0*	0.08	2.4	*0.32*	20.0	4.0	0.16	2.4	**0.08—64.0**
M-1-1	DAS	n.d.	*n.d.*	n.d.	n.d.	*n.d.*	12.0	—	—	—	**n.d. — 12.0**
	T-2	8.0	*12.8*	0.08	16.0	*0.8*	80.0	—	—	—	**0.8—80.0**
R-2301	DAS	n.d.	*n.d.*	n.d.	n.d.	*n.d.*	n.d.	—	—	—	**n.d.**
	T-2	24.0	*8.0*	0.16	1.6	*1.92*	100.0	—	—	—	**0.16—100.0**
RANGE (mg/l or mg/kg)	**DAS**	**n.d. — 8.0**	***n.d.***	**n.d.**	**n.d.**	***n.d.***	**n.d. — 12.0**	—	—	—	
	T-2	**8.0—120**	***8.0—64.0***	**0.08—0.16**	**1.6—48.0**	***0.32—64.0***	**12.0—120**	—	—	—	

Legend: n.d. — not detected (< 0.08 mg/l or mg/kg)

while in the case of T-2 toxin, 50% of samples, tested under conditions of lower aeration (150 rpm), yielded higher quantities of the same mycotoxin (0.32—64.0 mg/l).

Results presented in Table 1 show that in the case of same applied concentration of C atom source, glucose had more positive effect on type A trichothecene biosynthesis than succrose, although other authors (Ueno et al., 1975) did not observe the essential influence of sugar type on T-2 toxin and fusarenon-X production.

N atom source and mineral supplements. On the basis of literature data it can be hardly concluded how large is the influence of N atom source, as well as mineral and vitamin supplements on *Fusarium* fermentation process under laboratory conditions. According to Cullen et al. (1982) highest yields of T-2 toxin can be achieved during cultivation in Vogels' mineral medium with 5% of glucose after 12—14 days at 15°C. Contrary to this finding, Muntañola-Cvetković et al. (1991) did not observe the positive influence of microelements and vitamin supplements on T-2 toxin yield, according to Vogel (1956). We had similar doubts about the influence of these paramethers while analyzing present results (Table 1). In the case of addition of Zn (0.00009%) to basic ***GPY*** medium, in order to accelerate the sporulation, as well as DAS and T-2 toxin biosynthesis, the outstanding decrease of toxin yields was observed. Contrary to that observation, in ***GPYM*** medium (same Zn concentration, but higher content of peptone — 0.04%, and sugar — 20.0%, as well as presence of different mineral supplements) the best toxigenic results were obtained (Table 1).

CONCLUSIONS

All tested cultures of *F. sporotrichoides* were better T-2 toxin producers (max. 120.0 mg/l) than DAS producers (max. 12.0 mg/l).

Highest yields of both type A trichothecenes were obtained under higher aeration conditions (180 rpm), as well as during the fermentation of smaller media volume in cultivation flask (100/500 ml in comparison to 250/500 ml).

Glucose, in quantities ranging from 20% (***GPYM***) under 5% (***GPY***) in given cultivation conditions i.e. independently of applied aeration, influenced more favorably T-2 toxin, and DAS production than succrose did.

The addition of microelement Zn in standard glucose medium (***GPY***), with the aim to obtain better sporulation and biosynthesis of fusariotoxins, did not give the expected results. In the last case, very low quantities of T-2 toxin were detected, near the detection limits of applied TLC method.

It was demonstrated that submerged cultivation in nutrient media with glucose can be the most suitable, cheapest and most rapid method for large scale screening of *Fusarium* isolates regarding the trichothecene production.

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ОПТИМИЗАЦИЈА УСЛОВА ЗА БИОСИНТЕЗУ ТРИХОТЕЦЕНА ТИПА А У ЛАБОРАТОРИЈСКИМ УСЛОВИМА

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Резиме

Трихотецени групе А, Т-2 токсин и диацетоксисцирпенол — ДАС, представљају једну од најтоксичнијих група фузариотоксина. Они се у већим концентрацијама чешће могу наћи у хладнијим регионима Европе, али, у складу са њиховим карактеристикама и врстама обољења која изазивају, јасно је да и њихове мање количине могу довести до озбиљних здравствених поремећаја код људи и животиња у климатским условима Србије.

С обзиром на значај ових једињења, циљ овог истраживања је био да се изврши оптимизација лабораторијских услова у којима би се испитивала способност за биосинтезу Т-2 токсина и ДАС-а код *Fusarium* изолата из Србије.

Истраживањем су биле обухваћене 4 културе *F. sporotrichioides* пореклом из различитих земаља света: ИТМ-391 (Италија), КФ-38/1 (Пољска), М-1-1 (Јапан) и Р-2301 (Немачка), за које је претходно описано у литератури да су продуценти Т-2 токсина, а неке и ДАС-а. Испитан је утицај састава подлоге (различити извори атома угљеника и азота, микроелементи и сл.) као и аерације (у случају течних подлога) на процес биосинтезе ових микотоксина *in vitro* условима. Код већине изолата највећи приноси Т-2 токсина и ДАС-а су добијени у условима веће аерације и при употреби глукозе (5 или 20%) као извора угљениковог атома. Ферментација у течној подлози се показала као погоднија метода за тестирање токсигености гљивичних изолата од природног стерилног супстрата, због краћег периода култивације, добијања сирових екстраката токсина са мање пратећих материја, као и могућности прецизнијег дефинисања фактора који утичу на принос трихотецена.

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BASIC MECHANISMS OF THE CELLULAR ALTERATIONS IN T-2 TOXIN POISONING: INFLUENCE ON THE CHOICE AND RESULT OF THE THERAPY

ABSTRACT: T-2 mycotoxin, secondary metabolite of *Fusarium* fungi, is one of the most potent cytotoxic representatives of trichothecene mycotoxin type A. After ingestion, T-2 toxin affects actively dividing cells and irreversible post-mitotic cells. In our experiments, the best protective effects were produced by dexametasone (PI = 3.37) and different methylprednisolone formulations (PI = 2.43—2.64). Significant protective efficacy was shown by nimesulide (PI = 1.44) and N-acethyilcistein (PI = 1.29), but their values were higher in a combination with methylprednisolone (PI = 2.16—2.34). Radioprotector amifostine (WR-2721) expressed good protective effects (PI = 1.26) or/and different absorbent formulations, such as: activated charcoal (PI = 1.13) and variuos Min-a-zel® powder compounds, which are a well known zeolite clinoptilolite absorbents. Among the five zeolite regimens investigated, only Min-a-zel Plus® showed a significant protective effect (PI = 1.77). In summary, the steroidal anti-inflammatory drugs could be recommended as a regimen of choice for treatment of acute T-2 toxicosis, while nonsteroidal anti-inflammatory compounds, different absorbent formulations and their combinations with antioxidants or radioprotectors, could be important for the treatment of subacute and chronic T-2 toxin poisonings.

KEY WORDS: T-2 toxin, citotoxicity, inflamation, therapy

INTRODUCTION

T-2 toxin, as one of the most potent cytotoxic trichothecene mycotoxin type A, produces sometimes fatal toxic reactions in humans and animals (Pang et al., 1988; Anonymous, 2003; Josephs, 2004).

Acute high dose of T-2 toxin has a potentially inhibitory effect on the membrane structure, which consequently stimulates lipid peroxidation. T-2 toxin affects actively dividing cells and irreversible post-mitotic cells (Grizzle et al., 2004; Pestka 2004), interacts with a number of targets, including the ribosome and the mitochondria (Speijers and Speijers, 2004), and inhibits protein synthesis (Ueno, 1984) in all eukaryotic cells (Larsen et al., 2004). Pro-inflamatory actions of the T-2 toxin are probably the most important mechanisms of its acute toxicity, especially in permanent cells (Bondy and Pestka, 2000). They showed that trichothecene-mediated elevations in cytokines, chemokines, and other immune related proteins are preceded by up-regulation of mRNAs for these genes. The underlying mechanism for such effects is induction of COX-2 (Islam et al., 2002; Pestka et al., 2004). Also, in the heart tissue, T-2 toxin activates a large number of mast cells (Jaćević et al., 2001b; Jaćević, 2005; Jaćević et al., 2006), synthesis and degranulation of numerous mediators which play an important role in the pathogenesis of T-2 mycotoxicosis (Bondy and Pestka, 2000).

The aim of this study was to investigate the protective effects of different antidotes and their combinations on 24 hour survival and pathohistological changes in the gut and heart of the rats acutely poisoned with 1.0 LD_{50} T-2 toxin (T2).

MATERIAL AND METHODS

The experiment was performed on adult Wistar rats, weighing 180—220 g (Animal House, Military Medical Academy, Belgrade, Serbia). The animals were housed in plastic cages, under standard laboratory conditions (21°C, 12/24 hour light/dark cycle, commercial food and tap water *ad libitum*), before being randomized into experimental groups. One day before the experiment, animals were fasting. During the subsequent experiment, they were fed with standard laboratory food *ad libitum*. They were allowed access to fresh tap water *ad libitum*.

In order to obtain the optimal doses of dexamethasone (DM), methylprednisolone (Lemod-solu®, LS; Lemod-depo®, LD; and their combination, LS + LD), nimesulide (NM), N-acetylcysteine (Fluimucil®; NAC), Amifostine (WR-2721, AMI), Activated charcoal (AC) and different Min-a-zel® formulations (M, MP, MD, M32 and M+), a range of their doses was previously tested (Jovanović, 1992; Jaćević et al., 2001a; Jaćević et al., 2002; Jaćević et al., 2003; Jaćević et al., 2006) (Table 1).

Tab. 1 — Effects of various methylprednisolone regimens on 24-hour survival in rats poisoned with T-2 toxin

Regimens	T-2 toxin LD_{50} (mg/kg *sc*)	95% confidence limits	$f(LD_{50})$	Protective index (PI)
DM	1.65	1.35—1.80	1.67	3.37
LS	0.44	0.35—0.55	1.25	2.43
LD	0.48	0.36—0.63	1.32	2.64
LS + LD	0.45	0.30—0.45	1.48	2.48
NM	1.53	1.39—1.69	1.10	1.44
NAC	1.22	1.19—1.27	1.19	1.29
LS + NM	1.55	0.47—0.30	1.15	2.34
LS + NAC	0.76	0.51—1.12	1.48	2.16
NM + NAC	1.24	1.57—2.22	1.15	1.22
AMI	1.95	1.56—2.42	1.25	1.26
AC	1.31	0.95—1.74	1.27	1.13
M	1.31	1.05—1.33	1.37	1.17
MP	1.21	0.95—1.53	1.27	1.33
MD	0.95	0.75—1.19	1.26	1.04
M32	0.67	0.49—0.93	1.38	0.74
M+	1.61	1.42—1.81	1.13	1.77

The rats were randomly allocated to 18 groups, each of them consisting of 10 animals. Their treatments were:

(1) The control group, (2) T2, (3) T2 + DM, (4) T2 + LS, (5) T-2 + LD, (6) T-2 + LS + LD, (7) T2 + NM, (8) T2 + NAC, (9) T2 + LS + NM, (10) T2 + LS + NAC, (11) T2 + NM + NAC, (12) T2 + AMI, (13) T2 + AC, (14) T2 + M, (15) T2 + MP, (16) T2 + MD, (17) T2 + M32 and (18) T2 + M+.

Following the registration, 24 hour survival and pathohistological changes were monitored after 28 days. General health condition of the animals was monitored daily, throughout the whole experimental period (four weeks).

Study protocol was based on the Guidelines for Animal Study no. 282--12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia).

T-2 toxin (T2) used in these experiments was produced under the laboratory conditions from *Fusarium sporotrichoides* fungi, cultivated on synthetic GPY medium (glucose 5%, peptone 0.1%, yeast extract 0.1%, pH 5.4). Extraction and crude purification of the toxin were performed by filtration, while definite purification and determination of T-2 toxin content were performed by gas chromatography with electron capture detection (GC-ECD) (R o m e r, 1987). T-2 toxin was preliminarily tested an animals in order to obtain its LD_{50} value (L i t c h f i e l d and W i l c o x o n, 1949; J a ć e v i ć et al., 2001a). It was thereafter used in the current experiment as a single dose of 0.23 mg/kg sc (1 LD_{50}).

Dexamethasone was used in a single dose of 20 mg/kg *sc*. Commercially available formulation of methylprednisolone, Lemod-solu® or/and Lemod-depo®, was used in a single dose of 40 mg/kg *sc*. A dose of 30 mg/kg of nimesulide was dissolved in 1 ml of 0.9% NaCl before *im* application. Fluimucil® contains 200 mg of N-acetylcysteine in 1 ml. It was a single injected *im* dose in these experiments. Activated charcoal in a dose of 1 g/kg was dissolved in

1 ml of 0.9% NaCl before *po* application. Amifostine (WR-2721) was synthesized of the Chemical Department of Military Technical Institute, Belgrade. Amifostine, in a dose of 50 mg/kg, was also dissolved in 1 ml of 0.9% NaCl immediately before *im* use. Five different commercially available formulations of Min-a-zel® absorbents were used in these experiments. These absorbents were dissolved in 1 ml of 0.9% NaCl before *po* administration.

Animals were sacrificed, after 28 ended treatment days. The gut and heart were excised and their samples were fixed in 10% neutral formalin for 5 days. Transmural tissue samples were dehydrated in graded alcohol, xylol and embedded in paraffin blocks. Finally, 2-μm of thick paraffin sections were stained by haematoxylin and eosin (H & E) method and analyzed (Olympus-2 microscope).

RESULTS AND DISSCUSION

Registration of 24 hour survival rates revealed that all the regimens significantly antagonized the lethal effects of T-2 toxin. Based on the results shown in Table 1, it could be seen that the highest protective index was obtained with DM and LD, respectively.

During the 4 week period, in the gastrointestinal tract, T-2 toxin caused diffuse epithelium deficit, erosions, ulcerations, hyperemia, transmural edema, atrophy of intestinal villi, cystic deformation of the stomach, and small intestine glands with diffuse accumulation of polymorphonuclear cells (Figure 1).

Myocardial alterations detected in the poisoned animals ranged from degeneration to diffuse necrosis of all the myocardiocytes and included massive vascular changes, too. Such areas were most prominent in the inner part of the

Fig. 1. — The small intestine of rats treated by T-2 toxin, 28 day (HE, 20x)

myocardium, and in all layers of endocardium. The most striking finding was the presence of haemorrhagic foci in the interstitium that separates the bundles and fibres of myocardium. This haemorrhage appeared uniformly in each of the examined sections, and was located in the middle of myocardial or subendocardial areas (Figure 2).

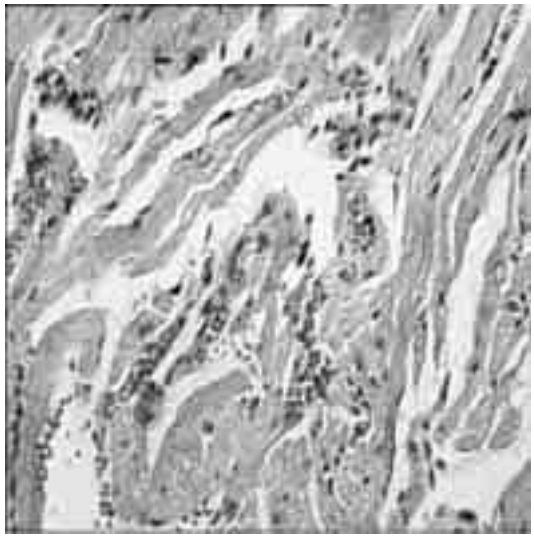

Fig. 2. — Miocardium of rats treated by T-2 toxin, 28 day (HE, 20x)

The histological changes observed from the gut section of these animals varied from intracellular edema to focal necrosis of the epithelial cells and mild hemorrhagic infiltration. These areas were present in the focal part of *the tunica mucosa* and some layers of *the tunica submucosa*. Dissolution and granularity of cytoplasm were observed in 50 percent of the stomach and small intestine. The presence of polymorphonuclear cell infiltration, diffuse hyperemia and hemorrhagic foci were more prominent in the poisoned group treated with LD, LS + LD, NM, NAC, AMI, AC, MD and M32. In the group of poisoned animals protected with LS, LS + NM or NAC, AMI + AC and M+, described histological changes were the smallest. After the 4 week period, the guts of rats treated with combination of LS and NM, or AMI + AC, and especially LS and M+ alone (Figure 3) had histological structure similar to the those of the control group.

The quality of pathohistological changes in this experimental group was similar to that observed in the poisoned rats, protected with solu form of methylprednisolone. However, the intensity of degeneration and vascular infiltration was stronger in NM, NAC, AMI, AC, MD and M32 groups. The presence of mononuclear cell infiltration, diffuse hyperaemia and haemorrhagic foci was more prominent in the inner part of the myocardium and all layers of endocardium. Single injection of LS in poisoned rats showed significant cardi-

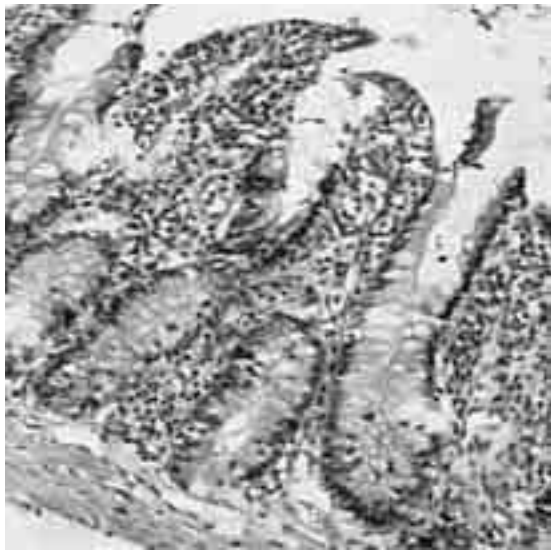

Fig. 3. — The small intestine of rats treated by T-2 toxin and M+, 28 day (HE, 20x)

oprotective efficiacy, in comparison with animals that received T-2 toxin only (Figure 4). During the whole experimental period, these values remained significantly higher than those in the control animals. Cardioprotective efficiacy was registered in the other protected groups, but the values obtained were significantly less than in the control group and LS.

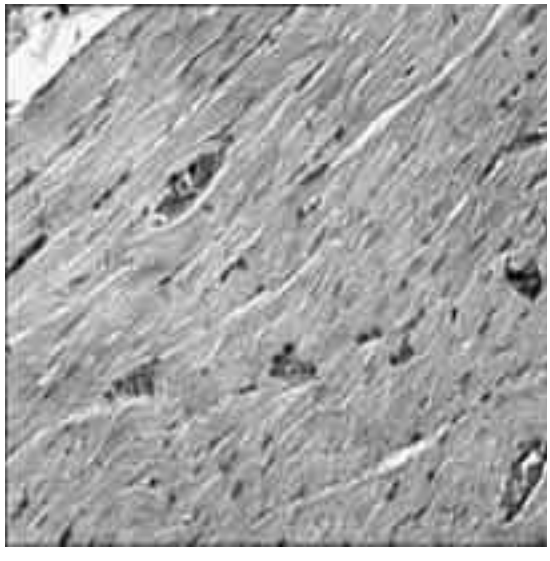

Fig. 4. — Myocardium of rats treated by T-2 toxin and LS, 28 day (HE, 20x)

CONCLUSION

In summary, the steroidal anti-inflammatory drugs could be recommended as a regimen of choice for treatment of acute T-2 toxicosis, while the nonsteroidal anti-inflammatory compounds, different absorbent formulations and their combinations with anti-oxidants or radioprotectors could be important for the treatment of subacute and chronic T-2 toxin poisonings.

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ОСНОВНИ МЕХАНИЗМИ ОШТЕЋЕЊА ЋЕЛИЈА КОД ТРОВАЊА Т-2 ТОКСИНОМ: УТИЦАЈ НА ИЗБОР И ИСХОД ТЕРАПИЈЕ

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Резиме

Т-2 микотоксин, секундарни метаболит гљивица из рода *Fusarium*, један је од најтоксичнијих представника трихотеценских микотоксина типа А. Његове основне особине, првенствено велика стабилност у природи, јефтина производња, тешка детекција и још увек непостојање адекватног антидота, чине га веома добрим потенцијалним бојним отровом. После уношења, у организму отроване јединке Т-2 токсин се у ћелијама везује за рецепторе на рибозомима и покреће серију каскадних реакција које за последицу имају смањење стабилности гРНК и повећану експресију проинфламаторних гена који су између осталог одговорни за настанак анорексије, губитак телесне масе, имуносупресију, аутоимуних ефеката и оштећење већине ткива. Токсично оштећење циљних органа, настало под дејством Т-2 токсина, последица је његовог цитотоксичног ефекта на лабилне ћелије и проинфламаторног ефекта на стабилне ћелије у организму животиња и

људи. С обзиром на напред изнете чињенице, јасно је што је у нашим истраживањима најбољи терапијски ефекат, код акутног тровања Т-2 токсином, постигнут применом антиинфламаторних лекова стероидне структуре, првенствено дексаметазона (ZI = 3,37) и различитих облика метилпреднизолона (ZI = 2,43—2,64). Осим тога, антиинфламаторни лекови нестероидне структуре испољили су значајан терапијски ефекат, нимесулид (ZI = 1,44) и N-acetlilcistein (ZI = 1,29), али се њихово заштитно дејство потенцира у комбинацији са метилпреднисолоном (ZI = 2,16—2,34). Терапијску ефикасност испољили су радиопротектор амифостин (WR-2721) (ZI = 1,26) и/или различити апсорбенси. Од примењених апсорбенаса, као што су активни угаљ (ZI = 1,13) и различити облици Мин-а-зел-а®, највећи протекивни ефекат испољио је Мин-а-зел Плус® облик клиноптиолинског зеолита (ZI = 1,77). На основу приказаних резултата, а у складу са чињеницом да је цитотоксично и проинфламаторно дејство Т-2 токсина у директној сразмери са његовом акутном токсичношћу, у потпуности је оправдано коришћење високих доза антиинфламаторних лекова стероидне структуре у терапији акутног тровања Т-2 токсином. Са друге стране, у терапији субакутних или хроничних тровања Т-2 токсином, препоручује се употреба антиинфламаторних лекова нестероидне структуре, различитих апсорбенаса, или њихове комбиноване примене са антиоксидансима или радиопротекторима.

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THE PRESENCE OF OCHRATOXIN, A RESIDUE IN BLOOD PLASMA OF SLAUGHTERED SWINE

ABSTRACT: The aim and task of this study was to determine the presence of ochratoxin A (OTA) residue in blood of swine, slaughtered regularly. The fact that ochratoxin A is heterogeneously distributed in a contaminated lot of feed material, makes the sampling problematic. It has been shown that an alternative method to monitor the presence of ochratoxin A in the feed is to analyse blood samples from swine, which reflect the toxin content of the ingested feed. With the aim of determining the presence of ochratoxin A residue in blood of swine slaughtered regularly, and originating from different areas of Vojvodina and Serbia, the samples were collected from the corresponding slaughter. During a three month investigation period, a total of 60 blood samples were analysed. Spectrofluorimetric method was applied for sample analysis. The presence of the OTA residue was found in 56,6% of the examined plasma samples. The average OTA concentration in plasma was 2.91 ± 4.91 ng/mL (0,0—33,3 ng/mL). The experiment showed that the average OTA concentration in plasma samples originating from different areas of Vojvodina and Serbia, was not significantly low ($p > 0,05$).

KEY WORDS: ochratoxin A, plasma, residue, swine

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several fungi of genera *Aspergillus* and *Penicillium*, *A. ochraceus* principally in tropical parts of the world (23) and *P. verrucosum* in temperate climates (19). OTA has been found predominantly in cereal and cereal products, but also in a variety of other food commodities (1, 22, 26, 28). OTA has been suggested to be a determinant of a disease known under the name of Balkan Endemic Nephrophaty (BEN). This mycotoxin has a special role in the genesis of swine mycotoxic nephropathy (12). Toxin production usually occurs during the storage. The distribute of OTA in the stored grains is very heterogeneous, making analysis and dietary

exposure assessment of animals difficult. However, exposure of animals may also be assessed from analysis of blood levels. The high concentration in a swine blood is due to a relatively long half-life of OTA in this tissue (3,7 ± 6 days) (13), as a consequence of the strong bindings between OTA and serum albumin (7). H u l t et al. (1979) demonstrated that the level of OTA in blood is very expressive of the general exposure of the individuals to this mycotoxin, and would be a useful tool in diagnosing ochratoxicosis.

It has also been demonstrated that OTA accumulates in blood and edible organs, especially kidneys. Therefore, pork products, especially those that include blood and kidney, are considered to be an important source of OTA in humans (5). Surveys of swine for OTA in blood and/or edible tissues have been carried out in several countries: Denmark (10), Germany (5, 15, 16, 17), Hungary (25), Norway (15). The purpose of this work was to monitor the presence of OTA in the blood of slaughtered swine, and to investigate its regional distribution.

MATERIALS AND METHODS

Reagents

OTA and Carboxypeptidase A enzyme were purchased from Sigma Chemical Co (St Louis MO, USA). Diluted standard solution of OTA was prepared from a stock solution (10^{-4} M OTA) in buffer, which was stored frozen. The concentration of the stock solution was determined spectrophotometrically at 380 nm, using a value of 5680 M^{-1} cm for an extinction coefficient. Carboxypeptidase A was prepared in 0,04 M tris (hydroxyl-methyl) aminomethane sulfuric acid buffer, pH 7,5, 1 M sodium chloride (100 U/mL). All other solvents and reagents were analytical grade.

Sample collection

Blood serum was collected from slaughtered swine (n-60) originating from three different areas of Vojvodina and Serbia, where a significant swine industry was, during a three month investigation period (april—june). Slaughtered swine were randomly sampled in the slaughterhouse. About 100 mL blood was sampled when slaughtered swine were bled by jugular puncture. Three sodium citrate (TCT) was used as an anticoagulant. Blood samples were centrifuged at 3000 g for 15 min. Serum was decanted and stored at –18°C prior to analysis. Spectrofluorimetric method was applied for sample analysis (9), with detection limit of 2 ng/mL of OTA and 78% recovery.

RESULTS AND DISSCUSION

The results obtained during a three month investigation period are presented in Table 1. The results of this study show that thirty four serum samples

(56,6%), out of 60, were found positive with toxin at levels ranging from 0,0 to 33,3 ng/mL. The average concentration was 2,91 ± 4,91 ng/mL. Of these positive samples, the highest incidence (73,3%), concentration (33,3 ng/mL), and mean level of OTA were found in the samples originating from Kovilj region (5,26 ± 8,22 ng/mL), while the lowest incidence (40%) and mean level of OTA residue were established in the samples originating from Šabac region (1,41 ± 1,86 ng/mL). The results of this study show that the mean level of OTA among the regions where samples were collected, are very similar, but the incidences of OTA are different (Figure 1 and 2). The highest incidence of ochratoxin A residue was established in the samples collected in june (80%), while the lowest incidence of ochratoxin A residue was established in the samples collected in may (20%). During the whole period of investigation, the average OTA differences between the samples originating from the studied regions were not significantly low ($p > 0,05$).

In comparison with other recently published data about the occurrence and concentration of OTA in blood serum, these results are similar to the studies carried out in other European countries (3, 4, 1, 15, 19, 21). This results indicate that pork products (meat, kidney, blood, and liver) are frequently contaminated with OTA (14, 27). Consequently, foods containing pork liver and blood or plasma, e.g. liver paste and processed meat products, could be important sources of OTA in humans (5, 8). However, when comparing data it should be remembered that factors such as climate conditions during harvest, practices for grain/feed storage, kinds of feed, etc. have influence on the ochratoxin A levels found in swine edible organs. The annual variation and regional differences were primarily due to the moisture content of the grain at the time of harvest. Seasonal variations were also observed after prolonged storage of the grain.

Tab. 1 — Occurrence of ochratoxin A residue ($\overline{X}$ ± Sd) in serum samples (ng/mL)

Region	n	Positives samples (n)	Positive samples (%)	$\overline{X}$ ± *Sd* ng/mL
Bačka Topola	15	10	66,6	2,17 ± 1,70 (0,0—5,2)
Kovilj	15	11	73,3	5,26 ± 8,22 (0,0—33,3)
Šabac	15	6	40	1,41 ± 1,86 (0,0—5,0)
Senta	15	7	46,6	2,66 ± 4,34 (0,0—16,0)
Total	60	34	56,6	2,91 ± 4,91 (0,0—33,3)

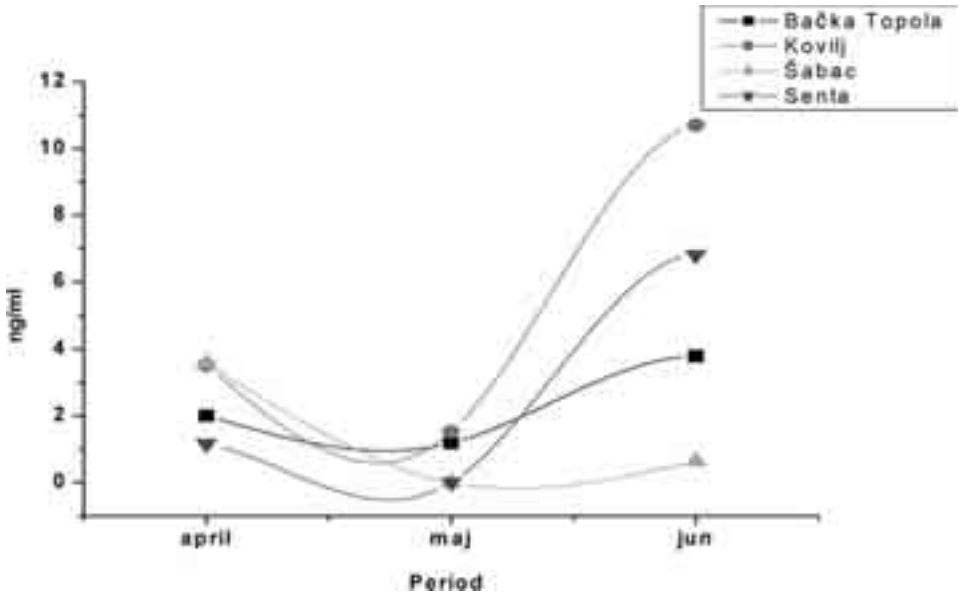

Fig. 1 — The average concentration of OTA residue during the investigation period

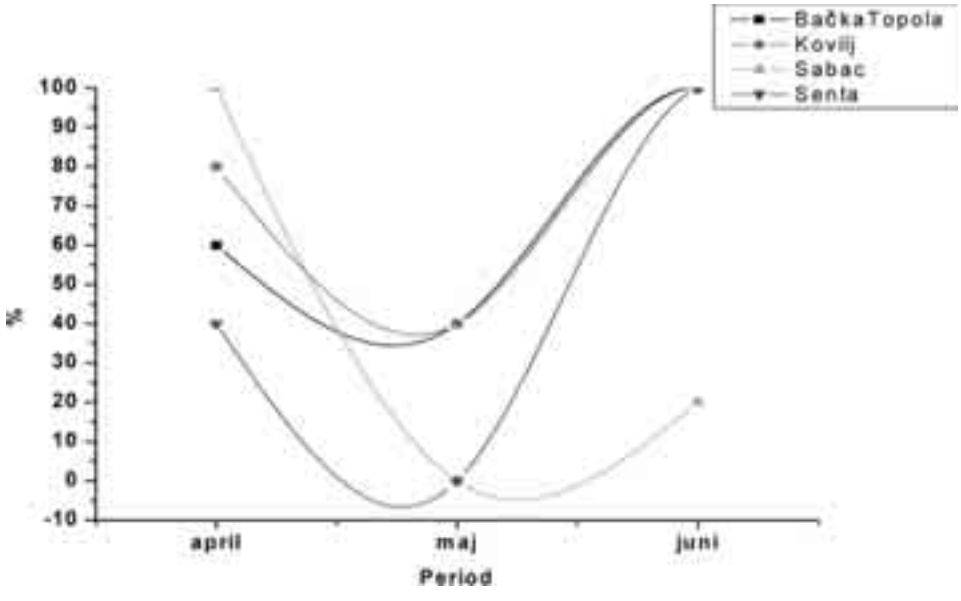

Fig. 2 — The average incidence of OTA residue during the investigation period

CONCLUSIONS

The results of this study show that a thirty four serum samples (56,6%), out of 60, were found contaminated with toxin at levels ranging from 0,0 to 33,3 ng/mL. The average concentration was 2,91 ± 4,91 ng/mL. Of these positive samples, the highest incidence (73,3%), concentration (33,3 ng/mL), and mean level of OTA residue were found in the samples originating from Kovilj region (5,26 ± 8,22 ng/mL), while the lowest incidence (40%) and mean level of OTA residue were established in the samples originating from Šabac region (1,41 ± 1,86 ng/mL). During the whole period of the investigation, the average OTA differences between samples originating from the studied regions were not significantly low ($p > 0,05$).

The results of this study demonstrate that the detected residue of OTA in blood serum of slaughtered swine, with incidence and mean level of OTA in blood plasma, is comparable to that from the other European countries, but a more extensive survey is advisable in order to obtain a more realistic overview. The distribution of OTA in stored grains is very heterogeneous, alternative method to monitor the presence of ochratoxin A in the feed is to analyse swine blood samples, which reflect the toxin content of the ingested feed. Also, assays of OTA in the blood can provide a level of OTA in other tissues, because blood concentrations are highly correlated with tissue levels. With regard to the national legislation on OTA in animal feed, maximum tolerable levels of OTA are established only for complete feedmixes, intended for swine and poultry, while for feed component they have not been proposed for established yet. In addition, it is necessary to harmonize the national legislation on sampling methods, and OTA limits in animal feed with EU regulations. In order to reduce the colonization of ohratoxigenic mold and toxin production in feed, there is a need for implementation of adequate control of moisture (a_w) and temperature, during transport and storage. Programs such as GAP, GMP, and GHP implemented a HACCP system which is a powerful tool for controling OTA in commodity system.

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ПРИСУСТВО РЕЗИДУА ОХРАТОКСИНА А У КРВНОЈ ПЛАЗМИ ЗАКЛАНИХ СВИЊА

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Резиме

У овом раду приказани су резултати испитивања присуства резидуа охратоксина А у крвној плазми, клинички здравих, редовно закланих, товних свиња. Дистрибуција охратоксина А (ОТА) у ускладиштеној храни је веома хетерогена, што доста отежава узорковање, а самим тим и правилну процену алиментарне изложености људи и животиња овом токсину. Један од алтернативних начина за праћење контаминације хране ОТА је анализа крви која доста поуздано рефлектује присуство ОТА у храни. За испитивање присуства резидуа ОТА у крвној плазми закланих свиња пореклом из Војводине и дела западне Србије, на линији клања током ветеринарско-санитарног прегледа закланих свиња, методом случајног узорковања узимани су узорци крви свиња. Током тромесечних испитивања укупно је анализирано 60 узорака крвне плазме.

Присуство резидуа ОТА је утврђено у 56,6% испитиваних узорака крвне плазме, док је просечан садржај ОТА у испитиваним узорцима крвне плазме био 2.91 ± 4.91 ng/mL (0,0—33,3 ng/mL). Највећа заступљеност (73,3%), просечан садржај (5,26 ± 8,22 ng/mL) и концентрација резидуа ОТА (33,3 ng/mL) забележени су у узорцима крвне плазме свиња пореклом са локалитета Ковиљ, док су нај-

мањи заступљеност (40%) и просечан садржај резидуа ОТА (1,41 ± 1,86 ng/mL) забележени у узорцима крвне плазме свиња пореклом са локалитета Шабац. Просечан садржај ОТА у узорцима крвне плазме са одговарајућих локалитета није се статистички значајно разликовао ($p > 0,05$). Анализа крви доста поуздано рефлектује присуство ОТА у храни и може се успешно користити као средство у дијагностици охратоксикоза, нарочито супклиничких које су најчешће забележене на нашим просторима. На бази познате концентрације ОТА у крви могуће је одредити садржај ОТА у осталим ткивима. Постојећи правилник о максималним количинама прописује максималне количине ОТА само за комплетне смеше и то за свиње и живину, док хранива нису обухваћена. Неопходно је да постојећа законска регулатива претрпи знатно веће промене и усклади се са одредбама ЕУ које стандардизују узимање узорака за анализу и садржај ОТА у храни за животиње. У циљу спречавања контаминације хране плеснима и ОТА неопходна је примена адекватних мера у контроли температуре и влаге (a_w) током транспорта и складиштења. Применом мера добре пољопривредне праксе (GAP), добре произвођачке праксе (GMP), добре хигијенске праксе (GHP), добре складишне праксе (GSP), имплементираних у систем HACCP представљају ефикасно средство у превенцији контаминације хране плеснима и њиховим токсичним продуктима

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GROWTH INHIBITION OF SOME *EUROTIUM* AND *ASPERGILLUS* SPECIES WITH SPICE EXTRACTS

ABSTRACT: The antifungal activity of spice extracts obtained from caraway seed, garlic and origanum was tested against antifungal activity of *Eurotium herbariorum*, *E. amstelodami*, *Aspergillus flavus* and *A. sydowii*. Caraway seed extract has been proved to possess the highest inhibitory effect on all investigated mould species. The concentrations of caraway extract, sufficient to inhibite the growth completely were: 0,5% for *E. herbariorum*, *E. amstelodami* and *A. sydowii*, and 1% for *A. flavus*; of garlic, 1% for *Eurotium* spp. and 2% for *A. sydowii*, and of origanum, 1% for *E. herbariorum* and 2% for *E. amstelodami*. The results of colonies diameter measuring showed that garlic and origanum extracts have no significant supressing ability on micellar growth of *A. flavus*, while garlic was more efficient in other test cultures.

KEY WORDS: spice extracts, antifungal activity, moulds

INTRODUCTION

Natural antimicrobic agents extracted from plants are very interesting, and their activity was investigated by many researches. In food production, their importance is increasing, as being a possible choice for food protection from microorganisms. In that way, the use of synthetic preservers would decrease (Arora and Kaur, 1999; Nielsen and Rios, 2000; Smith-Palmer et al., 2001; Lopez-Malo et al., 2002; Moreira et al., 2005). Most preservers are believed to have a bad influence on human health.

Some kinds of spice herbs, besides their use as flavour ingredients of food, possess antimicrobial properties. Mustard, sage, rosemary, and thyme are outstanding in their expressive antimicrobial activity. Essential oils, e.g. their active components (eugenol, carvacrol, thymol, cinnamaldehyde, allyn, limonene, geraniol, etc.) are the antimicrobial agents of spices (Savić and Danon, 1982; Montville and Matthews, 2005).

Numerous species of moulds cause food deterioration, however, some species are producers of different toxic substances. Aflatoxins (AF) and steri-

gmatocystine (ST) are toxic secondary metabolites of some *Aspergillus* and *Eurotium* species, which are important contaminants and potential causes of spoilage of intermediate and low-moisture content food (Pitt and Hocking, 1985; Guyenot et al., 2003; Dimić et al., 2005).

Antifungal activity of three kinds of spices against toxigenic moulds, *E. herbariorum*, *E. amstelodami* and *A. sydowii*, potential producers of sterigmatocystine and aflatoxigenic *A. flavus*, was investigated in this work. These moulds are related since aflatoxins and sterigmatocystine have a common precursor in their biosynthesis, and sterigmatocystine can be converted into aflatoxin B1 (Keller et al., 1997; Duraković and Duraković, 2003).

MATERIALS AND METHODS

Isolates of moulds

Moulds including *E. herbariorum*, *E. amstelodami*, *A. flavus* and *A. sydowii*, isolated from different spices, were used as test microorganisms. The cultures used in this experiment were cultivated on slant Saboraud maltose agar (SMA) for 7 days, at 25°C.

Spices

Extracts of spice herbs: caraway seeds, garlic, and origanum were supplied by "Etol Celje", Slovenija.

Medium

SMA medium was used to follow the growth inhibition of moulds. The medium was poured into 250 ml Erlenmayer flasks and autoclaved at 121°C, for 15 min. After sterilization, 0; 0,07; 0,1; 0,5; 1 and 2% concentrations of spice extracts were added into flasks.

Antifungal investigations

After the addition of spice extracts, equal portions of SMA medium were poured into sterile Petri plates. Each concentration was prepared in triplicate. Inoculation was performed by transfering conidia into the center of agar medium, as a point. Mould growth inhibition was estimated measuring the diameter of colonies every day during the incubation at 25°C, for 7 days.

RESULTS

Inhibitory effect of three kinds of spice extracts on two *Eurotium*, and two *Aspergillus* species was investigated. Lower concentration of caraway seed extract (0,07 and 0,1%) affected all four toxigenic moulds, during 7 days of incubation at 25°C (Table 1). The activity against *E. herbariorum* was higher (47 and 76,5%), while the growth inhibition of *A. flavus* was insignificant (5,1 and 18,6%). Compared to the control, the effect on *E. amstelodami* and *A. sydowii* was similar. Caraway seed extract inhibited completely the growth of *E. herbariourum*, *E. amstelodami* and *A. sydowii*, in concentration of 0,5%. The same concentration inhibited partially the growth of *A. flavus* (88,1%). Total growth inhibition was achieved with 1% concentration.

Tab. 1 — Antifungal activity of caraway extract on the growth of moulds

Conc. (%)	Micelial growth inhibition (%)			
	E. herbariorum	*E. amstelodami*	*A. flavus*	*A. sydowii*
0.07	47	22.2	5.1	25
0.1	76.5	50	18.6	39.3
0.5	100	100	88.1	100
1	100	100	100	100
2	100	100	100	100

No inhibitory effect of garlic extract (0,07 and 0,1%) (Table 2) on *A. flavus* was found. On the contrary, 0,07% of garlic extract insignificantly stimulates the growth of this mould. As shown in Table 2, 0,1% at garlic extract decreased the growth of *E. herbariorum* by 41,4%, *E. amstelodami* by 33,3% and *A. sydowii* by 10,7%. The concentration of 0,5% was not sufficiently efficient to prevent significantly the growth of the investigated moulds, *A. flavus* especially. Garlic extract inhibited completely the growth of both *Eurotium* species in concentration of 1%, insignificantly (32,2%) of *A. flavus*, and partially (85,7%) of *A. sydowii. A. sydowii* was completely inhibited at 2% level of garlic extract, but *A. flavus* was not (42,4%).

Tab. 2 — Antifungal activity of garlic extract on the growth of moulds

Conc. (%)	Micelial growth inhibition (%)			
	E. herbariorum	*E. amstelodami*	*A. flavus*	*A. sydowii*
0.07	11.7	27.8	0*	7.1
0.1	41.4	33.3	0	10.7
0.5	52.9	61.1	5.1	42.8
1	100	100	32.2	85.7
2	100	100	42.4	100

* stimulation of micelial growth

Neither *Eurotium*, nor *Aspergillus* species showed sensitivity to 0,07% extract of origanum (Table 3). Also, no inhibition activity against *A. sydowii* at low concentrations (0,07 and 0,1%) was found, moreover, 0,07% extract even

favoured the micellar growth. Origanum extract inhibited completely inhibited the growth of *E. herbariorum* and *E. amstelodami* at higher concentrations (1 and 2%), and almost totally (96,4%) the growth of *A. sydowii* at 2% level. No particular effect was exposed on *A. flavus*.

Tab. 3 — Antifungal activity of origanum extract on the growth of moulds

Conc. (%)	Micelial growth inhibition (%)			
	E. herbariorum	*E. amstelodami*	*A. flavus*	*A. sydowii*
0.07	0	0	0	0*
0.1	11.8	5.5	1.7	0
0.5	47	33.3	10.2	10.7
1	100	66.7	18.6	46.4
2	100	100	49.1	96.4

* stimulation of micelial growth

The effect of caraway seed extract on germination and growth rate of *E. herbariorum*, *E. amstelodami*, *A. flavus* and *A. sydowii* during seven days of incubation, is presented in Figure 1, having in mind that caraway expressed the strongest inhibition effect in our experiments.

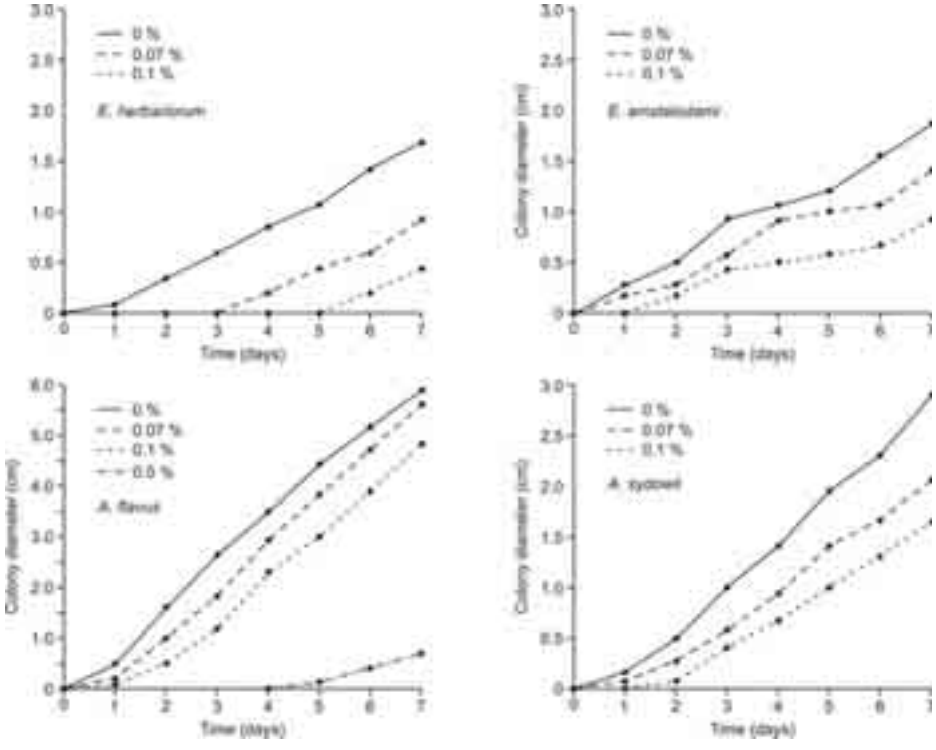

Fig. 1. — Inhibition of *E. herbariorum*, *E. amstelodami*, *A. flavus* and *A. sydowii* by caraway extract

The decrease of growth rate with increased content of caraway in the agar medium was more expressed in *E. herbariorum*, than in other mould species, both at the beginning and at the end of incubation. The beginning of germination, compared to the control, was delayed for three (0,07%) and six days (0,1%). In all caraway treatments, where *E. amstelodami* was growing, the rate of colonies spreading was higher, and the germination was inhibited only at concentration of 0,1%, in two days. The observation for *A. sydowii* growth was similar. At lower levels of caraway extract (0,07 and 0,1%), the growth of *A. flavus* was the same as in the control. The diameters of colonies differed only in 0,1 cm. Although the beginning of the growth was not affected by 0,07 and 0,1% of caraway, difference in growth rate was observed later. This difference was practically at the same level, till the end of the experiment. In case of 0,5% concentration, the colonies of *A. flavus* became visible only the fifth day after the inoculation of agar plates.

Sporulation of all moulds decreased with the increase of content of caraway, garlic and origanum extracts. The highest concentrations where growth of moulds was evident, caused greatest changes in appearance of colonies compared to the control.

DISCUSSION

These experiments have shown that caraway seed, garlic and origanum have antifungal activity against the tested toxigenic *Eurotium* and *Aspergillus* species. Caraway in concentrations higher than 0,5%, efficiently prevents the growth of moulds. *Eurotium* spp. exhibited higher sensibility. *E. herbariorum* was the most sensitive to caraway extract. Garlic extract is also a significant antifungal agent for the same kinds, however, for *A. sydowii*, hit was not so efficient as a caraway seed extract. The tested organisms were less sensitive to origanum, compared with garlic. Spice extracts had the lowest effect on *A. flavus*.

Garlic and origanum have some inhibitory effects, however, neither of them is significantly efficient in growth reduction of *A. flavus*. Neilsen and Rios (2000) investigated some spices and found garlic to be efficient in growth inhibition of *Emericella fibuliger*, *Penicillium commune*, *P. corylophilum*, *P. roqueforti* and *P. solitum*, however, the inhibition of *A. flavus* growth was very poor, similar to origanum. In general, the inhibitory potential of origanum for these species was week, whereas vanilla had no effect at all.

Caraway is the spice which has high inhibitory effect on growth of moulds. This effect can be compared to clove (Malbrouk and El-Shayeb, 1980). This activity can be attributed to the presence of carvon and limonene, present in caraway seed essential oil in significant quantities (50 to 85%, and 20 to 30%), respectively (Savić and Danon, 1982).

It was found that the essential oils of caraway, clove, onion, and garlic may affect preventively the synthesis of sterigmatocystin and aflatoxin (Hitokoto et al., 1980; Hasan and Mahmoud, 1993). The combined effect of 16 spices and different water activities (0,80 to 0,90 a_w) on *Eurotium*

spp. (*E. amstelodami*, *E. herbariorum*, *E. repens* and *E. rubrum*) was tested, and the results have shown a complete inhibitory effect of lemongrass, clove, cinammon, bay and thyme at all a_w values (Guyenot et al., 2003). The inhibitory effect of garlic was stated many times (Mei-Chin and Wen--Shen, 1998; Yin and Tsao, 1999; Benkeblia, 2004).

The essential origanum oil is rich in timol and carvakrol, and this spice is a very strong growth inhibitor of *A. niger* (Baratta et al., 1998) and *A. ochraceus*, procuder of ochratoxin A (Basilico and Basilico, 1999). Özcan (1998) found that origanum extract, besides thyme and savory (out of 31 tested ones), inhibits completely the growth *A. parasiticus*, in 2% concentration during incubation at 30°C, for 10 days.

The spice extracts of caraway seed, garlic and origanum, investigated in this work, caused morphological changes besides growth inhibition of colonies, and this points to the possible changes on a cell level. The investigation of cell ultrastructures of *A. niger*, after exposure to the effect of thyme essential oil, showed damages of cell wall, cellular membrane and organellas (Rassoli et al., 2006).

CONCLUSION

Having in mind the obtained results, the investigated spice extracts can be used to limit or prevent the development of harmful moulds in food (preserved, or fresh stored food), as additives, surface prevention, or use in products packed under modified atmosphere. A number of active components of spices and their derivates are being further investigated.

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ИНХИБИЦИЈА РАСТА НЕКИХ *EUROTIUM* И *ASPERGILLUS* ВРСТА ЕКСТРАКТИМА ЗАЧИНА

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Резиме

Екстракти зачина добијени из кима, белог лука и оригана били су испитани на антифунгални активитет према *Eurotium herbariorum, E. amstelodami*, *Aspergillus flavus* и *A. sydowii*. Екстракт кима имао је најјачи инхибиторни ефекат и то на

све три врсте плесни. Концентрације екстракта кима довољне да комплетно инхибирају раст биле су 0,5% за *E. herbariorum, E. amstelodami* и *A. sydowii* и 1% за *A. flavus*; за бели лук 1% за *Eurotium* spp. и 2% за *A. sydowii* и за оригано 1% за *E. herbariorum* и 2% за *E. amstelodami*. Резултати мерења дијаметра колонија су показали да екстракт белог лука и оригана не супресују значајно мицеларни раст *A. flavus*, док је бели лук био ефикаснији према осталим тест-културама.

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ANTHRACNOSE — A NEW STRAWBERRY DISEASE IN SERBIA AND ITS CONTROL BY FUNGICIDES

ABSTRACT: Anthracnose is a destructive disease of strawberry fruits in warm and continental climate. During 2004, in the vicinity of Valjevo, there were severe losses in two strawberry plantations due to fruit anthracnose. Two fungal isolates, GG-6A and GG-JUP were recovered from strawberry stolons, and fruits showing severe anthracnose symptoms. Based on morphological and pathological characteristics, and PCR analyses with specific primers of reference species, isolate GG-6A was identified as *Colletotrichum gloeosporioides*, and GG-JUP isolate as *C. acutatum.* This is the first identification of *C. acutatum* in strawberry in Serbia.

In order to control strawberry anthracnose, five fungicides and their combinations were applied four times during the flowering. The best fruit protection was achieved by fungicides Metiram + piraclostrobin (Cabrio top), Captan FL and Fludioksinil + ciprodinil (Swich). Less effective were Benomil (Benlate) and Krezoksim-metil (Stroby).

Pathogen is transmitted by planting material, so phytosanitary measures are extremely important in preventing the disease.

KEY WORDS: anthracnose, *Colletotrichum acutatum*, planting material, strawberry disease

INTRODUCTION

The strawberry (*Fragaria x ananassa* Duch.) production in Serbia, both in the field and under the plastic, is increasing. Deficiency of certified planting material is frequent, so the import is necessary. However, with importation of the planting material there are possibilities of introducing new strawberry diseases.

In two new strawberry plantations founded by the imported planting material, in the vicinity of Valjevo, a new disease was registered in 2004. The first visible symptoms of fruit rotting were in the maturity, and yield was reduced over 80%. In 2005 the appearance of the disease was mild, and in 2006 it was weak.

Anthracnose diseases of strawberry are caused by three fungal pathogens: *Colletotrichum acutatum* J.H. Simmonds, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz & Sacc. (teleomorph *Glomerella cingulata* (Stoneham.) Schrenk & Spaulding). All three species incite diseases which cannot be distinguished in the field by symptoms alone (Peres et al., 2005). *C. fragariae* is most often associated with anthracnose crown rot of strawberry grown in hot, humid areas, such as the southern United States. *C. gloeosporioides* usually cause petiole and stolon lesions and crown rot on a strawberry, but may also produce fruit symptoms (Smith, 1998).

Anthracnose, caused by *C. acutatum* is responsible for the major losses in strawberry production worldwide. Fruit rot and flower blight are common symptoms in fruiting fields (Howard et al., 1992), whereas lesions on stolons, petioles and leaves are particularly harmful in plant nurseries (Freeman & Katan, 1997). *C. acutatum* was first described as a separate species by Simmonds (Simmonds, 1968). The teleomorph of the fungi was recently described as *Glomerella acutata* Guerber & Correll (Guerber & Correll, 2001).

None of the three mentioned anthracnose diseases have been registered on strawberry in Serbia even though *gloeosporioides* has been present for many years as the pathogen of sour cherry, apple and other plants (Ivanović & Ivanović, 2005).

In order to study the causal agent of strawberry disease in new plantations, in the vicinity of Valjevo, and to optimize the control strategy for the disease, this research has been carried out.

MATERIAL AND METHODS

Pathgen isolation and maintenance. Strawberry stolons and fruits from naturally infected cv. Favet were collected in January and May in 2005 respectively. Fruit lesions were clear, frequently coalesced and sporulated quickly, but stolon lesions were atypical, unclear. Both fruit and stolon lesions were cut in small sections, their surface was sterilised in 70% ethanol, cultured on a laboratory prepared potato dextrose agar (PDA), and kept under laboratory ambient conditions until colony was developed. Mycelial fragments were cut from the edges of the developed colonies, and transferred to a new PDA to get a pure culture. The cultures were stored on PDA slants at 10°C and transferred every 4 weeks. Two isolates, GG-6A and GG-JUP recovered from stolons and fruits, respectively, were chosen for further research. Reference isolates of *C. acutatum* (TUT 137A) and *C. gloeosporioides* (AVO 37 4B), used in this study, were acquired from V. Trkulja, Banja Luka, Republic of Srpska.

Colony growthrate assessment and spore dimensions. The effect of PDA, carrot agar (CA), and oatmeal agar (OM) media on the colony growth of two isolates GG-6A and GG-JUP, and reference isolates (TUT 137A and AVO 37 4B) were studied under ambient laboratory conditions. The diameter of colonies was measured after 9 and 15 days. Morphology of the colonies, the

occurrence of sectors, and the vegetative and reproductive structures were described after 14 days of incubation.

For conidial measurements, isolates were cultured under continuous fluorescent light for 3 days at room temperature on PDA, to promote sporulation. The spores were suspended in sterile water using a sterile needle. The length and width of too condition were measured, using measuring program IM 1000, and conidial shape was recorded at x400 magnification (10 x ocular, 40x objective) using bright field microscope (Leica DMLS).

Pathogenicity test. Strawberry fruits were inoculated with previously mentioned four isolates. Spores were produced on PDA media. Cultures were flooded with sterile distilled water and filtered through four layers of cheesecloth to remove mycelia. Conidia concentration was determined using a haemocytometer, and adjusted with sterile distilled water to 4×10^5 conidia per ml. Strawberry fruits of cv. Favet were, just before the ripening, inoculated by injecting 20 µl of conidial suspension. Control fruits were inoculated with 20 µm of sterile distilled water. Inoculated fruits were held on the top of the metal screen in plastic containers at 25 ± 2°C. In other to maintain RH, hot water was added to the bottom of each container which was then sealed tightly. Lesion diameter was measured for four days, after inoculation. The test was repeated two times.

Nucleic acid extraction and PCR. Nucleic acid extraction was performed following the protocol described by Day and Shattock (1997). Extracted nucleic acids were resuspended in TE buffer and maintained at –20°C. PCR was performed using ITS4 universal primer in pair with, in separated reactions, primer CgInt specific for *C. gloeosporioides*, and CaInt2 specific for *C. acutatum.* PCR was performed in 25 ml total volume composed of: 1X PCR master mix (Fermentas, Lithuania) (0,625 U Taq polymerase, 2 mM $MgCl_2$, 0,2 mM of each dNTP, 1 ml of each primer (20 mM) and 1 ml of extracted DNA. PCR conditions were 35 cycles: 94°C 1 min (denaturation), 59°C 2 min (annealing) and 72°C 2 min (extension). Visualization of PCR products was performed in 1% agarose gel, stained with ethidium bromide, under UV light.

Control of strawberry fruits anthracnose in naturally infected plantation. Field trials for strawberry anthracnose control were conducted during 2005 and 2006 in naturally infected plantation in the vicinity of Valjevo. Five fungicides, single or in a combination of two, were supplied by market companies in Serbia. The plot for each treatment consisted of double rows, 50 m long (100 plants per each row planted in zigzag position). All trials were arranged in a completely randomised block design with three replications. Fungicide treatments, doses and application timing are listed in **Table 1**. Fungicide treatment was applied on 7 days schedule from the beginning of flowering till the beginning of ripening (April 23 till May 16). Disease incidence was measured at each of the four harvests by collecting the fruits with anthracnose, their measuring and comparing with the weight of diseased fruits in untreated control plot.

Tab. 1 — Treatments, doses and application timing of fungicides applied to control strawberry anthracnose

Fungicide — Generic name	Fungicide chemical name	Doses/ha	Data of application for all fungicides (in 2005)
Kaptan FL	Captan F	2,5 l	April 23; April 30; May 7, and May 16.
Benfungin	Benomil	1,0 kg	
Stroby DF	Krezoksim-metil	0,20 kg	
Cabrio top	Mertira + piraklostrobin	2,0 kg	
Swich 62, 5WG	Fludiksinil + Ciprodinil	0,80 kg	

RESULTS

Diseases symptoms. The most visible symptoms of anthracnose on strawberry were on fruits at maturation. At the beginning infected fruit form of brown, circular, sunken, initially water-soaked lesion (**Figure** 1). Under optimal temperature (25°C) and humidity they rapidly increased, giving lesion of 1 to 2 cm in diameter within 3 to 4 days (**Figure** 2). With warm weather the disease spreads fast causing fruit rotting. The symptoms on vegetative parts of plants, stolons, leaves during the vegetation were unlikely to be seen.

Fig. 1 — Water-soaked lesion on strawberry fruit caused by *C. acutatum*

Colony and spore morphology. Uniform colony growth of all investigated isolates was on the PDA and OM. The colonies filled Petri dishes, Ø 90 mm, after 15 days. Three isolates (GG-6A, GG-JUP and TUT 137 A) had significantly less growth on CA than on PDA and OM (**Table** 2). Only reference AVO 37 4B isolate (*C. gloeosporioides*) had the same colony diameter in all three media. The colonies of GG-6A and GG-JUP isolates were effuse, first

Fig. 2 — Typical anthracnose lesion on ripen strawberry fruit

white later becoming orange, then turning into greenish grey as the cultures aged and later became covered with pink to salmon conidial masses on PDA. Light orange spore masses were formed around the centre of the colony. Older cultures developed black acervuli in the centre of the colony.

Tab. 2 — Effect of different media on colony growth of *Colletotrichum* isolated from strawberry and two reference species *C. acutatum* and *C. gloeosporioides*

Fungi	**Media**	**Diameter of colony (mm) after days**	
		9	**15**
GG-6A	PDA	73.3 a	90.0 a
	CA	62.7 b	83.3 b
	OM	56.7 c	90.0 a
GG-JUP	PDA	67.3 a	90.0 a
	CA	34.3 b	45.0 b
	OM	63.3 a	90.0 a
TUT-137A	PDA	72.0 a	90.0 a
	CA	28.0 c	37.0 b
	OM	63.3 b	90.0 a
AVO 37 4B	PDA	90.0 a	90.0 a
	CA	86.3 b	90.0 a
	OM	85.7 c	90.0 a

The same alphabet are not statistically different by Duncan test (P = 0.05)

Colony reverse was brownish orange to black. There were no differences in the colony morphology among the mentioned isolates, nor among the two reference isolates of *Colletotrichum.*

Conidia were hyaline, unicellular, and cylindrical with obtuse apices and tapering base. Conidia of GG-JUP isolate are usually ellipsoid and fusiform at least at one end (**Figure** 3), while conidia of isolate GG-6A are typically having both end rounded. Setae are not produced in the culture but are present in diseased strawberry fruits. Pigmented appressoria are produced after the germination of the conidia and vary in shape and size. There was no registered sexual stage formation under the laboratory conditions.

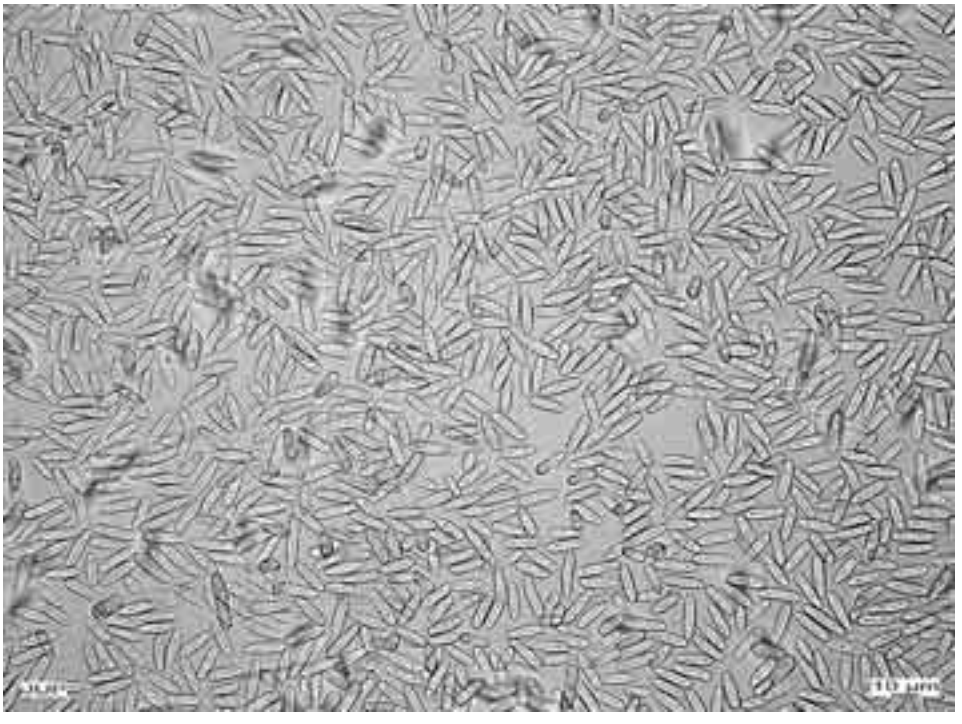

Fig. 3 — Conidial morphology of *C. acutatum*, isolate GG-JUP

Conidial size has been given in **Table 3**. Average conidial length of TUT 137A reference isolate is statistically shorter than the conidial length of other three isolates. However, GG-JUP and the referent isolate TUT 137A have statistically more similar conidia than GG-6A and AVO 37 4B. Concerning the ratio of conidial length:width, GG-JUP and TUT 137 A did not differ statistically, but GG-6A and AVO 37 4B did (**Table** 4).

Tab. 3 — Size of conidia of two isolates of *Colletotrichum* isolated from strawberry, and two reference isolates *C. acutatum* (TUT 137A), and *C. gloeosporioides* (AVO 37 4B).

Isolate	Length of conidia (µm)			Width of conidia (µm)		
	Min.	Average	Max.	Min.	Average	Max.
GG-6A	12,63	15,31 ± 0,49 a	22,03	4,98	5,66 ± 0,08 a	6,53
GG-JUP	12,96	15,49 ± 0,38 a	19,37	3,77	4,57 ± 0,08 c	5,22
TUT 137A	12,59	14,08 ± 0,39 b	17,49	3,53	4,32 ± 0,09 c	5,01
AVO 37 4B	12,57	15,07 ± 0,35 a	17,24	4,68	5,78 ± 0,17 a	7,82

The same alphabet are not statistically different by Duncan test (P = 0.05)

Tab. 4 — Ratio of conidia length/width of two isolates of *Colletotrichum* isolated from strawberry, and two reference isolates *C. acutatum* (TUT 137A), and *C. gloeosporioides* (AVO 37 4B).

Isolate	Min.	Average	Max.
GG-6A	2,56	2,98 ± 0,08 b	3,83
GG-JUP	2,76	3,42 ± 0,12 a	5,14
TUT 137A	2,78	3,27 ± 0,96 a	4,62
AVO 37 4B	1,63	2,65 ± 0,09 c	3,59

The same alphabet are not statistically different by Duncan test (P = 0.05)

Pathogenicity test on strawberry fruit. Both reference and our isolates caused lesions on artificially inoculated strawberry fruits. More pathogenic isolates were GG-JUP and TUT 137A, compared to the isolates GG-6A and AVO 37 4B (**Table** 5). Fruits challenged with distilled water did not develop lesions. On inoculated fruits, symptoms first appeared as whitish, water soaked lesions up to 3 mm in diameter. As lesions developed, they turned a light tan to dark brown and eventually became sunken and black within 2 to 3 days. After several days lesions may be covered with salmon-coloured spore masses. Infected fruits dried down to form hard, shrivelled mummies.

Tab. 5 — Pathogenicity of two isolates of *Colletotrichum* and two referent isolates of *C. acutatum* and *C. gloeosporioides* to strawberry fruits.

Isolates	Appearance of lesions	Diameter of lesion	Acervuli presence
GG-6A	Rounded cowered with sparse mycelia	13	No present
GG-JUP	Big, sunken, brownish, cowered with whitish mycelia	20	Present, creamy
TUT 137A	Big, sunken, brownish, cowered with whitish mycelia	20	Very present
AVO 37 4B	Smaller spot cowered with whitish mycelia	12	Not present

Molecular identification. Using CgInt primer specific for species *C. gloeosporioides*, in pair with ITS4 primer, expected length amplicons were obtained with isolates GG-6A which were, based on the conidia size, identified as *C. gloeosporioides.* Using CaInt2 primer specific for species *C. acutatum*, in pair with ITS4 primer, expected length amplicons were obtained with isolates GG-JUP which were, based on the conidia size, identified as *C. acutatum.*

Identification of fungal isolates was beside morphological characteristics, confirmed by using molecular technique PCR with primers specific for fungal species *C. gloeosporioides* and *C. acutatum.*

Control of anthracnose in strawberry fruits. Results obtained in this investigation showed that strawberry anthracnose can be controlled with fungicides application. The best fruit protection was achieved by fungicides Metiram + piraclostrobin, Captan FL and Fludioksinil + ciprodinil. Krezoksim-metil and Benomil did not protect strawberry fruit from anthracnose attack (**Table** 6).

Tab. 6 — Effect of the fungicides on anthracnose incidence in strawberry in 2005.

Date of harvesting	Weight of affected strawberry fruits (g)/treatment — (two-rows 50 m)					
	Market name of fungicides					
	Kaptan FL	Benomil	Stroby DF	Cabrio top	Swich	Control
May 21	20	20	300	0,0	0,0	1500
May 23	30	800	230	0,0	55	1250
May 26	150	500	700	150	300	1250
June 01	100	600	350	50	150	2750
Total (g)	**300**	**1920**	**1580**	**200**	**505**	**6750**

DISCUSSION

Our first objective was to identify the species of *Colletotrichum* causing strawberry anthracnose disease in our country. Morphological characteristics indicated that the causal agent of fruit rotting, isolate GG-JUP, could be *C. acutatum*. This statement was confirmed by fungus isolation from the infected fruits, pathogenicity tests, as well as molecular tests.

The isolates recovered from stolons, according to the morphological and molecular characteristics could be determined as *C. gloeosporioides*. Since this is the first finding of the pathogen on strawberry, further researches are needed to collect more isolates in order to study their morphological, pathological and molecular characteristics.

Other researches have shown that the characterisation of fungi isolated from strawberry plants, affected by anthracnose is complex. Lewis Ivey et al. (2004) found that the characteristics such as the colony morphology, the conidial shape, the presence or absence of setae and sclerotia, and the appressorium shape and size could be used for differentiation of the genus *Colletotrichum.* Morphological features, however, are highly variable among the isolates and often subject to interpretation. Concerning the same characteristics we did not find full consistency as well.

Since the anthracnose was first registered in 2004 in the plantations founded by the imported planting material, there are possibilities that the pathogen was introduced by the planting material from abroad.

Transmission of the pathogen by planting material is reported by Legard (2000) in the US. Investigation of Eastburn and Gubler (1990) suggested that the fungus is transmitted in infested soil attached to strawberry

crowns. Petioles of foliage harbour inoculum for fruit and flower for several *Colletotrichum* spp. (T i m m e r and B r o w n, 2000).

In OEPP/EPPO Bulletin (2004), it was emphasized that the infected planting material of strawberry is the main mean of introduction, but symptoms of anthracnose are unlikely to be seen on this material as the fungus is usually inactive in living vegetative tissues.

Several studies have pointed out that *C. acutatum* can develop quiescent infection on strawberry plants (H o w a r d et al., 1992). Production of secondary conidia and appressoria of *C. acutatum* on symptomless strawberry leaves, under a range of environmental conditions suggests that these processes also occur under field conditions and contribute to inoculum availability during the growing season (L e a n d r o et al. 2003).

Anthracnose is a serious disease of strawberry in Northern hemisphere. Three species of the *Colletotrichum* are responsible for strawberry anthracnose. *C. gloeosporioides* and *C. fragariae* usually cause petiole and stolon lesions and crown rot on strawberry but may also produce fruit symptoms (S m i t h, 1998). *C. acutatum* is predominantly on flowers and fruits inducing rotting, and causing the most yield reduction worldwide.

In other to develop recommendations for the management of the disease, we assessed the efficacy of several fungicides in reducing disease incidence. The best fruit protection was achieved by fungicides metiram + piraclostrobin, captan FL and fludioksinil + ciprodinil. Less effective were Benomil and Krezoksim-metil. Benomil was not good in anthracnose control in Ohio (E l l i s, 2004), nor was it in our trials in sour sherry anthracnose control (I v a n o v i ć & I v a n o v i ć, 1992). Various fungicides were assessed for their ability to control strawberry anthracnose caused by *C. acutatum* in Israel (F r e e m a n et al., 1997), and anthracnose of immature bell peppers in Ohio (M e l a n i e et al., 2004). In Florida, strawberry anthracnose management is based on the use of Captan or thiram by regular weekly application (M e r t e l y & P e r e s, 2005).

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АНТРАКНОЗА, НОВА БОЛЕСТ ЈАГОДЕ У СРБИЈИ И ЊЕНА КОНТРОЛА ФУНГИЦИДИМА

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Резиме

Антракноза је деструктивна болест плодова јагоде у топлим и континенталним климатским условима. Током 2004. године, на 2 плантаже јагода у близини Ваљева, било је великих губитака проузрокованих антракнозом. Два изолата гљива ГГ-6А и ГГ-ЈУП су изолована из столона јагоде, и плодова са израженим симптомима антракнозе. На основу морфолошких и патолошких карактеристика, и ПЦР анализе са специфичним прајмерима за референтне врсте, изолат ГГ-6А је идентификован као *Colletotrichum gloeosporioides*, а изолат ГГ-ЈУП као *C. acutatum*. Ово је први налаз *C. acutatum* на јагоди у Србији.

У циљу контроле антракнозе јагоде пет фунгицида и њихових комбинација су примењени 4 пута током цветања. Најбоља заштита плодова јагоде је постигнута применом фунгицида Metiram + piraclostrobin (Cabrio top), Captan FL и Fludioksinil + ciprodinil (Swich). Мање ефективни су били Benomil (Benlate) и Krezoksim-metil (Stroby).

Патоген се преноси садним материјалом па су фитосанитарне мере веома важне у превенцији болести.

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CHARACTERISTICS OF *PHOMOPSIS* SP. ISOLATES OF PLUM TREES ORIGIN

ABSTRACT: Twelve isolates of *Phomopsis* sp. were obtained from the branches and the trunk of plums (*Prunus domestica* L.) with decay symptoms in Valjevo, Ljig, Koceljeva and Ub vicinity during 2004—2006. Morphological, pathogenic and growing characteristics were studied. Pathogen caused tissue necrosis of branches around the inoculate seats, and wrinkling and watering of plum fruits. All media were suitable for pathogen development, except prune agar. The best growth of isolates was at medium pH 5,5. The optimal temperature for growth and germination of pycnidiospores was 25°C.

KEY WORDS: development, morphology, pathogenisity, *Phomopsis* sp., plum, *Prunus domestica*

INTRODUCTION

The plum (*Prunus domestica* L.) is the most frequent fruit tree in Serbia. During the middle of the '90s of the 20th century, a new plum disease occurred. It caused wilting and decay of young plum trees in the orchards. It has been first registered in the vicinity of Valjevo on cv. Stenly and Požegača. In some orchards more than 40% of plum trees were diseased. Later on, the disease was spread on the other cultivars, expecially on cv. Čačanska lepotica and Čačanska rodna. This disease occurred also in the other plum growing area. From the samples of diseased plum brunches, Stojanović et al. (2004) isolated fungi belonging to the following genera: *Cytospora*, *Phomopsis*, *Sphaeropsis*, *Seimatosporium* and *Fusarium*. As the obtained isolates showed extensive virulens and aggressivnes to plum branches, the investigations of some characteristics of pathogen isolates were conducted.

MATERIAL AND METHODS

The samples of diseased plum branches were collected in Valjevo, Ljig, Koceljeva and Ub vicinity during 2004—2006. Isolation of pathogen was carried out by cutting the fragments of cca 0.5 cm^2 from the border of diseased and healthy plum tissues. The fragments were surface sterilized with 3% NaOCL for 3 minutes, and transferred on potato dextrose agar (PDA). From numerous isolates obtained, 12 were chosen for further investigations and were designated as SL-1, SL-M, SL-T-1, SL-B-2, SL-Br-2, SL-K-1, SL-K-3, SL-K-4, SL-K-5, SL-K-7, SL-K-8 i SL-U-4.

Morphology of pathogen was studied on infected branches and on nutrient media. A variety of mycological media supported the growth of plum *Phomopsis* cultures, including PDA, Czapek-Dox agar (CA), oatmeal agar (OA), malt extract agar (MA) and prune agar (PA). The cultures were incubated in 90 mm Petri dishes at 25°C in darkness, for 7 days.

Pathogenicity studies were conducted on (1) young green branches of plum cvs. Stenly, Požegača, Čačanska rodna and Čačanska lepotica in the field, (2) one-year-old shoots of cv. Stenly 30 cm in length, which were drived into wet sterile sand in tin containers, and (3) plum fruits of cv. Stenly under laboratory conditions. A V-shaped incision was made at 45°-angle to expose the tissue under bark of green branches and shoots. An agar plug (0,5 cm^2), containing young hyphae from 10-day-old culture of a *Phomopsis* sp., was placed in the incision and wrapped with moist sterilized cotton wool and parafilm (Weingartner and Klos, 1975). Control branches and shoots were wounded in the same way and inoculated with blocks of sterile PDA. The parafilm and cotton wool were removed 10 days after the inoculation, and the disease development was evaluated by measuring the tissue necrosis length after 6 weeks. Plum fruits were washed with tap water, surface sterilized by 70%--ethanol, washed with distilled water, and mycelial fragments were put into wounds made with sterile needle. Control fruits were wounded in the same way and inoculated with blocks of sterile PDA. Inoculated plum fruits were placed in glass dishes (30 cm in diameter) and covered with wet filter paper. The dishes were wrapped in plastic bags for two days and then stored under laboratory conditions (Arsenijević et al.,1995).

The colony growth on PDA and the conidial germination were examined at temperature range from 5° to 35°C, with 5°C intervals, in the darkness. Effect of medium pH on the pathogen development was studied on PDA which was adjusted with 0,1 M NaOH or 0,1 M HCl to pH 3.5, 4.0, 5.5, 4.0, 8.5 and 9.5. Colony diameter was measured 7 days after the inoculation. The conidia in water drops (0,2 ml) on microscope slides were placed in Petri dishes with wet filter paper and incubated for 18 hours, since the high percentage of conidial germination at all temperatures, after the incubation of 24 hours, was found in the preliminary germination tests.

RESULTS

Symptoms. The first symptoms were expressed in the stage of flowering and leaf forming. Leaves were smaller and chlorotic, while later both leaves and flowers became necroted, if the infection was centered around the flower buds and leaf petioles. If the infection started around the vegetative buds, it resulted in young shoots becoming diseased and necroted (Fig. 1 a). On the stem and the main branches necrotic sunken lesions occurred, the bark longitudinally cracked, and cankers were formed (Fig. 1 b). The necrotic lesion expanded rapidly, disrupted the vascular tissues, and caused the shoot to wilt and die (Fig. 1 c).

Fig. 1 — Necrosis of current season's shoot of plum resulting from infection started around vegetative bud (a); Elongate canker and numerous pycnidia on diseased plum branches (b); Brown tissue necrossis under the bark removed from the diseased plum branches (c).

Morphology. Colonies were farinaceous, or woolly to cottony, white or whitish, pale to light brown or pale greyish (Fig. 2 a). Conidiomata was pycnidial, stromatic, dark brown to black, single or aggregated, often botryose in culture, uniloculate, over 500 mm in diameter (Fig. 3 a-d). The a-conidia in all 12 isolates were hyaline, fusiform to ovate, straight, aseptate, and frequently biguttulate, with average size 7,3 x 2,5 μm, and little variation in shape or size between the isolates. The β-conidia were filiform, sigmoidal, hyaline, with average size 23.7 x 1,3 μm (Fig. 2 b-c).

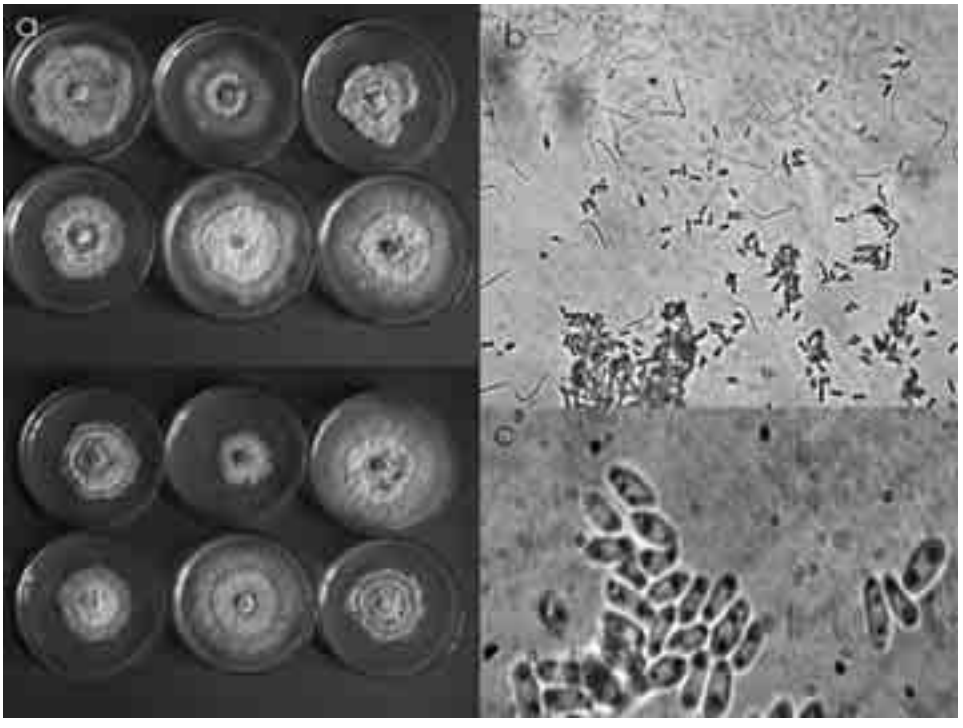

Fig. 2 — Colonies of *Phomopsis* sp. isolates from plum 7 days after incubation on PDA at 25°C (first upper row: SL-1, SL-M and SL-T-1, second row: SL-B-2, SL-Br-2 and SL-K1, third row: SL-K-3, SL-K4 and SL-K-5, fourth row: SL-K-7, SL-K8 and SL-U-4) (a); Alfa and beta conidia of isolate SL-U-4 (x 600) (b); Alfa conidia of isolate SL-K-1 (x 1000) (c).

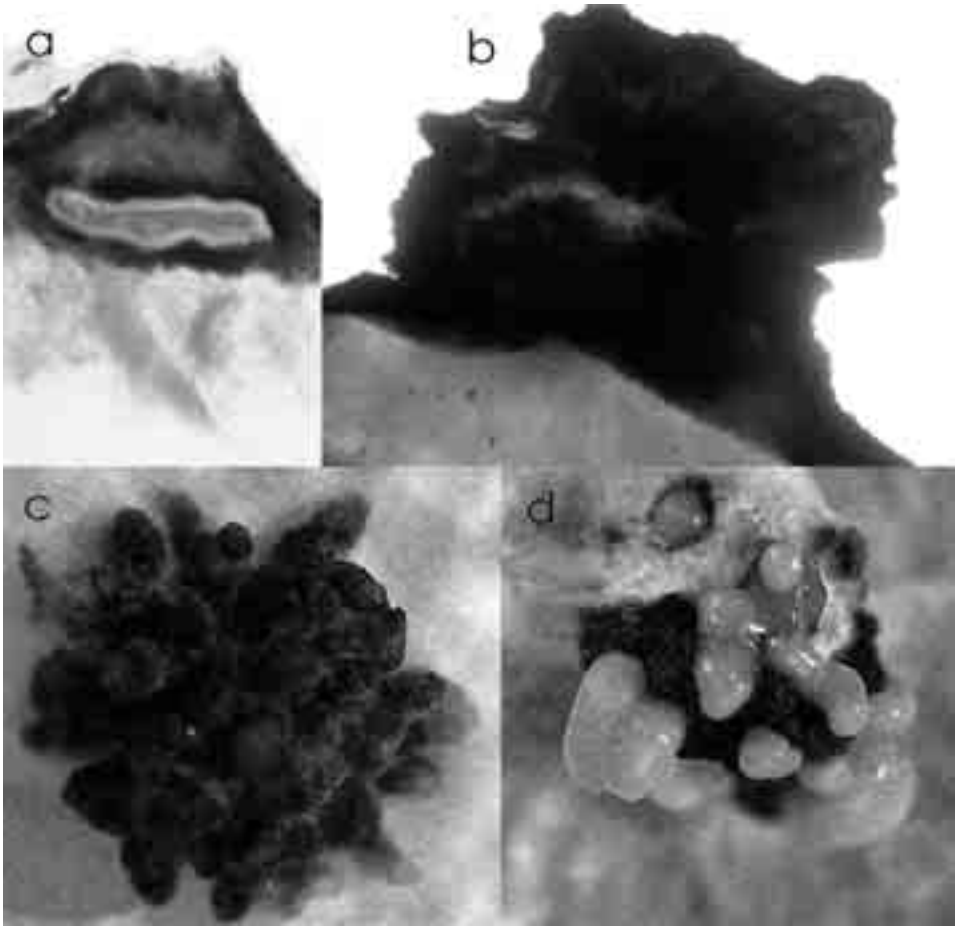

Fig. 3 — Cross section of pycnidium formed on plum branches (a); Cross section of pycnidium formed on stromatic structure on PDA (b); Botriose pycnidium on PDA (c); Cirri produced by pycnidia on PDA (d).

Pathogenicity. All isolates showed extensive virulens and aggressivnes to plum branches and fruits. Inoculated young green branches in the field, and the one-year-old shoots under laboratory conditions, showed necrosis of bark tissue around inoculate seats. Isolates SL-1, SL-T-1, SL-Br-2, SL-K-1, SL-K-3, SL-K-4, and SL-U-4 and SL-B-2, SL-Br-2, SL-M and SL-T-1 showed the greatest pathogenicity in the laboratory and field test respectively, after 6 weeks (Fig. 4 a-b). On the vertical sections of infected shoots the brown necrosis of woody tissue could be seen.

7 days after inoculation, plum fruits showed settled necrotic spot around the inoculation seat. Later on, the fruits became wrinkled, and after 15 days they were completely wrinkled and watery. Control fruits were healthy and fresh. The numerous pycnidia were formed on the inoculated plum fruits. Even the isolate SL-M, which did not form pycnidia in culture, formed abundant co-

nidimata on inoculated plum fruits. The pathogen was reisolated from the inoculated shots, branches and plum fruits (Fig. 4 c-d).

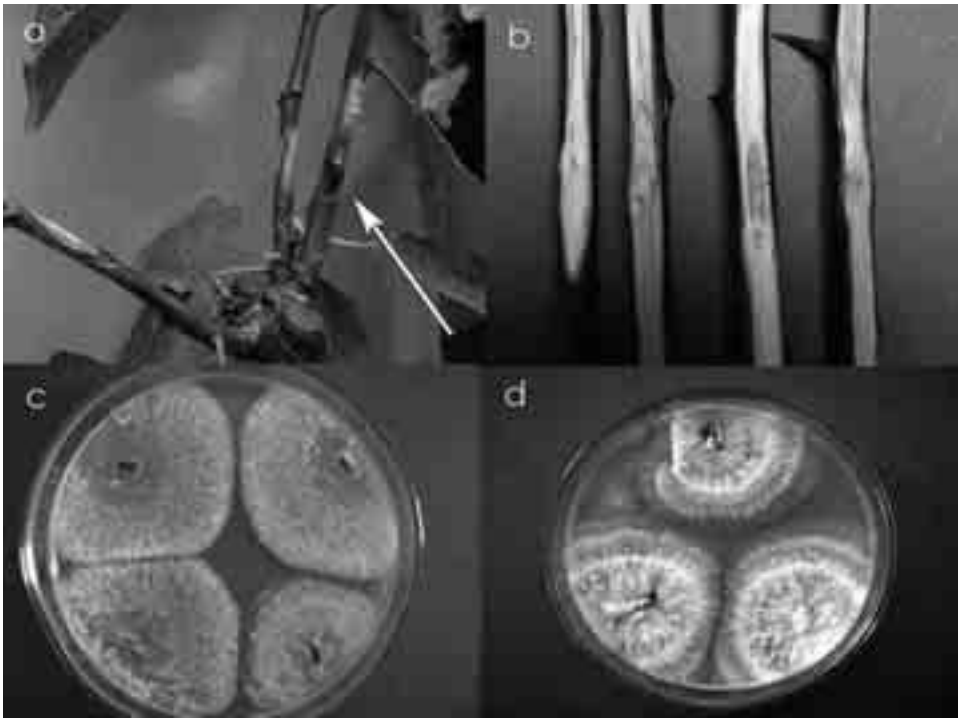

Fig. 4 — Necrosis of bark 6 weeks after artificial inoculation of green plum braches with isolate SL-B-2 in the field (a); Longitudinal section of plum shoots artificial infected in laboratory conditions (from left to right: control, SL-1, SL-M and SL-T-1) (b); Cultures obtained by pathogen reisolation from artificial inoculated green plum branches with isolate SL-B-2 (c) and plum shoots with isolate SL-K-3 (d).

Pathogen development *in vitro*. All media tested, except PA, were favorable for pathogen development (Sl. 5 d). The majority of isolates had the best growth on PDA. For isolates SL-T-1, SL-K-4 and SL-U-4, SL-B-2 and SL-K-7 and Sl-K-3 the most suitable media were CA, MA and OA, respectively. The average radial growth (mm) of tested isolates was 30.0—71.8, 27.5—60.7, 42.2—60.8, 41.7—71.2 i 25.7—63.3 on PDA, PA, OA, CA and MA, respectively. Isolate SL-K-4 had the slowest growth on all media tested. None of the mediums was suitable for pycnidial formation of isolates SL-M and SL-B-2. All isolates formed sparse pycnidia on PA. The abundant pycnidial formation was on CA (isolates SL-1, SL-K-3, SL-K-4, SL-K-7, SL-K-8 and SL-U-4), on OA (isolates SL-T-1, SL-K-1, SL-K-5 and SL-U-4), on MA (isolates SL-T-1, SL-K-1, SL-K-4, SL-K-5 and SL-U-4) and on PDA (isolates SL-K-4 and SL-K-5).

The optimal temperature for pathogen radial growth was 25°C, except for isolates SL-1 (20—25°C) and SL-U-4 (20°C). Minimal and maximal tempera-

tures for the growth of all isolates were 5°C and over 35°C, respectively (Sl. 5 a). The average radial growth (mm) of tested isolates was 5.0—5.5, 6.0—10.2, 14.2—36.7, 26.5—60.2, 35.0—71.8, 12.2—33.7 and 5.2—12.2 at 5, 10, 15, 20, 25, 30 and 35°C, respectively. Temperature in the range of 20—25°C was the most favorable for the formation of pycnidia, but for isolates SL-T-1, SL-Br-2, SL-K-5 and SL-U-4 temperature of 15°C was also suitable. The other tested temperatures were unfavorable for pycnidial production.

The pathogen growth was the largest at medium pH 5,5 (Sl. 5 c). The average radial growth (mm) of tested isolates was 11.5—29.0, 34.0—64.2, 56.7—88.3, 32.8—71.5, 21.5—53.8 and 18.5—42.5 at pH 3.5, 4.0, 5.5, 7.0, 8.5 and 9.5, respectively. The isolates SL-1, SL-K-1, SL-K3 and SL-K-5 did not produce pycnidia at pH 3,5. Medium pH seems to have no effect on the production of pycnidia. Mostly pH values in the range of 4.0—7.0 were equally suitable for pathogen sporulation, but for isolate SL-U-4 the maximal pycnidial formation occurred at pH 8,5.

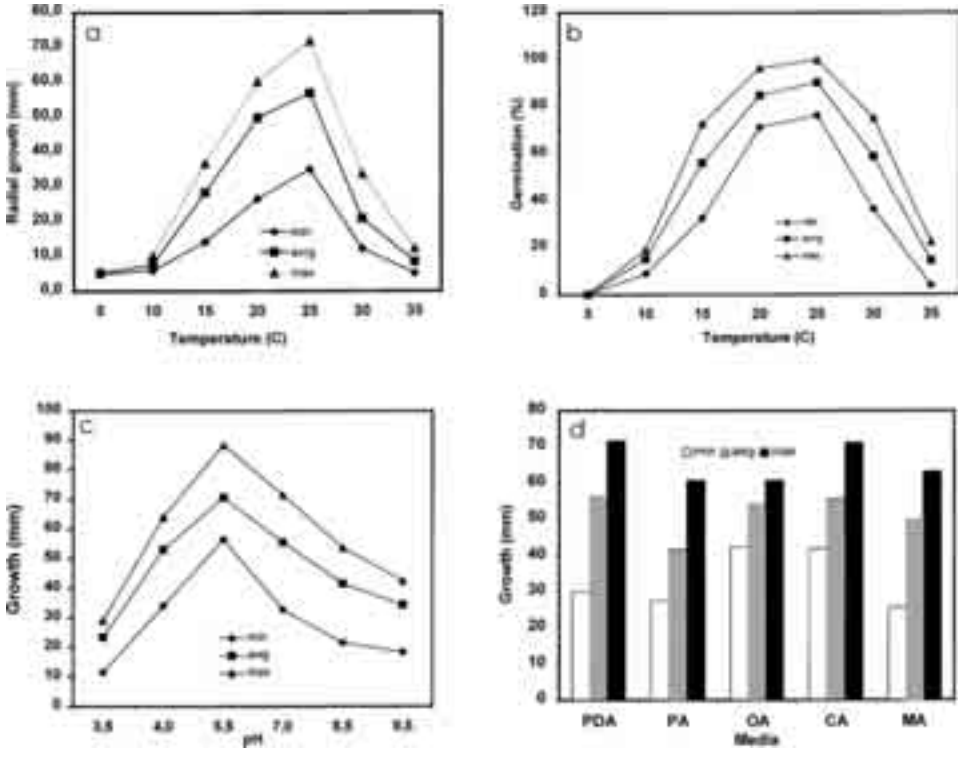

Fig. 5 — Pathogen development *in vitro*. Cultures growth at different temperature (a); Effect of temperature on conidial germination (b); Cultures growth at different pH of medium (c); Pathogen development on different media (d).

The greatest conidial germination occurred at 25°C, except for the isolate SL-1 (20°C). Minimal and maximal temperatures for conidial germination of all isolates were between 5 and 10°C and over 35°C, respectively (Fig. 5 b).

Germinating conidia produced 1—2, rarely 3, terminal, subterminal or basal, rarely lateral germ tubes. The average percentages of conidial germination were 9.0—19.0, 32.8—72.8, 71.4—96.4, 76.4—100.0, 36.4—75.0 and 4.0—22.4 at 10, 15, 20, 25, 30 and 35°C.

DISCUSION

Disease symptoms expressed on plum trees in Serbia were similar to those described by Harris (1988) in the United Kingdom, which were caused by *Diaporthe perniciosa*. Recently, an anamorph of this fungus (*Phomopsis perniciosa*) was described as a rot pathogen of storaged apple fruits in Serbia (Arsenijević and Gavrilović, 2005).

According to morphology, the isolates from plum trees in Serbia belonged to genus *Phomopsis*. The species from this genus were described as the bark pathogens of numerous hosts in our country (Arsenijević, 2005), but plum was not mentioned.

Distinct differences in the colony morphology (color, texture and fruiting), pathogenicity and development at different media, temperature and pH, were observed between the *Phomopsis* isolates from plum. Variability in *Diaporthe perniciosa* from plum was noted before (Caley, 1923 loc. cyt. Harris, 1988).

Diaporthe ambiqua was identified as a cause of cancer disease in apple, pear and plum rootsocks in South Africa (Smit et al., 1996). Uddin et al. (1998) showed that apple, plum and pear were susceptible to the pathogen *Phomopsis* sp., causing shoot blight of peach in Georgia, USA. They concluded that the isolates of *Phomopsis* from peach were not host-specific. *Phomopsis perniciosa* from fruits and *P. mali* from branches of plum, sour cherry and sweet cherry were isolated in Lithuania (Valiuškaite, 2002).

Further investigations should be conducted in order to identify pathogen at species level, as there are three species of genus *Phomopsis* isolated from plum.

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КАРАКТЕРИСТИКЕ ИЗОЛАТА *PHOMOPSIS* SP. ПОРЕКЛОМ СА ШЉИВЕ

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Резиме

Дванаест изолата *Phomopsis* sp. добијено је са грана и стабла шљиве (*Prunus domestica* L.) са симптомима пропадања у околини Ваљева, Љига, Коцељеве и Уба током 2004—2006. године. Проучене су морфолошке, патогене и одгајивачке одлике ових изолата.

Први симптоми уочавају се у време цветања и листања шљиве уколико се инфекција десила у близини пупољака и основе лисних петељки. У том случају листови су ситнији и хлоротични. Касније и листови и цветови некротирају и суше се. Тек формирани млади изданци обољевају, постају некротични и пропадају. На гранама и стаблу формирају се улегнуте некротичне пеге, које се брзо шире и у оквиру којих кора уздужно пуца, тако да се формирају рак-ране. Некроза се шири на дрвенасти део, где бивају захваћени спроводни судови, што доводи до увенућа и изумирања грана.

Патоген се добро развија на свим подлогама, осим на подлози од сувих шљива. Подлоге чија је pH 5,5 показале су се као најбоље за пораст патогена. Температуре од 25°C су најповољније за развој патогена и клијавост конидија.

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FREQUENCY OF TOXIGENIC *FUSARIUM* SPECIES AND FUSARIOTOXINS IN WHEAT GRAIN IN SERBIA

ABSTRACT: A total of 88 and 40 wheat samples collected immediately prior to harvest in 2005 and 2006, respectively, under different agroecological conditions, were studied in respect to the occurrence of *Fusarium* spp. and the production of fusariotoxins. The greatest number of samples was infected with species of the genera *Fusarium* (81.8 and 65.0%), and *Alternaria* (36.3 and 17.5%) with the intensity ranging from 9.4 to 84.0% in 2005 and from 23.4 to 80.6% in 2006. Out of 13 identified species belonging to the genus *Fusarium*, *F. graminearum* had the highest frequency (35.2 and 12.5%) and the intensity up to 67.2%, and 21.9%, in 2005 and 2006, respectively, followed by *F. poae*, but only in 2005 (20.4%), and *F. proliferatum* in 2006 (19.7%). The natural occurrence of mycotoxins in positive samples varied from 37 to 331 ppb for zearalenone and from 31 to 125 ppb for diacetoxyscirpenol (DAS) and T-2 toxin. The concentration of mycotoxins amounted, on average, to 133.4, 61.0 and 45.7 ppb for zearelenone, DAS and T-2 toxin, respectively.

KEY WORDS: diacetoxyscirpenol (DAS), *Fusarium* spp., T-2 toxin, wheat, zearalenone

INTRODUCTION

The *Fusarium* species predominantly found associated with Fusarium head blight (FHB) in wheat and other cereals all over Europe are *F. graminearum*, *F. avenaceum* and *F. culmorum*. Among the less frequently encountered species are several others which are less pathogenic or opportunistic, but also toxigenic. These include *F. cerealis* (Cooke) Sacc., *F. equiseti* (Corda) Sacc., *F. sporotrichioides* Sherb., *F. tricinctum* (Corda) Sacc., *F. acuminatum* Ell. & Ev., *F. subglutinans* (Wollenw. & Reinking) Nelson, *F. solani* (Mart.) Appel & Wollenw. and *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Scheldon) (Bottalico and Perrone, 2002). However, *F. poae* (Peck)

Wollenw. and *F. subglutinans* prevail in the Netherlands, Belgium, Switzerland, France and Austria, whereas *F. tricinctum, F. equiseti* and *F. acuminatum* are significantly less frequently encountered species (Mauler-Machnik and Suty, 1997; Muler and Reiman, 1997; Parry et al., 1995; Waalwijk, 2002). *F. avenaceum*, *F. poae*, *F. tricinctum* and *F. graminearum* are prevalent in Norway, Sweden and Finland (Kosiak et al., 2003; Langseth et al., 1997).

According to the global studies, approximately 25% of cereals are contaminated with mycotoxins, and this percentage is even higher if certain mycotoxins such as deoxynivalenol and fumonisin, are taken into consideration, or if unidentified mycotoxins, whose presence has been established by biological tests, are also included (Logrieco et al., 1992; Bottalico, 1998). The most frequently encountered *Fusarium* mycotoxins in FHB have proved to be deoxynivalenol (DON) and zearalenone (ZEA), produced by *F. graminearum* and *F. culmorum*. The increased production of the T-2 toxin and diacetoxyscirpenol (DAS) is attributed to sporadic epidemics of *F. sporotrichioides* and *F. poae* (Bottalico and Perrone, 2002).

The prevalence of the genus *Fusarium* has been determined on wheat grain in Serbia too, but the composition and the intensity of occurrence of certain species have been varying over years (Balaž et al., 2003; Dopuđa and Lević, 2004). *F. graminearum* or *F. culmorum* have been prevailing on wheat grain in various periods since the 1960s, although *F. graminearum* was encountered each year in higher or lower intensity, which was not the case with *F. culmorum* (Lević et al., 2004).

Although species of the genus *Fusarium* have been mainly isolated from maize and wheat grain in Serbia, studies on the natural occurrence of fusariotoxins in feed have been much more numerous (Lević et al., 2004). According to these authors the data related to the natural occurrence of zearalenone (ZEA) and T-2 toxins are the most numerous, while there is a smaller number of data related to diacetoxyscirpenol (DAS). Little work has been done in studying the natural presence of deoxynivalenol (DON); there have not been any studies on its derivates and just a few on fumosions, which is not in accordance with the widespread distribution of producers and favourable conditions for biosynthesis of these mycotoxins during certain years.

With the aim to determine the intensity of the occurrence of species of the genus *Fusarium*, and the natural appearance of fusariotoxins in wheat grain, studies were carried out on the samples collected under different agroecological conditions in Serbia in 2005, the year that favoured FHB, and in 2006, the year that did not favour FHB.

MATERIAL AND METHODS

Fungal isolation and determination

Samples of wheat spikes of 12 varieties were collected immediately prior to harvest at 22 and 10 locations in 2005 and 2006, respectively. The samples

were drawn diagonally from each plot, each sample from an area of 50 x 50 cm (0.25 m^2). Thirty two kernels from each sample were analysed in four replications. Eight kernels, each surface sterilised with 1% of sodium hypochlorite and rinsed with distilled water were placed on the water agar (WA) in 10-cm Petri dishes, and incubated under indoor conditions for seven days. Resulting colonies were purified by the procedure of obtaining single-spored cultures that were then used for the identification of *Fusarium* spp. Single-spored cultures were subcultured on the potato dextrose agar (PDA), carnation sterilised leaf-fragment agar (CLA) and synthetic nutrient agar (SNA). Cultures on the PDA were incubated in the dark at 25 ± 1°C, while cultures on the CLA and SNA were incubated under fluorescent and near ultraviolet light for 12 hours at 25 ± 1°C, and in the dark for 12 hours at 20 ± 1°C. The identification of the obtained species was done according to Nelson et al. (1983) and Burgess et al. (1994). Fungal cultures not belonging to species of the genus *Fusarium* were also grown on the three stated media and were identified according to Ellis (1971) and Watanabe (1994).

Identification of fusariotoxins

Mycotoxicological analyses were performed on the wheat samples that had been ground to powder granulation. Zearalenone (ZEA) was determined by the multitoxin method developed by Balzer et al. (1978). The type-A trichothecene (T-2 toxin and diacetoxyscirpenol-DAS) were isolated and purified by the method of Romer et al. (1978), whereas thin layer chromatography of T-2 toxin and DAS was done according to Pepeljnjak and Babić (1991).

RESULTS AND DISCUSSION

Mycopopulations on wheat grain

The mycopopulation on wheat grain differed over years in both the composition and the frequency of certain species, as it was expected due to weather conditions in these two years. Temperatures and precipitation in the wheat flowering period in 2005 favoured the FHB development, while the same period in 2006 was characterized with a much lower amount of precipitation than the long-term mean.

Out of 12 identified fungal species, species belonging to the genus *Fusarium* were isolated from the greatest number of samples in both years, 2005 and 2006 (81% and 65%, respectively) and with a high frequency (67.2% and 21.9%, respectively) (Table 1).

Tab. 1 — Frequency (%) of fungal species originating in wheat grain collected at different locations in Serbia in 2005 and 2006

No.	Species	2005			2006		
		PS[a] (%)	Frequency (%)		PS[a] (%)	Frequency (%)	
			Min	Max		Min	Max
1.	*Acremonium* spp.	7.9	0.78	2.30	10.0	1.6	14.1
2.	*Alternaria* spp.	36.3	9.4	84.0	17.5	23.4	80.6
3.	*Aspergillus* spp.	nd	nd	nd	5.0	1.6	7.8
4.	*Bipolaris* spp.	10.2	0.78	1.56	2.5	0.8	0.8
5.	*Cladosporium* spp.	6.8	0.78	3.90	2.5	0.8	0.8
6.	*Chaetomium* spp.	12,5	1.5	20.30	5.0	0.8	20.3
7.	*Epicoccum* spp.	4.5	0.78	1.56	10.0	1.6	7.8
8.	*Fusarium* spp.	81.8	0.78	67.20	65.0	0.8	21.9
9.	*Mucor* spp.	nd	nd	nd	7.5	1.6	7.8
10.	*Penicillium* spp.	nd	nd	nd	12.5	2.5	3.1
11.	*Phoma* spp.	2.2	0.78	1.56	nd	nd	nd
12.	*Trichoderma* spp.	nd	nd	nd	2.5	3.9	3.9

[a] Positive sample (PS) — percentage was estimated on the basis of samples in which the fungus had been identified
nd — no data

Although percentage of species of the genus *Alternaria* was not high (36% and 17.5%), the frequency (84% and 80.65%) was. Other pathogenic and toxigenic fungal species, such as *Penicillium* spp. and *Aspergillus* spp., were isolated in 2006 in the amount of 12.5%, i.e. 5% of samples with the intensity of 3.1%, i.e. 7.8%. Besides the stated species, *Chaetomium* spp. and *Acremonium* spp. (up to 20.3% and 14.1%, respectively) prevailed in certain samples.

Previous studies (Milošević et al.; 1995; Dopuđa and Lević, 2004) show a similar frequency of all three fungal genera on wheat grain in Serbia. Balaž et al. (2003) state a significantly lower frequency of *Fusarium* spp. on wheat grain (11.3—20.6%), as well as of *Asspergillus* spp. (0—1.2%) and *Penicillium* spp. (0—8.9%). According to these authors species of the genus *Alternaria* spp. (up to 86.8%) prevail.

The occurrence of species of the genus Fusarium on wheat grain

F. graminearum was a prevalent species of the genus *Fusarium*, and was isolated from the greatest number of samples in 2005 and 2006 (35.2% and 12.5%, respectively) with a very high frequency (up to 67.2%) (Table 2). Bočarov-Stančić (1996) also states that this species was prevalent on wheat grain harvested in semi-humid year of 1991. In contrast to these results, Stojanović et al. (2002) stated that *F. oxysporum* (19.44—25%) was a prevalent species, although they determined the presence of *F. graminearum* in wheat at all observed locations with the frequency of 2.78—15.38%.

Tab. 2 — Frequency of fungal species originating in wheat grain collected at different locations in Serbia in 2005 and 2006

No.	Species	2005			2006		
		PS[a] (%)	Frequency (%)		PS[a] (%)	Frequency (%)	
			Min	Max		Min	Max
1.	*F. arthrosporioides*	1.1	0.8	0.8	7.5	0.8	5.5
2.	*F. avenaceum*	2.2	0.8	3.3	5.0	0.8	3.1
3.	*F. equiseti*	2.2	0.8	1.6	5.0	0.8	0.8
4.	*F. culmorum*	0.0	0.0	0.0	2.5	0.8	0.8
5.	*F. graminearum*	35.2	7.1	67.2	12.5	3.9	21.9
6.	*F. poae*	20.4	0.8	6.3	7.5	0.8	2.4
7.	*F. proliferatum*	3.4	0.8	7.8	20.0	0.3	19.7
8.	*F. semitectum*	2.2	0.8	0.8	nd	nd	nd
9.	*F. sporotrichioides*	2.2	0.8	0.8	5.0	0.8	0.8
10.	*F. subglutinans*	4.4	0.8	3.2	2.5	0.8	0.8
11.	*F. tricinctum*	1.1	2.3	2.3	2.5	2.3	2.3
12.	*F. verticillioides*	7.0	0.8	3.2	7.5	0.8	2.4
13.	*Fusarium* spp.	33.0	0.1	0.2	7.5	2.4	3.9

[a] Positive samples (PS) — percentage was estimated on the basis of samples in which the fungus had been identified
nd — no data

The species *F. poae* was isolated in a higher percentage of samples (20.4%) and with a higher frequency (up to 6.3%) in the first, than in the second year of the investigation.

If the occurrence of the remaining *Fusarium* spp. is observed, the difference over years is perceived (Table 2). The species *F. proliferatum* was isolated in the range of 0.8%—7.8%, i.e. 0.3—19.7% in 2005, i.e. 2006, respectively. The number of samples infected with *F. verticillioides* was approximately equal (7.0% and 7.5%) in both years, but the intensity was higher in 2005 than in 2006 (3.2% vs. 2.4%). The occurrence of *F. arthrosporioides, F. avenaceum, F. culmorum*, *F. equiseti, F. sporotrichioides, F. subglutinans* and *F. tricinctum* was determined in the range from 0.8% to 3.2%.

Our results are in accordance with the results obtained by D o p u đ a and L e v i ć (2004) who studied the mycobiot of wheat grain during 2002 and 2003 especially in regard to varying of certain species during the years of the investigation. These authors determined that species of the genus *Fusarium* prevalent in 2002 were *F. graminearum* (up to 61%) and *F. verticillioides* (up to 10%), and in 2003 were *F. poae* (up to 12%) and *F. prolifearatum* (5%), whereas the prevalence of remaining *Fusarium* spp. ranged from 4—5% (*F. sporotrichioides*) to 1—3% (*F. acuminatum, F. avenaceum, F. culmorum*, *F. equiseti, F. oxysporum*, *F. tricinctum*).

Natural occurrence of fusariotoxins in wheat grain

The natural occurrence of zearalenone and trichothecene (DAS, T-2 toxin) in wheat grain varied in dependence on a variety and agroecological conditions of locations at which wheat was grown in 2005 (Table 3).

Tab. 3 — Natural occurrence of fusariotoxins in grain of different wheat varieties grown under various agroecological conditions in Serbia in 2005

Sample code	Variety	Location	Mycotoxin contents (ppb)		
			ZEA	Trichothecenes	
				DAS	T-2
234	Dragana	Pirot	74	31	31
164	Evropa	Sombor	0	75	50
167	Evropa	Gredetin	0	0	0
216	Evropa	Krsmanovci	0	37	0
208	Evropa-90	Bač	147	0	0
172	Evropa-90	Kikinda	331	0	0
162	Evropa-90	Sombor	37	0	0
169	Evropa-90	Lipnički Šor	0	62	31
212	Evropa-90	Veliki Radinci	0	0	62
222	Kg-20	Zobnatica	0	0	0
207	Kg-20	Zemun	184	0	0
210	Mina	Bački Petrovac	258	0	0
151	Pahulja	Zemun Polje	74	0	0
219	Pesma	Maglić	0	0	0
223	Pesma	Zobnatica	37	0	0
170	Pobeda	Runjani	147	0	0
150	Pobeda	Zemun Polje	0	31	31
175	Pobeda	Kovin	184	0	0
213	Pobeda	Nova Pazova	0	0	31
214	Pobeda	Stari Banovci	74	0	0
215	Pobeda	Sremska Mitrovica	37	0	0
155	Renesansa	Zemun Polje	0	125	50
163	Renesansa	Sombor	147	0	0
174	Renesansa	Kikinda	138	0	25
209	Renesansa	Rimski Šančevi	110	0	31
211	Renesansa	Veliki Radinci	147	62	0
217	Renesansa	Zobnatica	37	0	0
220	Renesansa	Despotovac	110	0	31
165	Simonida	Loznica	0	125	125
153	Takovčanka	Zemun Polje	138	1	50
152	Žitka	Zemun Polje	257	0	0

Zearalenone was determined in 20 samples in the range from 37 to 331 ppb or in 64.52% of the samples with the average for positive samples of 133.4 ppb. The presence of zearalenone was determined in three most cultivated varieties in Serbia (Evropa-90, Pobeda and Renesansa), in the majority of studied locations with the average for positive samples of 171.67, 110.50 and 114.83 ppb. Contamination in a high number of wheat grain samples (78%) with ZEA (160—500 ppb) was also recorded by Stojanović et al. (2002),

who analysed the three varieties (Evropa 90, Kg56S and Nora) in two trial spots. Mesterhazy (1997) states that a plant genotype is of a great importance in the accumulation of toxins and that generally less total toxins, are produced in more resistant cultivars.

The presence of zearalenone is not surprising, as *Fusarium* spp. (Tabela 2) were isolated from the analysed samples. According to Marasas et al. (1984) these species can biosynthesise the same fusariotoxin under certain ecological conditions. This statement is also confirmed by our previous *in vitro* studies on the ZEA production in the *F. oxysporum* isolates from which grain contaminated with the same mycotoxin in the amount of 1540 ppb, Bočarov-Stančić et al. (2003).

Out of 31 wheat grain samples, DAS was determined in nine samples in the range from 31 to 125 ppb, or in 29.03% of the samples with the average of 61.0 ppb for positive samples (Table 3). The T-2 toxin was determined in the same range as DAS in 12 samples, but its presence was greater (38.72%) and while the average for positive samples was smaller (45.7 ppb). DAS and T-2 toxin were simultaneously identified in six samples (19.35%), and individually in two, i.e. six samples, respectively.

The presence of these trichothecenes is not surprising considering that the species *F. avenaceum, F. equiseti, F. culmorum, F. sporotrichioides* and *F. tricinctum* (Table 2) isolated from wheat, harvested in 2005 and 2006, can biosynthesise type-A trichothecenes under laboratory conditions, as it was concluded in our previous study Bocarov-Stančić et al. (1986).

Gained data on the concentrations of zearalenone and T-2 toxin in wheat grain in 2005 were lower than those found in the literature. Stojanović (1999) determined the natural occurrence of zerelenone in 87.5% of wheat grain samples and with the concentration up to 500 ppb, while Bočarov-Stančić et al. (1998) detected T-2 toxins in 33 of the samples with the concentration in the range from 500 to 750 ppb.

CONCLUSION

The two-year studies (2005—2006) on the mycobiot of wheat grain show that *Fusarium* spp. are widespread and prevalent fungi in Serbia (81.8—65.0%), but that species of the genus *Alternaria* (84.0—80.6%) are more frequent.

F. graminearum is predominant *Fusarium* species that prevailed in wheat grain in 2005 and 2006, not only by its distribution (35.2% and 12.5%, respectively), but also by its intensity (67.2% and 21.9%, respectively). *F. poae* was more frequent in 2005 (20.4%) than in 2006 (7.5%), but the intensity did not exceed 6.3%. The species *F. proliferatum* was isolated in the range from 0.8% to 7.8%, i.e. from 0.3 to 19.7%, in 2005, and 2006, respectively. *F. verticillioides* was equally distributed during the both years (7.0% and 7.5%), but the intensity was somewhat higher in 2005 than in 2006 (3.2% vs. 2.4%). The distribution and intensity of the occurrence of remaining 10 identified species of the genus *Fusarium* varied during the years of investigation.

The analysis of 31 wheat samples, collected in 2005, shows that the natural occurrence of zearalenone, DAS and T-2 toxin was determined in 20 (64.52%), 9 (29.03%) and 12 samples (38.72%), respectively. The greatest range and concentration (37—331 ppb) of determined mycotoxins were detected in zearalenone (133.4 ppb on average). This is in accordance with the distribution and the intensity of the occurrence of *F. graminearum*, which is one of the most important producers of this mycotoxin. Although 11 species of the genus *Fusarium*, synthetising the type-A trichothecenes (DAS and T-2 toxin), were identified in this study (in 2005), the natural occurrence of these mycotoxins varied from 31 to 125 ppb. These results point out that the species of the genus *Fusarium* originating in wheat from Serbia have a low potential for synthesis of these fusariotoxins.

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УЧЕСТАЛОСТ ТОКСИГЕНИХ ВРСТА *FUSARIUM* И ФУЗАРИОТОКСИНА У ЗРНУ ПШЕНИЦЕ У СРБИЈИ

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Резиме

Осамдесет осам и 40 узорака пшенице, прикупљених непосредно пред жетву у 2005. и 2006. години у различитим агроеколошким условима у Србији, проучавани су ради праћења појаве *Fusarium* spp. и стварања фузариотоксина. Највећи број узорака био је заражен врстама рода *Fusarium* (81,8 и 65,0%), а затим са *Alternaria* spp. (36,3 и 17,5%) са интензитетом 9,4—84,0% у 2005. и 23,4—80,6% у 2006. години. Од укупно 13 идентификованих врста из рода *Fusarium* најучесталија је била *F. graminearum* (35,2 и 12,5%) са интензитетом до 67,2% у 2005. и до 21,9% у 2006. години, а затим *F. poae*, али само у 2005. години (20,4%), и *F. proliferatum* у 2006. години (19.7%). Природна појава микотоксина у позитивним узорцима је варирала од 37 до 331 ppb за зеараленон и од 31 до 125 ppb за диацетоксисцирпенол (ДАС) и Т-2 токсин. У просеку, концентрација микотоксина је била 133,4 ppb за зеарелонон, 61,0 ppb за ДАС и 45,7 ppb за Т-2 токсин.

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PATHOGENICITY AND DIVERSITY OF VEGETATIVE COMPATIBILITY OF *FUSARIUM VERTICILLIOIDES*

ABSTRACT: Pathogenicity of 10 *Fusarium verticillioides* isolates, originated from grain of wheat (five isolates) and maize (five isolates), were studied under greenhouse conditions. Based on different parameters of the pathogenicity estimate (a scale for % of nonemerged plants, % of survived plants, plant vigour — the growth and dry weight of roots and epicotyls and disease severity) it was determined that all *F. verticillioides* isolates expressed a different degree of pathogenicity. According to % of nonemerged plants six, three and one *F. verticillioides* isolates expressed low, moderate and high deegre of pathogenicity, respectively. All *F. verticillioides* isolates reduced the plant survival rate and vigour, while the disease severity ranged from 2.0 to 3.54. Two types of *nit* mutants, *nit1* and NitM, were obtained by the use of the method of vegetative compatibility. The frequency of *nit1* mutants was greater (58.79%) than the frequency of NitM mutants (5.77%). A total of 10 vegetative compatibility groups (VCGs) of *F. verticillioides* were established in the complementation tests. These results point out to a high genetic diversity of *F. verticillioides* population.

KEY WORDS: *Fusarium verticillioides*, pathogenicity, vegetative compatibility

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (syn. *Fusarium moniliforme* Scheldon) is a widely distributed pathogen of maize (*Zea mays* L.) and many other plant species. In Serbia, *F. verticillioides*, as a pathogen of grain, was identified on maize, wheat and sorghum up to 77.8% (Lević et al., 1997, 2003), 10% (Dopuđa and Lević, 2004) and 7.5% (Lević et al., 2006), respectively.

The epidemiological studies show that *F. verticillioides* has one comparative advantage over other species of the genus *Fusarium*, especially in relation to *F. graminearum* Schwabe, as it requires a greater range of temperatures (Reid et al., 1999) and humidity (Schneider and Pendery 1983) for

its development, hence the competitiveness of the fungus will not change in different environments. It is typical of this species to colonise the plant tissue and to remain at the dormant stage or at the endophytic stage, as long as the tissue is healthy and active (Munkvold and Desjardins, 1997).

It is difficult to discuss with certainty the role of *F. verticillioides* in the etiology of seedling diseases, as seedling infections and the disease development are produced by the seed affected by a disease or a contaminated soil, and depend on the temperature during the maize growing period (Kommedahl and Windels, 1981). *F. verticillioides* does not affect seed germination at the endophytic stage, but it affects the thickness, height, weight and leaf length of seedlings developed from infected seeds (Yates et al., 1997). On the other hand, some strains of this fungus can even stimulate an earlier growth of seedlings.

Lević (2000) established that the frequency of occurrence of *Fusarium* species is not always correlated with their effects on seed germination. According to this author, the following species are most often isolated from maize grain: *F. verticillioides* (50.2%), *F. subglutinans* (Wollenw. & Reinking) Nelson (45.6%) and then *F. proliferatum* (Matsushima) Nirenberg (7.9%). However, germination of seeds infected with *F. subglutinans*, *F. proliferatum* and *F. verticillioides* amounted to 15.3%, 23.4% and 32.6%, respectively.

The characterization of *F. verticillioides* isolates can be done on the basis of the seedling pathogenicity test or vegetative compatibility, since it was determined that isolates of the similar pathogenicity belonged to the same vegetative compatibility group (VCG) (Klein and Correll, 2001). Therefore, if a rapid method of the VCG identification is developed and rapid analyses of the population strain evaluation are provided, then the VCG pathogen strain identification can replace the pathogenicity test, which is time — consuming and requires specific, control led conditions, depending on a plant species.

Considering the economic importance of *F. verticillioides*, pathogenicity of *F. verticillioides* isolates, originating in maize and wheat grown at different locations in Serbia, to maize seedlings and their vegetative compatibility were observed in this study.

MATERIAL AND METHODS

Fungal isolates

Ten isolates of *F. verticillioides* were used to perform pathogenicity and vegetative compatibility tests. Five isolates originated from grain of commercial maize hybrids grown in the vicinity of Belgrade—Zemun, and five isolates originated from grain of wheat varieties grown at different locations in Serbia (Table 1). Isolates were identified as *F. verticillioides* using of the procedure outlined by Nelson et al. (1983) and Burgess et al. (1994).

Tab. 1 — *F. verticilioides* isolates tested for pathogenicity to maize seedlings under greenhouse conditions and vegetative compatibility

No.	Isolate	Origin	Host
1.	MGA-7	Belgrade—Zemun	Commercial maize hybrid
2.	MGD-4	Belgrade—Zemun	Commercial maize hybrid
3.	MGE-5	Belgrade—Zemun	Commercial maize hybrid
4.	MGG-13	Belgrade—Zemun	Commercial maize hybrid
5.	MGI-1	Belgrade—Zemun	Commercial maize hybrid
6.	MRIZP-201	Inđija	Evropa 90 (wheat variety)
7.	MRIZP-237	Inđija	Pobeda (wheat variety)
8.	MRIZP-570	Ruma	Renesansa (wheat variety)
9.	MRIZP-748	Loznica	Simonida (wheat variety)
10.	MRIZP-830	Sombor	Evropa 90 (wheat variety)

Selected cultures were initiated from single conidia and stored on PDA slants at 4°C, until use for the pathogenicity test and vegetative compatibility.

Pathogenicity test with maize seedlings

An insignificantly modified method described by Molot and Simone (1967) was followed for estimations of pathogenicity of *F. verticillioides* isolates. Petri dishes with the two-layer filter paper, instead of flasks, and sterile quartz sand, instead of soil, were used for the development of the fungus and artificial inoculation of seeds. A total of 45 maize seeds, surface-sterilised with sodium hypochlorite per isolate were inoculated in the sterile Petri dishes (ø 100 mm) with 30 ml of spore suspension (2—3 x 10^6 spore ml^{-1}). The spore suspension was prepared from 7—10 old isolates cultured on the PDA at room temperature. Inoculated and non-inoculated (control) maize seeds were incubated at 22°C for two days and at 10°C for three days, and then planted into flats (40 x 18 x 16 cm) with sterile quartz sand, watered and incubated at 24—26°C.

Maize seeds were inoculated for two weeks and the following was determined: degree of pathogenicity, length (cm) and dry weight (g) of seedling roots and epicotyls. In this study, the degree of pathogenicity was defined on the basis of nonemerged plants (%), which was an outcome of seeds that had never germinated, and germinated seeds with completely rotted shoots. According to this parameter, the isolates were classified into five categories based on the scale described by Maçka (1989) (Table 2).

Tab. 2 — The scale for the estimation of pathogenicity of *F. verticillioides* isolates

Percentage of nonemerged plants	Degree of pathogenicity
0—10%	not pathogenic
11—20%	very low pathogenic
21—40%	low pathogenic
41—60%	moderate pathogenic
61—80%	high pathogenic
81—100%	very high pathogenic

Disease severity was also used as a measurement of pathogenicity of isolates, and was rated by a six-class scale, in which 0 = healthy root and epicotyl, and 5 = nongerminated seed, or completely rotted root and shoot. The length of each seedling from the seed attachment site to the top of the longest root and leaves was measured (cm). The detached root and epicotyl per replicate were dried at 60°C for 24 hours and then, their weights (g) were measured. Means were compared by Duncan's multiple range test.

Vegetative compatibility groups

Methods described by Correll et al. (1987) and Kedera et al. (1994) were used to isolate and characterize *nit*-mutants and their mutual pairing in order to determine vegetative compatibility of the studied *F. verticillioides* isolates. The excised pieces of mycelia were planted on the minimum medium (a basal medium amended with 30 g $KClO_3$, 2 g $NaNO_3$, and 1.6 g L-asparagine) for the selection of mutants (sectors). The basal medium contains 1.0 g KH_2PO_4; 0.5 g $MgSO_4$ x $7H_2O$; 0.5 g KCl; 10 mg $FeSO_4$ x $7H_2O$; 0.2 ml sterile solution of microelemenats; 30.0 g of sucrose; 20.0 g of Difco agar; 1000 ml of distilled water. Pieces of hyphae and loose growing sectors were transferred to the basal medium with different nitrogen sources ($NaNO_3$, $NaNO_2$ and hypoxantine) in order to determine the type of the *nit*-mutant on the basis of a phenotype (Puhalla, 1985).

Complementary *nit1* and NitM mutants from each of 10 *F. vertillicioides* isolates were paired on the minimum medium (MM) in all possible combinations to perform complementation tests among the isolates. The *nit* mutants grew very sparsely across the medium, but complementation of auxotrophic mutants was indicated by a line of a vigorous growth where the mutants interacted.

RESULTS AND DISCUSSION

Pathogenicity

All observed *F. verticillioides* isolates affected the survival rate and vigour of plants. Out of 10 *F. verticillioides* isolates tested under greenhouse conditions six, three and one isolates were low (26.67—40.0% of nonemerged plants), moderate (48.87—55.53% of nonemerged plants) and high (62.20% of nonemerged plants) pathogenic (Table 3). The isolate MGG-13, originated from maize grain, was estimated as high pathogenic as it reduced germination by 62.20%. The same isolate was significantly more pathogenic than the remaining isolates, as determined disease severity (3.69) was the highest and the survival rate (37.80%) and plant vigour were the lowest (Table 4).

Tab. 3 — *F. verticillioides* isolates classified on the basis of a percentage of nonemerged plants

Isolate	Nonemerged plants (%)	Degree of pathogenicity
MGA-7	31.13	low pathogenic
MGD-4	40.00	low pathogenic
MGE-5	26.67	low pathogenic
MGG-13	62.20	high pathogenic
MGI-1	55.53	moderate pathogenic
MRIZP-201	48.87	moderate pathogenic
MRIZP-237	37.80	low pathogenic
MRIZP-570	51.13	moderate pathogenic
MRIZP-748	40.00	low pathogenic
MRIZP-830	35.54	low pathogenic
Control	4.47	

The survival rate of seedlings developed from inoculated seeds with different isolates of *F. verticillioides* varied from 37.80% (MGG-13) to 73.33% (MGE-5), which was significantly lower than in the control (95.53%) (Table 4). The observed isolates affected the reduction of the root growth in comparison with the epicotyl growth. The isolate MGI-1, originated from maize, as well as, isolates MRIZP-201 and MRIZP-570, originated from wheat, expressed similar pathogenicity, which was particularly established on the basis of the root growth. The isolate MGD-4 showed peculiar behaviour, as disease severity caused to seedlings was high (3.18), which made it similar to the high pathogenic isolate (MGG-13), but due to a relatively high survival rate of plants (60.00%), it was estimated as low pathogenic (Table 4).

Tab. 4 — Effect of *F. verticillioides* isolates on maize seedlings growing from artificially infected seeds under greenhouse conditions

No.	Isolate	Plant survival* (%)	Plant vigour*				Disease severity*
			Length (cm)		Dry weight (g)		
			Root	Epicotyl	Root	Epicotyl	
1.	MGA-7	68.87^{bc}	18.73^{bcd}	14.71^{bc}	2.10^{bcd}	0.800^{abc}	2.66^{bcd}
2.	MGD-4	60.00^{bcd}	15.87^{cd}	12.14^{bc}	1.37^{cde}	0.500^{bc}	3.18^{abc}
3.	MGE-5	73.33^{ab}	23.74^{b}	16.86^{ab}	2.23^{bc}	0.767^{abc}	2.00^{d}
4.	MGG-13	37.80^{d}	11.42^{d}	8.98^{c}	1.10^{e}	0.400^{bc}	3.69^{a}
5.	MGI-1	44.47^{cd}	13.79^{d}	9.87^{c}	1.33^{de}	0.367^{c}	3.41^{abc}
6.	MRIZP-201	51.13^{bcd}	11.26^{d}	12.15^{bc}	1.00^{e}	0.833^{ab}	3.54^{ab}
7.	MRIZP-237	62.20^{bcd}	23.22^{bc}	18.26^{ab}	2.00^{bcd}	0.833^{ab}	2.54^{bcd}
8.	MRIZP-570	48.87^{bcd}	15.34^{d}	13.52^{bc}	1.23^{de}	$0.56b^{c}$	3.02^{abc}
9.	MRIZP-748	60.00^{bcd}	17.77^{bcd}	13.41^{bc}	2.63^{b}	0.600^{bc}	2.40^{cd}
10.	MRIZP-830	64.46^{bcd}	13.13^{d}	13.18^{bc}	1.80^{bcde}	0.633^{abc}	2.48^{cd}
	Average	57.64	16.43	13.30	1.67	0.629	2.85
11.	Control	95.53^{a}	31.62^{a}	22.49^{a}	3.97^{a}	1.067^{a}	0.10^{e}
	LSD (0.05)	3.574	6.864	5.411	0.800	0.388	0.898
	LSD (0.01)	4.876	9.364	7.382	1.092	0.529	1.225

* Values of column followed by the same letter(s) are not significantly different (P = 0.05) according to Duncan's multiple range test.

Our results on pathogenicity of *F. verticillioides* are in accordance with the results obtained by Desjardins et al. (1995) and Munkvold and Carlton (1997).

Vegetative compatibility

Mutants *nit1* and NitM, with prevalence of *nit1* (58.79%) over NitM (5.77%) (Table 5), were isolated from the observed isolates of *F. verticillioides*. According to the literature data (Klittich and Leslie, 1988), the frequency of mutants *nit1* is higher than the frequency of other types of *nit* mutants.

Tab. 5 — Frequency of *nit1* and NitM mutants in the studied *F. verticillioides* isolates

Isolate	*nit1* (%)	NitM (%)
MGA-7	92.86	4.29
MGD-4	70.00	15.00
MGE-5	55.00	5.00
MGG-13	40.00	5.00
MGI-1	65.00	5.00
MRIZP-201	45.00	6.67
MRIZP-237	62.50	1.25
MRIZP-570	50.00	7.14
MRIZP-748	67.50	5.00
MRIZP-830	40.00	3.33
Average	58.79	5.77

Ten vegetative compatible groups (VCGs) of *F. verticillioides* were established on the basis of the complementation test among the isolates in all possible combinations. These results point out to a high genetic diversity of the population of this fungus pathogenic to maize. Similar results were stated by Chulze et al. (2000).

CONCLUSION

All studied *F. verticillioides* isolates originated from wheat (five isolates) and maize grain (five isolates) expressed pathogenicity to maize seedlings. According to the percentage of nonemerged plants, it was established that six, three and one isolates expressed low, moderate and high pathogenicity. The survival rate (%) and vigour (growth and dry weight of roots and epicotyls) of plants that were developed from inoculated seeds, were significantly reduced (approximately two times) in comparison with the control. There was a tendency for isolates from different hosts to have similar values for pathogenicity. These results are of a practical importance from the aspects of maize and wheat crop rotation and for the success of breeding for resistance to *F. verticillioides*.

The analysis of the results on pathogenicity obtained on the basis of the scale for the % nonemerged plants, plant survival rate (%), vigour (growth and dry weight of roots and epicotyls) and disease severity, shows a concurrence in defining moderate and high pathogenicity of isolates, while there was a certain nonconformance among these results in relation to low pathogenicity defining. Nevertheless, a selection of parameters, such as the scale for % of nonemerged plants, is a simple and good choice for the characterisation of pathogenicity degree of all *F. verticillioides* isolates.

Two types of *nit* mutants, *nit1* and NitM, were obtained by the use of the method of vegetative compatibility. The frequency of *nit1* mutants was greater (58.79%) than the frequency of NitM mutants (5.77%). A total of 10 VCGs of *F. verticillioides* were determined in the complementation tests. This number of vegetative compatible groups indicates a high genetic diversity of the observed *F. verticillioides* population.

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ПАТОГЕНОСТ И ДИВЕРЗИТЕТ ВЕГЕТАТИВНЕ КОМПАТИБИЛНОСТИ *FUSARIUM VERTICILLIOIDES*

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Резиме

Патогеност 10 изолата *F. verticillioides*, пореклом из зрна пшенице (5 изолата) и кукуруза (5 изолата), проучавана је у условима стакленика. На основу различитих параметара оцене патогености (скала за % неизниклих биљака, % преживелих биљака, вигора биљака — пораст и сува тежина корена и епикотила, и интензитета болести) установљено је да су сви испитивани изолати *F. verticillioides* испољили различит степен патогености. Према % неизниклих биљака 6 изолата испољило је ниску, 3 изолата средњу и један изолат *F. verticillioides* високу патогеност. Сви испитивани изолати *F. verticillioides* су проузроковали смањено преживљавање и вигор биљака, са интензитетом болести од 2.0 до 3.54. Применом методе вегетативне компатибилности изоловане су две врсте *nit* мутаната, *nit1* и NitM. Учесталост мутаната *nit1* је била већа (58.79%) у односу на NitM мутанте (5.77%). У комплементарним тестовима установљено је 10 вегетативно компатибилних група (VCG) *F. verticillioides* што указује на висок генетички диверзитет популације овог патогена.

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THE TOXIGENIC POTENTIAL OF *FUSARIUM POAE* ORIGINATED FROM WHEAT

ABSTRACT: Eleven isolates of *F. poae*, originated from wheat grain at 9 locations mainly in Vojvodina, were encompassed by the present study. The greatest number of samples was collected in 2005, in which the climatic conditions favoured a more intensive occurrence of *Fusarium* ear blight of wheat. In order to determine toxicological potential of this species, cultures of the selected isolates were grown in liquid media (GPY and SPY) on a rotary shaker (180 revolutions min^{-1}), at room temperature (21—26°C) for three days. Crude toxins were isolated from liquid culture filtrates of isolates by the use of ethyl acetate, while quantification of mycotoxins was done by the thin layer chromatography method. A liquid culture of the isolate GZ-LES (*F. graminearum*) was used as a control for the evaluation of the zearalenone biosynthesis potential. On the other hand, the liquid culture of the isolate KF-38/1 (*F. sporotrichioides*) was used as a control for both type-A trichothecenes (T-2 toxin and diacetoxyscirpenol — DAS).

The obtained results show that *F. poae*, in contrast to *F. graminearum*, has no potential for the zearalenone biosynthesis. The presence of DAS was determined only in one isolate of *F. poae* (MRIZP-666), and in the control isolate of *F. sporotrichioides* (KF-38/1/R), that were grown in the GPY liquid medium. The T-2 toxin was detected in the isolate MRIZP-666, grown in both media, and in the isolates MRIZP-37 and MRIZP-860, cultured in the GPY and SPY liquid medium, respectively. The control culture KF-38/1/R (*F. sporotrichioides*) produced the T-2 toxin at the concentration of 4,000 μg L^{-1}. According to the gained information, it can be concluded that the potential of *F. poae* for the type-A trichothecene biosynthesis was low, as the concentration of DAS or T-2 toxin did not exceed 80 μg L^{-1} or 240 μg L^{-1}, respectively.

KEY WORDS: diacetoxyscirpenol, *F. poae*, *in vitro* biosynthesis, T-2 toxin, wheat

INTRODUCTION

The occurrence of *Fusarium* head blight of stronger intensity in wheat was recorded not only in Europe, including Serbia (Lević et al., 2004), but worldwide during the last decade of the 20th century. The disease resulted in a

significant economic damage, due to the grain yield reduction, in a quality loss, due to shrunk grain, and in contamination with mycotoxins.

An enormous number of species of the genus *Fusarium*, including *F. poae* (Peck) Wollenw, was isolated from *Fusarium* damaged wheat grain (Waalwijk, 2002). Although a high percentage of *Fusarium poae* was isolated from the grain of wheat, barley and oats in certain years, its role in the aetiology of *Fusarium* head blight had not been yet completely clarified (Kestemont et al., 2002; Kryuchkova et al., 2002; Lew et al., 2001; Hornok and Toth, 2001; Hysek et al., 2000).

It is most often stated that only *F. graminearum* Schwabe and *F. culmorum* (W. G. Smith) Sacc. are important for the aetiology of *Fusarium* head blight (Teich, 1989). Parry et al. (1995) and Waalwijk (2002) have an opinion that *Fusarium* head blight could be caused by four species — two previously stated, *F. avenaceum* (Fr.) Sacc. and *F. poae*. Furthermore, in England, *F. poae* has been very often isolated from chaff spots, although the connection between its occurrence and head blight has not been confirmed (Nicholson et al., 1997).

The species *F. poae* is important from the toxicological aspect as it biosynthesises a great number of mycotoxins, such as: diacetoxyscirpenol (DAS), monoacetoxyscirpenol, nivalenol, fusarenone-X, T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, deoxysinivalenol, neosolaniol, beauvericin and enniatines (BEA) (Bottalico, 1998; Torp and Langseth, 1999; Nicholson et al., 2004; Chełkowski et al., 2007). Out of the stated mycotoxins, type-A trichothecenes (T-2 toxin and DAS), produced by these species, are the most important.

In Italy, it was determined that two main *Fusarium* species isolated from wheat, *F. poae* and *F. sporotrichioides* Sherb., biosynthesised the T-2 toxin and DAS in the amount that ranged from 6,200 to 120,000 ppb (Criseo et al., 1989). According to the results obtained by Gagkaeva et al. (2006), *F. poae* synthesises more DAS than *F. sporotrichioides*, 400 ng mL^{-1} vs. 62 ng mL^{-1}, while *F. sporotrichioides* produces more T-2 toxins (75,860 ng mL^{-1}) than *F. poae* does (158 ng mL^{-1}). Besides the type-A trichothecenes, it was determined that the species *F. poae*, originated from *Fusarium* damaged wheat grain in Canada, produced the type-B trichothecenes (Wong et al., 1995). In Poland, *F. poae* was determined in 35% of wheat samples, while chemical analyses showed no presence of the type-A trichothecene (T-2 toxins) but just the presence of the type-B trichothecenes (Grabarkiewicz-Szczesna et al., 2001). Contrary to these authors, Kosiak et al. (2003) found out that the same species in Norway was the best producer of the type-A trichothecenes (T-2 and HT-2 toxins). A somewhat lower frequency of *F. poae* on wheat grain (13.5%) was recorded by Muthomi et al. (2006) in Kenya. These authors determined only the presence of T-2 toxin and zearalenone in the same substrate.

According to the mycological studies carried out in Serbia by Bočarov-Stančić et al. (1991), only the type-A trichothecene (T-2 toxin), in the amount of 310—780 ppb, was present in wheat grain, originated from a macro-trail in Klek (Vojvodina). Škrinjar et al. (1996) detected zearaleno-

ne just in one sample of wheat, harvested during 1994. In both cases, not a single determined *Fusarium* spp. belonged to *F. poae*. Dopuđa and Lević (2004) and Stojanović et al. (2005) determined the presence of *F. poae* in 12% and 7% of the samples, respectively, but the authors did not present data on their toxigenicity. The results of Dopuđa and Lević (2004), obtained on the basis of studies performed on the samples of five wheat cultivars harvested during 2002 and 2003 at four locations, show that *F. poae* occurred more intensively in the year (2003) in which the frequency of *F. graminearm* was lower, and vice versa. Similar results were achieved by Schaafsma (1999) who stated that the presence of *F. poae*, *F. sporotrichioides* and *F. avenaceum* on wheat grain in Canada was reduced during the years with more intensive occurrence of *F. graminearum*.

Our previous studies performed on *Fusarium* isolates originated from wheat (harvested during 1982 and 1984), cultured on the natural solid substrate, showed that 22% of the observed *Fusarium* spp. biosynthesised DAS, and even 44% produced T-2 toxin, although not a single one belonged to the species *F. poae* (Bočarov-Stančić et al., 1986).

The potential of *F. poae* to biosynthesise one group of fusariotoxins — the type-A trichothecenes (T-2 toxin and DAS), and zearalenone (ZEA) — was observed under *in vitro* conditions in the present study. In our country according to the literature data, a little attention was paid, to the study of the toxicological profile of this species, considering its distribution and toxicogenic properties.

MATERIAL AND METHODS

Cultures of *Fusarium poae.* Isolates of the fungus *F. poae* were obtained from grain samples of wheat, collected in harvest at 20 locations in 2003, 2005 and 2006. A total of 103 isolates of this species were isolated and determined. Out of these 103, 11 isolates, designated as MRIZP-32, MRIZP-37, MRIZP-664, MRIZP-665, MRIZP-666, MRIZP-833, MRIZP-834, MRIZP-860, MRIZP-879, MRIZP-890 and MRIZP-897, were selected for toxicological studies.

Each sample of 32 wheat kernels (four replicates) was analysed. Eight surface sterilised kernels were placed on each water agar (WA) in the 10-cm Petri dishes, and incubated under indoor conditions for seven days. Resulting colonies were purified by the procedure of obtaining monosporous cultures that were then used for the identification of *Fusarium* species. Monosporous cultures were subcultured on the potato dextrose agar (PDA), carnation sterilised leaf-fragment agar (CLA), and synthetic nutrient agar (SNA). Cultures on the PDA were incubated in the dark at 25 ± 1°C, while cultures on the CLA and SNA were incubated under fluorescent and near ultraviolet light for 12 hours at 25 ± 1°C, and in the dark for 12 hours at 20 ± 1°C. The identification of the obtained species was done after Nelson et al. (1983) and Burgess et al. (1994). The identified isolates were stored on the PDA, CLA and

SNA slants in ampoules at +4°C, until studying of their toxicological potential in the liquid culture.

Control isolates. The following species for which it was previously determined (B o č a r o v - S t a n č i ć, unpublished data) to have the capacity to biosynthesise fusariotoxins five weeks after the cultivation on wet maize grain at 30°C, were chosen as the control isolates: a) *F. graminearum*, the isolate GZ-LES that synthesises ZEA and DON at the concentration of 4,420 ppb and 465,900 ppb, respectively; b) *F. sporotrichioides*, KF-38/1/R, a re-isolate of the original strain that biosynthesises T-2 toxin and DAS at the concentration of 2,400 ppb and 1,600 ppb, respectively.

Medium and conditions for the toxin production. All isolates of *F. poae* and both control isolates were grown in the glucose-peptone-yeast extract (GPY) liquid medium. Also, three isolates of *F. poae* and the control isolates were grown in sucrose-peptone-yeast extract (SPY) liquid medium. The GPY liquid medium (pH 5.8) contains 5% of glucose, 0.1% of peptone and 0.1% of yeast extract. The SPY liquid medium (pH 6.5) contains 5% of sucrose, 0.1% of peptone and 0.1% of yeast extract.

Media (100 ml each) were poured into 500-ml Erlenmeyer flasks, and cultured with five fragments (5 x 5 mm) of the fungus that were grown on potato dextrose agar (PDA) in the Petri dishes at 27°C, for seven days. In order to obtain submersed cultures, after inoculation of the medium the Erlenmeyer flasks were kept on the rotary shaker (180 rounds min^{-1}) at room temperature (21—26°C) for three days. The pH value was measured after the incubation of the isolate.

Determination of fusariotoxins. After the cultivation on the rotary shaker, liquid cultures were filtered. Qualitative and quantitative ZEA determinations in filtrates of mould cultures were carried out by applying the multitoxin thin layer chromatographic method, developed by C v e t n i ć et al. (2005). Crude extracts of the type-A trichothecenes (DAS and T-2 toxin) were obtained by the use of ethyl acetate. Each liquid culture (25 ml) was extracted twice with 15 ml of ethyl acetate. Organic extracts were recovered by filtration through the layer of anhydrous sodium sulphate, combined and evaporated almost to dryness, under the rotary evaporator. Further purification was done by the method of R o m e r et al. (1978). The crude oily extract of trichothecenes was dissolved in the methanol/water (1:1, v/v) extraction solvent, which tends to extract compounds of the polarity of trichothecenes, while it does not extract low polar compounds, such as fats and oils. Afterwards, 30% of aqueous ammonium sulphate was added to remove additional interferences. The further step was selective concentration of the analytes into chloroform, and removal of acidic interferences from chloroform extracts, by washing it with the aqueous potassium hydroxide solution. Thinlayer chromatography was performed according to P e p e l j n j a k and B a b i ć (1991) with toluole/ethyl acetate/formic acid developing solvent (5:4:1, v/v/v). All analyses were done in three repetitions.

RESULTS AND DISCUSSION

After the incubation period, with the exception of one *F. poae* isolate (MRIZP-890), the decreased pH value was determined in both liquid media, especially in the control isolates (Tables 1 and 2).

The results obtained in mycotoxicological studies show that *F. poae*, in contrast to *F. graminearum* (isolate GZ-LES), did not have the potential for the zearalenone biosynthesis (Tables 1 and 2). These results are in accordance with literature data (M a r a s a s et al., 1984). Generally, there is a very small number of authors stating that *F. poae* biosynthesises zearalenone, among others K o c i ć - T a n a c k o v (2004). By re-testing numerous toxicogenic isolates of the *Fusarium* species, M a r a s a s et al. (1984) determined that some results obtained on the production of mycotoxins were incorrect as the identification of fungi was not correct.

Tab. 1 — Yields (μg L^{-1}) of zearalenone and type-A trichothecene (DAS, T-2 toxin) in GPY liquid cultures of 11 *F. poae* isolates originated from wheat and control isolates of *F. graminearum* (No. 12) and *F. sporotrichioides* (No. 13)

No.	Isolate designation	Origin	pH	Fusariotoxin yields (μg L^{-1})		
				ZEA	DAS	T-2
1.	MRIZP-32	Inđija	5.30	n.d.*	n.d.**	n.d.**
2.	MRIZP-37	Erdevik	5.50	n.d.*	n.d.**	240
3.	MRIZP-664	Zemun Polje	5.23	n.d.*	n.d.**	n.d.**
4.	MRIZP-665	Zemun Polje	5.40	n.d.*	n.d.**	n.d.**
5.	MRIZP-666	Zemun Polje	5.41	n.d.*	80	n.d.**
6.	MRIZP-833	Lipnički Šor	5.04	n.d.*	n.d.**	n.d.**
7.	MRIZP-834	Stari Banovci	5.63	n.d.*	n.d.**	n.d.**
8.	MRIZP-860	Sombor	5.48	n.d.*	n.d.**	80
9.	MRIZP-879	Loznica	5.52	n.d.*	n.d.**	n.d.**
10.	MRIZP-890	Pazova	5.87	n.d.*	n.d.**	n.d.**
11.	MRIZP-897	Pirot	5.71	n.d.*	n.d.**	n.d.**
12.	GZ-LES	Leskovac	4.40	37	n.d.**	n.d.**
13.	KF-38/1/R[a]	Poland	4.20	n.d.**	240	4,000

[a] — from barley grains; n.d.* — not detected (F^{-1}); n.d.** — not detected (F^{-1})

Tab. 2 — Yields (μg L^{-1}) of zearalenone and type-A trichothecene (DAS, T-2 toxin) in SPY liquid cultures of three *F. poae* isolates (No. 1—3) originated from wheat and control isolates of *F. graminearum* (No. 4) and *F. sporotrichioides* (No. 5)

No.	Isolate designation	Origin	pH	Fusariotoxin yields (mg L^{-1})		
				ZEA	DAS	T-2
1.	MRIZP-666	Zemun Polje	6.09	n.d.*	n.d.**	80
2.	MRIZP-860	Sombor	5.88	n.d.*	n.d.**	80
3.	MRIZP-897	Pirot	5.84	n.d.*	n.d.**	n.d.**
4.	GZ-LES	Leskovac	4.73	n.d.*	n.d.**	n.d.**
5.	KF-38/1/R[a]	Poland	4.69	n.d.*	n.d.**	160

[a] — from barley seeds; n.d.* — not detected (F^{-1}); n.d.** — not detected (F^{-1})

The presence of DAS was recorded in the isolate MRIZP-666 (*F. poae*) and the control isolate KF-38/1/R (*F. sporotrichioides*) in the glucose-peptone-yeast (GPY) liquid medium at the concentration of 80 μg L^{-1} and 240 μg L^{-1}, respectively. In both cases, the DAS yield was low, close to the limit of detection (LOD) of the applied method. Marasas et al. (1984) concluded that some, but not all isolates in *F. poae*, were able to produce DAS, and the ability could be lost rapidly in culture.

The T-2 toxin was detected in the following three *F. poae* isolates: MRIZP-860 (in both liquid cultures — 80 μg L^{-1}), and MRZIP-37 (240 μg L^{-1}) and MRIZP-666 (80 μg L^{-1}) in the GPY and SPY liquid culture, respectively (Table 1). The control culture KF-38/1/R (*F. sporotrichioides*) in the glucose-peptone-yeast extract (GPY) liquid medium, produced this mycotoxin at the concentration of 4,000 μg L^{-1}. However, these values were significantly lower than those recorded with the original strain of this species, in which the production of the T-2 toxin at the concentration of 150,000 μg L^{-1} had been recorded (Mašić et al., 1997). The obtained results can be explained by the fact that a long-term passaging of microorganism isolates, even fungi isolates, on the artificial media leads to a gradual loss of their biochemical properties. Although *F. sporotrichioides* KF-38/1/R were reisolated (KF-38/1R) from sterile, wet maize grain, it is obvious that their initial potential for production was not completely recovered. On the other hand, a low T-2 toxin yield in the *F. poae* isolate (Table 1 and 2) can not be interpreted in such a way, considering that the majority of the isolates were from 2005, hence they were subcultivated under laboratory conditions only for a short period of time. Thus, these isolates of *F. poae* can be considered as non-toxic.

The gained results show that under such conditions of cultivation in the liquid media, the isolates of *F. poae* from wheat originated in Serbia express low potential for biosynthesis of the type-A trichothecenes. Similar results were obtained with isolates of other potentially toxigenic *Fusarium* spp. (*F. oxysporum* and *F. proliferatum*) also determined on wheat. A fairly weak potential for the production of fusariotoxins was evaluated when the cultivation was performed in the liquid medium: 250—320 μg L^{-1} ZEA, i.e. 320 μg L^{-1} DAS and 160 μg L^{-1} T-2 toxin (Bočarov-Stančić et al., 2003). According to our previous studies, greater amounts of the T-2 toxin in *Fusarium* spp. cultures originated from wheat grain from Serbia were recorded only in *F. sporotrichioides* and *F. culmorum* (Bočarov-Stančić, 1996).

Unlike low toxigenic potential of the isolates of *Fusarium* spp., originated from wheat from Serbia, the information gained in the countries of Northern Europe show that yields, especially those of T-2 toxin, were significantly higher at the cultivation of *Fusarium* spp. under laboratory conditions. In Norway, Torp and Langseth (1999) determined the biosynthesis of the T-2 toxin in all tested isolates at the concentration of 25,000—400,000 μg L^{-1}. A *Fusarium* species resembling *F. poae* (= *F. langsethiae* Torp and Nirenberg) was cultured on the PDA, or in the liquid medium with yeast extract and sucrose. Kroiakova et al. (1989) obtained T-2 toxin yields ranging from 50 to 600,000 ppb, when three isolates of *F. sporotrichioides* v. *poae*, originated from wheat grain, harvested in Moscow region, were *in vitro* cultivated. Hor-

nok and Toth (2001) state that the application of the thinlayer chromatography assay revealed no trichothecene producing strain among *F. poae* isolates originated from Hungary.

Considering the presented results, in order to obtain the final answer to the question on the toxicological profile of the *F. poae* isolates in Serbia, it is necessary to carry out additional studies, not only with new isolates of the coming years, but also under different cultivation conditions, first of all on the sterile natural substrates, such as wheat and maize. Marasas et al. (1984) brought forward examples in which differences in the trichothecene production occurred due to conditions and substrates of the *F. poae* cultivation.

CONCLUSIONS

According to the presented results, it can be concluded that *F. poae* isolates from wheat, in contrast to *F. graminearum*, have no potential for the zearalenone biosynthesis.

In the case of the type-A trichothecenes, the diacetoxyscirpenol, i.e. T-2 toxin production was detected only in one culture of *F. poae*, i.e. 15.38% of the studied isolates, respectively. The potential of *F. poae* from wheat, for the type-A trichothecene production was low, as the concentration of DAS and T-2 toxin did not exceed 80 μg L^{-1} and 240 μg L^{-1}, respectively.

An answer to the question on the toxicological profile of the *F. poae* cannot be made, unless other cultivation conditions are not observed, and unless a greater number of isolates of this species, originated from different harvest years of wheat and other cereals that are hosts of this species, are not studied.

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ТОКСИГЕНИ ПОТЕНЦИЈАЛ ИЗОЛАТА *FUSARIUM POAE* ПОРЕКЛОМ СА ПШЕНИЦЕ

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Резиме

У овом раду је у *in vitro* условима проучена способност изолата *F. poae* за биосинтезу једне групе фузариотоксина — трихотецена типа А (Т-2 токсин и диацетоксисцирпенол-ДАС), као и зеараленона (ЗЕА). Токсиколошки профил ове врсте је код нас недовољно испитан с обзиром на њену заступљеност и токсигена својства преме литературним подацима.

Проучавањима је било обухваћено 11 изолата *F. poae*, пореклом са пшенице из 9 локалитета, углавном са подручја Војводине. Највећи број узорака прикупљен је 2005. године, када су климатски услови погодовали интензивнијој појави фузариоза класа ове пољопривредне културе. За одређивање токсиколошког потенцијала *F. poae*, културе одабраних изолата су гајене у течним подлогама (ГПК и СПК) током 3 дана на собној температури (21—26°C) и на ротационој треси-

лици (180 обртаја мин$^{-1}$). Сирови токсини су изоловани из филтрата течних култура испитаних изолата помоћу етил ацетата, док је квантификација микотоксина извршена методом танкослојне хроматографије. Течна култура изолата ГЗ-ЛЕС (*F. graminearum*) је коришћена као контролна култура за утврђивање потенцијала за биосинтезу зеараленона, а КФ-38/1/Р (*F. sporotrichioides*) за оба трихотецена типа А (Т-2 токсин и ДАС).

Добијени резултати показују да *F. poae*, за разлику од *F. graminearum*, не поседује потенцијал за биосинтезу зеараленона. Присуство ДАС-а је утврђено само код једне културе *F. poae* (МРИЗП-666) и контролног изолата *F. sporotrichioides* (КФ-38/1/Р) који су гајени у течном ГПК медијуму. Т-2 токсин је детектован код изолата МРИЗП-666 при гајењу у обема подлогама, као и изолата МРИЗП-37 у ГПК, односно МРИЗП-860 у СПК медијуму. Контролна култура КФ-38/1/Р (*F. sporotrichioides*) производила је Т-2 токсин у концентрацији од 4000 μg L^{-1}.

На основу изнетих података може се закључити да је потенцијал *F. poae* за биосинтезу трихотецена типа А био низак у датим условима с обзиром да концентрација ДАС-а није прелазила 80 μg L^{-1}, односно Т-2 токсина 240 μg L^{-1}.

Имајући у виду приказане резултате, сматрамо да је за добијање коначног одговора на питање о токсиколошком профилу изолата *F. poae* у Србији неопходно предузети додатна испитивања, не само са новим изолатима из година које следе, него и у другим условима култивисања, у првом реду на стерилном природном супстрату као што су пшеница и кукуруз.

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INVESTIGATIONS OF THE CAPABILITY OF *FUSARIUM* ISOLATES FROM CORN FOR BIOSYNTHESIS OF FUSARIOTOXINS

ABSTRACT: The aim of this paper was to investigate the potential of zearalenone (ZEN), and type A trichothecenes (T-2 toxin and diacetoxyscirpenole — DAS) for biosynthesis by *Fusarium* spp. isolated from corn kernels contaminated by mycotoxins.

The samples of corn kernels (2004 and 2005 harvest) originating in different regions of Bačka (Vojvodina, Serbia) were tested. Mycotoxicological investigations showed in most cases a significant contamination with deoxynivalenol (DON 200 — 2,460 μg/kg) and ZEA (520 — 1,680 μg /kg).

Isolations and identifications of fusaria established only the presence of species *F. verticillioides*, after one month storage in freezer conditions, in fusariotoxin positive samples. The control cultures known as ZEA producers — *F. graminearum* GZ-LES, i.e. T-2 toxin and DAS producer — *F. sporotrichioides* KF-38/1/R were also tested. *In vitro* toxicological investigations of isolated fusaria were performed in liquid semisynthetic media (GPK or SPK), and on wet sterilized corn kernels, respectively.

Under testing conditions, analyzed *F. verticillioide* and *F. sporotrishioides* isolates were not ZEA producers. Contrary to them, *F. graminearum* GZ-LES pure culture was very good producer of fusariotoxins; it biosynthesized max. 465,900 μg/kg DON, and 4,416 μg/kg ZEA, respectively.

Cultivation conditions influenced a great deal of T-2 toxin production under laboratory conditions. In most cases, higher yields were obtained during the cultivation of *F. verticillioides* in liquid glucose medium (80—240 μg/L). Contrary to the control strain *F. sporotrichioides* KF-38/1/R that under the same conditions synthesized, besides T-2 toxin (4.000 μg/L) and DAS (240 μg/L), isolates of *F. verticillioides* from corn grain did not show that ability.

KEY WORDS: biosynthesis, corn grains, fusariotoxins, *Fusarium* spp.

INTRODUCTION

Corn is predominantly attacked by *Fusarium graminearum, F. verticillioides, F. proliferatum* and *F. subglutinans* (D o o h a n et al., 2003). These representatives of *Fusarium genus* differ in their climatic distribution, optimal conditions for growth and toxin production, as well as in pathogenicity to different cerealia. Several *Fusarium* species known as causal agents of corn ear rot are capable to biosynthesize toxic metabolites in infected kernels, some of which have notable impact on human and animal health. Zearalenone (ZEA) and deoxynivalenol (DON) are commonly found in red ear rot of corn, a disease essentially caused by species of Discolor section, usually *F. graminearum* (L o g r i e c o et al., 2002). Diacetoxyscirpenol (DAS) and T-2 toxin are mainly associated with the presence of representatives of Sporotrichiella section *F. poae* and *F. sporotrichioides*. According to the data presented by M a r a s a s et al. (1984) *F. verticillioides* can also biosynthesize ZEA, DAS and T-2 toksin, although these authors regarded them as unlikely.

MATERIAL AND METHODS

Fungal cultures. Four *F. verticillioides* isolates from corn grains of 2004 and 2005 harvest were investigated (Table 1). Isolations were performed from coarse corn grain after one month of storage in freezer conditions. Coarsed material was placed on the surface of Petri dish (10 cm diameter), on potato dextrose agar supplemented with straeptomycin sulphate (500 mg/L) (PDAS), and incubated at room temperature (23—26°C) for 7 days. Grown fungal colonies, were purified by the method of obtaining monosporous colonies which were further on used for the identification of *Fusarium* spp. Determination of the obtained species was done according to N e l s o n et al. (1983), and confirmed by dr J. L e v i ć (Maize Research Institute "Zemun Polje", Belgrade—Zemun). The obtained isolates were afterwards kept on PDA slants at 4°C, until *in vitro* investigation of their toxigenic potential was not performed in liquid culture.

Control isolates. *Fusarium* cultures, known as fusariotoxin producers, were also under present investigation: 1) *Fusarium graminearum* GZ-LES producer of ZEA, according to B o č a r o v - S t a n č i ć et al. (1986), and 2) *F. sporotrichioides* KF-38/1/R reisolate of original strain known as T-2 toxin and DAS producer (M a š i ć et al., 1997). Stock cultures of the fungi were maintained on potato dextrose agar at 4°C.

Conditions for toxin production. 1) Control isolates *Fusarium graminearum* GZ-LES and *F. sporotrichioides* KF-38/1/R were cultivated on naturaly dried corn grain (hybrid NS-444, locality Stajićevo — Vojvodina) with 53.12% of initial moisture. Substrate inoculation (250 g) in flasks (1750 ml) was performed with fungal fragments cultivated for 7 days at 26 ± 1°C in PDA Petri dishes (1/2 of the fungal growth, 10 cm in diameter). Cultivation was performed in darkness at 30 ± 1°C for 35 days. 2) All *F. verticilioides* isolates, as well as both control cultures, were parallely grown in GPY liquid medium

(5% of glucose + 0.1% of peptone + 0.1% of yeast extract) pH 5.8; and SPY medium (5% of saccharose + 0.1% of peptone + 0.1% of yeast extract) pH 6.5 respectively. Media were poured in Erlenmeyer flasks (500 ml) in volume of 100 ml each (A), or 250 ml each (B), and sowed with 5 fragments (5 x 5 mm) of fungus cultivated on PDA in Petri dishe at 26 ± 1°C for 7 days. After inoculation, Erlenmeyer flasks were cultivated on rotary shaker under submerged conditions (180 revolutions per min-rpm) at room temperature (23—26°C). *Fusarium graminearum* GZ-LES was also cultivated under stacionary conditions in 100/500 ml GPY for 13 days at room temperature (23—26°C) (**C**).

Determination of fusariotoxins. 1) After cultivation on corn grains, the samples were dried for 24 h or more at 60°C, until constant weight was not achieved. After pulverization of dried samples, DON determination was done according to Abramović et al. (2005), and ZEA by the use of multitoxin method applied by Balzer et al. (1978). Type A trichothecenes were isolated and purified according to the method of Romer et al. (1978). Thin layer chromatography of ZEA, T-2 toxin and DAS was was done according to Pepeljnjak and Babić (1991).

2) After cultivation on rotary shaker, liquid cultures were filtered. Qualitative and quantitative ZEA determination in filtrates of *Fusarium* cultures was carried out by applying multitoxin thin layer chromatographic method of Cvetnić et al. (2005). Crude extracts of type A trichothecenes (DAS i T-2 toxin) were obtained by the use of ethyl acetate. Further purification was done by the method of Romer et al. (1978). Thin layer chromatography was performed according to Pepeljnjak and Babić (1991) with toluole/ethyl acetate/formic acid developing solvent (5:4:1, v/v/v).

RESULTS AND DISCUSSION

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

Tab. 1 — Presence of deoxynivalenole (DON) and zearalenone (ZEA) in samples of corn kernels (harvest 2004 and 2005).

No.	Sample design.	Type of sample	Quantity of fusariotoxins (μg/kg)	
			DON	ZEA
1.	N001/06	Coarse corn grain KPUT4, Putinci 2005	1,130	1,680
2.	N002/06	Coarse corn grain KŠID, Šid 2005	200	520
3.	N003/06	Coarse corn grain 57002	—	n.d. (< 0.037)
4.	N004/06	Corn grain 12168, Kupusina 2004	2,460	800

Seventy five percentages of tested samples of coarsed/whole corn grain originating from 2004 and 2005 were contaminated with DON. Detected quantities of this mycotoxin ranged from 200 to 2,460 μg/kg (Table 1). Higher concentrations of fusariotoxin ZEA than DON were found in 2 samples (2005 harvest) out of three positive samples for this type B trichothecene. The obtained results can be explained by the assumption that in 2005 the harvest was

carried out in later vegetative phase of corn than in 2004. According to the investigations of Lew (1995), DON can be found very early and in high quantities in cereal grains attacked by *Fusarium* spp., contrary to ZEA, fusariotoxin which appears only in the later phase of fungal growth.

Tab. 2 — Yield of DON, ZEA and type A trichothecenes (DAS i T-2) in control isolates *F. graminearum* and *F. sporotrichioides* cultivated on sterilised corn grains under laboratory conditions

No.	Cultivation period	Control isolate	Moisture (%)	Yield of fusariotoxins (μg/kg)			
				DON	ZEA	DAS	T-2
1.	Starting sample	—	53.1	n.d.*	n.d.**	n.d.***	n.d.***
2.	17 days	GZ-LES	62.5	—	220	n.d.***	n.d.***
		KF-38/1/R	58.4	—	n.d.**	n.d.***	n.d.***
3.	35 days	GZ-LES	80.4	465,900	4,416	n.d.***	n.d.***
		KF-38/1/R	—	n.d.*	n.d.**	1,600	2,400

Legende: n.d* — not detected (< 45 μg/kg),
n.d.** — not detected (< 37 μg/kg),
n.d.*** — not detected (< 60 μg/kg).

The results obtained during cultivation of the control strains *F. graminearum* GZ-LES and *F. spototrichioides* KF-38/1/R on natural solid substrate at 30°C are shown in are Table 2. Corn kernels used as the substrate for *in vitro* investigations of toxin production were not contaminated with fusariotoxins. Isolate *F. graminearum* produced only type B trichothecene (DON) and ZEA in that conditions. During prolongated cultivation, the yield of ZEA increased from 220 μg/kg after 17 days to 4,416 μg/kg after 35 days. The obtained results are in accordance with the previously quoted opinion of Lew (1995). *F. sporotrichioides* KF-38/1/R biosynthesized only type A trichothecenes after 35 days on wet sterilized corn grain substrate (1,600 μg/kg DAS, and 2,400 μg/kg T-2 toxin, respectively). These results are in accordance with the data presented by Marasas et al. (1985) concerning toxigenic potential of *Fusarium* spp., as well as with data concerning optimal temperature conditions for the production of fusariotoxins presented by other authors. Bočarov-Stančić et al. (1986) point out that most (50%) of *F. graminearum* isolates biosynthesized ZEA during cultivation at rather high constant temperature (28°C). Similar temperature conditions favoured DON production by the same fusaria species: 28°C (Llorens et al., 2004) and 25°C (Hope et al., 2005, Ramirez et al., 2006) respectively.

Tab. 3 — Yield (μg/L) of zearalenone and tipe A trihotecenes (DAS, T-2 toxin) in **GPY** liquid culture of four *F. verticillioides* originated from corn grains and control isolates *F. graminearum* (No. 5) and *F. sporotrichioides* (No. 6)

No.	Isolate designition	Aeration	pH	Yield of fusariotoxins (μg/L)		
				ZEA	DAS	T-2
1.	N001/06	A	4.71	n.d.*	n.d.**	80
		B	4.29	n.d.*	n.d.**	240
2.	N002/06	A	4.77	n.d.*	n.d.**	n.d.**
		B	4.72	n.d.*	n.d.**	240
3.	N003/06	A	4.96	n.d.*	n.d.**	160
		B	4.33	n.d.*	n.d.**	n.d.**
4.	N004/06	A	4.76	n.d.*	n.d.**	n.d.**
		B	4.55	n.d.*	n.d.**	80
5.	GZ-LES	A	4.40	37	n.d. **	n.d. **
		C	5,70	75	—	—
6.	KF-38/1/R	A	4.20	n.d. *	240	4. 000

Legende: n.d.* — not detected (< 37 μg/L),
n.d.** — not detected (< 80 μg/L)

Tab. 4 — Yield (μg/L) of zearalenone and tipe A trihotecenes (DAS, T-2 toxin) in **SPY** liquid culture of four *F. verticillioides* originated from corn grains and control isolates *F. graminearum* (No. 5) and *F. sporotrichioides* (No. 6)

No.	Isolate designition	Aeration	pH	Yield of fusariotoxins (μg/L)		
				ZEA	DAS	T-2
1.	N001/06	A	4.95	n.d.*	n.d.**	n.d.**
		B	4.22	n.d.*	n.d.**	120
2.	N002/06	A	5.14	n.d.*	n.d.**	n.d.**
		B	4.80	n.d.*	n.d.**	80
3.	N003/06	A	5.17	n.d.*	n.d.**	80
		B	4.07	n.d.*	n.d.**	n.d.**
4.	N004/06	A	5.04	n.d.*	n.d.**	n.d.**
		B	4.45	n.d.*	n.d.**	80
5.	GZ-LES	A	4.73	n.d.*	n.d.**	n.d.**
6.	KF-38/1/R	A	4.69	n.d.*	n.d.**	160

Legende: n.d.* — not detected (< 37 μg/L),
n.d.** — not detected (< 80 μg/L)

On the basis of the results presented in Table 1, high concentrations of DON and ZEA, it should be expected that one can isolate from these corn kernels mainly species *F. culmorum* or *F. graminearum* which are, according to the literature data, the best producers of the same mycotoxins (M a r a s a s et al., 1984). However, having in mind storage conditions of the samples and low viability of *F. graminearum* conidia in the first place, as well as higher viability of conidia of other fusaria pathogenic to corn (D o o h a n et al., 2003), it is not so surprising that *F. verticillioides* was obtained solely.

pH value. During submerged cultivation (Tables 3 and 4), with the exception of the control isolate *F. graminearum* GZ-LES, the pH value decrease was observed in all other liquid cultures after incubation. In both cases of liquid media (GPY/SPY) more outstanding changes of the same paramether were noted in higher volume of fermentation media (250/500 ml).

ZEA biosynthesis. Although Cvetnić et al. (2005) found that some *F. verticillioides* isolates from agricultural regions of Croatia can produce ZEA, during our investigation the production of this fusariotoxin was noted only in liquid GPY culture of *F. graminearum* (Table 3), but in much lower quantities than during incubation on the natural solid substrate (37 and 75 μg/L, and 220 and 4,659 μg/kg, respectively) (Tables 2 and 3). Higher yield of ZEA was obtained during stationary cultivation in the liquid medium of the same composition. Last result is in accordance with our previous investigations of different liquid nutrient media (Bočarov-Stančić and Škrinjar, 1995).

DAS biosynthesis. It was not recorded in any of the tested *F. verticillioides* isolates, but was found in liquid GPY culture of the control isolate *F. sporotrichioides* KF-38/1/R — 240 μg/L (Table 3). Although again, much lower yields of DAS were achieved in liquid medium than on sterilized corn grains (Tables 2 and 3), the period of incubation was much shorter (3 days in comparison with 35 days). That fact can become a great advantage in the case of screening toxicity of a large number of *Fusarium* spp. under laboratory conditions.

T-2 toxin biosynthesis. It was observed (Tables 3 and 4), besides control strain *F. sporotrichioides* KF-38/1/R, in all tested *F. verticillioides* isolates from corn (80 do 240 μg/l in GPY, and 80—120 μg/l in SPY, respectively). In the last case, found concentrations were rather low, i.e. near the detection limit. During submerged cultivation in both liquid media in 75% of the tested cases, higher yields of this type A trichothecene were achieved under the conditions of smaller aeration (250/500 mL). An exception was the isolate N003/06 that produced more T-2 toxin during cultivation in higher aeration conditions (Tables 3 and 4). The obtained results are in accordance with the investigation of Mašić et al. (1997). These authors recorded, with one *F. verticillioides* isolate from corn grains, T-2 toxin biosynthesis (500 μg/L) under similar laboratory conditions.

Influence of the sugar type. Eighty percentages of the tested isolates, including the control one KF-38/1/R, regardless of volume of fermentation medium, achieved higher yields of T-2 toxin in glucose medium GPY (80 — 4,000 μg/L) than in sucrose medium SPY (80 — 160 μg/L). In contrast to our findings, Ueno et al. (1975) didn't observe the same influence of sugar type during cultivation of other fusaria in the liquid media with the same composition.

On the basis of the presented results it can be concluded that isolates of *F. verticillioides* originating from Vojvodina possess weak potential for T-2 toxin production.

CONCLUSIONS

Only *F. verticillioides* cultures were isolated from corn kernels harvested in 2004/2005, contaminated with rather high quantities of DON (200 — 2,460 μg/kg) and ZEA-a (520 — 1,680 μg/kg).

During submerged cultivation of these isolates in liquid media (GPY and SPY) all 4 tested *F. verticillioides* cultures biosynthesized T-2 toxin (80 — 240 μg/L), as well as the control strain *F. sporotrichioides* KF-38/1/R.

Production of DAS and ZEA was not observed with *F. verticillioides* isolates, but it was with control cultures *F. graminearum* (ZEA) and *F. sporotrichioides* (DAS) during cultivation in the same liquid media (GPY and SPY).

The influence of glucose, as sugar source, was more favourable than saccharose on T-2 toxin biosynthesis in most (80%) of the tested samples.

After 35 days of cultivation on solid natural substrate, the control strain *F. graminearum* GZ-LES produced high DON (469,900 μg/kg) and ZEA (4,416 μg/kg) quantities. Under the same cultivation conditions another control isolate *F. sporotrichioides* KF-38/1/R, biosynthesized only type A trichothecenes (1,600 μg/kg DAS, and 2,400 μg/kg T-2 toxin, respectively).

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ИСПИТИВАЊЕ СПОСОБНОСТИ ЗА БИОСИНТЕЗУ ФУЗАРИОТОКСИНА КОД *FUSARIUM* ИЗОЛАТА СА КУКУРУЗА

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Резиме

Иако припада групи најмање токсичних трихотецена (тип Б), фузаријумски микотоксин дезоксиниваленол (ДОН) је од великог значаја за квалитет хране и сточне хране, с обзиром да може бити индикатор присуства других, токсичнијих једињења као што је нпр. зеараленон (ЗЕА). С друге стране значајно је и праћење присуства трихотецена типа А (Т-2 токсин и ДАС) с обзиром на њихову изразиту токсичност и у мањим концентрацијама.

Циљ овог истраживања је био да се испита потенцијал за биосинтезу зеараленона (ЗЕА) и трихотецена типа А (Т-2 токсин и ДАС) код *Fusarium* култура изолованих са зрна кукуруза контаминираног микотоксинима.

Истраживањем су били обухваћени узорци кукуруза рода 2004/2005. год. пореклом из различитих региона Бачке. Микотоксиколошка истраживања датих узорака су код већине показала значајну контаминацију са ДОН-ом (200 — 2.460 μg/kg) и ЗЕА-ом (520 — 1.680 μg/kg).

Изолације и идентификације фузарија су показале искључиво присуство врсте *F. verticillioides*. Испитивања токсигености ових изолата су обављена у течним семи-синтетичким подлогама са глукозом (ГПК) и сахарозом (СПК), као и на влажном, стерилном зрну кукуруза.

У датим условима испитани изолати нису биосинтетисали ЗЕА. За разлику од њих контролна култура *F. graminearum* ГЗ-ЛЕС показала се као веома добар продуцент фузариотоксина — производила је максимално 465.900 μg/kg ДОН-а, односно 4.416 μg/kg ЗЕА.

На биосинтезу Т-2 токсина у лабораторијским условима у великој мери су утицали услови култивирања. У већини случајева су код изолата *F. verticillioides* добијени већи приноси у подлози са глукозом (80—240 μg/L). За разлику од контролног соја *F. sporotrichioides* КФ-38/1м који је у истим условима биосинтетисао поред Т-2 токсина (4.000 μg/L) и ДАС (240 μg/L), изолати *F. verticillioides* са зрна кукуруза нису испољили ту способност.

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PRESENCE OF DEOXYNIVALENOL IN MAIZE OF VOJVODINA

ABSTRACT: By applying previously established optimal conditions for the determination of deoxynivalenol (DON) by liquid chromatography with DAD detector, in this work, its content was determined in maize samples collected during the past 3 years (2004—2006) from different locations in Vojvodina. Analyzing 103 maize samples in total, the presence of deoxynivalenol was established in 42.7% of the samples. Only 3 samples of maize contained DON in concentrations that exceeded the maximum permitted level (1 μg/g) legislated in most countries.

KEY WORDS: deoxynivalenol, liquid chromatography, maize

INTRODUCTION

DON is the most widely spread mycotoxin from the trichotecene group, produced by fungi from the genera *Fusarium*, most frequently *Fusarium graminearum* and *Fusarium culmorum.* The most sensitive to the presence of these fungi are wheat and maize, a disease of wheat caused by this fungus is known as *Fusarium* head blight, while of corn, it is known as *Gibberella* ear rot (JECFA, 2001). Infection of these cereals causes a decrease in the grain size and the protein content in the grain, and has harmful effect on germination. The final result is a decrease in the yield and the feed quality. Its presence in feed is manifested by rejection of feed, vomiting, diarrhea and finally, the weight loss in livestock (Kuiper-Goodman, 2002). The most sensitive to the presence of deoxynivalenol are pigs, hence already at concentrations of 1 mg/kg in feed a certain percentage of these animals refuse food. That is exactly why this deoxynivalenol doses is the maximum permitted dose in feed intended for this animal species in most countries worldwide.

The maximum permitted level of deoxynivalenol in feed for milk cows is also 1 mg/kg, while the level considered to be permitted for cattle and sheep is

5 mg/kg, although these species can tolerate levels above 10 mg/kg. The least sensitive species to the presence of this mycotoxin in feed is poultry, although the maximum permitted level of deoxynivalenol in this case is 5 mg/kg (Canadian Grain Commission, 1999).

The maximum permitted levels for this mycotoxin, however, have not been yet legally regulated in our country, either in foodstuffs or in feed.

Most frequently used for quantitative determination of DON are chromatographic methods, *i.e.* liquid chromatography (LC) with or without derivatization of DON, as well as gas chromatography with almost compulsory derivatization, and somewhat less commonly used thin layer chromatography (TLC), immunochemical method and others (JECFA, 2001; Krska, 2001; Lombaert, 2002; Jajić, 2004). All quantitative methods for deoxynivalenol determination (except immunochemical) require clean-up of crude sample extract by solid phase extraction (SPE). To that purpose, columns with different sorbents are used: activated charcoal, alumina and celite (Eppley et al., 1986), fluorisil (Sano et al., 1987), silica-gel, ion exchange resins, as well as different combinations of the above sorbents (Lauren and Greenhalgh, 1987). Lately, the most frequently used are multifunctional, so called MycoSep columns (Weingaertner et al., 1997; Mateo et al., 2001) and to some extent less often immunoaffinity columns filled with antibodies specific for an individual mycotoxin (Cahill et al., 1999).

In the study that encompassed the available data from the entire world, performed by JECFA (2001), deoxynivalenol was found as a frequent contaminant of cereal grains such as wheat (11444 samples, 57% contaminated), maize (5349 samples, 40% contaminated), oats (834 samples, 68% contaminated), barley (1662 samples, 59% contaminated), rye (295 samples, 49% contaminated), and rice (154 samples, 27% contaminated).

The aim of this work was to determine the DON content in maize samples collected in Vojvodina by applying previously established optimal conditions for DON determination by liquid chromatography with DAD detector (Abramović et al., 2005). In addition, this paper attempted to compare the data with those found in relevant literature about the incidence of this mycotoxin in countries of our region.

MATERIAL AND METHODS

Materials

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

Deoxynivalenol calibrant solutions. Deoxynivalenol (Biopure, Tulln, Austria) was purchased as an analytical standard. Calibrant solution was prepared in ethyl acetate-methanol (19:1, v/v) at the concentration of 85.05 μg/cm^3 from crystalline substance, according to AOAC method 986.17. Stock solution con-

taining DON at 17.01 μg/cm^3 was prepared by measuring 2.00 cm^3 calibrant solution of DON into a 10 cm^3 volumetric flask, and diluting to volume with ethyl acetate-methanol (19:1, v/v). Working calibrant solutions were prepared by evaporation of the appropriate volume of the stock solution and dilution with the appropriate volume of methanol. Standard solutions were stored at 4°C.

Sample and preparation. Maize samples were collected during past 3 years (2004—2005) from different locations in Vojvodina. Immediately after the sampling, each sample was prepared by grinding in a laboratory mill. After that, the sample was homogenized by mixing. Sample prepared in such a way was packed in plastic bags and stored in a freezer at –20°C until analysis. Prior to each analysis, the samples were allowed to reach room temperature.

Apparatus

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

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

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

Procedure

Principle. DON was extracted from maize with the mixture of acetonitrile (ACN) — water. After filtration, the crude extract was cleaned-up on CACC column. The cleaned-up extract was evaporated up to dryness, residue redissolved in methanol and analyzed by liquid chromatography with DAD detection.

Extraction and clean-up. 25.0 g of the sample were extracted with 100 cm^3 of ACN-water (84:16, v/v) and shaken on a magnetic stirrer for 60 minutes. After filtration through Advantec filter paper, 6.0 cm^3 of the extract were applied to the prepared column. The column was then washed with 5 cm^3 of the solvent mixture comprising of ACN-water (84:16, v/v) at about 0.6 cm^3/

min. The cleaned-up extract was evaporated to dryness, dissolved in 3 cm^3 of ethyl acetate and quantitatively transferred to an evaporation vessel by triple washing with 1.5 cm^3 ethyl acetate. The eluate was evaporated up to dryness.

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

RESULTS AND DISSCUSION

Samples of maize, collected over the period 2004—2006. in Vojvodina were analyzed and the results are presented in Table 1. As it can be seen, the DON content was above the LOQ in 50% maize of samples collected in 2004. The number of samples from the 2005 harvest was much higher (76) and the percentage of DON positive samples was 42.1%. During 2006, 17 samples were collected and analyzed, and 41.2% was positive. Only 3 samples of maize contained DON in concentrations that exceeded the maximum permitted level (1 µg/g) legislated in most countries.

Tab. 1 — Content of DON in maize samples collected during 2004—2006

Year	No. of samples	No. of positive samples (%)	Concentration in samples	
			Average (mg kg^{-1})	Range (mg kg^{-1})
2004	10	50.0	0.54	0.05—2.46
2005	76	42.1	0.36	0.045—2.21
2006	17	41.2	0.26	0.19—0.36

In case of maize, the infection of the ear most frequently takes place through the tip of the ear, when the fungi penetrate through the silk in the phase of maize flowering (Sutton, 1982). Exceptionally humid weather in the period from silking to ripening, enables ear contamination (Vigier et al., 1997). The ear is the most sensitive to contamination at the beginning of silking, while this sensitivity lowers with silk aging (Reid et al., 1992; Reid and Hamilton 1996). The silking period in the climatic region of Vojvodina and Serbia takes place within about 60 days from the moment of plant sprouting (during the month of July and the first half of August).

According to the reports of the Republic Hydrometeorological Service of Serbia (2004; 2005; 2006) the average monthly spring and summer temperatures were somewhat higher (2004), i.e. around the average value (2005) in comparison to the appropriate long-term average value (1971—2000). With respect to humidity, the year 2004 was somewhat more humid than the appropriate long-term average (1971—2000), while 2005 can be classified as "highly humid" because of frequent rains, especially in the period July-August, which

is, as it has already been said, the critical period for the development of fungi in maize. In July 2006, the average daily temperature values were higher, with deficit rainfall, while in August, it was colder and more humid than the appropriate long-term average (1971—2000), especially in the south of Vojvodina and central Serbia.

Aforementioned data infers that favourable conditions for the growth of *F. graminearum* and subsequent DON toxin production might have occurred, especially in 2005. However, the similar results, regarding the contamination with DON during 2004 and 2005, and in some cases, even higher contamination of the 2004 harvest (although the climatic conditions were somewhat less favourable for the fungal growth), are most likely due to the fact that the samples were analyzed one year after storage in barns, which enabled further mycotoxin production.

In the study, performed by JECFA (2001), deoxynivalenol was a frequent contaminant of maize (5349 samples, 40% contaminated) in the concentration range 3—3700 μg kg^{-1}. The european study on the occurrence of *Fusarium* toxins (EC, 2003) revealed that 57% of the samples of cereals from 11 countries (11022 samples) were positive for DON. A high frequency of DON was found in maize (89%).

In the period between 1991 and 1998, R a f a i et al. (2000) investigated maize (760) for the presence and concentration of DON in Hungarian cereals. The incidence rate of DON in maize was 10.8%.

C u r t u i et al. (1998) analyzed samples of maize (30), collected in 1997 after the harvest in western Romania, by enzyme immunoassays. Frequency of DON contamination was 46% (median value 890 μmg kg^{-1} and maximum concentration 160.000 μmg kg^{-1}). Climatic conditions prevailing in the summer months of 1997 were characterized by heavy rainfall before harvest.

As it can be seen, in respect to the obtained results of the incidence rate of DON in analyzed samples, it can be said that they mostly fluctuate. Namely, differing from the results for Hungarian maize samples (R a f a i et al., 2000), in which low incidence rate of DON (10.8%) was observed, in Romanian and our maize samples it is significantly higher (46 and 42.7%, respectively). Similar results, 40%, were presented in JECFA (2001), while the EC (2003) reports a significantly higher contamination of maize, even 89%.

Such differences in contamination by DON in maize of the region are most likely due to several factors. Data are not from the same years and impact of the climatic factors, as it can be seen, is considerable. Some authors like R a f a i et al., (2000), did not provide data about the climatic conditions during the period of their research (1991—1998). Also, the limit of detection or limit of quantitation of the analytical method used for DON determination also influence the incidence rate of DON in maize. We believe that different agrotechnical conditions among the countries of region are not important, because land treatments and growing crops are performed in similar manner. This is particularly the case if comparisons are made in respect to the results presented in the studies of JECFA (2001) and EC (2003).

In conclusion to the occurrence of the range of DON in maize in Vojvodina and Serbia, on the basis of the foregoing discussion of our findings, with

reference to data in relevant literature, it can be said that although the incidence rate of DON in Vojvodina-grown maize is occasionally considerable, the position of the country is not worse than the average of the surrounding countries. Besides, the concentration range of DON is low or medium, while the concentration was higher than the maximum level adopted by EC only in three cases. By regulating the maximum permitted level of DON in feed and food in Serbia, as well as by establishing monitoring programs the risk for the consumer could be minimized.

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ПРИСУСТВО ДЕОКСИНИВАЛЕНОЛА У КУКУРУЗУ ВОЈВОДИНЕ

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Резиме

Примењивањем претходно утврђене оптималне услове за одређивање деоксиниваленола течном хроматографијом са DAD детектором, у овом раду је одређен његов садржај у узорцима кукуруза који су током протекле 3 године (2004—2006) сакупљени са различитих локалитета у Војводини. Анализом укупно 103 узорка кукуруза утврђена је присутност деоксиниваленола у 42,7% уз констатацију да је свега 3 узорка имало садржај изнад 1 μg/g, количине која је максимално дозвољена у већини земаља.

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ANTIGENIC CHARACTERISTICS AS TAXONOMIC CRITERION OF DIFFERENTIATION OF *ALTERNARIA* SPP., PATHOGENIC FOR CARROT AND PARSLEY

ABSTRACT: Identification of *Alternaria* genus species is a very complicated process which demands broadly designed investigations and studying of great number of properties which together can be considered as satisfying taxonomic criteria. The main objective of these investigations was examining the possibilities of applying the antigenic characteristics of *Alternaria* spp. phytopathogenic fungi as a taxonomic criterion, as well as introducing the serological methods for their identification. Conducting the examination of *Alternaria* spp., pathogenic for Apiaceae plants in Serbia, several isolates were obtained and identified as *Alternaria radicina, A. petroselini, A. dauci* and *A. alternata*, based on the conventional mycological methods and host range, as well as on molecular detection and partial characterization.

The investigation included 12 isolates from plant leaves, seeds and soil, which were pathogenic mainly to carrot and parsley and were identified as *A. radicina, A. petroselini, A. dauci* and *A. alternate*. Investigated isolates were compared with each other, as well as with standard isolates for the mentioned species (a total of 5 isolates, originating from USA and EU).

During the investigation of serological characteristics of *Alternaria* spp., firstly a polyclonal antiserum was prepared against one isolate from Serbia, identified as *A. dauci*. This antiserum was specific to *Alternaria* genus, while there was no reaction with antigens from other phytopathogenic fungi genera (*Fusarium, Rhizoctonia* and *Agaricus*). Antiserum titer, determined by slade agglutination test, was 1/32. Antigenic characteristics of *Alternaria* genus fungi were examined by Electro-Blot-Immunoassay serological method (EBIA, Western blot), i.e. their protein profiles were compared.

Investigated *Alternaria* spp. isolates showed different protein band profiles in gel and on nitrocellulose paper, and the observed differences were in complete correlation with the results of the previous identification. All investigated isolates, both domestic and the standards, were similar to each other, and they could be correctly identified to the species level using EBIA. Besides grouping to the species level, antigenic characteristics indicated similarities and differences among the isolates within the same and different species, showing their complex relationships which properly reflect their diversity in nature.

In all the previous investigations of *Alternaria* genus fungi up to now, there have been no data about their serological characteristics as possible taxonomic criteria. Introduction of this group of characteristics represents an important contribution both to the

taxonomy and implementation of fast and accurate methods of phytopathogenic fungi identification.

KEY WORDS: *Alternaria* spp., antigenic characteristics, EBIA, polyclonal antiserum, taxonomic criterion

INTRODUCTION

Alternaria species are probably the most common fungi that mycologists deal with in different scientific fields. There is a huge number of species names in literature which are, in fact, synonims for undetermined taxonomic accuracy. That is the reason why it is so difficult even to collect any reliable data about how many species belong to this genus. It is estimated that this number varies from 100 up to several hundred species (R o t e m, 1994). Since they are extremly variable and widely distributed, *Alternaria* spp. have been described under different names and that is why their taxonomy, classification, and systematics are very complex and mostly incompletely solved today (S i m m o n s, 1992, P r y o r and M i c h a i l i d e s, 2002). Diagnostic characteristics of *Alternaria* genus include the formation of chains of dark-coloured, multi-celled conidia with beaks of tapering apical cells. However, these characteristics are not common to all the species belonging to this genus, which led to taxonomic confusion among researchers during the history of *Alternaria* classification (R o t e m, 1994).

Because of the complex differentiation of particular species within this genus, as well as the differences among particular isolates of the same species, some authors have suggested and used different criteria for determination of taxonomic relationships among the species (host range, cultural and morphological properties, pigment and crystal formation, etc.). In this way, for example, P r y o r and G i l b e r t s o n (2002) differentiated some isolates of *A. radicina* and *A. petroselini*, which had been previously improperly classified as undetermined nomenclature. The criteria which they used were the growth rate on PDA, yellow crystal formation and host range.

According to R o t e m (1994), *Alternaria* spp. taxonomy is very complex because of the variability of their morphological features, which are affected by the environmental conditions and other factors. Whatever the nature of these factors is that variability of *Alternaria* species has created a taxonomic dilemma, and led to the description of some species which have never been confirmed by other researchers. This situation confuses plant pathologists who investigate physiology, epidemiology, or the possibilities of control measures, and expect from taxonomy the proper and correct identification of the concerned organisms. Taking into consideration the possible influence of the environmental conditions, R o t e m (1994) suggests that a researcher should give his opinion only after examining a great number of isolates originating from different localities. S i m m o n s (1992) states that taxonomy and classification of fungi from any genus, including genus *Alternaria*, have to, at least at the beginning, undoubtedly match a species name with its constant morphological characteristics, as well as with its biology, pathogenicity, reproduction, physiology and other properties.

The main objective of this investigation was the comparison of chosen *Alternaria* spp. isolates on the basis of their antigenic characteristics. The isolates were obtained from tissue, soil and seed of host plants from Apiaceae family, and their proper and precise identification and characterization had been previously done up to the species level, using conventional and molecular detection methods. Since serology has not until now been applied in *Alternaria* genus investigations, the obtained results could contribute to a more precise determination of the relationships and taxonomic status of *Alternaria* fungi, pathogenic for hosts from Apiaceae family, as well as to the development of a new group of methods which could be applied in routine detection and identification of fungi.

MATERIAL AND METHODS

Isolates. In the period 2004—06, the inventory of *Alternaria* spp. fungi included diseased leaves and petioles, soil and seed of carrot, parsley and other plants from Apiaceae family in Serbia. A total of 17 isolates were chosen for the investigation, 12 of which from the Republic of Serbia (8 from seeds, 2 from plant tissue and 2 from soil), and 5 standard isolates originating from USA (B. M. Pryor, University of California, Davis) and Europe (J. van Bilsen, Bejo Zaden B. V., Warmenhuizen, Holland). On the basis of examination and identification, using conventional methods and host range, as well as molecular detection and partial characterization, the obtained isolates were classified as *Alternaria radicina*, *A. petroselini*, *A. dauci* and *A. alternata* (B u l a j i ć, 2007).

Antigen preparation. Antigens for rabbit immunization were prepared from isolate 108 mycelium, originating from Serbia, which had been previously identified as *A. dauci*. Antigen was prepared according to the method of A d a m s and B u t l e r (1979). The chosen isolate was first cultured on a solid nutrient media for three times, and then on a liquid synthetic GNA. The obtained mycelium was dried using vacuum pump, frozen at –18 to –20°C, or –80°C and stored until usage. Amount of 8 g of frozen mycelium was homogenized in a pestle and mortar with 40 ml of 0,1 M Tris-HCl buffer pH 8,0 (1,211 g Trizma base, Sigma, Chemical Company, dissolved in 100 ml distilled water, and pH adjusted with concentrated HCl to pH 8,0). After 2 hours of horizontal stirring, the obtained extract was centrifuged for 40 min at 11000 rpm in Eppendorf centrifuge (5804 R). Pellet was eliminated and supernatant was used as an antigen for rabbit immunization.

Antiserum production. Antiserum was produced by rabbit immunization, by daily intramuscular injecting of increasing volume of previously prepared mycelium suspension, for 10 days. Total of 21,25 ml of isolate 108 mycelium suspension was injected. After blood coagulation, the liquid phase was separated from clot, and it was used as antiserum in further investigation of *Alternaria* spp. antigenic properties.

Determination of antiserum titer. Antiserum titer was determined using serological method of agglutination on a glass slide. Antigen dilutions were

prepared in 0,01M phosphate buffer pH 7,0 and antiserum dilutions in physiological solution. Agglutination on a glass slide was carried out according to Noordam (1973) and Krstić and Tošić (1994) method. Formation of precipitates was observed in dark field (DF) using Olympus CX41 microscope (magnification 100x).

Determination of antigenic characteristics. *Alternaria* spp. antigenic characteristics were investigated using Electro-Blot-Immunoassay serological method (EBIA, Western blot). For EBIA, fungi samples were prepared by adding 250 µl of loading buffer (Laemmli, 1970) into 500 µl of mycelium suspension prepared in the same way as for the antiserum production. Then, the samples were boiled in water bath for about 10 min, and after cooling stored, at –20°C until usage. Electro-Blot-Immunoassay serological method (EBIA, Western blot) was used for comparing the fungi isolates on the basis of their antigenic characteristics, as well as their protein profiles. This method (O'Donnell et al., 1982; Rybicki and Von Wechmar, 1982) includes: antigen electrophoresis in polyacrylamide gel, adsorption from the gel to nitrocellulose paper, and immunoenzyme detection of antigen on NC paper. SDS polyacrylamide gel electrophoresis was performed by using discontinuous gel, 5% for protein concentration, and 12,5% for protein separation. About 40 µl of antigen sample was added per each slot of the gel. Electrophoresis was carried out by Protean II xi Cell (Bio-Rad) vertical slab gel apparatus with cooling. Components were conducted through the gel for protein concentration at constant 30mA, and through the gel for protein separation at 50mA. After electrophoresis had been completed, proteins were immediately adsorbed on NC paper (Protran, Nitrocellulose Transfer Membrane, pore size 0,45 µm, Whatman, Scheicher and Schuell, EU). Antigen adsorption was performed at room temperature. NC paper was processed using immunosorbent method, according to the catalogue manual No 170-6545 Bio-Rad, as O'Donnell et al. (1982) had previously described, and Shukla et al. (1989) modified. In the investigation of isolate antigenic characteristics, antiserum dilution was 1:250, and Anti-rabbit IgG peroxidase conjugate affinity isolated antibody developed in Goat (Sigma Immuno Chemicals) was diluted 1:2500 in skimmed milk. Paper development was performed in the mixture of 50 ml TBS with 10 ml methanole solution of 20 mg 4-chloro-1-naphthol (Bio-Rad) and 30 µl 30% hydrogen peroxide. Colour development was followed visually. The presence of bluish-purple band was considered as a positive reaction, and its absence as a negative reaction between antigen and antiserum.

RESULTS

Polyclonal antiserum was prepared against isolate 108 which, according to the previously obtained results, belongs to species *A. dauci.* Antiserum was prepared by rabbit intramuscular immunization with the total of 21,25 ml of mycelium suspension. Antiserum, in dilution 1/1, reacted positively in slide agglutination test with homologous antigen, i.e. with prepared antigen of isolate 108. In additional agglutination tests, negative reactions were observed with

the following antigens: MLE isolate of double-nucleate *Rhizoctonia* AG-A (V i c o, 1997), and *Fusarium* sp., both from the Collection of Department of Plant Pathology, Faculty of Agriculture, Belgrade, and *Agaricus* sp. (Basidiomycotina) prepared from fresh carpophora. In this way it was confirmed that antiserum shows specific reactions only with *Alternaria* species. According to the obtained results, the antiserum titer was 1/32, while the antigen titer was 1/256 (Table 1). Reactions were visualized about 2h after mixing the antiserum with the antigen.

Tab. 1 — Determination of polyclonal antiserum titer, prepared against *Alternaria* isolate 108

Antigen dillution	Dillution of polyclonal antiserum prepared against isolate 108							
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	K_f
1/1	++++[a]	++++	+++	++	++	+	—	—
1/2	++++	++++	+++	+	++	+	—	—
1/4	++	+++	+	±	++	±	—	—
1/8	++	+++	++	—	—	—	—	—
1/16	++	+++	+	—	—	—	—	—
1/32	+++	+++	++	—	—	—	—	—
1/64	++	+	—	—	—	—	—	—
1/128	±	+	—	—	—	—	—	—
1/256	±	—	—	—	—	—	—	—
1/512	—	—	—	—	—	—	—	—
K_t	—	—	—	—	—	—	—	—

[a] — reaction intensity: — = negative reaction, ± = very weak reaction, + = weak reaction, ++ = medium reaction, +++ = strong reaction i ++++ = very strong reaction,
K_f = negative control, physiological solution, K_t = negative control, Tris-HCl buffer

Electro-Blot-Immunoassay serological method (EBIA, Western blot), applied in these investigations, made possible, to a considerable extent, the study of antigenic characteristics and serological relationships of 12 domestic and 5 standard *Alternaria* spp. isolates. Figure 1 shows the presence of bluish-purple bands, which indicates positive reactions between the antigen and the antiserum. It is clear that the prepared antiserum reacted with all *Alternaria* fungi isolates, but not with *Agaricus* spp., which proves its specificity. The presence and intensity of bands on NC paper were estimated visually, and the results were summarized in Table 2.

According to the protein band profiles on NC paper, antigenic characteristics of examined *Alternaria* spp. isolates indicate that they belong to four different species: *A. radicina, A. petroselini, A. dauci* and *A. alternata*. *A. dauci* isolates (68-5, 94 and 108, as well as the standards) reacted by forming the greatest number of bands (9—14, depending on the isolate), which was expected, considering that antiserum was prepared against isolate 108. These five isolates can be clearly separated from the others by their protein band profiles. It should be emphasized that studying the antigenic characteristics under the given conditions revealed some additional differences among the isolates from the same species. Therefore, besides great similarities, some differences were also observed among some isolates within this compact group-species. The

Tab. 2 — Serological reactions of selected *Alternaria* spp. isolates by EBIA with polyclonal antiserum against isolate 108

Band No	Antigen (isolate marks)														
	BMP155	NL2D	108	94	68-5	Agaricus	13	BMP79	89	NL1R	68-1	106	BM P139	95	69
1	++++	++++	++++	++++	++++	—	++++	++++	++++	++++	++++	++	++	++	++
2	++++	++	++++	++++	++++	—	++++	++	++++	++++	++++	++	++	++	++
3	++	++	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	++	++	++++	—	—	—	—	—	—	—	—	—	—
5	—	—	++	++	++++	—	+++	++	++	++	+	+	+	+	+
6	+++	++	+++	+++	++++	—	—	+++	+++	+++	—	—	—	—	—
7	++	+	+++	+	++++	—	+++	+	+	+	+	+	+	+	+
8	+++	+	++	+	+++	—	+	—	—	—	—	—	—	—	—
9	—	—	—	++	++++	—	+++	—	—	—	—	—	—	—	—
10	+++	+	++++	++++	++++	—	++++	++++	++++	++++	+	+	+	+	++
11	++	—	++	++	++	—	+++	+	+	+	+	—	—	—	—
12	—	—	—	—	++++	—	—	—	—	—	—	—	—	—	—
13	++++	++++	++++	++++	++++	—	++	++	++	+++	+++	—	—	—	—
14	—	—	—	—	++	—	+	—	—	—	—	—	—	—	—
15	—	—	—	—	+	—	+	—	—	—	—	—	—	—	—

[a] — reaction intensity: — = negative reaction, + = weak reaction, ++ = medium reaction, +++ = strong reaction i ++++ = very strong reaction

isolate 68-5 has protein bands 14 and 15, which are omitted in the other isolates from this species. Only this isolate forms protein band 12 and shows some similarity with isolate 13, identified as *A. alternata*. Among the all investigated, only these two isolates, 68-5 (*A. dauci*) and 13 (*A. alternata*), although belonging to different species, form protein bands 14 and 15. Nevertheless, isolate 13 (*A. alternata*) clearly differs from all the other isolates included in the investigations, by omitting the protein band 6, which is common to all *A. dauci* and *A. radicina* isolates, except for the isolate 68-1. The group of isolates which belong to species *A. radicina* (68-1, 89, BMP 79 and NL1R), with a few exceptions, showed smaller number of protein bands in profile, i.e. revealed differences between particular isolates. Thus, only isolate 68-1 does not form band 6, which is present in all the others in this group, and does not completely correspond to a common profile. The group of isolates belonging to *A. petroselini* (69, 95, 106 and BMP 139) also exhibited great uniformity of characteristics, but reacted with smaller number of bands, which were of weaker intensity, compared to the other *Alternaria* spp. All the isolates from this group formed five bands each, while variations within this group were not observed under the investigations conditions.

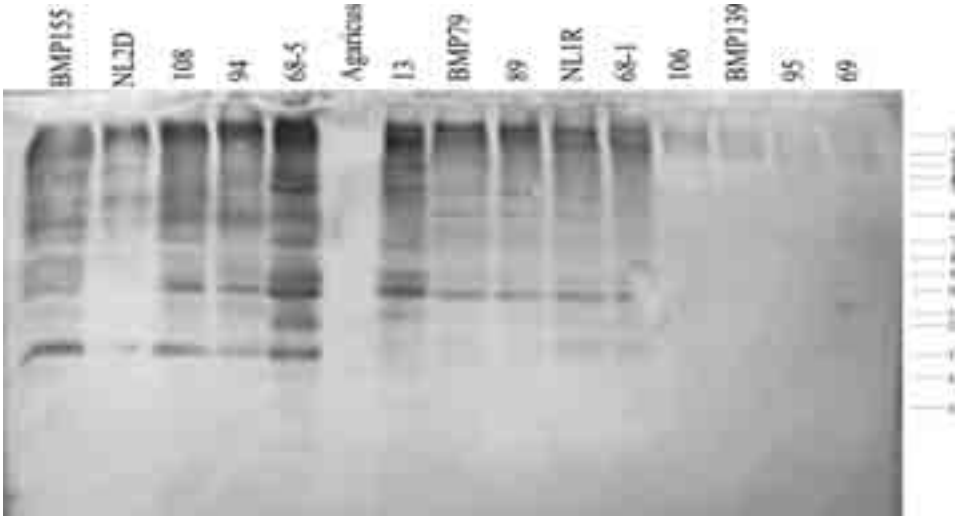

Fig. 1 — Serological reaction of *Alternaria* spp. isolates using EBIA method

BMP 155 and NL2D standards for *A. dauci*, 108, 94 i 68-5 (*A. dauci*), *Agaricus* sp. (-) control, 13 (*A. alternata*), BMP79 and NL1R standards for *A. radicina*, 89 i 68-1 (*A. radicina*), 106, 95 i 69 (*A. petroselini*) and BMP 139, standard for *A. petroselini*

DISCUSSION

In comparison to other features, serological characteristics in mycology are investigated and used less frequently. Nevertheless, there are examples of a very successful application in distinguishing or diagnostics of particular plant pathogenic species of fungi, even the categories lower than species level (Mohan, 1989; Mathew and Brooker, 1991; Benson, 1992; Bowen et al., 1996; Dewey and Cole, 1996; Hahn and Werres, 1996; Heppner and Heitefuss, 1996; Stcherbaukova and Umnov, 1996; Ueli and Walsh, 1996; Srivastava and Arora, 1997; Vico, 1997; Williams and Fitt, 1999; Kesari et al., 2005). Antigenic characteristics of particular fungi have been successfully used even for detection of the presence of some mycotoxins in different substrates (Szurdoki et al., 1996). This experience shows that studying of phytopathogenic fungi serological characteristics could lead to the development of very powerful and fast methods for detection or diagnostics, which could be routinely used.

Serological features of *Alternaria* species have not been studied almost at all. These fungi have drawn the greatest attention as powerful allergens, and the greatest number of published papers is related to studying of their antigenic properties in human medicine (Scumacher et al., 1975; Vijay et al., 1997; Bush et al., 1983; Chang et al., 1989; Weber, 2001). Guilong (1995) studied the possibility of application of standard serological test ELISA, which is routinely used in diagnosis of plant pathogen categories other than fungi, for *A. alternata*, detection on tobacco seed. Antiserum used in his investigation was highly specific to *A. alternata* and it did not react with fungi from other genera, which is in complete accordance with the results obtained in this investigation. Our results, obtained by using EBIA, showed the presence of bands in protein profiles, which could be specific for particular species. Specificity of the produced antiserum, if necessary, could be improved for diagnostic purposes. However, since the objective of this investigation was comparison of taxonomic relationships among *Alternaria* isolates, the fact that the produced antiserum reacted with all the examined isolates made the comparison of their protein profiles possible, so that their common or specific features could be observed.

Study on the antigenic characteristics of the examined *Alternaria* isolates, pathogenic for Apiaceae plant hosts, provided a new insight into their taxonomic relationships. Serology, as a taxonomic criterion, succeeded in grouping the isolates based on the appearance of their protein profiles in the way which completely corresponds to all other criteria, used in this investigation. Except the grouping to an assumed species level, antigenic properties indicated both similarities and differences among the isolates within the same and different groups-species, revealing their extremely complex relationships which genuinely reflect the diversity of these fungi in nature.

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АНТИГЕНЕ ОСОБИНЕ КАО ТАКСОНОМСКИ КРИТЕРИЈУМ ЗА РАЗЛИКОВАЊЕ *ALTERNARIA* SPP. ПАТОГЕНИХ ЗА МРКВУ И ПЕРШУН

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Резиме

Идентификација врста у оквиру рода *Alternaria* је веома сложен процес који захтева широко постављена испитивања и проучавање већег броја особина које заједно представљају задовољавајуће таксономске критеријуме. Основни циљ овог рада је био да се испита могућност примене антигених особина фитопатогених гљива из рода *Alternaria* као таксономског критеријума, као и увођење серолошких метода за њихову идентификацију. Проучавајући гљиве из рода *Alternaria* које су патогене за гајене биљке из фам. *Apiaceae* у нашој земљи, добијено је више изолата који су, на основу проучавања конвенционалним методама и круга домаћина, као и молекуларне детекције и делимичне карактеризације, разврстани у четири врсте: *Alternaria radicina*, *A. petroselini*, *A. dauci* и *A. alternata*.

У испитивања је било укључено 12 изолата пореклом са листа, семена или из земље, који су испољавали патогеност првенствено према мркви и першуну и идентификовани да припадају врстама *A. radicina*, *A. petroselini*, *A. dauci* и *A. alternata*. Испитивани изолати су међусобно упоређивани као и са стандардима за наведене врсте (укупно 5 изолата, пореклом из САД и ЕУ).

Приликом испитивања серолошких особина *Alternaria* spp., прво је припремљен поликлонални антисерум на изолат из Србије, који је идентификован као *A. dauci*. Антисерум је испољио специфичност за род *Alternaria*, док није реаговао са антигенима из других родова фитопатогених гљива (*Fusarium*, *Rhizoctonia*, *Agaricus*). Титар антисерума, испитиван методом аглутинације на плочици, био је 1/32. Антигене особине гљива из рода *Alternaria* проучаване су применом електрофоретско-адсорпционо-имуноензимске серолошке методе (EBIA, Western blot), односно упоређивани су њихови протеински профили.

Испитивани изолати *Alternaria* spp. испољили су различите профиле протеинских трака у гелу и на нитроцелулозном папиру, а уочене разлике су, у потпуности, одговарале резултатима претходне идентификације. Наведени изолати, домаћи као и стандарди, испољили су међусобне сличности и могли су бити правилно идентификоване до нивоа врсте, применом ЕБИА. Поред груписања до нивоа врста, антигене особине су указале на постојање сличности и разлика

између изолата унутар истих, односно различитих врста, указујући на њихове веома сложене међуодносе који верно осликавају диверзитет постојања ових гљива у природи.

У досадашњим проучавањима гљива из рода *Alternaria*, не постоје подаци о њиховим серолошким особинама као могућим таксономским критеријумима. Увођење ове групе особина, представља значајан допринос како у таксономији, тако и у примени брзих и тачних метода идентификације фитопатогених гљива.

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RESISTANCE OF SOME COMMERCIAL WINTER WHEAT CULTIVARS TO *TILLETIA TRITICI*

ABSTRACT: This paper deals with the resistance of twenty commercial winter wheat cultivars to common bunt causal agent (*Tilletia tritici*). Significant differences among the cultivars concerning the infection percent were observed, as well as the differences in the level of commercial cultivars' resistance to *T. tritici*. Most of the studied cultivars belonged to susceptible categories, and just few of them to the resistant ones. Cultivar Lasta was classified as highly resistant during the both investigation years in Kragujevac, while in Leposavić Lasta and Tiha were classified as resistant. The other studied cultivars were more or less susceptible.

KEY WORDS: wheat, *Tilletia tritici*, common bunt, resistance, cultivar

INTRODUCTION

One of the earliest known wheat diseases is bunt, which is caused by various fungi species from genus *Tilletia*. The first report about the appearance of this disease in our country was published by Ranojević (1912). Until 1960, it was very frequent and harmful disease in Serbia (Kostić et al., 1966), and in the early '90s of the last century this almost forgotten disease massively appeared again (Stojanović et al., 1993, 1994; Jevtić et al., 1997a, 1997b). Thanks to the intensive seed disinfection it appears rarely (Matijević et al., 1994).

Among integral wheat protection measures against common bunt causal agent, creation resistant cultivars of and their growing could be of importance

(W i e s e, 1987). Thus, the aim of this research was to study the resistance of some important commercial winter wheat cultivars to *T. tritici* and to point out their importance.

MATERIAL AND METHODS

They studies were carried out in 2005 and 2006 at the experimental field of the Institute for Small Grains in Kragujevac, as well as in 2006 at the location Leposavić (Kosmet). Resistance of twenty commercial winter wheat cultivars to *T. tritici* was investigated. Overdose method was used for inoculation of hundred grains per each studied wheat cultivar, by application of dry teleutospores, after which excessive teleutospores were removed by sifting through a sifter. This method ensured the presence of over 60 000 teleutospores on each grain.

Sowing in Kragujevac was done on October 26, 2005 and October 15, 2006, and in Leposavić on October 28, 2006. Inoculated seeds were sown in consecutive rows, one cultivar per row. Row length was 1 m, and inter-row distance was 25 cm. Common agrotechnical measures for wheat were applied during the vegetation, and weed plants were destroyed mechanically.

In full ripening stage, spikes of the studied cultivars were cut off and their health status was investigated in the laboratory. Total number of both analysed and attacked spikes was evaluated.

A scale with values 0-IV was used for establishing the level of cultivars resistance to *T. tritici* (K r i y c e n k o and M j a g k o v a, 1977):

0 — highly resistant cultivar (every spike healthy);
I — resistant cultivar (number of affected spikes up to 10%);
II — medium resistant cultivar (number of affected spikes from 11 to 25%);
III — medium susceptible cultivar (number of affected spikes from 26 to 50%);
IV — highly susceptible cultivar (number of affected spikes over 50%).

RESULTS

On the basis of the data presented in Table 1, one can see that the average infection intensity in Kragujevac was 25.64% in 2005, and 70.62% in 2006, while in Leposavić it amounted to 51.06%. That points to better conditions for infection during the autumn 2005, in regard to 2004. The highest infection intensity in 2006 in Kragujevac was shown by susceptible cultivars Kraljevica (92.21%) and Evropa (90.48%), and in Leposavić by cultivar Evropa (95.71%).

Tab. 1 — Resistance of some commercial winter wheat cultivars to *Tilletia tritid*

Cultivar	2005.			2006.					
	Kragujevac			Kragujevac			Leposavić		
	I	II	III	I	II	III	I	II	III
Pobeda	86	11	12.79	82	72	87.80	26	17	65.38
Evropa	32	15	46.88	63	57	90.48	70	67	95.71
NS Rana 5	42	19	45.24	57	47	82.46	24	17	70.83
Lasta	31	0	0.00	63	0	0.00	49	4	8.16
Tiha	67	20	29.85	77	65	84.42	77	5	6.49
Balkan	56	8	14.29	78	60	76.92	66	39	59.09
KG-100	40	18	45.00	83	48	57.83	34	20	58.82
KG-56S	62	25	40.32	62	44	70.97	41	24	58.54
Vizija	18	4	22.22	69	43	62.32	56	32	57.14
Ana Morava	82	30	36.58	50	33	66.00	38	23	60.53
Kraljevica	59	20	33.90	77	71	92.21	9	4	44.44
Partizanka	24	1	4.17	61	39	63.93	70	41	58.57
Jugoslavija	44	11	25.00	93	69	74.19	17	5	29.41
Rodna	45	3	6.67	71	41	57.75	43	17	39.53
Kruna	45	17	37.78	83	71	85.54	97	70	72.16
PKB Krupna	31	7	22.58	83	63	75.90	36	22	61.11
Dejana	76	0	0.00	64	42	65.63	18	8	44.44
Danica	16	5	31.25	90	78	86.67	33	19	57.58
Toplica	75	26	34.67	99	65	65.66	27	8	29.63
Rana Niska	38	9	23.68	70	46	65.71	55	24	43.64
MEAN			25.64			70.62			51.06

I — number of analysed spikes; II — number of infected spikes; III — infection percent

The study results also point to the existence of significant differences in the infection percent of various cultivars, as well as to the different resistance level to *T. tritici* of the investigated commercial cultivars. Most of the studied cultivars belonged to susceptible categories (III and IV), and just few of them to the resistant ones (0, I and II).

Cultivar Lasta was the only one classified as highly resistant during the both investigation years in Kragujevac. In 2005 cultivar Dejana was highly resistant, cultivars Partizanka and Rodna belonged to the resistant category, while medium resistance was shown by cultivars Pobeda, Balkan, Vizija, Jugoslavija, PKB Krupna, and Rana Niska. At Leposavić location in 2006, only cultivars Lasta and Tiha were classified as resistant.

DISCUSSION

The obtained results show significant differences among cultivars regarding the infection percent, which points to their different resistance to *T. tritici*. Cultivar Lasta was highly resistant during the both investigation years in Kragujevac, as well as in Leposavić. Resistance of this cultivar, similar to that observed in this research, was pointed out in many previous reports (S t o j a n o v i ć et al., 1996, S t a l e t i ć et al., 2002, G u d ž i ć et al., 2006). Culti-

var Lasta has a satisfactory level of resistance to common bunt causal agent, which could be of importance for its spreading in the production, or using in the selection programmes as a gene donor. Peresipkin (1979) found that the mycelium *T. tritici* relatively easily, penetrates into shoot tissue, but in the heading stage it becomes disorganized in the resistant host cultivars, so most of the spikes remain without any visual disease symptoms.

A high number of susceptible cultivars was expected, regarding the fact that no organized selection for getting resistance to this pathogen was done in our country, which points to the necessity of creating new cultivars that would show, besides other positive production traits, a high resistance to *T. tritici*. When talking about wheat protection from common bunt, one ought to have in mind that this fungus has a large number of physiological races, each having a different virulency. Roderhiser and Holton (1937) reported the first data about the existence of *T. tritici* physiological races. Virulency structure of this pathogen is not known in our country.

Differences in the cultivar resistance level within years can be explained by different conditions for infection development, but it need not mean that this cultivar has factors of resistance (Staletić et al., 2002). In order to estimate active resistance of a cultivar, long term studies are neccessary.

Although an efficient wheat protection from common bunt can be achieved by fungicide application, future selection programmes for creation and growing resistant cultivars will enable a more efficient, ecologically clear, and cost effective wheat protection.

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ОТПОРНОСТ НЕКИХ КОМЕРЦИЈАЛНИХ СОРТИ ПШЕНИЦЕ ПРЕМА *TILLETIA TRITICI*

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Резиме

У раду је приказана отпорност двадесет комерцијалних сорти пшенице према проузроковачу главнице (*Tillecia tritici*). Утврђено је да постоје значајне разлике у проценту инфекције појединих сората, као и то да је отпорност комерцијалних сората пшенице према *T. tritici* различита. Већина испитиваних сората припадала је осетљивим, а мањи број отпорним категоријама. Врло отпорна у обе године проучавања у Крагујевцу била је сорта Ласта, а у локалитету Лепосавић у категорији отпорних биле су сорте Ласта и Тиха. Остале проучаване сорте су биле мање или више осетљиве.

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IDENTIFICATION OF *RHIZOCTONIA SOLANI* ISOLATES FROM SUGAR BEET ROOTS BY ANALYSING THE ITS REGION OF RIBOSOMAL DNA

ABSTRACT: *Rhizoctonia solani* (Kühn) is one of the most important sugar beet pathogens. *Rhizoctonia solani* anastomosis groups (AGs) 2-2 and 4 are proven to be the most common pathogenic strains on sugar beet. AG 2-2 (intraspecific groups IIIB and IV) can cause root and crown rot while damping-off of seedlings is most frequently attributed to AG 4. Four isolates of *R. solani* from sugar beet roots showing characteristic crown and root rot symptoms, collected from different localities in Vojvodina Province, were chosen and compared to the well-characterized *R. solani* isolate R9, AG 2-2 IV, from the USA. All Vojvodinian isolates showed medium level of pathogenicity and were able to cause crown and root rot symptoms on inoculated sugar beet roots. Based on anastomosis reaction, isolates from Vojvodina did not belong to the AG 2-2 group. Sequencing of the ITS (internal transcribed spacer) region of ribosomal DNA was performed on the Vojvodinian isolates from R9 in order to determine their relatedness. Sequence analysis showed that these isolates were different than R9 and were closely related (99—100% sequence homology) to anastomosis group 4, subgroup HG II.

KEY WORDS: Sugar beet, *Rhizoctonia solani*, anastomosis group, ITS region, PCR

INTRODUCTION

Rhizoctonia solani Kühn, teleomorph *Thanatephorus cucumeris* (Frank) Donk is a soilborne fungus with a worldwide distribution. In the USA more than 24% of acres planted with sugar beet have an economic damage from this pathogen, while in Europe only 5—10% of the planted area is considered to have economic losses from this pathogen (J a c o b s e n, 2005). There are no

data about economic damages of *Rhizoctonia* crown and root rot in Vojvodina (Jasnić et al., 2006). However, this fungus was isolated from 0—18.2% of beets showing root rot during the period from 2000 to 2005 (Stojšin et al., 2006). This specy is highly heterogeneous and produces damping-off, root rot and foliar blight symptoms on many plant species. *Rhizoctonia* is typically a sterile fungal genus and has been characterized by division into binucleate and multinucleate groups. *Rhizoctonia solani* is multinucleate and on the basis of hyphal anastomosis between different isolates, it is divided into 14 anastomosis groups: AG 1-13 and AG BI (Carling et al., 2002). Groups -1, -2, -3, -4, -6, -7, -8 and -9 are additionally divided into subsets (intraspecific groups) based on their morphological characteristics, frequency of hyphal fusion, virulence, host range, nutritional requirements, biochemical characteristics, thiamine requirement, pectic isozymes, fatty acids and molecular characteristics (Carling et al., 2002; Guillemaut et al., 2003). On sugar beet, this fungus causes damping-off, root, and crown root as well as foliar blight of sugar beet. According to Windels and Nabben (1989) *R. solani* anastomosis groups -1, -2-2, -4 and -5 can cause damping-off of sugar beet. Additionally, AG 3 and AG 5 were isolated from sugar beet with symptoms of dark discoloration on petiole basis (Windels et al., 1997). The causal agent of *Rhizoctonia* crown and root rot of sugar beet is typically characterized as belonging to AG 2-2 with individual isolates being placed into intraspecific groups IIIB and IV. Both of these intraspecific groups are found worldwide, although AG 2-2 IIIB is more common on sugar beet in Europe in rotation with maize (Jacobsen, 2005). Recently, besides the system of anastomosis grouping of *R. solani* isolates based on hyphal fusion, different molecular methods have been developed and proven to be very useful for analysis of the evolutionary homology between isolates in the *R. solani* complex. Genetic heterogenicity between, and within anastomosis groups was evaluated by Fenille et al., 2003, using sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA. Comparison of the ITS region is significant not only for the determination of anastomosis groups, but these sequences are also useful for verifying subsets. Polymorphism between AGs was revealed in the ITS1 and ITS2 sequences of ribosomal DNA, while 5.8s rDNA sequence is comletely conservative across all AGs (Kuninaga et al., 1997).

In this study, the ITS region of four *R. solani* isolates, which originated from sugar beet root with typical symptoms of crown and root rot, was analysed in order to determine sequence variations between the isolates, and to identify the anastomosis group and subset of examined isolates.

MATERIALS AND METHODS

Four isolates of *Rhizoctonia solani*: RhKZ, RhBG, RhGL and RhVR were isolated from the sugar beet plants with typical symptoms of *Rhizoctonia* crown and root rot in Vojvodina Province. These isolates were pathogenic on the susceptible sugar beet variety Delta (Budakov et al., 2006), and caused characteristic crown and root rot symptoms.

Characterization of the isolates. Isolates were checked for number of nuclei by staining mycelia with safranin-0 and observed microscopically at 400X (B a n d o n i, 1971). Anastomosis tests were performed using the method which was set up by L i u and S i n c l a i r (1991). Before microscopic examination, samples were dyed with cotton blue in dilute lactophenol (P a r m e t e r et al., 1969). These isolates were tested for anastomosis with determined culture of *Rhizoctonia solani*, which was isolated from sugar beet in the USA and belonged to AG 2-2 IV. Hyphae were checked in, at least, 15 microscopic fields at a magnification of 400X, while anastomosis was rated at 600X (V i c o, 1997). Examination of the reaction between hyphae was categorized according to C a r l i n g (1996) — Table 1.

Tab. 1 — Categories of hyphal interaction in *Rhizoctonia solani*

Category	Hyphal interaction
C0	No interaction
C1	Hyphal wall contact
C2	Hyphal wall and membrane fusion, death in fused and adjacent cells
C3	Hyphal wall and membrane fusion, no death in fused and adjacent cells (perfect fusion)

DNA isolation. Prior to DNA extraction, *R. solani* isolates were cultured on potato-dextrose agar (PDA, Difco Laboratories, Detroit, MI) at 25°C for 4—5 days. The mycelium was harvested by removing excess of the solid media using steril scalpel and then ground up in liquid nitrogen. DNA was extracted from 100 mg of ground fungal tissue using the E.Z.N.A. Fungal DNA Kit (Omega Bio-Tek Inc., Lilburn, GA, USA) and following the protocol recommended by the manufacturer.

PCR amplification. The ITS region of the rDNA was amplified using ITS 1F and ITS 4 primers (Table 2). The PCR reaction was performed in 50 µl total volume consisting of 25 µl of PCR Master Mix (Promega Corporation, Madison, WI, USA), 5 µl of $MgCl_2$, 2 µl of each primer (concentration 10 pmol/µl), 2 µl of DNA template and 14 µl of PCR Grade water. The amplification was performed in PCR thermal cycler (Whatman Biometra, Goettingen, Germany). The cycle parameters were as follows: an initial denaturation at 95°C for 2 minutes, followed by 38 cycles consisting of denaturation at 95°C for 1 minute, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute. Final extension was at 72°C for 5 minutes. Following the PCR reaction, the amplified products were loaded in a 2% agarose gel stained with ethidium-bromide, together with 100 bp DNA marker (Promega Corporation, Madison, WI, USA). Before loading, both samples and marker were dyed with Blue/Orange 6X Loading Dye used for tracking migration during electrophoresis. Electrophoresis was run at 80 V for 2 hours. The DNA bands were visualised using a AlphaImager 2200 Imaging System (Alpha Innotech Corporation, San Leandro, CA).

Tab. 2 — Primer ITS 1F and ITS 4 sequences, base count, molecular weight, melting temperature and GC content

	Sequence (5' to 3')	Base count	Molecular weight	Melting temperature (°C)	GC content (%)
ITS 1F	CTT GGT CAT TTA GAG GAA GTA A	22	6813.32	56.60	36.37
ITS 4	TCC TCC GCT TAT TGA TAT GC	20	6033.78	61.50	45.00

DNA sequencing. After the amplification of the ITS region of the rDNA, each product was purified using the QIAquick PCR Purification Kit and protocol (Qiagen Inc., Valencia, CA, USA). The purified rDNA was shipped by overnight mail to DNA Sequencing Facility at University of California, Berkeley where isolates were sequenced.

Data analysis. ITS sequence analysis was performed using on-line softwares CLUSTAL W and BLAST via http://www.ncbi.nlm.nih.gov, www.bioservers.org. CLYSTAL W is a multiple sequence alignment program which calculates the best match for the selected sequences, and lines them up so that similarities and differences can be seen. The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide sequences to the sequence databases and calculates the statistical significance of the matches. BLAST can be used to infer functional and evolutionary relationships between sequences, as well as to identify the members of the gene families. Sequence database from the National Center for Biotechnology Information (NCBI), which was downloaded from http://www.ncbi.nlm.nih.gov, was used for sequence information on selected *R. solani* isolates (Table 3).

Tab. 3 — *Rhizoctonia solani* isolates whose ITS sequences were recovered from the GenBank (National Center for Biotechnology Information — NCBI) and used for determining complementarity with tested Vojvodinian isolates RhKZ, RhBG, RhGL and RhVR

AG and subset	Host and geographic origin	GenBank accession number of ITS sequence
AG 1-IA	*Oryza sativa*, Japan	AB000017
AG 1-IB	*Beta vulgaris*, Japan	AB000038
AG 1-IC	*Beta vulgaris*, Japan	AB000029
AG 2-1	*Solanum tuberosum*, USA	AB000026
AG 2-2IIIB	*Beta vulgaris*, USA	AB054856
AG 2-2 IV	*Beta vulgaris*, USA	AB054859
AG 2-3	*Glycine max*, Japan	U57740
AG 2-4	*Zea mays*, USA	AB054878
AG 3	*Beta vulgaris*, USA	AB019006
AG 3PT	*Solanum tuberosum*, USA	AB019013
AG 3TB	*Nicotiana tabacum*, USA	AB000001
AG 4 HGI	*Beta vulgaris*, Japan	AB000028
AG 4 HGII	*Beta vulgaris*, Japan	AB000033
AG 4 HGIII	*Beta vulgaris*, USA	AF354075
AG 5	*Beta vulgaris*, Japan	AF153777

AG 6	*Pterostylis acuminata*, Australia	AF153784
AG 6GV	Soil, Japan	AF354101
AG 6HGI	Soil, Japan	AG153779
AG 7	Soil, Japan	AB000003
AG 8	*Triticum aestivum*, Australia	AB000011
AG 8ZGI-1	Soil, Australia	AF153795
AG 8ZGI-2	Soil, Australia	AF153797
AG 8ZGI-3	*Hordeum vulgare*, Australia	AF354068
AG 8ZGI-4	*Hordeum vulgare*, Scotland	AF354066
AG 9	*Solanum tuberosum*, USA	AF354109
AG 9TX	*Solanum tuberosum*, USA	AB000037
AG 9TP	*Solanum tuberosum*, USA	AB000046
AG 10	*Hordeum vulgare*, Australia	AF354071
AG 11	*Glycine max*, USA	AF354114
AG 12	*Pterostylis acuminata*, Australia	AF153803
AG BI	Soil, Japan	AB000044

RESULTS

Anastomosis reaction. *Rhizoctonia solani* isolates RhKZ, RhBG, RhGL and RhVR were multinucleate and did not anastomose with a well-characterized isolate R9, AG 2-2 IV, from the USA (Figure 1.b). However, isolates RhKZ, RhBG, RhGL and RhVR anastomosed with each other (Figure 1.a) indicating that they belonged to the same anastomosis group (Table 4).

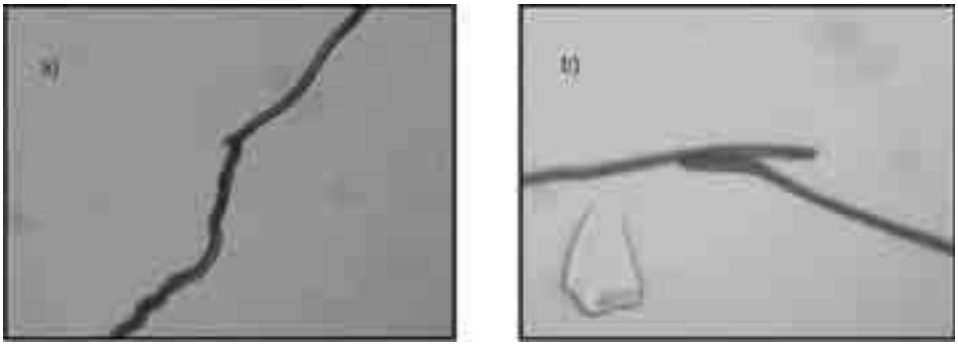

Fig. 1 — a) Hyphal fusion between two Vojvodina isolates RhKZ and RhVR at 400X; b) Contact between hyphae belonging to isolates RhR9 and RhGL at 400 X

Tab. 4 — Categories of hyphal interacton between all isolates

Isolate	Rh KZ	Rh GL	Rh BG	Rh VR	Rh R9
Rh KZ	C3	C3	C3	C3	C0
Rh GL		C3	C3	C3	C0
Rh BG			C3	C3	C0
Rh VR				C3	C0
Rh R9					C3

Gel elecrophoresis. After PCR reaction, the presence of amplified products were tested by running an agarose gel in TAE buffer. DNA bands were approximately 740—750 base pairs long (Figure 2).

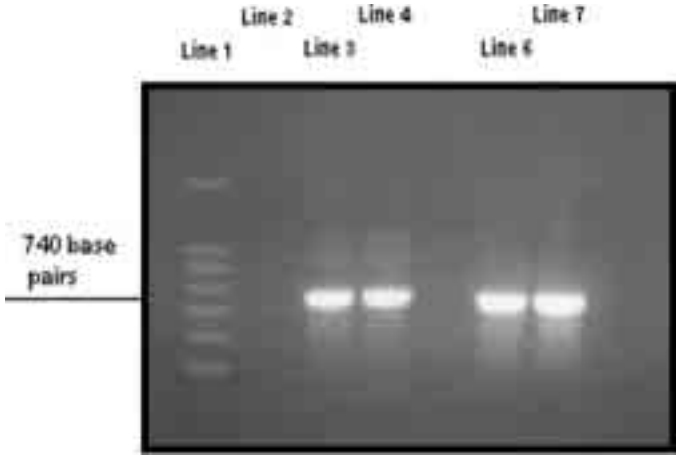

Fig. 2 — Amplification of DNA from *Rhizoctonia solani* isolates RhKZ, RhBG, RhGL and RhVR with primers ITS 1F and ITS 4 shows bands 740 base pairs long (line 1 — 100 bp marker, line 2 — control, line 3 — RhKZ, line 4 — RhBG, line 6 — RhGL and line 7 — RhVR)

rDNA sequence analysis. Sequencing of rDNA of isolates RhKZ, RhBG, RhGL and RhVR showed that sizes of the ITS region varied from 713 to 716 base, pairs and their sequences were identical (100%) in the ITS region. Similiarity of the ITS region of Vojvodinian isolates and the American isolate R9 (AG 2-2 IV) was 93%. The ITS sequences of 31 isolates of *R. solani*, which belonged to AG 1-12 and AG BI were recovered from the GenBank (Table 4). The comparison between sequences registered in GenBank and sequences of Vojvodinian tested isolates showed that all isolates had sequence homology between 90 and 100% with the referent isolates (Table 5). ITS sequences of the tested isolates showed 100% complementarity with representative of anastomosis group 4, intraspecific group HGII.

Tab. 5 — Sequence similarity between isolates RhKZ, RhBG, RhGL and RhVR and other AGs isolates of *Rhizoctonia solani*

AG and subset (GenBank)	ISOLATES			
	RhKZ	RhBG	RhGL	RhVR
AG 1-IA	94%	94%	94%	94%
AG 1-IB	91%	91%	91%	91%
AG 1-IC	93%	93%	93%	93%
AG 2-1	94%	94%	94%	94%
AG 2-2IIIB	92%	92%	92%	92%
AG 2-2 IV	93%	93%	93%	93%
AG 2-3	93%	93%	93%	93%

AG 2-4	93%	93%	93%	93%
AG 3	93%	93%	93%	93%
AG 3PT	93%	93%	93%	93%
AG 3TB	95%	95%	95%	95%
AG 4 HG-I	96%	96%	96%	96%
AG 4 HG-II	100%	100%	100%	100%
AG 4 HG-III	91%	91%	91%	91%
AG 5	92%	92%	92%	92%
AG 6	94%	94%	94%	94%
AG 6-GV	95%	95%	95%	95%
AG 6HG-I	93%	93%	93%	93%
AG 7	95%	95%	95%	95%
AG 8	95%	95%	95%	95%
AG 8ZGI-1	95%	95%	95%	95%
AG 8ZGI-2	95%	95%	95%	95%
AG 8ZGI-3	95%	95%	95%	95%
AG 8ZGI-4	91%	91%	91%	91%
AG 9	94%	94%	94%	94%
AG 9TX	94%	94%	94%	94%
AG 9TP	94%	94%	94%	94%
AG 10	90%	90%	90%	90%
AG11	92%	92%	92%	92%
AG12	93%	93%	93%	93%
AG BI	94%	94%	94%	94%

DISCUSSION

The present study shows that ITS sequencing is a powerful tool in understanding and determinating the relationship between anastomosis groups and subgroups of *R. solani*. Techniques which rely on molecular markers are very important in understanding *R. solani* complex because they are more accurate, easier and more rapid than conventional techniques for determining anastomosis group. The aim of the research was to analyse the less conserved part of the rDNA region, since it is proven that 5.8s rDNA sequence is completely conserved across all AGs, whereas the ITS1 and ITS2 rDNA sequences show significant differences between AGs (Fenille et al., 2003). The sequence homology in the ITS regions is above 96% for isolates of the same subgroup, 66—100% for isolates of different subgroups within an AG, and 55—96% for isolates of different AG (Kuninaga, 1997). The four isolates from Vojvodina Province which were isolated from sugar beet root with symptoms of crown and root rot, were shown to belong to AG 4 HGII, with ITS sequence homology with referent AG 4 HGII isolates of 100%. In general, AG 4 isolates are known to be pathogenic on wide variety of hosts (Kuninaga, 1997), among which is sugar beet (Windels and Nabben, 1989). AG 4 HG-II is pathogenic on soybean seedlings, causing damping-off and hypocotyl rot (Fenille et al., 2000), on turfgrass (Hsiang, 2000), coffee seedlings (Kuramae et al., 2000), and sugar beet (Sneh et al., 1991; Gonzalez et al., 2006). Besides sequencing of the ITS part of the rDNA, other molecular biology techniques have been developed. For example, restriction fragment

length polymorphism (RFLP) analysis of ITS region of rDNA was developed for a rapid and accurate analysis of large number of isolates of *R. solani* (Guillemaut et al., 2003). RFLP analysis can be very fast and can be carried out with 10 to 40 isolates simultaneously (Burns et al., 1991). Another example is the use of random amplified polymorphic DNA-RAPD used for detection of differences between *Rhizoctonia solani* AGs (Duncun et al., 1993), then for determinating AG 3 and its subsets (Justensen et al., 2003; Bounou et al., 1999), and for identification of a uninucleate *Rhizoctonia* sp. (Lilja et al., 1996). Analysis of the ITS region of ribosomal DNA has proven in this instance to be an excellent tool for the identification and determination of *R. solani*, on the level of anastomosis group and subset. Since conventional methods for assigning AGs, such as observation of hyphal anastomosis, can be complicated, time-consuming and subjective, PCR assays provide a more accurate and rapid detection. This research shows the importance of identifying the correct AG of *R. solani* associated with crown and root rot of sugar beet, since the host range of AG 2-2 IIIB and IV are much different than AG 4 (Sneh et al., 1991; Windels and Nabben, 1989). AG 2-2 has a reported host range that includes sugar beet, bean, soybean, corn, rice, mat rush, turf grasses, ginger, gladiolus, burdock, chrysantemum, konjack and chinese yam, whereas AG 4 has a host range that includes sugar beet, tomato, pea, spinach, potato, slash and lobolly pine, and snap bean. These differences can affect the recommended crop rotations used to control partially the crown and root rot disease of sugarbeet. For example, potato would be a host for AG 4, but not AG 2-2.

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ИДЕНТИФИКАЦИЈА ИЗОЛАТА *RHIZOCTONIA SOLANI* СА КОРЕНА ШЕЋЕРНЕ РЕПЕ АНАЛИЗОМ ITS РЕГИОНА РИБОЗОМАЛНЕ ДНК

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Резиме

Rhizoctonia solani (Kühn) је један од најважнијих патогена шећерне репе. Познато је да су анастомозне групе (АГ) 2-2 и 4 најпатогеније на шећерној репи. АГ 2-2 (интраспецифичне групе IIIБ и IV) могу проузроковати трулеж корена и главе шећерне репе, док је палеж и пропадање клијанаца карактеристично за АГ 4. Одабрана су четири изолата *R. solani* изолованих са корена шећерне репе са карактеристичним симптомима мрке трулежи и који су пореклом са различитих локалитета у Војводини. Ови изолати су упоређени са *R. solani* изолатом P9, АГ

2-2 IV, пореклом из САД. Сви изолати пореклом из Војводине су показали средњи ниво патогености и проузроковали симптоме мрке трулежи на инокулисаном корену шећерне репе. На основу реакције анастомозе утврђено је да изолати из Војводине не припадају АГ 2-2. У циљу утврђивања сродности између изолата из Војводине и САД извршено је секвенционирање ITS (internal transcribed spacer) региона рибозомалне ДНК. Утврђено је да су наши изолати другачији од P9, а да су блиски (хомологија секвенци 99—100%) изолатима из анастомозне групе 4, подгрупе ХГ II.

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THE EFFECT OF *NEPETA RTANJENSIS* ESSENTIAL OIL ON TEST MICROMYCETES MYCELIA GROWTH

ABSTRACT: The antifungal activity of *Nepeta rtanjensis* Diklić et Milojević essential oil on mycelia growth has been performed by macrodilution method. The most efficient impact of *N. rtanjensis* essential oil on mycelia growth *in vitro* was found in *Alternaria* species with the same value of minimal inhibitory quantity (MIQ) of 0.6 µl/ml. *Bipolaris spicifera* and *Cladosporium cladosporoioides* had MIQ values of 1.0 µl/ml whereas *Trichoderma viride* with MIQ value of 1.6 µl/ml showed the most efficient defense against the essential oil examined. The values of minimal fungicidal quantity (MFQ) in *Alternaria* sp. 2, *B. spicifera* and *C. cladosporioides* match the MIQ values, whereas MFQ values in *Alternaria* sp.1 is 0.8 µl/ml, and in *T. viride* 1.8 µl/ml.

KEY WORDS: Antifungal activity, essential oil, micromycetes, *Nepeta rtanjensis*

INTRODUCTION

Nepeta rtanjensis (*Lamiaceae*) is an endemic and critically endangered (CR B_{2c}) aromatic plant which grows only on few localities on the Rtanj mountain in southeastern of Serbia (Diklić, 1999).

Nepeta species are widely used in folk medicine because of their medical properties. The essential oil of *N. rtanjensis* possesses strong antibacterial effect against different strains of *Staphylococcus aureus*, even more stronger than most synthetic antibiotics (Stojanović et al., 2005). The main component of essential oil of *N. rtanjensis* is 4aα, 7α, 7aβ nepetalactone. In wild population of *N. rtanjensis* oil amount of 4aα, 7α, 7aβ nepetalactone is 86.4%, while in oil of cultivated plants this component is presented with 77.9% (Chalchar et al., 2000; Stojanović et al., 2005). It is well known

that fungal infection can be a great threat to plant, animal and human health. Medicinal plants are good source of natural products with strong antimicrobial activities without any harmful effects. The use of natural antimicrobial compounds is important in the control of human, animal and plant diseases of microbial origin.

The aim of our investigation was to evaluate the antifungal activity of *N. rtanjensis* essential oil against mycelia growth of the selected fungi.

MATERIALS AND METHODS

N. rtanjensis was collected on the experimental fields of the Institute for Biological Research "Siniša Stanković", Belgrade. The plants were rapid micropropagated *in vitro*, transferred to the Greenhouse for acclimatization, and subsequently planted in an experimental field (M i š i ć et al. 2005). Herbal material was deposited at the Herbarium of Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, Belgrade (16064 BEOU).

The essential oil was isolated from air-dried aerial part of *N. rtanjensis* by hydrodistillation in a Clevenger type apparatus within two hours. Analyses of this oil were performed by GC (-FID) and GC/MS on fused silica capillary column PONA (crosslinked methyl silicone gum, 50 m x 0.2 mm, 0.5 μm film thickness). For these purposes Hewlett-Packard, model 5890, series II gas chromatograph, equipped with split-splitless injector, was used. Sample solution in ethanol (0.2%) was injected in split mode (1:100) at 250°C. Detector temperature was 300°C (FID), while column temperature was linearly programmed from 40°—280°C, at a rate of 2°C/min. In the case of GC/MS analysis, Hewlett-Packard, model 5971A MSD was used. The transfer line was kept at 280°C. The identification of each individual compound was made by comparison of their retention times with those of pure components, matching mass spectral data with those from Wiley library of 138 000 MS spectra. For library search PBM based software package was used.

Among the tested organisms were two groups of micromycetes: the autochtonous species (*Alternaria* sp. 1 from leaves and *Alternaria* sp. 2 from seeds of *N. rtanjensis*) and selected fungal species (*Cladosporium cladosporioides*, *Trichoderma viride* and *Bipolaris spicifera*) from Mycotheca at the Department of Algology, Mycology and Lichenology, Faculty of Biology, University of Belgrade.

The fungi were maintained on malt agar (MA). The cultures were stored at +4°C and subcultured once a month. In order to investigate the antifungal activity of essential oil, the mycelial growth test with malt agar was used (I s h i i, 1995). The minimum inhibitory quantity (MIQ) of oil necessary for the inhibition of mycelial growth of the fungal strain was determined. Different concentrations of essential oil (0.6—1.4 μg/mL) were diluted in Petri dishes with malt agar (MA). All fungal species were tested in triplicate. Essential oils were added into molten malt agar (MA) and poured into Petri dishes. The tested fungi were inoculated at the centre of the plates. Plates were incubated for three weeks at room temperature, and after this period MIQ and

MFQ were determined. Petri plates with commercial fungicide, Quadris (0.6—6.0 μg/mL), were used as a control.

RESULTS AND DISCUSSION

The analysis of chemical composition of essential oil showed the prevalence of 4aα, 7α, 7aβ nepetalactone (79.89%). It was shown that the essential oil isolated from *N. rtanjensis* has strong antifungal activity against all examined micromycetes (MIQ 0.6—1.6 μg/mL), (Table 1).

Tab. 1 — Minimal inhibitory quantity (MIQ) and minimal fungicidal quantity (MFQ) of the tested micromycetes

Micromycetes	MIQ (μl/ml)	MFQ (μl/ml)
Alternaria sp. 1	0.6	0.8
Alternaria sp. 2	0.6	0.6
Bipolaris spicifera	1	1
Cladosporium cladosporioides	1	1
Trichoderma viride	1.6	1.8

The oil quantity in amount of 0.6—0.8 μg/mL inhibited the growth of *Alternaria* species mycelia. The minimal inhibitory quantity of oil for *C. cladosporioides* and *B. spicifera* was 1.0 μg/mL. The highest MIQ (1.4 μg/mL) of oil was against *T. viride*. The commercial fungicide, Quadris, showed lower antifungal activity than *Nepeta* oil, with MIQ of 3.0—4.0 μg/mL. Quadris inhibited mycelial growth of *C. cladosporioides, B. spicifera* and *Alternaria* species at 3.0—4.0 μg/mL. *T. viride* was also the most resistant fungi on Quadris, with MIQ higher than 6.0 μg/mL.

In previous investigations of antifungal activity of different oils it can be seen that *A. alternata* was more sensitive than *T. viride* (Sokvić et al., 2002). The strong resistance of *T. viride* was also observed in previous investigations of essential oil antifungal activity. Analyses of antifungal activity of some essential oils, *Achillea atrata* and *Lauraceae* plants, showed that *T. viride* is the most resistant fungi (Ristić et al., 2004; Simić et al., 2004).

Our research proved that essential oil from *N. rtanjensis* has strong antifungal activity, and that it can inhibit the growth of mycelia of some fungi. The antifungal activity of essential oils isolated from other *Nepeta* species are also reported. Iridodial b-monoenol acetate isolated from the essential oil of *Nepeta leucophyla*, and actidine isolated from *Nepeta clarkei*, showed strong antifungal activity. Iridodial b-monoenol acetate was the most effective against *Sclerotium rolfsii*, while actidine was highly active against *Macrophomina phaseolina*. Both fungi are soybean pathogens. The essential oil from *Nepeta hindostana* has inhibitory effect on *Pythium aphanillermatum*, *P. debaryanum* and *Rhyzoctonia solani* (Saxena et al., 1996).

Because of low mammalian toxicity and biodegradable abilities as well as strong antimicrobial activity of essential oils, they can be used as bioagents (Oxenham et al., 2005).

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УТИЦАЈ ЕТАРСКОГ УЉА *NEPETA RTANJENSIS* НА РАСТ МИЦЕЛИЈЕ ТЕСТ МИКРОМИЦЕТА

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Резиме

Макродилуционом методом тестиран је утицај етарског уља ендемичне биљке *Nepeta rtanjensis (Lamiaceae)* на мицелијални раст микромицета: *Alternaria* sp. 1, *Alternaria* sp. 2, *Bipolaris spicifera, Cladosporium cladosporioides* и *Trichoderma viride*. Хемијска анализа етарског уља *N. rtanjensis* показала је апсолутну доминацију 4aα, 7α, 7aβ непеталактона (79.89%). Најефикаснији утицај на раст мицелије *in vitro* забележен је код врста рода *Alternaria* са истом вредношћу минималне инхибиторне количине (МИК) од 0.6 μl/ml. *Bipolaris spicifera* и *Cladosporium cladosporioides* су имали МИК 1.0 μl/ml, док је *Trichoderma viride*, са вредношћу

МИК од 1.6 μl/ml, показала највећу отпорност на дејство испитиваног уља. Вредности минималне фунгицидне количине (МФК) се код *Alternaria* sp. 2 (0.6 μl/ml), *B. spicifera* (1.0 μl/ml) и *C. cladosporioides* (1.0 μl/ml) поклапају са вредностима МИК, док је код *Alternaria* sp. 1 МФК 0.8 μl/ml а код *T. viride* 1.8 μl/ml. Етарско уље *N. rtanjensis* показало је јако антифунгално дејство на раст мицелије тестираних микромицета.

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ANTHROPOGENIC EFFECTS ON SOIL MICROMYCETES

ABSTRACT: This paper is a synthesis of long-term investigations based on the effect of different authropogenic pollutants (mineral and organic fertilisers, heavy metals, contaminated irrigation water, nitrification inhibitor and detergents) on the dynamics of soil fungi number.

The investigations were performed at the Microbiology Department and trial fields of the Faculty of Agronomy in Cacak on smonitza and alluvium soils in field and under greenhouse conditions. Maize, wheat, barley and red clover were used as test plants in these studies. The quantitative composition of the fungi in the soils investigated was determined by the *Czapek* selective agar dilution method.

The study results show that the number of soil fungi was dependent on the type and rate of agrochemicals used, on the growing season, and the soil zone the samples were taken from for the analysis. Lower nitrogen fertiliser rates (80 and 120 kg × ha^{-1}) and organic fertilisers stimulated the development of soil fungi, unlike the rate of 150 kg × ha^{-1}. Heavy metals, mercury and cadmium in particular, as well as high rates of the N-serve nitrification inhibitor, inhibited the development of this group of soil microorganisms. Generally, the adverse effect of contaminated irrigation water on the soil fungi was recorded in both soil types, and particularly in the smonitza under red clover. Low detergent (Meril) concentrations did not have any significant effect on this group of microorganisms.

In this respect, it can be concluded that the soil fungi number dynamics can be used in monitoring soils polluted by different toxinogenic substances.

KEY WORDS: contaminated irrigation water, detergents, fungi, heavy metals, mineral fertilisers, nitrification inhibitor, organic fertilisers

INTRODUCTION

One of the greatest scientific contributions made by Evgenii Nikolaevich Mishustin is a study and description of environmental-geographic distribution of soil microorganisms (Mishustin, 1966; Mishustin, Yemtsev, 1978). The results of these studies have been cited ever since in all textbooks on soil and applied microbiology throughout the world and are considered basal (Atlas, Bartha, 1992). The environmental-geographic distribution of

microscopic soil fungi was investigated in great detail in soils of different natural zones of former USSR and described in works by Mircink (M i r c i n k et al., 1981; M i r c i n k, 1988). As showed by these investigations, irrespective of the fact that the nominal composition of isolated fungi species in different soils can be fairly similar, fungal groups with a specific species structure (ratio of predominant, typical, rare and accidental species) are nevertheless created in concrete non-disturbed zonal soils. This thesis has been confirmed in numerous works by other authors (B a b j e v, S i z o v, 1983; Y e g o r o v, 1986; V e l i k a n o v, 1997 and others).

The overall development of biocenological researches in the seventies of the last century discovered the specificity of the soil fungi complex, not only from the biogeographic point of view, but also for specific zonal biotopes. It was determined that soil fungi formed typical complexes in zonal soils under specific plant communities, geographically very distant — the European part of Russia, Canada, the north of the USA (M i r c i n k, 1988; C h r y s t e n s e n, 1969, 1989).

The last decades of the XX century were characterised by increased diversity and intensity of anthropogenic factors in the biosphere, due to which occurred a change in the structure and function of terrestrial and aquatic communities of organisms (M i l l e r, 1990). Whereas these disturbances related to animals and plants were convincingly described as early as in the 60s—70s of the last century (O d u m, 1986), the communities of soil microorganisms, on the other hand, were long considered to be more resistant to anthropogenic factors. These issues, related to the complexes of soil microorganisms, became more intensified due to methodical problems with isolating microorganisms from soils and difficulties in identifying them at the species level.

The aim of our long-term investigations (1990—2006) was to determine whether the effect of different anthropogenic factors (mineral and organic fertilisers, heavy metals, nitrification inhibitors, detergents, contaminated irrigation water) could bring about a change in the quantitative composition of fungi, and whether there was a possibility of mycological indication of the effect of any specific anthropogenic factor.

MATERIAL AND METHODS

The investigations were conducted at the Microbiology Department and trial fields of the Faculty of Agronomy in Čačak. Field, laboratory and mathematical-statistical research methods were used in the study. Indication of the anthropogenic effects of mineral fertilisers, heavy metals, contaminated irrigation waters, nitrification inhibitors and detergents on the number of soil fungi was performed on smonitza and alluvium soils under field and greenhouse conditions. Maize, wheat, barley and red clover were used as test plants in these investigations. The quantitative fungi composition in the soils investigated was determined by the dilution method on the Czapek selective substratum by culturing 10^{-5} soil dilution. Following a seven-day incubation, their average number was determined and calculated per gram of absolutely dry soil.

The obtained results were assessed by the analysis of variance method, and the statistical significance of individual and interactive media was determined by the Lsd test.

RESULTS AND DISCUSSION

It was determined that a decrease in the qualitative diversity of natural ecosystems could be one of the most important consequences of the anthropogenic effect on them, which was confirmed several times via different plant and animal communities (Bigon et al., 1989). The results of our investigations showed that the anthropogenic factors affected the quantitative fungi composition in different soil types and under different crops (Đukić, Mandić, 1997; Mandić et al., 2004; Mandić et al., 2005a, b; Đukić et al., 2006).

For most microscopic soil fungi, the distribution range is wide, hence the isolation of stenotopic species is made very difficult. The most illustrative example of such species is *Mortierella ramanniana*, known as a species typical for non-disturbed podzolic zonal soils (Mircink, 1988). Under the effect of a number of anthropogenic factors (pollution with heavy metals, recreation and pasture plant cover degression etc.) the number of this species is decreased (Marfenina, 1985, 1997; Marfenina, Popova, 1989; Jemtsev, Đukić, 2000), the fungal complex "loses" shape, characteristic of a specific zonal soil type, and the frequency of its distribution is reduced (Tab. 1). Special experiments have determined that under conditions of anthropogenic disturbances the maturation of sporangia and spores in them does not occur (Marfenina, Lukina, 1989).

Tab. 1 — The change in the distribution frequence of the *Mortierella ramanniana* species in different soils under anthropogenic effects

Soil	Type of effect	Distribution frequency, %	
		Control	Trial
Podzol	Recreation degression	65*	10
Turfy-podzolic cultivated	Long-term NPK fertilisation	70*	40
	Liming	70*	20
	Pollution with Cd, 100 mg/kg	100*	—
Turfy-podzolic uncultivated	Recreation degression	60*	10
	Transport-associated pollution	40*	—
Brown forest soil	Pasture degression	80*	20
	Recreation degression	40*	—

* Reliable differences with probability > 0.95

In anthropogenically disrupted soils, far more transparent are resistant fungi species which are often simultaneously resistant to several anthropogenic effects, which are preserved in the soils and begin to predominate in them (Tab. 2). They are, generally, eurytopic species with a wide distribution range (Domsh et al., 1992) and a high sporogenesis level.

It is a known fact that one of the aspects of the natural zonal diversity loss is "trivialisation" of the flora and fauna, i.e. an increase in the density of the so-called "weed" species in communities (B i g o n et al., 1989). These tendencies can be manifested not only in higher organisms, but also in soil fungi. Table 2 lists soil fungi and their qualitative composition in the soil which is increased under the effect of certain anthropogenic factors. It should be particularly emphasised that all these species are eurytopic.

Tab. 2 — Microscopic soil fungi species resistant to different types of anthropogenic effects

Type of effect	**Resistant species**
Acid precipitations	*Penicillium spinulosum*
Heavy metals: Lead	*Mucor hiemalis* *Penicillium funiculosum*
Cadmium	*Aspergillus niger* *Paecilomyces lilacinus* *Penicillium funiculosum*
Transport-associated pollution	*Aspergillus fumigatus* *Aspergillus niger* Dark-coloured fungi: *Aureobasidium pullulans* *Cladosporium cladosporioides* *Alternaria alternata*
Urbanisation	*Aspergillus fumigatus* *Aspergillus flavus* *Aspergillus niger* *Fusarium oxysporum* *Fusarium moniliforme* *Paecilomyces variotii* *Penicillium vulpinum*
Recreation soil degression	Dark-coloured fungi: *Ulocladium botrytis* *Cladosporium cladosporioides*
Livestock grazing	*Aspergillus flavus*
Long-term nitrogen fertilisation	*Penicillium funiculosum*
Liming	Dark-coloured fungi: *Alternaria alternata* *Cladosporium cladosporioides*

Bearing in mind that **fertilisation** is one of the most important meliorative measures in modern production of agricultural crops, it can be also considered as a serious anthropogenic attack on soil microorganisms.

Soil fungi, as an important indicator of soil biogenity, can be used, among others, as indicators of the economic justification of using different types of fertilisers, particularly their higher rates. An increase in the number of this group of microorganisms, under the effect of nitrogen fertilisers, can be considered positive within certain limits. However, their excessive activation can be also harmful, because the processes focused on establishing the disturbed equilibrium lead to a mineral fertiliser loss, degradation of the soil physico-chemical and biological characteristics and other serious environmental

consequences (Knowles, 1982). For instance, according to the data by Đukić (1992) and Milošević et al. (1993), the long-term use of nitrogen fertilisers leads to a change in the structure of soil microorganism complexes and to an increase in the number of phytopathogenic microorganisms, particularly when the monoculture cropping system is used.

According to our long-term investigations (Đukić, Mandić, 1997; Đukić, Mandić, 2001; Mandić, Đukić, 2004), the number of soil fungi depended not only on the species and fertilisation concentrations used, but also on the soil zones the samples for analysis were taken from and on the crop growing period, as well as on their interactive effects (Tab. 3).

In general, the most pronounced stimulatory effect on soil fungi is produced by organic fertilisers, solid manure in particular, which may result from an increase in the organic matter amount in the soil, as well as from an improvement in the soil water-air relationship and nutrient regime (Jarak et al., 1991).

High nitrogen rates (N_3-150 kg × ha^{-1}) have a destimulatory effect on this group of microorganisms, particularly in initial vegetative phases (Tab. 3), whereas lower rates (N_1-80 kg × ha^{-1} and N_2-120 kg × ha^{-1}) statistically stimulate highly significantly the development of soil fungi.

Given that metabolites of plant and microbiological origin are important regulators of biological value of the soil (Govedarica and Jarak, 1995), the significantly higher number of soil fungi in the rhizosphere soil, compared to that in the edaphosphere soil, is a completely expected occurrence.

A decline in the number of soil fungi in final phases of maize development is a consequence of an increase in the amount of precipitations in these phases, which in the soil with high clay amount creates unfavourable conditions for the development of all aerobic groups of microorganisms (Derkačev and Balog, 1979).

Tab. 3 — Numbers of fungi (10^5 g^{-1} dry soil) in the soil under maize as affected by the fertilisers applied (A), sampling zone (B) and vegetation period (C) — Mandić et al., 2004

A		Control		N_1		N_2		N_3		Solid manure		Liquid manure		$\overline{X}$
B		Ed.	Rh.	Ed.	Rh.	Ed.	Rh.	Ed.	Rh.	Ed.	Rh.	Ed.	Rh.	
Periods (C)	I	18.0	27.0	38.3	39.0	30.0	40.3	12.6	15.3	25.0	34.0	24.3	30.6	28.28
	II	31.3	45.3	33.3	39.3	41.3	40.0	21.0	30.3	50.3	91.3	37.0	44.6	42.19
	III	7.3	8.6	19.3	21.7	27.0	29.6	11.3	21.0	11.3	15.3	7.0	8.6	15.14
$\overline{X}$		22.94		31.83		34.72		18.61		37.88		25.38		
$\overline{X}$	Edaphosphere							25.19						
	Rhizosphere							31.88						

Lsd	A	B	C	A × B	A × C	B × C	A × B × C
0.05	2.18	1.17	1.44	3.11	3.80	2.04	5.38
0.01	2.88	1.55	1.91	4.11	5.03	2.69	7.13

Ed. — Edaphosphere; Rh. — Rhizosphere

Heavy metals also significantly affect the number, species composition and viability of soil fungi (Duxbury, 1985, Đukić et al., 1999; Đukić, Mandić, 2000 a, b). They inhibit the processes of mineralisation and synthesis of different substances in the soil, suppress soil fungi respiration, cause a fungistatic effect and the like (Skvorcova et al., 1980).

At increased concentrations, most heavy metals reduce the number of soil fungi (Tab. 4). In laboratory examination conditions, in this respect, a particularly inhibitory effect is exhibited by lead and cadmium (Đukić, Mandić, 2006). However, it should be pointed out that concentrations of heavy metals, which undoubtedly inhibit the activity of soil fungi, differ significantly under laboratory and field conditions, so that a minimum reliable reaction under field conditions is evident at metal concentrations 10—50 times higher than the basal ones (Duxbury, 1985).

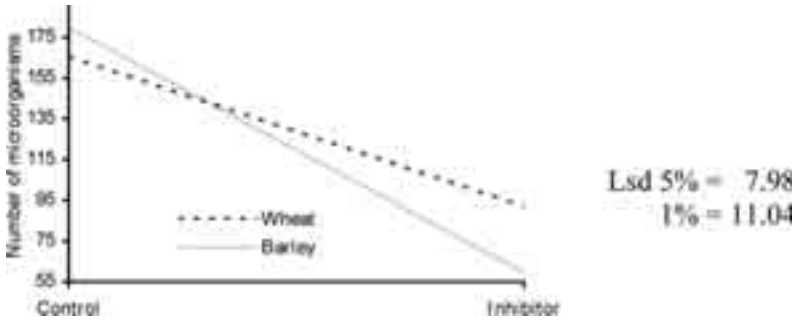
Tab. 4 — Effect of diverse concentrations of Pb, Cu, Cd, Hg on the number of fungi (10^5/1g absolutely dry soil) Đukić et al., 1996

Heavy metals	Concentrations	Number of fungi
	Control	18.0
Pb	6.25 $mg \cdot l^{-1}$	12.0
	0.625 $mg \cdot l^{-1}$	13.3
	0.125 $mg \cdot l^{-1}$	13.6
Cu	6.00 $mg \cdot l^{-1}$	10.0
	0.60 $mg \cdot l^{-1}$	16.3
	0.160 $mg \cdot l^{-1}$	16.6
Cd	2.70 $mg \cdot l^{-1}$	6.3
	0.27 $mg \cdot l^{-1}$	11.6
	0.027 $mg \cdot l^{-1}$	12.0
Hg	2.220 $mg \cdot l^{-1}$	1.1
	0.220 $mg \cdot l^{-1}$	6.6
	0.022 $mg \cdot l^{-1}$	11.0

The **nitrification inhibitor** effect on soil fungi depends on the inhibitor species and concentration, time of application, soil type and its characteristics, as well as on environmental factors determining the direction, rate, speed and products of microbiological transformation (Kanivec and Kiselj, 1978; Smirnov et al., 1981; Graceva, 1982; Muravin et al. 1985; Mandić, Đukić, 1997).

Our investigations (Đukić, Mandić, 1999) indicated that the number of soil fungi depended not only on the used inhibitor type (N-serve), but also on the soil type and the crop cultivated. A continual decline in their number, under the effect of the used inhibitor, was more pronounced in the soil under barley than in that under wheat (Graph. 1).

The use of **polluted waters** for irrigation and waste waters for soil fertilisation as well, due to the range of pollution of most watercourses, has a number of advantages, but also the shortcomings. The former include soil amendment and increase in the activities of microorganisms, increase in soil capacity to bind water and biological soil activation. The shortcomings of using pollu-



Graph. 1 — The interaction between nitrification inhibitor and crop effects on the fungi number in the soil (10^5/1 g absolutely dry soil) — Mandić, Đukić (1999)

ted waters are as follows: the risk of the presence of heavy metals, toxins and pathogenic bacteria, and the unproportional percentage of nutritive substances (as a rule, K supplements are necessary), certain nutrients can be found in unsoluble form, heavy metal ions and sulphuric compounds lead to a change in soil microbial cenosis (Yevdokimova, Mozgova, 1976; Mandić et al., 1994; Đukić, Mandić, 1996) etc. Waste water irrigation has an adverse effect on the organic matter evolution on surface soil horizons, being exhibited in a low organic matter humification degree, and a proportionally lower content of highly polymerised humic acids (Konecka-Betley and Zebrowski, 1978) and therefore its biological value.

The investigations by Đukić et al. (1999) indicate that soil fungi can be used as a parameter for evaluating the quality of waters to be used for irrigating agricultural crops.

Tab. 5 — An overview of physico-chemical characteristics of the water used for irrigating agricultural crops (Đukić et al., 1999)

Indicators	Value registered	Indicators	Value registered
pH	7.34	Ca	96.18 mg · dm^{-3}
NH_3	0.156 mg · dm^{-3}	Mg	14.59 mg · dm^{-3}
NO_3	66.00 mg · dm^{-3}	Mn	0.00 mg · dm^{-3}
NO_2	0.775 mg · dm^{-3}	Cu	0.0008 mg · dm^{-3}
$KMnO_4$	340.00 mg · dm^{-3}	Zn	0.03 mg · dm^{-3}
O_2	1.42 mg · dm^{-3}	Pb	0.031 mg · dm^{-3}
BPK_5	3.57 mg · dm^{-3}	As	0.008 mg · dm^{-3}
HPK	2.99 mg · dm^{-3}	Ni	0.028 mg · dm^{-3}
Suspended matter	556.00 mg · dm^{-3}	Hg	0.0018 mg · dm^{-3}
Total hardness	18.05 mg · dm^{-3}	Cr^{6+}	0.030 mg · dm^{-3}
Chlorides	1.37 mg · dm^{-3}	Cr (total)	0.042 mg · dm^{-3}
Sulfates	71.70 mg · dm^{-3}	Phenols	0.002 mg · dm^{-3}
Phosphates	0.314 mg · dm^{-3}	Detergents	0.090 mg · dm^{-3}
Fe	0.525 mg · dm^{-3}	Mineral oils	0.170 ml · dm^{-3}

The water used for irrigating the agricultural crops selected (Tab. 5) was loaded with different pollutants including primarily different agents entering it through industrial and municipal waste waters. Low O_2 concentration (1.42%)

and values of BPK_5 (3.57 mg × dm^{-3}), NO_2 (0.775 mg × dm^{-3}), NO_3 (65 mg × dm^{-3}), NH_3 (0.156 mg × dm^{-3}), PO_4 (0.314 mg × dm^{-3}), $KMnO_4$ consumption (340 mg × dm^{-3}) and pH above 7 (7.34) indicated that intensive organic matter decay processes were under way. Of the toxic substances, the following were registered: phenols (0.002 mg × dm^{-3}), mineral oils (0.17 ml × dm^{-3}), detergents (0.09 ml × dm^{-3}), Pb (0.031 mg × dm^{-3}), As (0.008 mg × dm^{-3}), Ni (0.028 mg × dm^{-3}) etc.

Generally, the irrigation water had an adverse effect on soil fungi in both soil types, and particularly on the smonitza under red clover (Tab. 6). During the growing season, a rise in the fungi number was observed, which was especially noticable towards the end of the growing season, which was due to microbiological inactivation of toxicants from the irrigation water, improvement of soil structure and balancing of the water-air relationship (U h r e c k y, Z v a n o v e c, 1956). Absolute fungi number values were higher in the smonitza under all three crops.

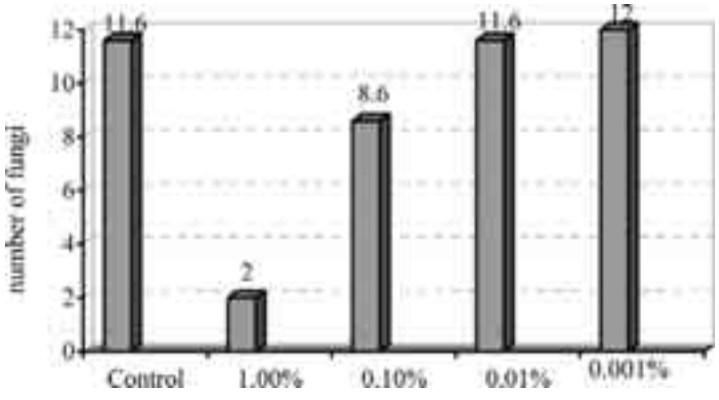
Tab. 6 — The effect of polluted irrigation water on the number of soil fungi (10^{-5}) — Đ u k i ć et al., 1999

Sampling phases	ALLUVIUM						SMONITZA					
	Wheat		Barley		Clover		Wheat		Barley		Clover	
	∅	trial	∅	trial	∅	trial	∅	trial	∅	trial	∅	trial
I	14	12	10	6	20	12	14	13	12	13	22	16
II	14	16	16	30	28	38	24	36	40	40	74	42
III	46	38	84	74	136	100	72	51	51	74	150	144
$\overline{X}$	74	66	120	110	184	150	110	100	103	127	242	202
	70.00		115.00		165.00		105.00		115.00		222.00	

The uncontrolled discharge of **detergents** into water and arable soil can cause reduced biological production of these ecosystems, leading to adverse consequences, both environmental and economic ones. Soil fungi, in this respect, can affect, up to a certain limit, their biodegradation, converting them into less toxic or often energetically important nutrient sources (S t o j a n o v i ć et al., 1990). Basically, increased detergent concentrations or their accumulation in the soil (G o n c a r u k and S i d o r e n k o, 1986) bring about a rapid decline in the number of these microorganisms, and so an analysis of the chemical compound load limits in the soil would provide a far more realistic picture in the neochemistry-effluents-soil system.

Based on our laboratory investigations (M a n d i ć et al., 2006), it can be concluded that the number of soil fungi had a high correlation with the concentration of the detergent introduced (Graph. 2). As a matter of fact, low concentrations of detergents evidently did not have any significant effect on this group of microorganisms. This particularly referred to low (0.001%), and even tenfold detergent rates (0.01%), the effect of which was mildly stimulatory or at the control variant level. This is associated with a familiar trait of soil fungi having strongly developed enzymic system (S t o j a n o v i ć et al., 1995) securing them the capacity to degrade and intoxicate different xenobiotics in the

soil, as well as clear adaptability to live even under the conditions of increased concentrations of not only detergents, but also pesticides, heavy metals, mineral fertilisers etc. (U m a r o v, 1980). As opposed to the mentioned, higher concentrations of the detergent (0.1 and 1%), despite the soil fungi features mentioned, caused a significant fungicidal effect.



Graph. 2 — The effect of different "Meril" detergent concentrations on the number of soil fungi (10^5 g^{-1} absolutely dry soil) — M a n d i ć, Đ u k i ć, 2006

CONCLUSION

Based on the results of the long-term investigations of the effect of different technogenic pollutions (mineral and organic fertilisers, heavy metals, polluted irrigation water, nitrification inhibitor and detergents) on the dynamics of the soil fungi number, the following conclusions can be drawn:

— the number of soil fungi depended on the species and rate of agrochemicals used, growing season and soil zone the samples were taken from for analysis;

— lower nitrogen fertiliser rates (80 and 120 kg × ha^{-1}) and organic fertilisers stimulated the development of soil fungi, which was not the case with the 150 kg × ha^{-1} rate;

— heavy metals, particularly mercury and cadmium, inhibited the development of this group of soil microorganisms;

— the use of high N-serve nitrification inhibitor rates gave rise to a more emphesized continual decline in the number of fungi under barley, compared to wheat;

— generally, the polluted irrigation water had exerted an adverse effect on soil fungi, in both soil types, particularly in the smonitza under red clover;

— low (Meril) detergent concentrations did not have any significant effect on this group of microorganisms. As opposed to the mentioned, its higher concentrations (0.1 and 1%) caused a significant fungicidal effect;

— generally, the number of soil fungi was significantly higher in the rhizosphere of the crops investigated than in the edaphosphere;

— in terms of the mentioned, we conclude that the soil fungi number dynamics can be used in monitoring soils polluted with different toxinogenic substances.

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АНТРОПОГЕНИ УТИЦАЈИ НА МИКРОМИЦЕТЕ У ЗЕМЉИШТУ

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Резиме

У овом раду дата је синтеза вишегодишњих истраживања базираних на утицају различитих антропогених загађења (минерална и органска ђубрива, тешки метали, загађена заливна вода, инхибитор нитрификације и детерџенти) на динамику бројности земљишних гљива.

Истраживања су обављена на Одељењу за микробиологију и на огледним пољима Агрономског факултета у Чачку на земљиштима типа смонице и алувијума у пољским условима и у стаклeнику. Као тест-биљке у овим истраживањима коришћени су кукуруз, пшеница, јечам и црвена детелина. Квантитативни састав гљива у испитиваним земљиштима одређиван је методом разређења на селективној подлози Чапека.

Резултати истраживања показују да бројност земљишних гљива зависи од врсте и дозе коришћених агрохемикалија, вегетационог периода и земљишне зоне из које су узимани узорци за анализу. Ниже дозе азотних ђубрива (80 и 120 $kg \cdot ha^{-1}$) и органска ђубрива стимулишу развој земљишних гљива, што се не може рећи за дозу од 150 $kg \cdot ha^{-1}$. Тешки метали, посебно жива и кадмијум, инхибирају развој ове групе земљишних микроорганизама, као и високе дозе N-serve инхибитора нитрификације. Укупно гледано, негативан учинак на земљишне гљиве загађена заливна вода имала је у оба типа земљишта, а посебно у смоници под црвеном детелином. Ниске концентрације детерџента (Мерил) немају значајног ефекта на ову групу микроорганизама.

Сходно наведеном, констатујемо да се динамика бројности земљишних гљива може користити у мониторингу земљишта загађених различитим токсиногеним материјама.

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MYCOPOPULATION OF MARSHMALLOW (*ALTHAEA OFFICINALIS* L.)

ABSTRACT: Marshmallow is an important medicinal plant in Serbia. Because of increasing demands on the market, cultivation has been started. Through regular quality control of commercial seeds and plantations, mycopopulation of marshmallow was recorded in the period 2000—2006. Seeds of marshmallow were dominated by *Alternaria alternata* and species from the genus *Fusarium* (*Fusarium verticillioides*, *F. proliferatum*, *F. semitectum*, *F. oxysporum* and *F. solani*). Species belonging to *Fusarium* genus are the cause of rot of seeds and roots of marshmallow, causing chlorosis and fading, and therefore deterioration and necrosis of plants, as well as decrease of seed germination of seeds. Leaves and stalks of marshmallow were from time to time under massive attack of *Puccinia malvacearum*, and that was the reason why leaves were unuseful as a herbal drug. On roots and lower part of the stalks, massive appearance of *Sclerotinia sclerotiorum*, a causal agent of the white rot, in cases when marshmallow was cultivated after sunflower, was recorded, too. From other fungi in roots, species belonging to the genus *Fusarium* (*F. oxysporum*, *F. solani* and *F. verticillioides*) were dominant.

KEY WORDS: *Althea officinalis*, marshmallow, diseases, micopopulation

INTRODUCTION

Marshmallow has been used in popular medicine from ancient times. As a perennial plant, it is characterised by branchy root system and could reach the height of 1.5—2 meters. It is cultivated because of medical properties of roots (*Althaeae radix*), leaves (*Althaeae folium*) and flowers (*Althaeae flos*). That was the reason why the Institute for Medicinal Plant Research "Dr Josif Pančić" started the cultivation of marshmallow in cooperation with other producers, as well as on its own land.

The plantations of marshmallow were exposed to attack of a number of phytopathogenic fungi in different stages of plant development, during the vegetation. Numerous fungal diseases in our country decrease the yield and quality of herbal raw material every year.

Marshmallow is the host of a dozen fungal species in our country (Pavlović and Stojanović, 2001; Pavlović et al., 2002; Pavlović et al., 2006). Considering the relatively poor scientific data recorded so far about the diseases of marshmallow in our country, the present study was carried out with the aim to get better insight into mycoflora of seeds, overground and underground parts of this medicinal plants.

MATERIAL AND METHODS

Sampling. Plantations of marshmallow were examined at the localities of Pančevo, Banatsko Novo Selo, Ruma and Zrenjanin for several times, during the vegetation, in the period 2000—2006. Plant parts expressing pathological changes were collected, packed into paper bags, and transferred to laboratory for further testing. Samples of seed were collected from the plantation subjected for marshmallow seeds production in Pančevo.

Isolation. Isolation of fungi from the seeds was carried out according to the procedure supplied by ISTA (Mathur and Kongsdal, 2003), using methods for incubation on filter paper and nutritive medium. These methods were applied to 4600 seeds. After incubation of seeds for 5—10 days, formed mycelia were transferred to PDA and WA with carnations leaves (CLA). From collected samples of leaves, leaf stalks, stems and roots, isolation was accomplished in common manner, taking fragments from the zones between healthy and diseased tissues. Before transferring to PDA, plant material was sterilised by 2% of NaOCl solution during 2 minutes, washed with distilled water, and incubated for 7—10 days at 25°C ± 1°C. Formed fungal colonies were again put on PDA, to get clean cultures and for further characterization of fungal isolates. The collection of 53 *Fusarium* isolates, 4 *Alternaria* isolates, 2 *Phoma* isolates and 2 *Sclerotinia* isolates, was formed and was chosen for determinations.

Morphological characteristics of fungi. The morphology of fungi was evaluated on natural substrate and nutritive media. Growth of colonies of *Fusarium* spp. was measured on PDA after incubation for 3 days, at 25°C and 30°C. To stimulate developed sclerotia to form apotetia and ascospora, cooling in refrigerator to +4°C was applied. After 30 days, sclerotia were put to wet filter paper at laboratory temperature. Morphological characteristics of obligatory pathogens (*Puccinia malvacearum*) were tested on the fresh material only.

Determination of fungi. Determination of fungi was conducted on the basis of morphological, biometric, and growing properties of the tested fungi, taking into account registered symptoms of the disease, described by Nelson et al. (1983) for *Fusarium* spp., Savulescu et Savulesku (1953) for *Puccinia malvacearum*, Sutton (1980) for *Phoma* sp., Mordue and Holliday (1976) for *Sclerotinia sclerotiorum.*

RESULTS

1. *Alternaria alternata* (Fr. ex Fr.) Keissel was the most abundant fungus in commercial marshmallow seeds. From total number of tested seeds, on average 45,6% of seeds (22—78%) were attacked by this fungus, and the range of infection was 22—78%. On the seed this fungos forms white aerial mycelium that later becomes dark grey, and may cover the whole seed, causing mechanical inhibition of germination. On PDA it develops fast, filling the Petri dish within 7—10 days. Aerial mycelium is olive-greyish, more abundant in the middle, while the edges are wavy and white. Conidiophores are simple, septate, dark. Conidia are formed in long branched chains, dark, different in shape, with or without beaks.

This fungus was isolated from dark, elongated (up to 1 cm), necrotic flakes formed at root necks of marshmallow originating at the localities of Pančevo, Banatsko Novo Selo and Inđija. At root and stem, this fungus is often found in combined infection with species from the genus *Fusarium*.

2. *Fusarium verticillioides* (Sacc.) Nirenberg was isolated from 9% of seeds, stems and roots of marshmallow. On seeds, this species forms white, abundant, woolly mycelia, with blue nuances. Around diseased seed, filter paper is coloured ink-blue. At the lower part of the stem, filthy white to beige colouring of mycelia of velvet consistency or white mycelia which covers bigger part of the root, can be observed. Diseased root is grabbed by dry or wet rot, because of necroses of conductory vessels.

Colonies on PDA are abundant, velvet-like, woolly, white at the beginning later decorated with different nuances of violet. There are variabilities between the isolates, regarding the colour of mycelia and nutritive medium. An average radius of 35 mm at 25°C, and 37,5 mm at 30°C was recorded. Macroconidia are abundantly formed at tips of monophialides, in longer or shorter chains (Fig. 1), or in false heads, after three days of growing on media. These are hyaline, obovoid with truncate base, not-septated, rarely with one septum. Macroconidia are formed in monophialides in pale orange sporodochia on CLA, rarely in hyphae, hyaline, delicate, somewhat folded, almost cam, with 3—5 septa, with apical cell narrowed and foot-shaped basal cell. None of the isolates formed chlamidospores.

3. *F. proliferatum* (Matsuhima) Nirenberg was recorded in all tested seed samples, an average 7% of the seeds were infected, and isolated from marshmallow root samples from all the localities. Aerial mycelium colonies on PDA were at first white, abundant, woolly, loose on the edges. Isolates of *F. proliferatum* may vary regarding the colour of aerial and substrate mycelium. An average increase of colonies of 35 mm at 25°C, and 37 mm at 30°C was recorded. Microconidia on CLA are usually formed on mono or polyphialides, in chains or in false heads (Fig. 2). They are hyaline, single celled, rarely with one septa, obovoid with truncate bases. Macroconi-

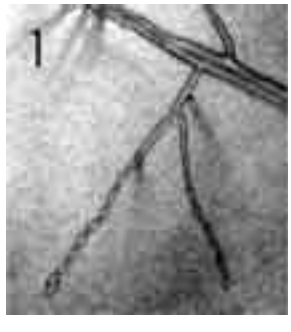

Fig. 1 — *Fusarium verticillioides*. Chains of microconidia

dia are rarely formed in monophialides and sporodochia. Sporodochia are pale orange, formed after 7—10 days of growing on CLA, in intermittent UV light/dark conditions (12/12 h). Macroconidia are hyaline, long, falcate to almost straight, delicate, thin walled, with 3—5 septa. Basal cell is foot-shaped. Chlamidospores were not present in any of the isolates.

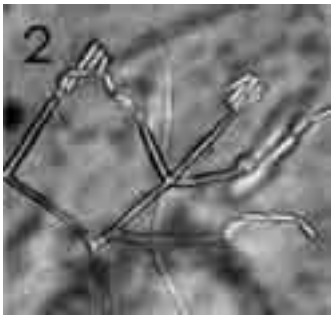

Fig. 2. *F. proliferatum*. Microconidia in false heads

4. *F. semitectum* Berk. & Rav produces abundant, woolly, beige coloured aerial mycelium around the seed on moist filter paper. On average 3% of the seeds were infected. Still, the infection does not stop the germination, but soon it causes the coverage of whole seed by mycelium, which leads to seed wilt. An average radius of colonies of 40, 5 mm at 25°C, and 39, 5 mm at 30°C was recorded. Colonies on PDA with abundant, rich, woolly, aerial mycelium, initially white to salmon, becoming peach colour with age. Substrate mycelium is of peach colour, or pale to dark brown pigment which develops in the agar. Microconidia are rarely produced. Macroconidia are formed in polyphialides (Fig. 3 and 4) in aerial mycelium on CLA, just 4 days after the inoculation. These conidia are straight, rarely slightly curved, spindle-shaped at the ends with 3—5, rarely 6—7 septa. Chlamidospores were present in all the isolates.

Fig. 3—4 — Macroconidia (3) and polyphialide of *F. semitectum* (4)

5. *Fusarium oxysporum* Schecht. Emend. Snyder & Hansen was present on 4% of the marshmallow seed. It forms rich, abundant, snow-white mycelium that covers the whole seed. Around diseased seed filter paper is coloured ink-blue. Especially heavy contamination of root by these fungi was observed at the locality of Banatsko Novo Selo, during 2005, causing fading and deterioration of shoots. The cross section of the diseased root clearly shows the necrosis of vessel elements (Fig. 8). The leaves of the diseased plants become yellow and fade, so the root becomes unuseful as a herbal drug.

Simultaneously, there is a great variability between the same isolates, regarding the morphology of the colonies and pigmentation of media. An ave-

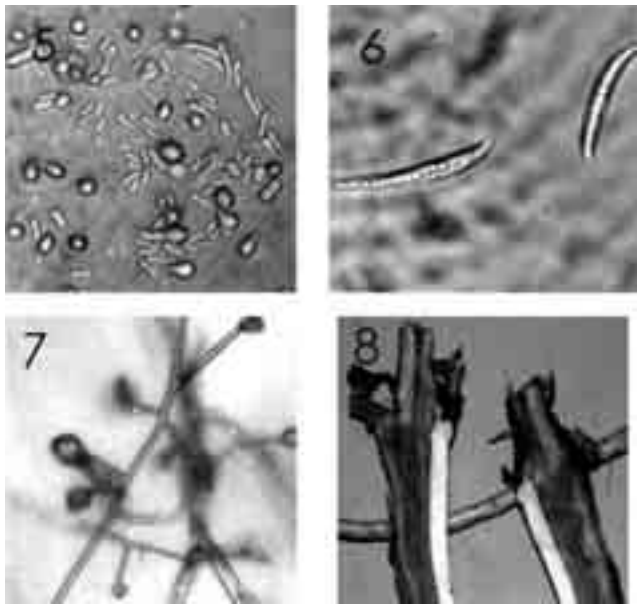

Fig. 5—8 — *Fusarium oxysporum*. Microconidia and chlamidospore *in situ* (5), macroconidia (6), short monophialide and false heads in situ (7), necrosis of diseased marshmallow rot (9)

rage radius of 40,36—41,18 mm at 25°C, and 40,81—40,90 mm at 30°C was recorded. Some isolates form stromatic-like bodies, resembling the peritecia. Microconidia (Fig. 5) are abundantly formed in false heads on short monophialides (Fig. 7) on CLA, four days after growing. Macroconidia (Fig. 6) in monophialides are formed on mycelium, but mainly in dark sporodochia that are hyaline, single celled or with one septa, oval to ellipsoid or kidney shaped. Macroconidia covered with mycelium on CLA, after seven days, and on PDA, in older cultures. They are hyaline, sometimes slightly sickle-shaped, apical cell narrowed, with 3—5 septa. Each isolate formed chlamidospores

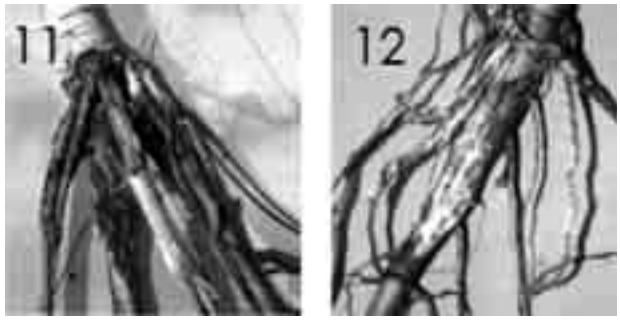

Fig. 11—12 — Mixed infection of marshmallow roots by *Fusarium solani* and *F. verticillioides*

6. *Fusarium solani* (Mart.) Appel & Wollenw. emend. Snyder & Hansen was isolated on 2% of the marshmallow seed, and the root as well (Fig. 11 and 12) during the autumn 2005. White, flourish mycelium was formed on the root within the dark brown, almost black necrotic tissue. With few plants, necroses took over the whole root. In these cases, leaves are chlorotic and die.

Colonies are with white, rare, zoned aerial mycelium, with slightly wavy edges. Pigmentation at the centre was filthy white to beige, almost white toward outside. An average radius of colonies of 24,8 mm at 25°C, and 29,6 mm at 30°C was recorded.

F. solani on CLA produces microconidia, macroconidia and chlamidospores (Fig. 9 and 10). Microconidia are abundantly formed in false heads on long monophialides. They are hyaline, single celled or with 1—2 septa, oval to ellipsoid or kidney shaped. Macroconidia are formed on monophialides in dark sporodochia (on PDA) on older cultures or on mycelium (on CLA) 7 days after growing. Macroconidia are hyaline, sometimes slightly sickle-shaped, apical cell narrowed, with 3—7 septa. Each isolate has abundantly formed chlamidospores.

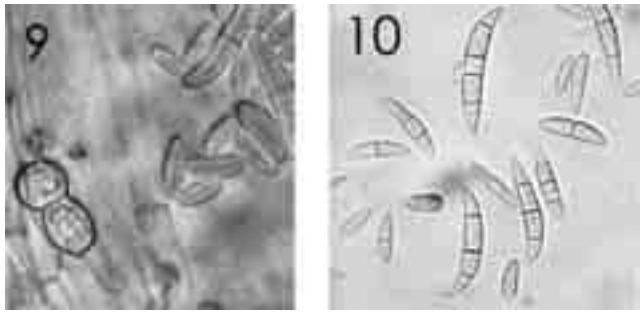

Fig. 9—10 — *Fusarium solani*. Chlamydospore (9), micro and macroconidia (10)

7. *Puccinia malvacearum* Mont. causal agent of the rot on the leaves and stalks of marshmallow was detected at the localities of Pančevo, Banatsko Novo Selo and Alibunar, and on wild-growing plants of both types of marshmallow near Belgrade as well.

During the first part of May, on the upper side of the leaves, pale yellow chlorotic spots, 0,5—3 mm in radius, were recorded, and inside of the leaves, covered with warts teliosorusy with orange-brown coloured teliospores, which later became dark purple. Over 100 of teliosoruses could be formed on a single leave. Teliosoruses formed on leaf stalks and stems are rare and less, they join together, so they become straigh. In a case of heavy attack, the leaves fade and die, that inhibits the production of quality herbal raw material for pharmaceutical needs.

Teliospores are formed as double cells, on long stalk, spheroidly alternated, pale yellow, with smooth, thick walls (Fig. 13). Lower cell is wider, and upper cell is slightly oval, sometimes apical narrowed. Teliospores can germinate only after being put under influence of alternating low and high temperatures under natural conditions.

8. *Sclerotinia sclerotiorum* (Lib.) de Bary was detected only on a private plantation near Bela Crkva (Banatsko Novo Selo), where marshmallow was cultivated after sunflower during July and August 2001. Pathogen attacks the root, neck of the root and stem, at first forming dark water-like spots. Within these spots, an abundant, white mycelium is developed, and later dark to black, large sclerotia. Under wet conditions, the lesions are growing fast, taking over the plant ring by ring, causing fading and actual deterioration of plants. Sclerotia are formed in the stem core of the diseased plant. Mycelium is taking over flower branches too, spreading the infection onto the seed.

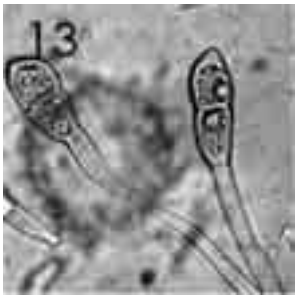

Fig. 13 — Teliospores of *Puccinia malvacearum*

Fungi produce sclerotia after 10 days of growing on PDA. They are alternated as spheroid to irregular shape, 2—5 mm in radius. Sclerotia form apotetia (Fig. 15) with ascus and ascospora (Fig. 16), after it was kept for 60 days on wet filter paper at laboratory temperature.

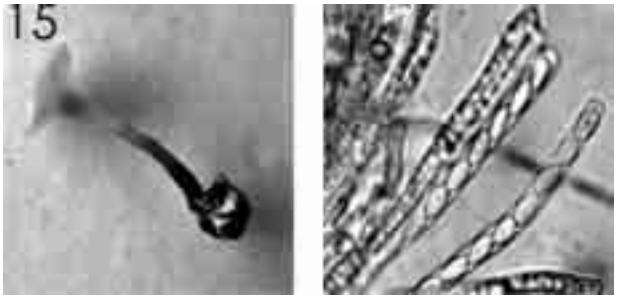

Fig. 15—16 — *Sclerotinia sclerotiorum*. Apothecium formed on sclerotium (15), asci and ascospores (16)

9. *Phoma* sp. was isolated from ground part of marshmallow stem, originating from Zrenjanin. On the stem dark, oval spots are formed and joined together. Tissue within the spots is creaking, thus forming numerous pycnidia. At first, aerial mycelium is white, later, in central part, the colour is changing to dark. Numerous individually, concentrically placed pycnidia are formed on PDA after 10 days (Fig.14). Pycnidiospores are small, hyaline, single celled, straight, ellipsoid or egg--shaped.

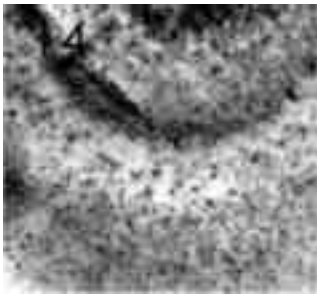

Fig. 14 — Pycnidia of *Phoma* sp. formed on PDA

Besides the previously mentioned fungi, some other fungi from genera *Epicoccum*, *Cladosporium*, *Penicillium*, *Aspergillis* and *Rhizopus* were also isolated from the marshmallow seed. They were present in low percentage (1—4%).

RESULTS AND DISCUSSION

Fourteen species from 10 genera were identified on the seed, leaf, stem and root of marshmallow. Because of the rich content of nutritional material, the seed presents the adequate substrate for growth. The dominant population on the seed are fungus from the genus *Alternaria* (22—78%) and *Fusarium* (18—25%), and in smaller percentage (1—4%) fungus from the genera *Phoma*, *Epicoccum*, *Cladosporium*, *Penicillium*, *Aspergillis* and *Rhizopus*.

Alternaria alternata is constantly present on the seed. It is also isolated from dark spots on the root, where it most often combines with the species from the genus *Fusarium*. The genus *Alternaria* forms different groups of isolates, from genuine saprophytes, facultative pathogens to virtual, for the host specific pathotypes (Scheffer, 1992). Otani and Kohmoto (1992) state that isolates of *A. alternata* have the capability of invading the herbal tissue, that leads to a disease on numerous hosts. In our country it was identified on chamomile, feverfew, valerian and balm.

Five species from genus *Fusarium* (*Fusarium verticilloides*, *F. proliferatum*, *F semitectum*, *F. oxysporum* and *F. solni*) were isolated from seed of marshmallow. The diseased seed is small and poor, with changed colour. Parts of germinated seeds fade and die. *Fusarium proliferatum*, *F. oxysporum* and *F. solani* often attack marshmallow root, causing its deterioration. Morphological characteristics of the obtained isolates of *Fusarium* spp. (appearance, micro and macroconidia, way of formation, presence or absence of chlamidospores) originating from marshmallow, as well as huge variability regarding the colour and pigmentation of nutritive medium, were in good agreement with the description of other authors (Nelson et al., 1983; Burgess et al., 1994).

Mallow rust (*Puccinia malvacearum*) is known as a disease of wild growing mallow (*Malva silvestris* L.) found at several localities in Serbia (Stojanović and Kostić, 1956). As a host of that pathogen in Montenegro *Malva rotundifolia* and *Althaea rosea* were reported (Mijušković, 1956).

In Serbia none of the species belonging to the genus *Phoma* were recorded, though Sutton (1980) quoted *Althaea rosea* as its host.

Moist rot was recorded only in one case, when marshmallow was cultivated after sunflower culture, that is the main host of *Sclerotinia sclerotiorum* (Marić et al., 1988). Damages were highly present since all the diseased plants deteriorated during the vegetation. Pathogen sclerotia are able to retain vitality in soil for a very long period of time (3—8 years), so, from this reason crop rotation by cultivation of marshmallow should be taken into account. Three-year lasting crop rotation in beans did not reduce the disease severity (Schwartz and Steadman, 1977).

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МИКОПОПУЛАЦИЈА ГЉИВА (*ALTHAEA OFFICINALIS* L.)

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Резиме

Редовном контролом квалитета комерцијалног семена и плантажних засада регистрована је микопопулација белог слеза у периоду 2000—2006. Четрнаест врста гљива из 10 родова идентификовано је на семену, листу, стаблу и корену белог слеза. Са семена су изоловане следеће врсте: *Alternaria alternata* и пет врста рода *Fusarium* (*F.verticillioides*, *F. proliferatum*, *F. semitectum*, *F. oxysporum* и *F. solni*), а у мањем проценту (1—4%) гљиве из родова *Epicoccum*, *Cladosporium*, *Penicillim*, *Aspergiluis* и *Rhizopus*. Врсте рода *Fusarium* проузрокују трулеж семена и корена слеза, доводе до хлорозе и увенућа, а самим тим и до угињавања биљака и утичу на смањење клијавости семена. Листови и стабло белог слеза су 2002. године били масовно су инфицирани са *Puccinia malvacearum*, због је чега лишће било неупотребљиво као биљна дрога. Влажна трулеж регистрована је само у једном случају када је бели слез гајен након сунцокрета. Пошто склероције патогена задржавају виталност у земљишту више година, у производњи белог слеза потребно је водити рачуна о плодореду.

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LYOPHILIZATION AS A METHOD FOR PATHOGENS LONG TERM PRESERVATION

ABSTRACT: Lyophilization (freeze-drying) is one of the most suitable methods used for a long term peservation of pathogens. The aim of this paper was the application of lyophilization for storage of three significant plant pathogens: *Fusarium graminearum, Helmintosporium gramineum*, and *Pseudomonas syringae* pv. *gylicinea*, respectively. The plant material was collected continuously (during a four year period 2002—2006), depending on a plant development stage, from different localities in Vojvodina. Pathogens were isolated from diseased parts with characteristic symptoms, and placed on nutritive media specific for a certain pathogen, using standard phytopathological methods. Lyophilization was carried out in marked and coded ampoules by freezing and drying of pathogen suspension and nutritive medium. Revitalization of lyophilized isolates was done after four days. High percentage of revitalization was characteristic for all studied isolates, and it ranged from 85—92%, confirming that lyophilized pathogens would be capable of keeping viability for a long time in the collection. Besides above mentioned pathogens, there were 200 isolates in the collection, originating mostly from field and vegetable crops. Each isolate that was put into the Collection, was followed by all the necessary data such as: name of the pathogen, number of isolates, locality, host plant, year of isolation, name of the researcher and other relevant data.

KEY WORDS: lyophilization, long time preservation, plant pathogen collection, fungi, bacteria.

INTRODUCTION

The study of microorganisms often involves the use of living cultures. The cultures need to be kept for a long period, and included into the microbiological collection. Purpose of the Collection is to maintain the biological material in vital and stable state, with all its original traits preserved. Lyophilization (freeze-drying) is one of the most suitable techniques used for long-term pathogen preservation (The Preserevation and Maintenance of Living Fungi — Smith and Onions, 1994).

MATERIAL AND METHODS

The plant material was collected continuously, from different localities in Vojvodina, depending on a plant development stage. Pathogens were isolated from diseased parts with characteristic symptoms, and placed on nutritive media, specific for certain pathogens, using standard phytopathological methods. These isolates originated from different localities in Vojvodina (Futog, Begeč, Bačka Palanka, Rimski Šančevi, Čenej, Pančevo, Srbobran, Tovariševo, Njegoševo, Bačka Topola, Laćarak and Čalma, respectively) and were collected during the period 2002—2006.

Fusarium graminearum was isolated from diseased maize and soybean seed, and from diseased leaves and ear of barley. Standard PDA medium was used. The obtained fungi colonies, belonging to *Fusarium* genus, were subcultured on CLA medium for better sporulation (Fisher et al. 1982). Isolates were incubated at 25°C under artificial ultraviolet light ("black light") with a 12hr photoperiod.

Helminthosporium gramineum was isolated from diseased seed and leaf of barley and placed on standard PDA medium. Incubation was done at 25°C, with 12 hour light/dark cycle during seven days. The obtained isolates were transferred to PDA and WA media in order to obtain pure cultures.

On basis of fungus morphological characteristics of colonies and reproductive organs (conidia, conidiophores, and chlamidiospores), determination of species was done (Nelson et al., 1983; Burgess et al., 1994; Mathur and Kongsdal, 2003).

Pseudomonas syringae pv. *glycinea* was isolated from diseased soybean leaves with characteristic symptoms of bacterial spot, placed on meat extract (MPA) and nutritive agar medium, enriched with sucrose (NSA), using standard surface smearing method (Arsenijević, 1997; Schaad, 1980). Two days after development in thermostat at 26°C, individual colonies were transferred to slope medium with glycerol (Klement et al., 1990). Biochemical-physiological characteristics and pathogenic traits of the obtained isolates were determined by using tests and usual procedures for this kind of testing (Fahy and Persley, 1983; Arsenijević, 1988; Klement et al. 1990; Schaad, 2001).

Preparation of the isolates for lyophilization

F. graminearum and *H. gramineum* were lyophilized using colonies 7—10 days old, while bacterial colonies were 24—48 hours old.

Lyophilization was done according to Smith and Onions (1994). Prepared material was lyophilized in Modulyo 4K freeze dryer (Edwards, UK) under vacuum (Fig. 1). Lyophilization processed ampoules were constricted using flame, in order to prevent the contact of lyophilized material with air, which could cause damage (Rey, 1977) (Fig. 2).

Sealed ampoules were kept in a special compartment of lyophilization chamber.

Fig. 1 — Modulyo 4K freeze dryer (Edwards, UK)

Fig. 2 — Ampoule with lyophilized isolate

Viability of lyophilized isolates was checked four days after the lyophilization. Growing and morphological characteristics (shape, colour, and size) of fungal and bacterial colonies were observed on nutritive media during the revitalization process.

RESULTS

Ten isolates of *F. graminearum* fungus were obtained by isolation. Five isolates originated from soybean seed (FG-5, FG-8, 56/13, 4/21, 5/18), three from maize seed (FG-1, FG-2, FG-3), one from barley leaf (FG-6) and one from barley ear (FG-7).

Four isolates of *H. gramineum* originating from barley seed, were obtained (H-1, H-1 (F), HG-1 and HG-2), and one originating from barley leaf (HG-3).

Three to four days after the completion of lyophilization, 22 isolates of *P. syringae* pv. *glycinea* (B2/4, B2/5, B2/6, B2/7, B2/8, B11/1, B13/1, B13/2, B13/4, S5/1, S5/2, S5/3, R9/1, R9/3, R10/1, R10/4, R10/5, R10/6, R12/2, P15/1, P15/2 i P15/3) were obtained.

Besides the above mentioned pathogens, the Collection contains 200 isolates originating mainly from field and vegetable crops. Each isolate contained in the Collection was put into the data base with all the relevant data, such as name of the pathogen, number of isolates, locality, host plant, year of isolation, name of the researcher and other relevant data (Tab. 1).

Tab. 1 — Form of table containing data on isolates and localities

Catalog number of isolate	
Name of pathogen	
Locality	
Host and part of plant used for isolation	
Year of introduction of isolate into the Collection	
Other data: name of institution, name of researher, original mark of isolate, origine of isolate, year of isolation and other relevant data	

Percentage of revitalized isolates four days after lyophilization was 85% for *F. graminearum* and *H. gramineum* fungi, and 92% for *P. syringae* pv. *glycinea* bacterium. These results point out to a high degree of isolate viability after lyophilization, confirming that the studied pathogens are capable of long term preservation by application of this method.

Morphological and growing characteristics of the colonies of revitalized fungal and bacterial isolates, observed on nutritive media, were identical with the original. On PDA, medium isolates of *F. graminearum* formed abundant, thick, and pinkish white mycelia, with grayish margines. The isolates formed burgundy pigment in the agar. On CLA medium, the isolates formed pink aerial mycelium, and dark red pigment in the agar. Macroconidia were formed on branched monophialides with 3—5, rarely 6 clearly visible septa. Apical macroconidial cell was tapered, while basal cell was foot-shaped. The isolates formed neither microconidia nor chlamidospores. (Fig. 3).

H. gramineum formed grayish to olivegreen black colonies with characteristic lobed margins, after revitalization on PDA medium. Conidiophores were branched and septate, formed individually or in groups. Cylindrical dark yellow conidia with 1—7 septa were formed laterally or terminally on conidiophores (Fig. 3).

Bacteria were revitalized on nutritive medium enriched with sucrose (NSA). Characteristic slimy, large, shiny, white and distinctively convex bacterial co-

Fig. 3 — Revitalized isolates of *H. gramineum* and *F. graminearum* on PDA medium

lonies of *P. s.* pv. *glycinea* were formed three to four days after revitalization (Fig. 4).

Fig. 4 — Revitalized isolate of *P. s.* pv. *glycinea* bacterium on NSA medium

DISCUSSION

Plant pathogens including *F. graminearum*, *H. gramineum* and *P. s. pv. glycinea* can cause significant damage to the plant production, and for that reason they should be precisely determined and preserved. Plant pathogens, which are the part of agro-ecosystem, are influenced by great number of factors, causing the change of their characteristics among which pathogenicity and virulence are the most significant ones. This fact revealed the need for a systematic collection and a long-term preservation of pathogens. Due to the changes caused by different conditions of environment, it is very important, especially from the aspect of breeding for resistance, to have a great number of isolates of a certain pathogen originating from different localities and different years. Lyophilization method enabled the biological material to preserve its original traits for long periods of time (Klement, 1990; Agrios, 1997).

Results obtained in our studies confirmed that the method of lyophilization is a very suitable way for plant pathogen preservation.

Microorganisms can survive conditions of lyophilization process, and preserve viability and original traits. Success of lyophilization and vitality of lyophilized isolates can vary between the isolates of the same species (Smith and Onion, 1994). Tan et al. (1991) mentioned that the optimal results can be obtained in case of lyophilization of young colonies, and that this method is proposed for preservation of fungi belonging to *Ascomycetes* class, to which *H. gramineum* and *F. graminearum* also belong.

The length of time allowed for rehidratation is one of the major factors in the process of revitalization, and can be very different for individual isolates (Haskins, 1957, Butterfield et al. 1974).

All studied isolates showed high percentage of revitalization and survival. Morphological and growing characteristics of lyophilized *F. graminearum* and *H. gramineum* fungi, and *P. s.* pv. *glycinea* bacterium, were identical to the original isolates after revitalisation.

The need for constant observation, study and preservation of plant pathogens, with the aim of improvement of cultivated plant production, first of all by developing less sensitive i.e. resistant varieties, genotypes, and hybrids comes from all the above mentioned.

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ЛИОФИЛИЗАЦИЈА КАО МЕТОД ДУГОРОЧНОГ ЧУВАЊА ПАТОГЕНА

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Резиме

Лиофилизација (freeze-drying) је једна од најпогоднијих метода која се користи за дугорочно чување патогена. Циљ рада је примена метода лиофилизације у чувању три значајна биљна патогена: *Fusarium graminearum, Helminthosporium gramineum* и *Pseudomonas syringae* pv. *gylicinea*. Прикупљање узорака биљног материјала вршено је у континуитету (током четири године, 2002—2006) са различитих локалитета на подручју Војводине, у зависности од развојне фазе биљака. Изолација патогена вршена је из оболелих биљних делова са карактеристичним симптомима на хранљиве подлоге специфичне за одређеног патогена коришћењем стандардних фитопатолошких метода. Поступак лиофилизације вршен је у обележеним и шифрираним ампулама смрзавањем и сушењем суспензије патогена и хранљивог медијума. Ревитализација лиофилизованих изолата извршена је четири дана након лиофилизације. За све проучене изолате карактеристичан је висок проценат ревитализације и износи 85—92%, што потврђује да ће лиофилизовани патогени дугорочно задржати виталност у колекцији.

У оквиру колекције поред наведених патогена постоји 200 изолата који потичу углавном са ратарских и повртарских биљних врста. Сваки изолат који се налази у колекцији унет је у базу података са свим потребним подацима, као што су: назив патогена, број изолата, локалитет, биљка домаћин, година изолације, име истраживача и други релевантни подаци.

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THE QUALITY OF SILAGE OF CORN GRAIN AND SPENT *P. OSTREATUS* MUSHROOM SUBSTRAT

ABSTRACT: The chemical composition, fermentation quality, mycological and mycotoxicological analyses of silage mixture, made of ground corn grain and spent *P. ostreatus* mushroom substrate, were investigated in this paper. Dry matter content in high moisture ground corn, at the time of ensiling was 70%, and in the spent substrate (on the Salt Cedar wood shaving basis) was 52.7%. Corn grain to spent substrate ratio in trials was: 100:0% (I), 90:10% (II), 80:20% (III) and 70:30% (IV) respectively. Content of the lignocellulose fractions in silage was slightly increased, and protein content was slightly decreased with the increase of spent substrate content. Contents of the VFA (volatile fatty acids) in silage, pH value, and NH_3-N content were for the silage of very good quality. In the spent substrate 9 mold species were found, from which the most frequent were genus *Penicillium, Paecilomyces variotii*, and *Trichoderma harzianum.* In ground corn grain silage (I) presence of the yeasts was dominant (90.000/g). In combined trials (II—IV) only *Penicillium* (*P. brevicompactum* and *P. echinulatum*) mold species were found. Presence of molds and yeasts in investigated trials was within tolerated values for ensiled feedstuffs. Mycotoxin presence in silage was not determined.

KEY WORDS: silage, *P. ostreatus* mushroom, substrat, mycotoxins

INTRODUCTION

P. ostreatus mushroom is grown on the compost made of various types of straw, sawdust, wood shavings or wood crust, seed shells, and other materials rich in cellulose and lignin. After the fungus fruiting and harvesting, spent compost remains, which is utilized by fungus as nutritive substrate. The advantage of *P. ostreatu*s mushroom production is utilization of cheap waste materials. Certain authors, among them Bano et al. (1984), Kakkar et al. (1990), Platt et al. (1984), Rajarathnam and Bano (1989), Adamović,

M. et al. (1996; 1998; 1999 and 2001, A d a m o v i ć, O. et al. (2000; 2001 and 2003, Z a d r a z i l, 1993), found that spent compost could be used as feedstuff for domestic animals, either natural or ensiled.

During mycelia development and *P. ostreatus* mushroom fruiting substrate is changed. The biggest changes are in N content which is decreasing. The content of NDF (neutral detergent fiber) and hemicellulose is also decreased, which is indicating that *P. ostreatus* mushroom enzymes (cellulases, hemicellulases, celobiases, ligninases, etc.) an effect degradation of easy degradable fraction of lignocellulose complex. On the other hand content of ADF (acid detergent fiber), ADL (acid detergent lignin) and cellulose (low degradable components of lignocellulose complex) is not changed significantly. Increased moisture content in spent *P. ostreatus* mushroom substrate (50—70%) causes rapid spoiling and difficult storage. Therefore, possibilities for its use in animal nutrition are lower.

The goal of this experiment was to investigate possibility for Salt Cedar wood shavings spent substrate in combination with high moisture ground corn ensiling, and obtain new information on ways of using spent substrate in cattle nutrition.

MATERIAL AND METHOD

The substrate for *P. ostreatus* mushroom growing was made on the basis of Salt Cedar wood shavings from New Mexico (USA). Content of dry matter at the time of ensiling was 52,7%. Particles of the wood shavings were heterogeneous (diameter 1—7 mm, length 5—100 mm). Content of dry matter in corn grain at the moment of harvesting was 70%. Particle size of grounded corn was between 0.1 and 2 mm. Ground corn grain and spent substrate were mixed and placed into the plastic buckets of 3 kg volume capacity. Ratio between high moisture ground corn and compost is shown in table 1.

Tab. 1 — Plan of the experiment

Silage component	Silage trials			
	I	II	III	IV
High moisture ground corn	100	90	80	70
Spent *P. ostreatus* mushroom substrat	0	10	20	30

After compressing of the ensiled material and remove air buckets were covered with black nylon foil (0.2 mm thickness) and sealed with plastic lid. The buckets were stored at the temperature of 20°C for the first 28 days, and at room temperature of 16—20°C for the last 15 days. The buckets were opened after 43 days. After the opening the surface layer (2—3 cm thick) contaminated with molds, was removed. The samples for laboratory analyses were taken from the remained content in the bucket. Silage samples (0.6 kg per sample) were placed in nylon bags, cooled in the freezer (–18°C), and kept until the beginning of the laboratory analyses. Determination of total number of saprobic microorganisms/g (bacteria, molds and yeasts) was done according

to Official Gazette of SFRY, No. 25/80. For mold isolation and identification following media were used: Czapek's agar (3% of succrose) — *Aspergillus* and *Penicillium*, and potato succrose agar — *Fusarium* spp. and all other fungal genera. Identification of mold species was done according to the following authors: Nelson et al. (1983) for *Fusarium*, Ellis (1971) for *Dematiaceous Hyphomycetes, Samson & van Reenen — Hoekstra* (1988) and Domsh et al. (1980) for all other fungal genera. Multidetection procedure (Official Gazette of SFRY, No. 15/87) was done for chemical analyses of aflatoxin B_1, ochratoxin A, and zearalenone. Qualitative and quantitative determination of trichothecene type A mycotoxins (T-2 toxin and diacetoxyscirpenole — DAS) was done by thin layer chromatographic method (Pepelnjak and Babić, 1991).

RESULTS AND DISCUSSION

Chemical composition. Chemical composition of high moisture ground corn and spent substrate is shown in Table 2. Chemical composition of ground corn grain and spent *P. ostreatus* mushroom substrate significantly differed. Spent *P. ostreatus* mushroom substrate had significantly higher content of ash and lignocellulose fractions (ADF, ADL and cellulose), and protein content was lower. Chemical composition of silage (trials I—IV) changed depending on the ratio of high moisture ground corn and spent substrate. With the increase of spent *P. ostreatus* mushroom substrate ratio in the trials (from 10 to 30%) the trend of lignocellulose fractions content was slightly increased and the trend of protein content was decreased.

Tab. 2 — Chemical composition, % (on the dry matter basis)

Item	High moisture ground corn	Spent *P. ostreatus* mushroom substrat	Silage trials			
			I	II	III	IV
Dry matter (DM)	76,41	52,07	65,88	63,36	59,07	57,19
Ash	1,71	13,12	1,47	2,14	2,82	3,55
Protein	8,51	3,81	8,70	8,46	8,07	7,81
Fat	3,81	0,65	3,28	3,07	2,76	2,55
NDF	17,88	65,47	15,41	18,16	20,65	23,64
ADF	3,17	52,98	2,73	5,70	8,78	11,99
ADL	0,43	14,19	0,37	1,19	2,05	2,94
Hemicellulosa	14,71	12,51	12,68	12,46	11,87	11,65
Cellulosa	2,74	30,79	2,36	4,02	5,74	7,54

NDF — Neutral detergent fiber; ADF — Acid detergent fiber; ADL — Acid detergent lignin

By the organoleptic evaluation of silage (I—IV) characteristic smell for high moisture ground corn silage was found. In the trials III and IV, with 20 and 30% of spent *P. ostreatus* mushroom substrat, slight appearance of spent substrate scent was detected.

Microbiological analysis. Total bacteria count was significantly higher in corn grain silage than in spent *P. ostreatus* mushroom substrate (Table 3). This is logical regarding to the ensiling process.

Tab. 3 — Total count of saprobic microorganisms in spent *P. ostreatu*s mushroom substrat and silage

Parameter	Spent *P. ostreatus* mushroom substrat	Silage trials				Maximum quantities*
		I	II	III	IV	
Total count of bacteria (No/g)	2.400.000	78.000.000	—	—	—	100.000.000
Total count of molds (No/g)	1.100.000	20	300	100	40	300.000
Total count of yeasts (No/g)	< 10	90.000	300	200	600	

Legend: * according to Regulation (Official Gazette of SFRY, No. 2/1990)

Regarding the mold count in the same samples, the situation was completely different. Unlike spent *P. ostreatus* mushroom substrate where more than 10^6 molds per gram was found (Table 3), in silage samples mold count was far lower, from 20 (silage I) to 300 (II). From 9 identified species of mold (Table 4) in spent *P. ostreatus* mushroom substrat, dominant species were genus *Penicillium, Paecilomyces variotii*, and *Trichoderma viride* typical mycobita for lignocellulose materials (D o m s h et al., 1980). In the silage only *Penicilllium* (*P. brevicompactum, P. echinulatum*, *P. funiculosum* and *P. variabile*) species were found. These species are regularly found on corn grain (N o o r y, 1983).

Tab. 4 — Fungal species identified in spent *P. ostreatus* mushroom substrat and silage

No	Species	Spent *P. ostreatus* substrat	Silage trials			
			I	II	III	IV
1.	*Acremonium* sp.	+				
2.	*Alternaria alternata*	+				
3.	*Aspergillus* sp.	+				
4.	*Cladosporium cladosporioides*	+				
5.	*Mortierella bainiery*	+				
6.	*Paecilomyces variotii*	+				
7.	*Penicillium brevicompactum*	+		+	+	+
8.	*P. echinulatum*		+			
9.	*P. funiculosum*		+			
10.	*P. variabile*			+	+	
11.	*Penicillium* sp.	+				
12.	*Trichoderma harzianum*	+				
	TOTAL	9	2	2	2	2

Total count of yeasts was the highest in ensiled corn (90.000/g), which does not surprise regarding the ensiling process. In trials with different ratio of spent *P. ostreatus* mushroom substrate this count did not exceed 600/g, which is understandable regarding their absence in the spent substrate. Determined

values were lower than maximum values for saprobic microorganisms in plant feedstuff (Official Gazette of SFRY, No. 2/1990).

The presence of pathogenic bacteria (genus *Salmonella*, *Staphylococcus*, *Proteus*, *E. coli* and *Clostridium*) was not found in silages. Having in mind this fact, and previously presented parameters, it could be said that microbiological quality of silage was satisfying.

Mycotoxicological analysis. By the thin layer chromatographic method, presence of mycotoxins (aflatoxin B_1, ohratoxin A, zearalenone, diacetoxiscirpenol-DAS and T-2 toxin) was not detected in silages. This confirms the fact that the detected molds were either not toxigenic, or the produced mycotoxin levels were below detection ability of methods used for mycotoxin identification (aflatoxin B_1, 0,8 μg/kg, ohratoxin A — 8 μg/kg, zearalenone — 12 μg/kg, DAS and T-2 31 μg/kg). Based on the presented results of mycotoxicology, the quality of silage was satisfying.

Silage quality. Determined pH values of the investigated silages (Table 5) show decreasing tendency of the content of spent *P. ostreatus* mushroom substrat was increased. Nevertheless, pH value in trial IV was above 3,5. Among the volatile fatty acids (VFA) the lactic acid was dominant. The content of lactic acid in trials was in range 3,16 to 3,77%. The content of acetic acid was about 4—5 times lower than to lactic acid (0,61—0,83%). The presence of butyric acid was not detected. Content of NH_3-N (nitrogen ammonia) was optimal (0,071—0,083% of dry matter, or 5,24—6,40% of total nitrogen), and slightly increased in trials III and IV. These results indicate that for all four silages the optimal conditions for good quality fermentation existed, which resulted in a good quality of silage.

Tab. 5 — Silage qulaity

Parameter	Silage variant			
	I	II	III	IV
pH	3.77	3.79	3.64	3.59
Lactic acid, % DM	3.16	3.23	3.77	3.55
Acetic acid, % DM	0.61	0.68	0.83	0.69
Butyric acid, % DM	0.00	0.00	0.00	0.00
NH_3-N, % DM	0.074	0.071	0.078	0.080
NH_3-N, % TN	5.31	5.24	6.04	6.40
Digestibility, % DM[1]	86.80	85.61	80.69	77.91

[1] Tilley, J. M. A., Terry, R. A. (1963): J. Br. Grassland Soc. 18:104.

Silage dry matter digestibility was slightly decreasing, from 86,80% (I) to 77,91% (IV), with the increase of spent *P. ostreatus* mushroom substrat ratio in silage. The reason for digestibility decreasing trend is high content of low digestible lignocellulose fractions, which increased in trials respectively. Investigating the taste palatability (silage II) it was graded quite good. After 20—30 seconds of getting used to the scent and taste of silage, cows consumed all offered silage quantity in 60 seconds.

CONCLUSION

With the increase of spent *P. ostreatus* mushroom substrate ratio in silage (from 10 to 30%) values for NDF, ADF, hemicellulose, cellulose and lignin, linearly increased. Total count of microorganisms in trials was among the tolerant values. The presence of mycotoxins was not detected. The content of volatile fatty acids in silage, silage pH, and NH_3-N content were within values characteristic for silage of a very good quality. With increased ratio of spent *P. ostreatus* mushroom substrat in silage, digestibility of silage dry matter decreased from 86,80% (I) to 77,91% (IV). Silage consumption was good. Microbiological and mycotoxicological analyses indicate that all the investigated silages had satisfying quality.

Use of this kind of silage in smaller quantities (up to 10% of dry matter in diets for cows and fattening bulls) could be reasonable in diets with low ADF and NDF content, as well as for the cattle, with lower genetic potential, under extensive conditions of nutrition.

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КВАЛИТЕТ СИЛАЖЕ ОД ЗРНА КУКУРУЗА И СУПСТРАТА ГЉИВЕ *P. OSTREATUS*

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Резиме

У раду су испитивани хемијски састав, квалитет ферментације, миколошка и микотоксиколошка слика силажа на бази прекрупе влажног зрна кукуруза и супстрата за гајење гљиве *Pleurotus ostreatus* (буковача). Садржај суве материје у влажном зрну кукуруза у време силирања износио је 70%, а у супстрату (на бази пиљевине бора) 52,7%. Силирање је обављено у лабораторијским условима, у пластичним кофама запремине 3 kg. Тежински удео прекрупе влажног зрна кукуруза и супстрата у силажи је износио: 100:0% (I), 90:10% (II), 80:20% (III) и 70:30% (IV). Узроци силаже узимани су 6 недеља након силирања. Хемијски састав силажа (I—IV) се мењао у зависности од удела прекрупе влажног зрна кукуруза и супстрата. Са повећањем удела супстрата (са 10 на 30%) количина лигноцелулозних фракција у силажи имала је тренд благог пораста, а протеина тренд опадања. Садржај испарљивих масних киселина у силажи, вредност pH силаже и садржај NH_3-N били су у оквиру вредности које су карактеристичне за силажу веома доброг квалитета. У супстрату је идентификовано 9 врста плесни од којих су највећу учесталост имали представници рода *Penicillium, Paecilomyces variotii* и *Trichoderma harzianum*. У силажи од прекрупе влажног зрна кукуруза (I) било је доминантно присуство квасаца (90.000/g). У узорцима комбинованих силажа (II—IV) уочене су искључиво *Penicillium* врсте гљива (*P. brevicompactum* и *P. echinulatum*). Присуство плесни и квасаца у испитаним узорцима је било у толерантним границама за силирана хранива која се користе за сточну исхрану. Токсиколошким претрагама није утврђено присуство афлатоксина B_1, охратоксина А, зеараленона и трихотецена типа А (DAS и Т-2 токсин).

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INFLUENCE OF THE EXTRACTS ISOLATED FROM *GANODERMA LUCIDUM* MUSHROOM ON SOME MICROORGANISMS

ABSTRACT: *Ganoderma lucidum* (Leyss.: Fr.) Karst, a mushroom-like fungus is one of the most famous traditional Chinese medicinal herbs. It received wide popularity as a healthy food and medicine in the Far East for more than 2000 years, because of its high medicinal value. One of very interesting aspects of *G. lucidum*'s performance is antimicrobial effect due to the extracts derived from this mushroom, which contain bacteriolitic enzyme, lysozyme and acid protease. The effects of these extracts depend on their composition, extraction mode and refining. Bioactive components isolated from several *G. lucidum*'s strains showed different effects on the investigated microorganisms. In some cases, the influence was very intensive, with inhibitory or stimulating effect, while some of them did not show any influence on the investigated microorganisms.

KEY WORDS: antimicrobial effect, extraction, extracts, *Ganoderma lucidum*

INTRODUCTION

For more than 2000 years, *Ganoderma lucidum* has been regarded as a popular folk medicine in the Far East, used to treat various human diseases, such as hepatitis, hypertension, hypercholesterolemia, gastric cancer and many others. Due to its ability to cure many different diseases it received names like "Elixir of life", "Food of Gods", "Mushroom of the Universe". Its intracellular and extracellular polysaccharides showed inhibition of the growth of several types of cancer cells (Sone et al., 1985, Wang at al., 2002, Zhang et al., 1994). Besides, it also produces many oxygenated triterpenes, especially ganoderic acid, with various biological functions such as cytotoxicity to hepatoma cells, inhibition of histamine release, inhibition of cholesterol synthesis and absorption, stimulation of platelet aggregation, and inhibition of thrombin induced platelet aggregation (Shiao et al., 1994).

Antimicrobial drugs derived from different kinds of microorganisms have long been used for prophylactic and therapeutic purposes. However, using the same antibiotic for a long period may cause the resistance of microorganisms

to that antibiotic. So, the investigation of influences of different kinds of polysaccharides derived from mushrooms on microorganisms and their effects on host's immune system are very important today. Such compounds would be expected to function by mobilising the body's humoral immunity to ward off viral, bacterial, fungal and protozoal infections resistant to the current antibiotics (S m i t h et al., 2002).

MATERIALS AND METHODS

Maintenance of microorganisms

Antimicrobial effects of the extracts of *G. lucidum* on certain strains of microorganisms were investigated:

1. *Escherichia coli* ATCC
2. *Bacillus cereus* ATCC
3. *Staphylococcus aureus* ATCC
4. *Salmonella enteritidis* — Faculty of Agriculture collection
5. *Proteus mirabilis* — Faculty of Agriculture collection
6. *Saccharomyces cerevisiae* ATCC
7. *Aspergillus niger* — Faculty of Agriculture collection

ATCC — American Type Culture Collection, Rockville, Maryland

Bacterial strains of *E. coli*, *B. cereus*, *S. aureus*, *S. enteritidis* and *P. mirabilis* were maintained on nutrient agar. The slant was inoculated and incubated at 37°C for 24 h, than stored at 4°C.

Investigated yeast *S. cerevisiae* and mould *A. niger* were maintained on malt agar. The slant was inoculated and incubated at 30°C, 48h for yeasts and at 25°C, 7 days for mold, than stored at 4°C.

Preparation of microorganisms

Bacterial strains of *E. coli*, *B. cereus*, *S. aureus*, *S. enteritidis* and *P. mirabilis* were inoculated in nutrient broth and incubated at 37°C for 24 h, to reach the concentration of 10^6 cells/ml.

Yeast *S. cerevisiae* and mould *A. niger* were inoculated in malt broth and incubated at 30°C for 24 h for yeast and at 25°C for 24 h for mold, to reach the concentration of 10^6 cells/ml.

Used strains of Ganoderma lucidum

For this experiment ten different extracts, derived from different strains of *G. lucidum* were used. Strains Gl-I, K1 were isolated naturally from Serbian woods, strains Gl-7 and Gl-349 were taken from the Research Plant International collection, Holland and strain Gl-K originated from China.

Hot extraction of bioactive compounds from dried Ganoderma lucidum *mushroom*

Powdered tissue (1—9 g) was washed with 96% ethanol (300 ml), than filtered and dried in vacuum (at 40°C for 60 min) up to getting powder. Dried filtercake was mixed with deionized water (300 ml) and glucans were extracted by autoclaving at 120°C for 20 min. Material was cooled down and centrifuged (10000 rpm, at 4—9°C for 10 min). Supernatant was mixed with 2 vol. 96% ethanol and left at 4°C untill precipitate was formed. After centrifuge (10000 rpm, at 4—9°C for 10 min) the collected pellet were dried in vacuum (at 40°C for 60 min) and the powder was dissolved in Tris buffer 0.01 M (50 ml). The suspension was dialyzed for 24 h at room temperature. Dialyze is necessary for refining because low-molecular weight molecules will pass through the membrane in solution, while high-molecular weight molecules, β-glucan will stay inside the membrane. After dialyzing the content was centrifuged (10000 rpm, at 4—9°C for 10 min) and 2 vol. 96% ethanol was added to supernatant and left at 4°C for a couple of hours. To remove supernatant centrifugation (10000 rpm, at 4—9°C for 10 min) was repeated and the pellets were dried in vacuum (at 40°C for 60 min). The dried pellets were dissolved in PBS and used for further examination on microorganisms.

Room temperature extraction of water-soluble bioactive compounds from dried mushroom Ganoderma lucidum

Powdered tissue (10 g) was mixed with water (300 ml) and steered on magnetic stirrer at room temperature for 24 h. After filtration, the supernatant was removed and $(NH_4)_2SO_4$ was added to 90% saturation. Centrifugation (10000 rpm, at 4—9°C for 10 min) was done and Tris 0.01 M was added to the pellets for dialyzing (at room temperature for 24 h). This is the way to obtain the lectins which will stay within the membrane and will be separated by the centrifugation (10000 rpm, at 4—9°C for 10 min). The obtained dry lectins were dissolved in PBS and used for the investigation of their influence on the observed microorganisms.

Influence of the extracts on microorganisms

Petri dishes were inoculated with 0.2 ml suspension of certain microorganism strains, and overlayed with 20 ml of medium. For bacterial growth, Mueller Hinton agar was used, and for yeast and mould, malt agar was used. Three filter disks (Schleicher & Schuell), 6 mm in diameter were placed on each agar, and 10 μl of appropriate mushroom extract was added. Blind probe contained only PBS, without any mushroom extract. The bacteria were incubated at 37°C for 24 h, the yeast was incubated at 30°C for 48 h, and the mould was incubated at 25°C for 7 days.

After incubation, inhibition zones around the filter disks were measured.

All experiments were performed in duplicate, for three times. The analysis of variance test ($P < 0.05$) was used to determine the statistical significance.

RESULTS AND DISCUSSION

Examination showed that the bioactive compounds derived from *G. lucidum* mushroom have had some influence on the observed microorganisms. PBS did not show any influence on the observed microorganisms.

The results of influence of ten different extracts derived from *G. lucidum* mushroom on the microorganisms are shown in the Table 1.

Tab. 1 — Influence of different extracts derived from *Ganoderma lucidum* on microorganisms

Microorganisms	Inhibition or stimulation zone in diameter (mm)									
	Mushroom extract									
	1	2	3	4	5	6	7	8	9	10
Salmonella enteritidis	2.66	2.66	—	1	2.66	3	—	2	3	2.66
Escherichia coli	2.33	1.33	—	—	2	2	—	—	3	2.5
Bacillus cereus	14.67*•	15*•	14.67*•	14*•	14.33*•	13.33*•	12*•	14*•	14*•	14*•
Proteus mirabilis	16*•	16*•	15*•	16*•	16*•	15.33*•	15*•	14*•	15*•	14.33*•
Staphylococcus aureus	—	—	—	—	—	—	—	—	—	—
Saccharomyces cerevisiae	—	—	—	—	—	—	—	—	—	—
Aspergillus niger	—	—	—	—	—	—	—	—	—	—

* stimulation of growth
— with no influence on growth
• significant difference was found between the treatment and the control ($p < 0.05$)

All investigated extract were derived by water extraction and alcohol precipitation, except the extract number 8 which was derived by hot water extraction.

The extracts derived from different strains and parts of fruitbodies of *G. lucidum* mushroom were used. These extracts were labeled with numbers 1—10:

1. Extract derived from the fruiting body of *G. lucidum*
2. Extract derived from the spore broken cell walls of *G. lucidum* Gl-K
3. Extract derived from the powdered fruiting body of *G. lucidum* K_1
4. Extract derived from the micro powdered fruiting body of *G. lucidum* Gl-K
5. Extract derived from the *G. lucidum* Gl-K hypha
6. Extract derived from the *G. lucidum* Gl-K powdered spores
7. Extract derived from the fruiting body of *G. lucidum* Gl-I by water extraction at room temperature

8. Extract derived from the fruiting body of *G. lucidum* Gl-I by hot water extraction

9. Extract derived from the fruiting body of *G. lucidum*-7

10. Extract derived from the fruiting body of *G. lucidum*-349

It was established that the extracts derived from *G. lucidum* Gl-K powdered spores (Fig. 4) and fruiting body of *G. lucidum*-7 had significantly (PT on bacteria *S. enteritidis*. The extracts derived from the fruiting body of *G. lucidum* Gl-I, the spore broken cell walls of *G. lucidum* Gl-K, the *G. lucidum* Gl-K hypha and the fruiting body of *G. lucidum*-349 (Fig. 2) showed reduced inhibition effects. The extracts derived from the powdered fruiting body of *G. lucidum* K_1 and from the fruiting body of *G. lucidum* Gl-I by water extraction at room temperature did not influence the growth of bacteria *S. enteritidis*.

The strongest inhibition effect on the growth of bacteria *E. coli* was observed in the case of the extract derived from the fruiting body of *G. lucidum*-349 (Fig. 1), and the extract derived from the fruiting body of *G. lucidum* Gl-I, while the extracts derived from the powdered fruiting body of *G. lucidum* K_1, the micro powdered fruiting body of *G. lucidum* Gl-K, the fruiting body of *G. lucidum* Gl-I by water extraction at room temperature and the fruiting body of *G. lucidum* Gl-I by hot water extraction, did not influence the growth of bacteria *E. coli*.

All investigated extracts derived from different kinds of *G. lucidum* mushroom showed significantly strong stimulating effects ($P < 0.05$) on the growth of bacteria *B. cereus* (fig. 5 and 6). The strongest effect showed the extract derived from the spore broken cell walls og *G. lucidum* Gl-K. The most reduced stimulating effect showed the extract derived from the fruiting body of *G. lucidum* Gl-I by water extraction at room temperature.

All investigated extracts had significantly strong stimulating effects ($P < 0.05$) on the growth of bacteria *Proteus mirabili*. The extracts derived from the fruiting body of *G. lucidum* Gl-I, the spore broken cell walls of *G. lucidum* Gl-K, the micro powdered fruiting body of *G. lucidum* Gl-K and *G. lucidum* Gl-K hypha (Fig. 3) were the strongest. The lowest stimulating effect showed

Fig. 1 — Inhibitory effect of extract derived from the fruiting body of *Ganoderma lucidum*-349 on the growth of bacteria *Escherichia coli*

Fig. 2 — Inhibitory effect of extract derived from the fruiting body of *Ganoderma lucidum*-349 on the growth of bacteria *Salmonella enteritidis*

Fig. 3 — Stimulating effect of extract derived from *Ganoderma lucidum* Gl-K hypha on the bacteria *Proteus mirabilis*

Fig. 4 — Inhibitory effect of extract derived from *Ganoderma lucidum* Gl-K powdered spores on the bacteria *Salmonella enteritidis*

Fig. 5 — Stimulating effect of extract derived from *Ganoderma lucidum* on the bacteria *Bacillus cereus*

Fig. 6 — Stimulating effect of extract derived from *Ganoderma lucidum* Gl-K, by hot water extraction, on the bacteria *Bacillus cereus*

the extract, derived by hot water extraction, from the fruiting body of *G. lucidum* Gl-I.

The investigated extracts did not show any effect on the growth of bacteria *S. aureus*, yeast *S. cerevisiae*, and mould *A. niger*.

CONCLUSIONS

G. lucidum, one of the oldest salutary remedy known for more than 3000 years, became a subject of interest in many contemporary science researching papers. Numerous experiments showed different possibilities of using of extracts derived from this mushroom in various disease treatments, improving immune system function which results in improving the general condition of an organism. One of the possible ways of utilising these extracts is their action on microorganisms which endanger human and animal health very often. Standard procedures in repression of harmful microorganisms are applications of antibiotics, but too much usage of antibiotics, and the ability of microorga-

nisms to develop resistance to them, result in their decreased effects on microorganisms. On the other hand polysaccharides derived from mushrooms became very interesting due to their influence on immune system and on microorganisms, that can be used in a struggle against them. In this work, the influence of some polysaccharides derived from *G. lucidum* mushroom on several microorganisms was investigated. In some cases the examined extracts showed very intensive influence, inhibitory or stimulating, while some of them did not show any influence on the examined microorganisms.

We believe that this investigation is of a current interest and should be continued with more microorganisms and numerous chemically defined fractions derived from the extracts of *G. lucidum* mushroom.

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УТИЦАЈ ЕКСТРАКАТА ИЗОЛОВАНИХ ИЗ ГЉИВЕ *GANODERMA LUCIDUM* НА НЕКЕ МИКРООРГАНИЗМЕ

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Резиме

Ganoderma lucidum (Leyss.: Fr.) Karst је једна од најважнијих традиционалних кинеских гљива. Према писаним подацима старим више од 2000 година користи се на Далеком истоку као здрава храна и лековита гљива. Један од врло интересантних аспеката коришћења гљиве *Ganoderma lucidum* је и употреба ове гљиве као антимикробног средства, захваљујући изолованим екстрактима који садрже бактериолитичке ензиме, лизозиме и киселе протеазе. Ефекти ових екстраката зависе од њиховог састава, начина екстракције и пречишћавања. Биоактивне компоненте изоловане из неколико сојева гљиве *Ganoderma lucidum* показале су различите ефекте на испитиване микроорганизме. У неким случајевима утицај екстраката је био врло интензиван, инхибишући или стимулишући, док неки од њих нису показали никакав утицај на испитиване микроорганизме.

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ABILITY OF *PLEUROTUS ERYNGII* MYCELIUM TO ABSORB SELENIUM DEPENDING ON THE SELENIUM SOURCE AND CONCENTRATION IN MEDIUM

ABSTRACT: The aim of our research was to investigate the influence of three different inorganic Se sources, added to the synthetic medium, on the *Pleurotus eryngii* mycelium ability to absorb and retain this microelement. All investigated *P. eryngii* strains were good Se absorbers from the media enriched with all studied Se sources. Strain HAI 711, which was cultivated in the Na_2SeO_4-enriched medium at Se concentration of 1.0 and 1.3 mgl^{-1}, respectively, showed extraordinarily high Se concentrations in the mycelium (725 μgg^{-1} of dry weight and 575 μgg^{-1} of dry weight, respectively).

KEY WORDS: *Pleurotus eryngii*, mycelium, selenium absorption

INTRODUCTION

Pleurotus eryngii (DC.: Fr.) Quél is economically highly praised species due to its nutritional and medicinal value. This species has: anti-hypertensive effect, because of low sodium and great potassium concentration; antioxidant effect, because of the presence of phenolic compounds; anti-hypercholesterolic effect, due to a significant amount of dietary fibers, β-glucans, chitin, and chitosan; anti-hyperglycemic, immunomodulating, antitumor, antibacterial, antiviral, antifungal, antiinflammatory, and antiosteoporotic effects (Manzi et al., 1999; 2004; Manzi and Pizzoferrato, 2000). Although selenium (Se) is not an essential microelement for yeast and plants (Flohé et al., 2000), it plays an essential nutritional role in some animals and humans. Se is an integral component of several enzymes, selenoprotein P and W, albumin, and so-called 15 kDa selenoprotein and selenoprotein N, whose functions are still elusive (Birringer et al., 2002). In accordance with the roles of Se dependent-enzymes and proteins, that trace element is antioxidant and an antimuta-

genic agent, which prevents the malignant transformation of normal cells and the activation of oncogenes (S t a p l e t o n, 2000).

Pleurotus species have the ability to absorb this microelement from medium and may present an excellent dietary Se source (S t a j i ć et al., 2002; D u l e t i ć - L a u š e v i ć et al., 2005).

The aim of this research was to investigate the influence of three different inorganic Se sources, added to the medium in different concentrations, on the mycelium ability to absorb it.

MATERIALS AND METHODS

The investigated *P. eryngii* strains and their origin are presented in Table 1. These cultures were obtained from the culture collection of the Institute of Evolution, University of Haifa (HAI), Israel.

Tab. 1 — Investigated *Pleurotus eryngii* strains

Scientific name of species	HAI number of strains	Origin of strains
P. eryngii (DC.: Fr.) Quél. var. *eryngii*	193	Ukrainian SSR, Kherson region, Chaplinka district, Askania-Nova, on *Stipa* sp.
	201	Israel, Menahemya, on *Ferula* sp.
	356	Israel, Gevaot Merar, near Gedera, on *Ferula* sp.
	507	Cultivated strain, Hawaii, Nextlab
	616	Israel, Tabor, on *Ferula* sp.
	711	Israel, Tel Hazor, on *Ferula* sp.
	716	Israel, Gilboa, on *Ferula* sp.
	728	Israel, Lahav, on *Ferula* sp.
P. eryngii var. *tingitanus* Lewinsohn et al.	555	Israel, Sataf, on *Ferula tingitana* L.

Selenium was used in the forms of sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), and selenium dioxide (SeO_2), in the concentrations of 0.3 mgl^{-1}, 0.7 mgl^{-1}, 1.0 mgl^{-1}, and 1.3 mgl^{-1}. Synthetic medium of the following composition: glucose, 10.0 gl^{-1}; NH_4NO_3, 2.0 gl^{-1}; KH_2PO_4, 0.8 gl^{-1}; Na_2HPO_4 x $7H_2O$, 0.75 gl^{-1}; $MgSO_4$ x 7 H_2O, 0.5 gl^{-1}; yeast extract, 2.0 gl^{-1}, pH 6.0 (50 ml per flask), was inoculated and incubated at room temperature (22 ± 2°C), on the rotary shaker at 180 rpm, for 28 days. The medium without Se was used as the control. Three repetitions for each Se source and concentration per strain were performed. After the cultivation period, mycelia were filtrated and dried at 30°C during the night. For Se extraction, dry mycelia were treated with: 70% of nitric acid, 70% of nitric acid + 70% of perchloric acid (3:1), and 6 M HCl (37%), in a mineralizator at 120°C. Se concentrations in mycelia were measured by graphite furnace Atomic Absorption Spectrometer (VARIAN, Australia).

RESULTS

Na_2SeO_3 was a good Se source for the absorption by mycelium and incorporation of Se compounds in the cell, in all investigated *P. eryngii* strains (Fig. 1).

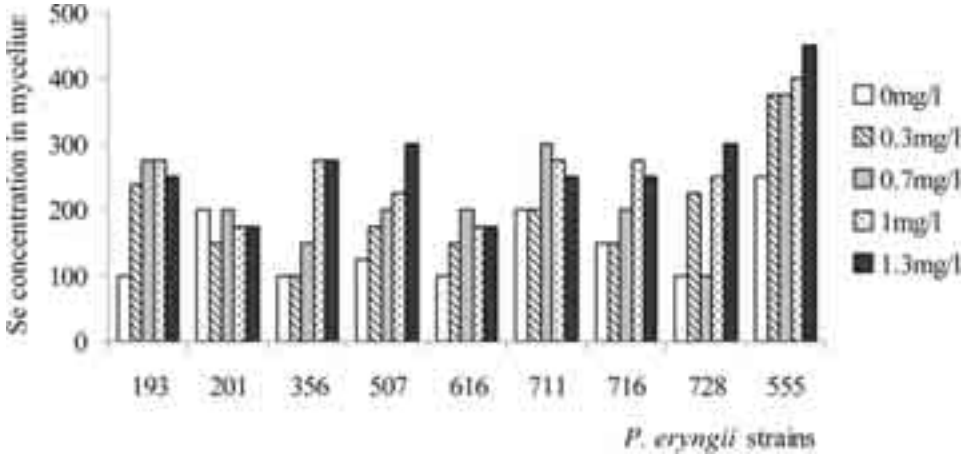

Fig. 1 — Se content in mycelia (μgg^{-1} of dry weight) of investigated *Pleurotus eryngii* strains cultivated in medium with Na_2SeO_3 as Se source

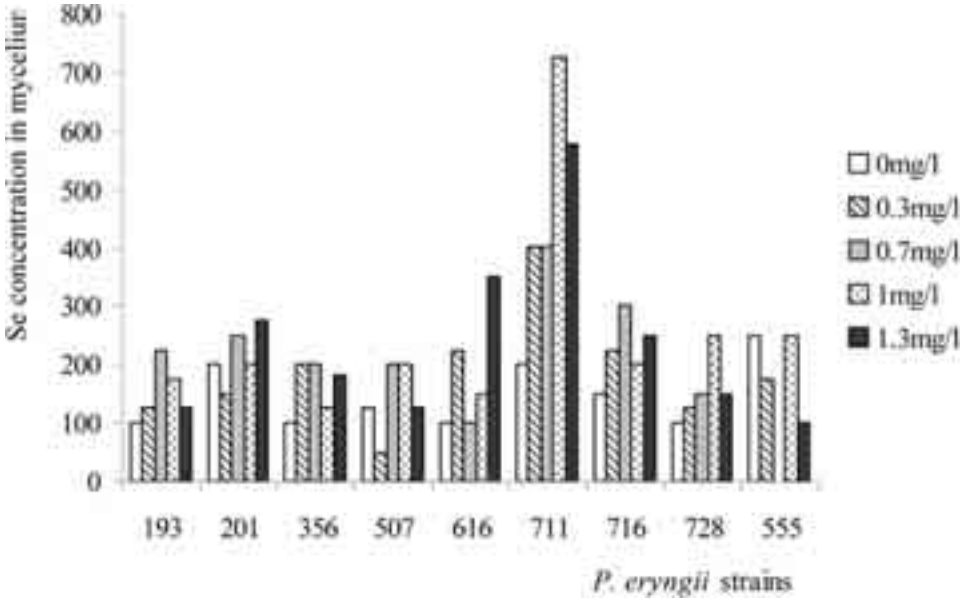

Fig. 2 — Se content in mycelia (μgg^{-1} of dry weight) of investigated *Pleurotus eryngii* strains cultivated in medium with Na_2SeO_4 as Se source

Among the investigated strains of *P. eryngii* var. *eryngii*, two groups were distinguished according to their ability to absorb Se from Na_2SeO_4-enriched medium, and to retain it. Strains HAI 201 and HAI 507 fit into the first group, where Se content in the mycelium decreased, compared to the control, at the Se concentration of 0.3 mgl^{-1}, while in the presence of higher Se concentrations in the medium, mycelium content increased. In the second group, other investigated *P. eryngii* var. *eryngii* strains showed more or less increase in Se concentration in the mycelium with its addition to the medium. Strain HAI 711, which was cultivated in the Se-enriched medium with 1.0 and 1.3 mgl^{-1}, respectively, showed extraordinarily high Se concentrations in the mycelium (725 μgg^{-1} of dry weight and 575 μgg^{-1} of dry weight, respectively). However, in *P. eryngii* var. *tingitanus* HAI 555, it was not noted only a decrease in Se concentration in the mycelium, compared to the control, but also its absence when it was present in the medium at the concentration of 0.7 mgl^{-1} (Fig. 2).

SeO_2, as well as Na_2SeO_3, were shown as good sources for Se absorption and retention by mycelia of *P. eryngii* strains. All investigated *P. eryngii* var. *eryngii* strains, as well as *P. eryngii* var. *tingitanus*, easily absorbed Se from medium, when it was present at the concentration of 1.3 mgl^{-1}, except strain HAI 201 where the highest concentration of absorbed Se was at its medium concentration of 0.7 mgl^{-1} (Fig. 3).

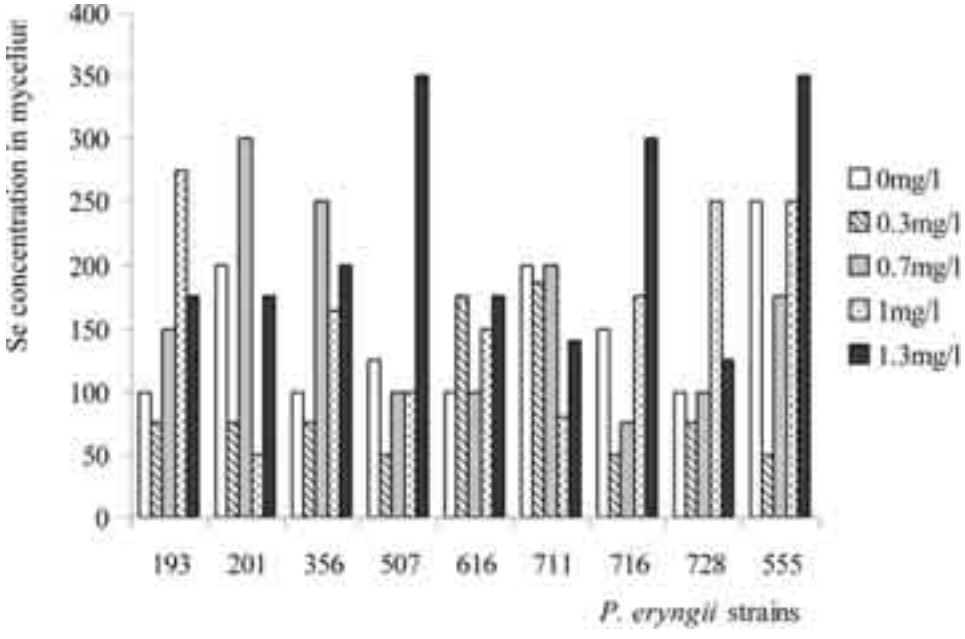

Fig. 3 — Se content in mycelia (μgg^{-1} of dry weight) of investigated *Pleurotus eryngii* strains cultivated in medium with SeO_2 as Se source

DISCUSSION

The obtained results showed that all the investigated *P. eryngii* strains were good Se absorbers from the medium enriched with all studied Se sources. However, the strains had different abilities to absorb and retain Se in the mycelium, and some of them had lower Se concentration in mycelium growing in Se-enriched medium, than in the control one. These may be explained by the following facts:

— the increased Se concentration in the control could be explained by using the wort agar medium for preserving the *P. eryngii* cultures, concerning that wort itself is rich in Se, containing between 0.24 and 0.66 mg/kg of dry weight, depending on the Se concentration in soil (Mihailović, 1996);

— all organisms can assimilate selenites and selenides, while only terrestrial plants and bacteria can assimilate selenates (Birringer et al., 2002);

— the absorption of Na_2SeO_3 from aquatic environments is a passive process distinguished from the absorption of Na_2SeO_4 (Barceloux, 1999);

— the resorption of Se from selenites is 3 times higher than from selenates (Butler and Peterson, 1967);

— for the Se incorporation into selenoproteins, it needs the absorbed selenites or selenates to be reduced to the selenide form. As distinguished from selenates, which are reduced to selenites, firstly in presence of ATP sulfurylase, selenites are readily reduced to selenide by flavine-dependent disulfide reductases (Birringer et al., 2002). SeO_2 in the presence of H_2O gives selenious acid, which with 4 molecules of glutathione, and by releasing 3 molecules of H_2O, gives selenoglutathione which is reduced to selenide (Spallholz, 1994). The obtained selenide will go either into biosynthesis of selenocysteine which will be incorporated into selenoproteins, or into methylation to methaneselenol or dimethyl selenide which lead to excretion or volatilization of Se (Combs and Gray, 1998). On the other hand, both synthesized selenoproteins are degraded after a certain period of time, and selenocysteine may go into one of three metabolic pathways (Rooseboom et al., 2001): *(i)* oxidative deamination, which products are NH_3, pyruvate, and $HSeO_3$ — that may oxidize to selenate; *(ii)* β elimination, which products are selenol, pyruvate, and NH_3; *(iii)* selenoxidation, which products are selenious acid and 2 — aminoacrylic acid that hydrolyse into pyruvate and NH_3.

Unfortunately, knowledge on Se metabolism in fungi is limited, because investigations have only been done with yeasts. The literature data about Se form in filamentous fungi is lacking. In the previous studies of yeast grown in Se-enriched medium, it was reported that yeast contained 15.7% of elemental Se, 5.5% of inorganic, and 76.8% of organic Se (Živković, 1989).

Shamberger (1985) studied the effects of sodium selenite, sodium selenide, and sodium selenate on suppressing spontaneous mutagenesis at lysine and histidine locus in yeast. Contrary to selenite and selenide, which completely suppressed mutagenesis, selenate inhibited mutagenesis only at the lysine locus, which needed lower quantities of selenite and selenide than the histidine locus mutagenesis. Do these results show that yeast has a lower ability to use selenate than selenide and selenite from media?

Capacity of Se absorption by mycelium, also depends on medium where it was grown. This was shown by previous experiments with *P. ostreatus* HAI 387 (Stajić et al., 2002) and *Ganoderma lucidum* (Duletić-Laušević et al., 2005). Se content in mycelium of both investigated species was significantly higher at their cultivation in potato-dextrose Se-enriched medium with Na_2SeO_3, than in malt medium, and especially in synthetic medium with the same Se source. Potato-dextrose medium was also better for production of mycelial biomass, compared to the synthetic medium.

Results obtained here set new goals for further investigations: Se metabolite pathways in higher Basidiomycetes, the forms of absorbed Se in mycelium, as well as finding the best cultivation medium, and Se source for its absorption and incorporation of the organic Se compounds in the cell.

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СПОСОБНОСТ МИЦЕЛИЈЕ *PLEUROTUS ERYNGII* ДА АПСОРБУЈЕ СЕЛЕН У ЗАВИСНОСТИ ОД ИЗВОРА И КОНЦЕНТРАЦИЈЕ СЕЛЕНА У МЕДИЈУМУ

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Резиме

Циљ истраживања је био проучавање утицаја три различита неорганска извора селена додата у синтетичку хранљиву подлогу на способност мицелије *P. eryngii* да апсорбује и задржава овај микроелемент. Сви проучавани *P. eryngii* изолати су добро усвајали селен из подлоге обогаћене одабраним изворима селена. Сој HAI 711 који је био култивисан у Na_2SeO_4 — обогаћеној подлози при концентрацији селена од 1.0 односно 1.3. mgl^{-1} имао је изузетно високу концентрацију селена у мицелији (725 μgg^{-1} суве масе односно 575 μgg^{-1} суве масе).

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MORPHO-PHYSIOLOGICAL CHARACTERISTICS AND INTERACTIONS OF ISOLATES OF *MYCOGONE PERNICIOSA* (MAGNUS) DELACR.

ABSTRACT: *Mycogone perniciosa* (Magnus) Delacr., which causes wet bubble disease of *Agaricus bisporus* Lange (Imb), results in a considerable crop loss on mushroom farms in Serbia. The isolation and identification of five isolates of *M. perniciosa* from diseased fruit bodies of white button mushroom from mushroom units in Serbia, Bosnia and Herzegovina and Holland were made. Morpho-physiological characteristics and inter-relationships of the obtained isolates were studied. Macroscopic and microscopic investigations of different zones between colonies of the isolates of *M. perniciosa* revealed the phenomenon of the hyphal interference between different isolates. The obtained results suggest that hyphal interference could serve as an additional parametar for a more reliable determination of fungal specifity.

KEY WORDS: *Mycogone perniciosa*, mycopathogen, hyphal interference

INTRODUCTION

Wet Bubble Disease, caused by mycopathogen *Mycogone perniciosa* (Magnus) Delacr., is still considered as one of the most important diseases of the cultivated mushrooms *Agaricus bisporus* Lange (Imb), wherever white button mushroom are produced commercially (Sisto et al., 1997, Sharma and Kumar 2000, Bora and Özaktan 2000, Nanagulyan and Yesayan 2002). Mushroom cultivation in Serbia is still less developed than in other countries, and *M. perniciosa* has a significant influence on quality and yield of mushrooms. *Mycogone perniciosa* produces small thin-walled phialoconidia on *Verticillium*-like conidiophores, together with much larger bicellular

conidia (aleuriospores) that develop on short, lateral hyphae, consisting of dark, spherical thick-walled, verrucose apical cell and thin-walled basal cell. Besides vegetative mycelium, both phialospores and aleuriospores of *M. perniciosa* are infectious (H o l l a n d and C o o k e 1990). Studies of the *M. perniciosa* isolates directed toward the distinction of different isolates, is of great importance from both theorethical and practical point of view. This fungi is known by extremly high mycopathogenic potencial and frequently attacks crops of white button mushroom, a common edible mushroom with major economic value and a cosmopolitan distribution (K e r r i g a n, 1995).

According to W e b s t e r (1970) hyphal interference can be of great significance if the hypotheses that it represents the most obvious and most clear form of interspecific competition, and that it could serve as an additional parametar for determination of different isolates of *M. perniciosa*, are true.

In this study, we examined the morpho-physiological characteristics and inter-relationships of the obtained *M. perniciosa* isolates.

MATERIALS AND METHODS

Samples of diseased mushrooms were collected from mushroom farms in Serbia, Bosnia and Hercegovina and Holland. Pure culture of the *M. perniciosa* was isolated from *A. bisporus* in the Mycological Laboratory, Institute for Biological Research "Siniša Stanković". Fungal isolates used in this work were as follows: MPS from mushroom farms in Bosnia and Herzegovina (Sarajevo); 2 from mushroom farms in Serbia: MPPS (Padinska Skela) and MPR (Ripanj) and 2 (MPH1 and MPH2) from the Mushroom Experimental Station, Wageningen, Holland. The isolates were maintained on potato dextrose agar (PDA). The cultures were stored at 4°C and subcultured once a month (B o o t h 1971).

Morpho-physiological characteristics of the isolates were recorded on the colony, grown on Petri plates with PDA medium, during 10 days, at 25°C. Colony characteristics and growth measurements were made daily. Hyphae, phialoconidia and aleuriospores were placed on microscopic slides and stained with Lactophenol cotton blue (J o h a n s e n, 1940). Measurements (at least 30 of each spore type) and photographs were made on Reichert microscope with Canon power Shot S40.

Different combinations each consisting of three fungal isolates, were inoculated in Petri plates containing PDA medium. The three isolates were inoculated equi-distant from the others in all possible combinations in the same Petri plates. The cultures grown under laboratory conditions (day light, at 25°C) were examined after 5 and 10 days of age.

RESULTS AND DISCUSSION

Colony characteristics of all isolates varied (Table 1). The colonies were very regular in growth with either dense aerial mycelium MPH2, MPPS and

MPR, or sparse MPH1. Only MPS isolate had colony, which grew in sectors compact and aerial, irregular in growth. Colony colour, which to some extent indicates the production of aleuriospores, varied from white MPS to dark brown MPPS (Table 1).

Tab. 1 — Some morphological characteristics of isolates of *Mycogone perniciosa*

Isolates	Colony tipe	Colony size (mm)	Hyphae max-min mostly (µm)	Phyalospores max-min mostly (µm)	Aleuriospores Upper cell (max-min, mostly) Lower cell (max-min mostly) (µm)
MPS	white, growth in sectors, compact and aerial mycelium, irregular growth	75x76x77	4—8 6	2x6—6x18 2x12	12x16—22x24 (18x20) 6x10—10x18 (10x14)
MPPS	dark brown, with white edge, aerial mycelium	68x69x68	3,75—5 3,75	3,75x12,50 — 5x18,75 3,75x12,50	15x17,50—21,25x23,75 (18,75x20) 7,50x11,25—13,75x15 (10x13,75)
MPR	light brown, with white edge, aerial mycelium	70x50x76	2,50—5 3,75	3,75x7,50 — 3,75x11,25 3,75x12,50	12,50x17,50—23,75x25 (18,75x21,25) 7,50x10—12,50x13,75 (12,50x12,50)
MPH1	white, supstrate mycelium	74x70x74	2,50—5 3,75	2,50x7,50 — 2,50x13,75 2,50x11,25	12,50x13,75—22,50x22,50 (20x20) 6,25x6,25—15x17,50 (10x10)
MPH2	amber brown, with white edge, aerial mycelium	80x80x79	2,50—5 3,75	2,50x7,50 — 3,75x14 3,75x12,50	15x17,50—22x22 (18,75x20) 6,25x8,75—12,5x17,50 (8,25x11,25)

Growth rates and sizes of the colonies of all isolates were similar (Table 1). All isolates produced both phialospores and aleuriospores. The most intensive sporulation was detected in MPPS and MPR, then in MPH2, and the lowest intensity was in MPS and MPH1. Phialospores size varied within the range 2x12 µm (MPS) — 5x18,75 µm (MPPS). Aleuriospores varied within the range 23,75 x 25 µm (MPR) to 12,50 x 13,75 µm (MPH1) for the upper cell, and 10 x 18 µm (MPS) to 6,25 x 6,25 µm (MPH1) for the lower cell (Table 1). Our isolates of *M. perniciosa* came from mushroom farms from Serbia, Bosnia and Herzegovina and Holland but differences between them were not so obvious when compared to the other literature data (Gray and Morgan-Jones, 1980; Umar et al., 2000; Sharma and Kumar, 2000 and Potočnik, 2006). In contrast to the data of Smith (1924), Atkins and La Touche (1948), Hsu and Han (1981) and Fletcher et al. (1995) which reported two-cell phialospores, we could not find this form in our isolates.

Albouy and Lapierre (1972) and Fletcher et al. (1995) found that some pathogenic strains of *M. perniciosa*, which were slow-growing on agar, were highly pigmented and produced numerous aleuriospores. Other

were weakly pathogenic, producing much vegetative growth and little pigmentation. The slow-growing forms were found to contain numerous virus like particles.

We observed interactions between all tested isolates after 10 days. There were several types of interaction: detaining of growth at the site of contact without visible changes, overgrowth of mycelium of one isolate and demarcation lines between the isolates (Table 2). According to the morpho-physiological characteristics and hyphal interferences of five *M. perniciosa* isolates, the isolates from Serbia were similar; the isolates from Holand showed mutually similar characteristics, but they were different from the isolates from Serbia. Isolate from Bosnia and Herzegovina was different from these two groups (Figure 1—4).

Tab. 2 — Interactions appearing between colonies of isolates of *Mycogone perniciosa*

Izolat	**MPH2**	**MPH1**	**MPR**	**MPPS**	**MPS**
MPS	detaining of growth at the site of contact	overgrowth of mycelium	demarcation line 4—5 mm	demarcation line	detaining of growth at the site of contact
MPPS	detaining of growth at the site of contact	detaining of growth at the site of contact	demarcation line	demarcation line	
MPR	detaining of growth at the site of contact	overgrowth of mycelium	detaining of growth at the site of contact		
MPH1	overgrowth of mycelium	overgrowth of mycelium			
MPH2	detaining of growth at the site of contact				

Earlier investigations of hyphal interference phenomenon on other fungal species showed different interspecies interaction. As demonstrated previously (Franić-Mihajlović et al., 1996), the isolates of *Diaporthe/Phomopsis* known for their extremely phytopathogenic potential, showed different reactions during the investigation of hyphal interference. Demarcation lines were formed between the isolates which originated from one plant. Formation of the lines between the colonies of the same morphological group of isolates was named as inter-species antagonism by Brayford (1990 a, b). Phenomenon of demarcation line formation results from the incompatibility between genetically different colonies. On the basis of comparison of interactions between the different isolates *M. perniciosa*, we can conclude that these belong to different groups. Genetical difference between these isolates based on molecular-genetic characteristics (Glamočlija, 2006), supports such a conclusion.

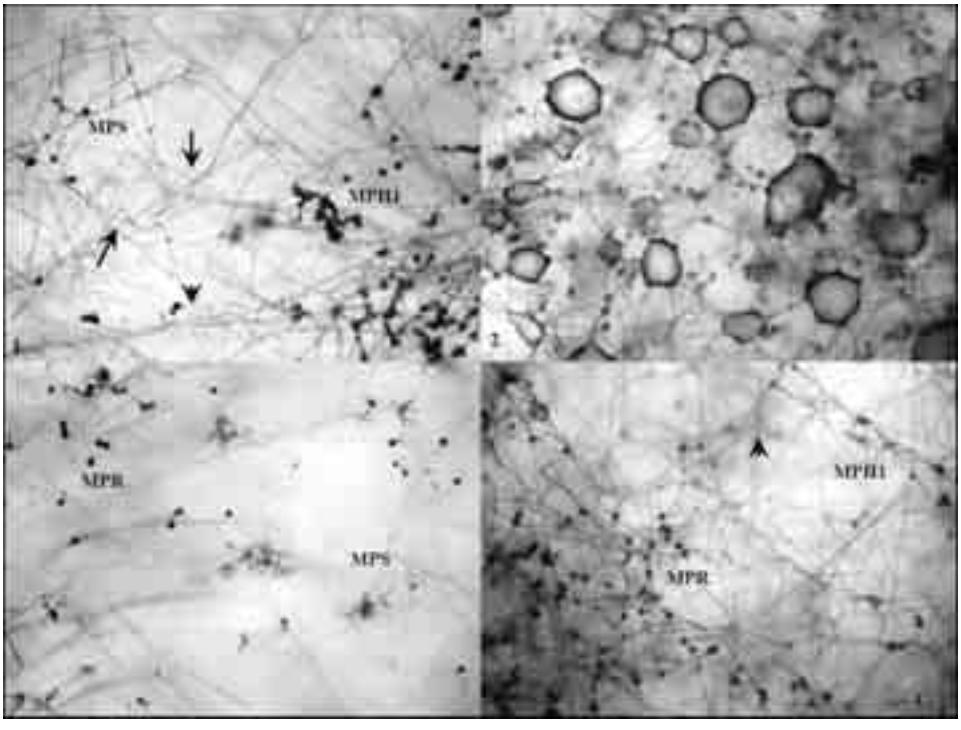

Fig. 1—4 — Types of interactions between isolates of *M. perniciosa*: 1. anastomosis (arrows) of hyphae MPH1 and MPS; 2. overgrowth of MPH1 and MPH2 with exudation; 3. demarcation line between MPR and MPS; 4. anastomosis of hyphae MPH1 and MPR

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МОРФО-ФИЗИОЛОШКЕ КАРАКТЕРИСТИКЕ И ИНТЕРАКЦИЈЕ ИЗОЛАТА *MYCOGONE PERNICIOSA* (MAGNUS) DELACR.

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Резиме

Mycogone perniciosa (Magnus) Delacr., изазивач обољења мокре трулежи, најчешћи је узрочник губитака у гајилиштима *Agaricus bisporus* Lange (Imb) у Србији. Извршена је изолација и идентификација 5 изолата *M. perniciosa* са оболелих плодоносних тела шампињона из гајилишта у Србији, Босни и Херцеговини и Холандији. Испитиване су морфо-физиолошке карактеристике као и степен сродности проучаваних изолата на основу анализе међусобног деловања колонија, односно коришћењем феномена хифалне интерференције. Макроскопска и микроскопска истраживања односа изолата и добијени резултати указују да хифална интерференција може представљати додатни параметар у разликовању изолата *M. perniciosa*. Изолати добијени из гајилишта у Србији слични су међусобно, као и изолати из Холандије који су показали међусобну сличност али се разликују од претходних. Изолати из Босне и Херцеговине разликовали су се и од српских и од холандских.

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CHARACTERISTICS OF LOCAL PULMONARY RESPONSE FOLLOWING INTRANASAL APPLICATION OF *ASPERGILLUS FUMIGATUS* CONIDIA

ABSTRACT: In this study, histopathology of local pulmonary response following intranasal inoculation of different doses of *Aspergillus fumigatus* conidia in laboratory rats was evaluated. Development of response was evaluated on days seven and twenty one, following conidia inoculation by cell infiltration and by presence of *A. fumigatus* conidia in homogenates of lung tissue. Total and differential peripheral blood leukocyte counts and state of leukocyte adhesion/aggregation were monitored to estimate the presence of systemic response in infected individuals. Mycological examination revealed the presence of conidia in lung tissue homogenates of infected animals, with high number of non-germinating spores on day twenty one, following the inoculation of lower conidia dose. Histopathological examination revealed the presence of lymphocytes perivascularly and in vascular lumen in the lungs. Presented data demonstrate pulmonary immune response following *A. fumigatus* conidia administration.

KEY WORDS: *Aspergillus fumigatus*, histopatology, laboratory rats, lung homogenates, lungs, lymphocytes

INTRODUCTION

The genus *Aspergillus* includes more than 200 species, which are abundant in every region in the world. *Aspergillus* can be differentiated from other fungi by the presence of thin, parallel walls, dichotomous branching, septate hyphae, and characteristic conidiophores. The most species of *Aspergillus* cause infections/diseases (aspergilloses), but 90% of aspergillosis are caused by opportunistic fungi *Aspergillus fumigatus* (L a t g e, J. P., 1999). *A. fumigatus* has conidia 2—3 mm in diameter which enter the respiratory tract by inha-

lation and reach distal alveoli (the lungs are the most common site of infection).

Pulmonary aspergillosis is the most common and involves allergic bronchopulmonary aspergillosis, aspergilloma and invasive pulmonary aspergillosis (S h i b u y a, K. et al., 2004). As manifestations of the infection depend on host's immune response, the increase in numbers of immunocompromised and susceptible hosts fueled interest in aspergillosis. The interaction between weakened defense mechanisms and pathogenic conidia of *A. fumigatus* cause pulmonary lesions. *A. fumigatus* is not pathogenic in immunocompetent individuals, where innate immunity is considered as the main defense against this fungus (R o m a n i, L., 2004).

In almost all investigations of *A. fumigatus* infections and aspergillosis, immunocompromised/immune suppressed animals were used. The aim of this study is to get the initial data about local host lung immune response in a model of experimental pulmonary infection in apparently healthy, immunocompetent rats. With this aim, local pulmonary response to intranasal inoculation of *A. fumigatus* conidia was evaluated by histological evaluation and by determining the number of germinating spores in lung tissue homogenates. The state of peripheral blood leukocytes was evaluated to monitor the presence of systemic response.

MATERIALS AND METODS

Conventionally housed Dark Agouti (DA) male rats, bred at the Institute for Biological Research "Siniša Stanković" were used. Animal treatment has been carried out in adherence with the Ethical Comittee of Institute for Biological Research "Siniša Stanković".

Human isolate *A. fumigatus* Fresenius (Institute of Public Health of Serbia "Dr Milan Jovanović Batut") was subcultured on standard mycology slant. Inoculum was prepared by flooding the surface of agar slants with sterile 0,85% of NaCl/0,1% of Tween 80. The suspension containing 10^6 and 10^7 conidia was applied intranasally, which reflects the natural route of *Aspergillus* infection in humans. The animals were assigned to two groups and sacrificed on days 7 and 21, following the inoculation.

Blood was collected (in citrate buffer 1:5 as anticoagulant) for counting peripheral blood leukocytes and leukocyte adhesion/aggregation assay (LAA). Cell counts were performed by differentiating 500 cells from blood smears stained with May-Grünwald-Giemsa.

Following exsanguination lung lobes were removed. One of the lobes was fixed in 4% of buffered formaline (pH 6.9), embedded in paraffin and 5mm sections were stained with hematoxylin and eosin for histopathological examination. The other was homogenized (in 1ml PBS/PMSF) in order to check the presence of *Aspergillus* by standard mycological identification method (Sabouraud Maltose Agar, SMA, medium) and micromycete identification. Homogenates were prepared in PBS (1:1 and 1:2) and seeded on solid (SMA) and liquid medium for quantitation of germinating and non-germinating spores.

Statistical evaluation of the data was performed using the Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

In this study, histological data revealed the presence of local pulmonary response 7 days after the inoculation at both doses: predominance of lymphocytes perivascularly and in vascular lumen with increased bronchial secretion and tickened intersticium. No histologically evident changes were noted 21 days following the inoculation. Histopathological changes in lungs were presented in Figure 1.

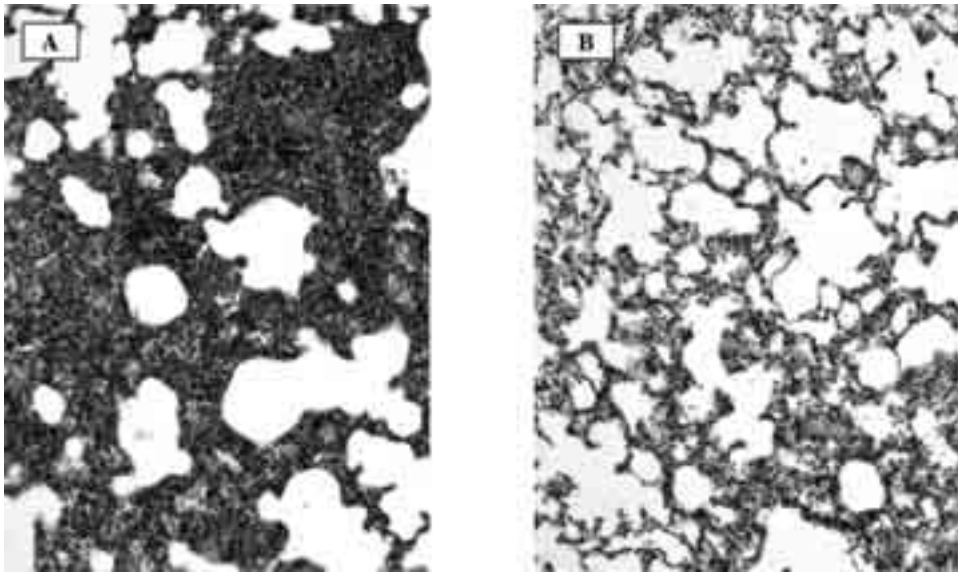

Fig. 1 — Histological picture of lungs from rats following application of 10^7 spores of *A. fumigatus* (A) and healthy (immunocompetent) animal (B)

Application of *A. fumigatus* led to increased, but not statistically significant dose dependent on peripheral blood leukocyte activity (adhesion and aggregation) 7 days following the inoculation. No such changes were noted on day 21 following the infection. There were no differences in total and differential peripheral blood leukocyte numbers between the control and the inoculated animals.

Mycological evaluation of lung homogenates documented the presence of both germinating and non-germinating spores of *A. fumigatus* in all rats, excluding the controls. On day seven, significant number of non-germinating spores was noted at both applied doses of conidia (Figure 2). On day 21, numbers of non-germinating conidia increased further in group of animals which received 10^6 conidia, while rise in germinating spores was noted in individuals challenged with 10^7 conidia.

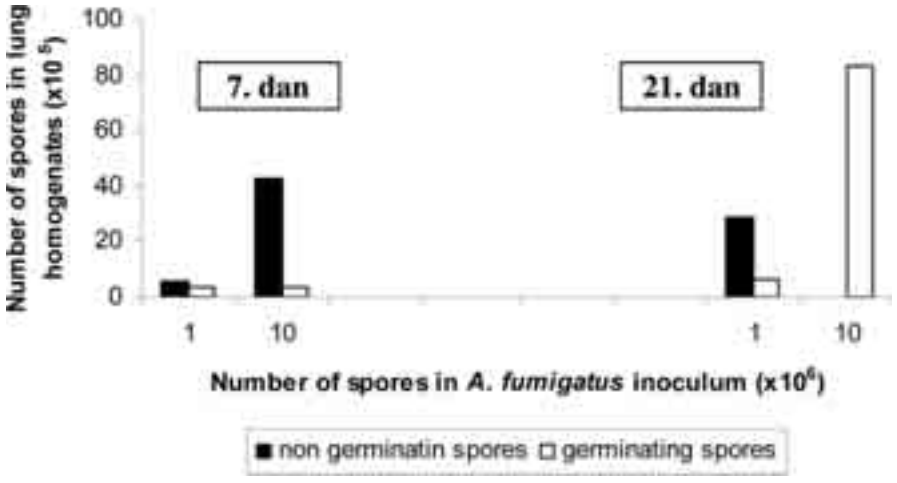

Fig. 2 — Number of spores in lung homogenates on Sabouraud Maltose Agar medium 7 and 21 days following *A. fumigatus* inoculation

DISCUSSION

The increased number of non-germinating spores, on day seven, in lung homogenates at both conidia doses, and on day 21 at lower dose, could be explained by the presence of lymphocytes in cell infiltrate in lungs and their activity *in situ*. The presence of increased number of germinating conidia at higher dose on day 21 reflects, presumably, the capacity of small fraction of spores to germinate later in lung microenvironment. Investigation of possibility to over-ride local immune mechanisms deserves future attention.

Although no quantitative changes were noted in peripheral blood leukocytes, qualitative changes were noted. Increased state of leukocyte aggregation/adhesion (LAA) suggest that local leukocyte lung response was accompanied by systemic response as well.

CONCLUSION

The presented data demonstrated histologically evident lung immune response following intranasal *A. fumigatus* conidia inoculation. This response is a possible mechanism of decreased numbers of germinating conidia at lower applied conidia dose.

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КАРАКТЕРИСТИКЕ ЛОКАЛНОГ ОДГОВОРА У ПЛУЋИМА ПАЦОВА НАКОН ИНТРАНАЗАЛНЕ АПЛИКАЦИЈЕ КОНИДИЈА *ASPERGILLUS FUMIGATUS*

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Резиме

Aspergillus fumigatus је опортунистичка гљива која најчешће доводи до пулмонарних аспергилоза код имунокомпромитованих организама. У овом раду су приказани резултати истраживања изазивања инфекције аспергилусом код пацова. У циљу добијања података о присуству и карактеристикама локалног инфламаторног одговора у плућима одређен је диференцијални састав леукоцита периферне крви и тестирана је њихова активност, извршени су хистопатолошки преглед плућа и провера присуства спора *A. fumigatus* у хомогенатима плућа. Наши подаци су показали присуство локалног одговора у плућима после 7 дана, док подаци добијени после 21 дан не показују значајне разлике у односу на контролну групу. Повећани број неклијајућих спора у хомогенату плућа седмог дана након инокулације може да се објасни присуством лимфоцитног инфилтрата у плућима и активношћу лимфоцита *in situ*. Повећан број клијајућих спора 21. дана након инокулације може да буде последица капацитета малог броја преосталих спора да клијају.

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EXPERIMENTALLY INDUCED DERMATOMYCOSES AT RATS AND TREATMENT WITH *LAVANDULA ANGUSTIFOLIA* ESSENTIAL OIL

ABSTRACT: The *in vivo* evaluation of antifungal activity of the *Lavandula angustifolia* essential oil was made on two-month old male Wistar rats. We examined the therapeutic potency against experimentally induced dermatomycoses in rats, using the most frequent dermatomycetes, *Trichophyton mentagrophytes*. The therapeutic efficacy of 1% solution of essential oil as well as commercial preparation-bifonazole, was evaluated. During the 13-day observation period the oil-treated animals were cured completely.

KEY WORDS: essential oil, *Lavandula angustifolia*, Dermatomycetes, antufungal activity

INTRODUCTION

During the past several years numerous antifungal agents have been formulated and evaluated for use in the management of fungal infections (R y a n, 1994). The emerging resistance of microorganisms to some synthetic antibiotics makes it necessary to continue the search for new antimicrobial substances. With the increasing acceptance of traditional medicine as an alternative form of health care, the searching for active compounds in medicinal plants became very important. Human infection diseases have been markedly increasing during the past ten years, especially in immunocompromised patients. Consequently, as high as 10% percent of hospital acquired systemic infections are caused by fungi.

The increasing resistance of human pathogens to current commercial drugs is a serious medical problem, and has resulted in the need for novel antimicrobial agents.

Natural products derived from plants have traditionally been used in ethnomedicine. In Western medicine, substances derived from higher plants constitute ca. 25% of prescribed medicines and 74% of the 121 bioactive plant-derived compounds currently in worldwide use, which were identified via research based on leads from ethnomedicine (Sokmen et al., 1999). Recent researches showed that higher plants may serve as promising sources of novel antimycotics with no side effects on human and animals (Clark and Hufford, 1993). Essential oils play a great role in these investigations. Studies over the last hundred years have demonstrated the antimicrobial properties of several common spice oils (Bullerman et al., 1977). Maruzzela and Balter (1959) found that 100 essential oils out of 119 spice oils, tested, possessed an antagonist effect an at least one of 12 pathogenic fungi, and 50 of these samples showed a wide spectrum of activities against all fungi tested.

The purpose of this study was to investigate *Lavandula angustifolia* essential oil for potential antifungal activity. The selection of the plant for evaluation was based on traditional use of this plant in treatment of various infection diseases (Jančić et al., 1995; Kovačević, 2000).

MATERIAL AND METHODS

Plant material

Lavandula angustifolia was collected during May in 1999 at the fields of the Institute for Medicinal Plant Research in Pančevo, Belgrade, Serbia. Voucher specimens (No. 04071970 and 25072) were deposited in the Herbarium of Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade.

Isolation of essential oils

Composition of essential oils was investigated using analytical GC/FID and GC/MS techniques. For this purpose, HP 5890 series II gas chromatograph, equipped with split-splitless injector, fused silica capillary column (25 m x 0.32 mm), coated with cross-linked methyl silicone gum (0.5 μm film tickness), and FID was employed. Essential oil solutions in ethanol (1%) were injected in split mode (1:30). Injector was heated at 250°C, FID at 300°C, while column temperature was linearly programmed from 40—280°C (4°C/min).

GC/MS analysis was carried out on a HP-GCD, equipped with split-splitless injector, fused silica capillary column (50 m x 0.2 mm) PONA, coated with cross-linked methyl silicone gum (0.5 μm film tickness) and mass selective detector. The chromatographic conditions were as above. Transfer line (MSD) was heated at 280°C. EMS spectra (70 eV) were acquired in scan mode in m/e range 40—300.

The identification of individual constituents was carried out by comparison of their retention times with those of analytical standards, and by computer searching, matching the mass spectral data with those held in Wiley/NBS library of mass spectra. For quantification purposes area percent reports obtained by FID were used.

Bioassays

Toxicology: in order to determine the non toxic concentration of the essential oil investigated, we used male Wistar rats (17—24 g). 0.5 ml of prepared stock solution of essential oil, and a component diluted in ethanol (0.01—1% v/v) were injected intraperitoneal in male Wistar rats (*Pharmacopea Jugoslavica*, 1984). The concentration which was not toxic for the animals investigated, was used for further investigation.

Animals. Two-month-old male Wistar rats were maintained at 21°C, and were allowed access to feed and water *ad libitum*.

In vivo Fungitoxicity Assay. The *in vivo* investigation of antifungal activity of *Lavandula angustifolia* essential oil was made according to Adam et al., (1998). We used *Trychophyton mentagrophytes* as an infectious agent. The organism was isolated from patients at the Center for Preventive Medicine, MMA, Belgrade, Serbia.

The micromycetes were maintained on Sabouraud Dextrose Agar (SDA), containing 40 g of glucose, 10 g of agar and 10 g of peptone in 1 l of distillate H_2O. The cultures were stored at +4°C, and subcultured once a month (Booth, 1971).

On the back of each animal, the areas of 4 cm^2 were cleaned and depilated. The infectious inoculum was prepared from a 7-day-old culture of *Trychophyton mentagrophytes*. The inoculum was applied on the animals' back immediately after the depilation and left for 3 days. The establishment of active infection was confirmed on the 4^{th} day, by isolation of the pathogens from skin scales cultured from infected loci on SDA plates, containing 100 units/ml of penicillin and streptomycin. Infections were, also, confirmed by visual examination of animals. In the animals in which active infections were confirmed, treatment was initiated on the 5^{th} day post inoculation and continued until complete recovery from the infection was achieved. The ointments contained 1% (v/v) of essential oil and component mixed in petroleum jelly. The commercial fungicide, bifonazole, was used as a control. Animals were treated once a day, and the infected areas were scored visually for inflammation and scaling as, well as for the presence of the pathogens by cultivating skin scales from infected loci in SDA plates containing 100 units/ml of penicillin and streptomycin, each day.

RESULTS AND DISCUSSION

The qualitative and quantitative composition of the essential oils of *Lavandula angustifolia* was presented in Table 1. The essential oil is characteri-

zed with high content of linalool (27.21%) and linalool acetate (27.54%), while limonene is presented with 8.5%.

Tab. 1 — Chemical composition of *Lavandula angustifolia* essential oil

Components	%	RI
tricyclene	0.04	301
α-thujene	0.58	307
α-pinene	0.19	319
p-cymene	0.25	471
limonene	8.50	481
1,8-cineole	3.34	485
cis-linalool oxide	2.44	574
camphenylon	—	594
fenchon	0.59	605
linalool	27.21	632
endo-fenchol	0.09	664
camphor	1.07	734
borneol	2.51	789
terpine-4-ol	2.09	820
p-cimene-8-ol	—	837
α-terpineol	4.30	852
myrtenal	—	864
fenchyl acetate	—	930
carvon	—	984
linalool acetate	27.54	1023
bornyl acetate	0.06	1099
lavandulyl acetate	6.54	1111
trans-pinokarvyl acetate	0.16	1135
neryl acetate	2.02	1303
geranyl acetate	2.95	1352
β-selinene	—	1608
δ-cadinene	—	1700
viridiphlorol	—	1859
Total	97.47	

* In elution order on DB-5 column (6)

The essential oil was tested for its potential toxicological activity in 0.1% and 1% (v/v) solutions in ethanol and petroleum jelly, separately. There is no toxicological activity for 0.1% solutions on the rats. However, in this work the animals were treated topically, and according to the literature (A d a m et al., 1998), for further investigation 1% solutions were used.

The therapeutic efficacy of the ointments was evaluated daily by macroscopic examination of lesions, and by screening for the presence of the infections by culturing skin scales from the infected area. The lesions were treated as cured only when the infected area was free of macroscopic lesions, and when the cultures were negative.

First symptoms (small vesicles) at the rats inoculated with *T. mentagrophytes* were observed on the 5th day of the experiment, while, later (8th day), these were exhibiting in bloody wounds, 20 mm in diameter. We started

with the treatment on the 5th day of the experiment. On the 13th day of the treatment with solution of *L. angustifolia* essential oil, the rats were completely cured, there were no visually observed symptoms and the cultures were negative. Animals treated with the commercial drug, bifonazole, were cured after 15 days of treatment.

During the 13-day observation period the treated animals were cured completely. It should be noted that in many cases macroscopic lesions disappeared long before the elimination of the infectious agent, indicating that long treatment periods of application and evaluation are necessary (Adam et al., 1998). It is normal because dermatomycetes infections typically resolve on their own over a variable time period (18 months to 4 years) depending on the immune response. Many of dermatomycetes rarely cause strong inflammatory reactions, making it very difficult for the immune system to recognize and eliminate the fungus.

The animals treated with the commercial drug, bifonazole, were cured after 15 days of treatment.

From the above results it can be concluded that essential oil of *Lavandula angustifolia* has a good therapeutic and antifungal effect *in vivo*, and could represent possible alternative for the treatment of patients infected by dermatomycetes. Even more, because of the side effects of commercial fungicides and possible resistance of pathogens to the synthetic mycotics, the preparation with natural products has an advantage in the treatment of disseases caused by fungi.

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ЕКСПЕРИМЕНТАЛНО ИНДУКОВАНА ДЕРМАТОМИКОЗА КОД ПАЦОВА И ТРЕТМАН ЕТАРСКИМ УЉЕМ *LAVADULA ANGUSTIFOLIA*

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Резиме

Инфекције људи узроковане гљивама су у знатном порасту у последњих 10 година, посебно код имунокомпромитованих пацијената. Чак 10% инфекција код хоспитализованих пацијената изазвано је гљивама. С обзиром да је појава резистенције на комерцијалне фунгициде учестала, јавља се потреба за новим алтернативним антифунгалним агенсима. Природни продукти добијају све већи значај у третману инфективних обољења због свог нетоксичног карактера, високе биодеградибилности и ефикасности. Етарска уља изолована из биљака показују веома добру антимикробну активност.

Експериментална дерматомикоза код Wistar пацова у овом раду изазвана је дерматомицетом *Trichophyton mentagrophytes*. Први симптоми појавили су се након 5 дана од инокулације. Тремтан етарским уљем врсте *Lavandula angustifolia*, 1% раствором почео је одмах након појаве првих симптома. Побољшање симптома примећено је након два дана третмана, а после 13 дана од почетка третмана животиње су у потпуности излечене, на SDA подлогама није било забележено присуство патогена. Бифоназол је довео до излечења након 15 дана третмана. Етарско уље *Lavandula angustifolia* показало је изузетно јак антифунгални потенцијал у третману експериментално индуковане дерматомикозе код пацова, боље од комерцијалног микотика, бифоназола, који је коришћен као позитивна контрола.

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EXPERIMENTALLY INDUCED INVASIVE ASPERGILLOSIS IN MICE

ABSTRACT: In this study systemic response to intravenous administration of *Aspergillus fumigatus* conidia was investigated. The intensity of response was evaluated by a survival rate and by histopathological tissue analysis. Administration of all doses (10^6 — $5x10^7$) of *Aspergillus fumigatus* conidia caused mortality, but the highest mortality and the shorter time of survival were noted at higher doses applied. At the highest applied dose, the presence of spores and hyphae was noted in lungs and kidneys. Histological analysis revealed the presence of intense inflammatory reaction in lungs, kidneys and spleen. Functional and histological changes observed provide means to study both mechanisms and drug interventions in systemic *Aspergillus* infection.

KEY WORDS: *Aspergillus fumigatus*, mice, systemic aspergillosis, urinary obstruction

INTRODUCTION

Species of genus *Aspergillus* are representatives of saprophytic filamentous fungi found in most environments. The most common species of *Aspergillus* causing invasive diseases include *A. fumigatus*, *A. flavus*, *A. niger*, *A. clavatus*, *A. glaucans*, *A. nidulans*, *A. terreus* and *A. versicolor*. *Aspergillus* sp. is weak pathogen, but might cause a disease in an immunocompromised host. Aspergillosis comprises a variety of infection, manifestation including invasive aspergillosis, pulmonary aspergilloma and allergic bronchopulmonary aspergillosis (D e n n i n g, 1998). Diseases that cause *A. fumigatus* are very difficult to diagnose, because the most diagnostic features are not specific, and patients are usually asymptomatic.

Animal experimental models offer an approach for *Aspergillus* infection studies. By using animal individuals, with defenses impaired by glucocorticoids, cyclophosphamide etc. (immunosupressed animals), valuable informa-

tion concerning pathogenesis of *Aspergillus* infections was obtained. Studies on immunocompetent hosts, on the other hand enable investigations of the mechanisms of resistance to *Aspergillus*.

In this study we investigated a response to systemically applied *A. fumigatus* conidia. With this aim, survival and histopathology of distinct organs were analyzed, following the intravenous injection of conidia.

MATERIALS AND METHODS

Conventionally housed female C57BL/6 mice, eight to twelve weeks old, are used in the experiments. Animals were housed under constant conditions (temperature 19—21°C, daily-night rhythm 12 h), with food and water *ad libitum*. The experiments were conducted with adherence to Ethical Committee of Institute for Biological Research "Siniša Stanković".

A. fumigatus, human isolate, from the Institute of public health of Serbia "Dr Milan Jovanović Batut" was subcultured on standard mycological slants (B o o t h, 1971). Inoculum was prepared by flooding the surface of agar slants with sterile 0,85% NaCl with 0,1% Tween 80. The suspension of spores was prepared in apyrogenic sterile physiological saline and doses of $1x10^6$, $1x10^7$ and $5x10^7$ conidia were applied intravenously into each mouse. The control mice received saline solely.

Animals were inspected two times a day. All mice were observed for a total 14 days after the infection. Mice that survived until the day 14, were euthanized.

Presence of fungi in organism of mice was established by histological analyses. Tissue specimens were fixed in 4% formalin (pH 6, 9). Fixed material was dehydrated in graded ethanol series. Material is then embedded in paraffin at 57°C. Sections 5 μm thick, were stained with hematoxylin-eosin (H & E).

Specific gravity, protein and haemoglobin content in urine were determinated by test strips Combur^{10} Test®M (Roche Diagnostics GmbH, Germany) as parameters of renal function.

Results were statistically processed by Mann-Whitney U test. As significant was considered $p < 0,05$.

RESULTS AND DISCUSSION

All doses of applied conidia induced mortality in experimental animals. Mortality rate was proportional to the injected inoculum. Inoculation of $1x10^6$ and $1x10^7$ conidia per mouse caused mortality in 40 and 60% of mice respectively. Dose of $5x10^7$ conidia caused mortality in all treated animals, by day five following inoculation (Figure 1). Higher doses also reduced time of survival (the higher dose, the shorter time of survival). Mortality/survival data are in accordance with the study showing high mortality at the dose of 10^7 of infected immunocompetent mice (C e n c i et al., 1997).

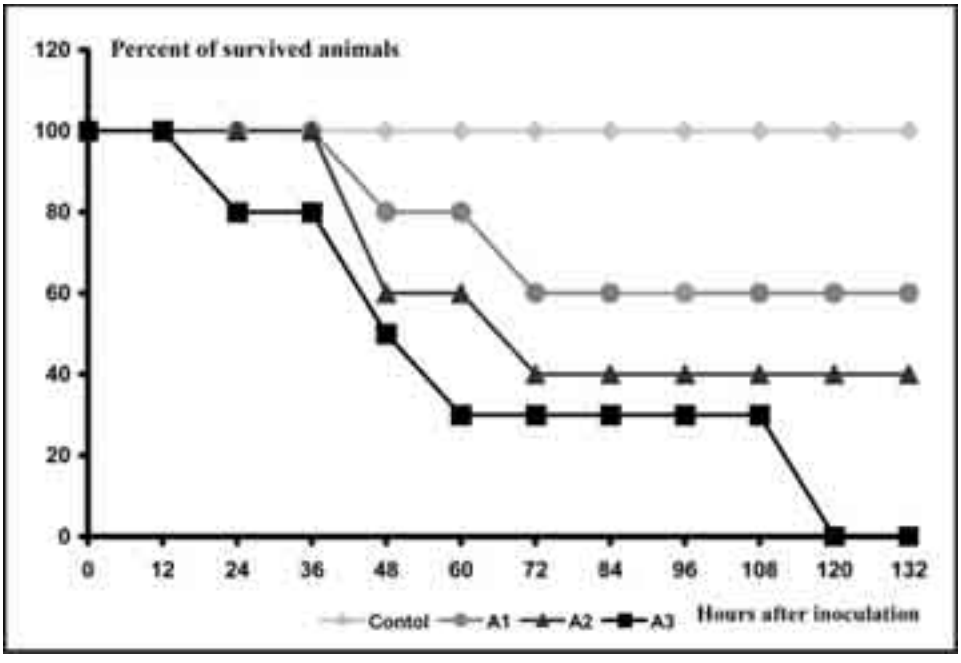

Fig. 1 — Survival curve (A1 — mice inoculated with $1x10^6$ conidia of *A. fumigatus*; A2 — mice inoculated with $1x10^7$ conidia; A3 — mice inoculated with $5x10^7$ conidia)

Presence of infection was assessed by observation of animal prostration. Pronounced hypodynamic state and piloerection were noted in infected mice, in accordance with the data from the studies of invasive aspergillosis in mice (Duong et al., 1998). Mice in hypodynamic state died shortly after these signs appeared.

Histological data revealed the presence of inflammatory pulmonary response (presence of lymphocytes in peribronchial and/or perivascular sites) in all experimental groups. Microabsceses were noted in lungs, liver, kidneys and spleen of animals. In animals which received the highest dose the presence of conidia and hyphae in lungs and kidneys was noted.

Renal aspergillosis, at highest dose was accompanied with compromised renal function as judged by the changes in selected urinary parameters (Figure 2). In all control individuals, specific gravity values were 1,015 (test strips values ranged from 1,000 to 1,030). In 62% of treated mice, this value was 1,030, and in the rest value of 1,025 was detected. Difference between the control and treated groups is statisticaly significant ($p = 0{,}0066$). Significantly increased haemoglobinuria ($p = 0{,}023$), and a tendency of increase in urine protein content ($p = 0{,}089$), were noted in treated individuals. Renal disfunction observed in these animals, is in agreement with the studies which showed that kidneys are the primary target organs for intravenous *A. fumigatus* infection (Latge, 1999) in animals and humans, and with reports which de-

monstrated urinary obstruction as a consequence of renal infection detected in humans (De Medeiros, 1999; Bisi, 2003).

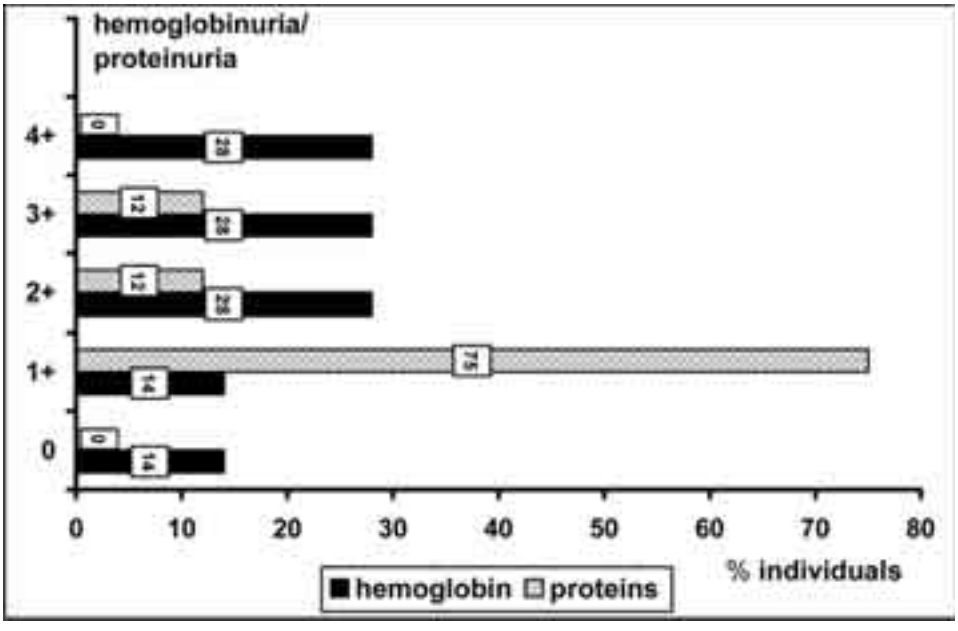

Fig. 2 — Hamoglobin/protein values in infected individuals

CONCLUSION

In conclusion, presented data demonstrated both functional and histological changes in organs of mice following systemic application of *A. fumigatus*. This model might provide means to study mechanisms of invasive aspergillosis, as well as drug testing in prelinical trials.

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ЕКСПЕРИМЕНТАЛНО ИНДУКОВАНА АСПЕРГИЛОЗА КОД МИШЕВА

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Резиме

Aspergillus fumigatus је опортунистичка гљива која се може наћи у свим срединама. Опортунистичке гљиве проузрокују појаву болести код имунокомпромитованих особа, односно код особа са ослабљеним имунским системом. Најчешће болести које изазива *A. fumigatus* су: плућна аспергилоза, аспергиломи и алергијска бронхопулмонарна аспергилоза. Ове болести су тешке за дијагнозу јер су симптоми неспецифични.

Интравенска апликација конидија *Aspergillus fumigatus* изазива промене у преживљавању и понашању животиња. Са повећањем концентрације конидија смањује се време преживљавања. Највећа апликована доза ($5x10^7$ конидија) доводи до угинућа свих животиња до петог дана након инокулације. Инфициране животиње карактеришу промене у понашању (акинезија, атонија) и пилоерекција.

Хистолошком анализом је показано присуство интензивне запаљенске реакције у плућима, бубрезима и слезини третираних јединки, као и присуство спора и хифа у бубрезима и плућима јединки које су примиле највећу дозу *A. Fumigatus*. Код ових јединки запажен је и поремећај бубрежне функције на основу промена у специфичној тежини урина, pH вредности, присуству протеинурије и хемоглобинурије.

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PRESENCE AND IMPORTANCE OF SAPROPHYTE FUNGAL ORGANISMS ON DOG SKIN

ABSTRACT: Dogs are animals that are most often kept as pets in the cities. Their health problem may be the cause of infections of humans and animals. Skin changes and etiology factors present important segment of the diseases that disturb health of the pets.

The objective of this work was mycology examination of scarifications and skin swabs from dogs with clinical symptoms. The aim was to find out which fungi species can be isolated from the changed parts of the skin, and whether is possible that, besides dermatophyte, saprophyte fungi from the environment may also be the cause of the changes, and to reveal their effect on the host.

During a one year period, 67 swabs and scarifications from dogs were examined to detect the presence of fungi. The samples were streaked on Sabourdaud's dextrose agar and incubated for 10—21 days at 25°C. In microscopis examination according to their shape, and color, the colonies were identified as conidia, macroconidia and conidiaophora.

From 59, of total 67 samples, the following saprophyte fungi were isolated: *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Mucor* sp. and *Fusarium* sp. Occurrence of these fungi means that a considerable increase of this microbiological flora may be expected in homes of the owners. This may be the cause of systemic mycosis and allergies in animals and humans, as well as a possibility of contaminated food and incidence of mycotoxicosis.

KEY WORDS: fungi, dogs, mycosis, pets

INTRODUCTION

Changes on dog skin are frequent disorders. It is not easy to determine etiology and therapy. Skin is a large organ. Depending on animal species and age, it presents 12—24% of body mass (Aiello, Susan, 1998, Popović, N., Lazarević, M. 1999). Having in mind its size and a manifold role, it serves as a barrier and protection from the external factors and infective agents. It helps thermoregulation, produces pigments and vitamin D, contains receptors of all the senses (Popović, N., Lazarević, M. 1999). Every change influences its features and normal functioning. Changes on skin may

occur as a consequence of different biological agents, bacteria, fungi, parasites and viruses, but may also be a consequence of allergy, immunology disorders, endocrine disturbances, inborn diseases, environmental factors and nutritive deficits (P o p o v i ć, N., L a z a r e v i ć, M. 1999).

Scope of our work was mycology examinations of scarifications and skin swabs taken from dogs with clinical symptoms. The aim was to determine what kind of fungi are present on the changed parts of the skin and see whether, besides dermatophyte, saprophyte fungi from the environment may also be the cause of changes important for the host.

MATERIAL AND METHODS

The samples originated from the dogs with different on skin changes. Clinical findings proved changes that ranged from dermatitis with amplified pigmentation and depigmentation, and to a red color change, petechia and echimosis. Itching, as a clinical finding, was not dominant, but loss of hair on fur was obvious, and on some parts it was total. There were 67 swabs and dog scarifications used also for other analyses (C a b a n e s, F. J. et al., 1996). The samples were streaked on Saboraud dextrose agar and incubated at 25°C for 10—21 days. The colonies were identified on the basis of their shape and color; in microscopic analyses they were determined as conidia, macroconidia and conidiaphora (Q u i n n, J. P. et al., 2002).

RESULTS AND DISCUSSION

Presence of dermatophyte was revealed in 11.9% out of 67 examined samples. The identification proved that there was only one dermatophyte that belonged to Mycrosporum genius. Saprophyte fungi that do not belong to dermatophyte, were present in 95.6%. All the data on fungi in the examined samples are displayed in Table 1.

Tab. 1 — Findings of fungi in the examined sample of swabs and scarifications of dog skin

Isolate	No. of examined samples	No. of positive samples
Mycrosporum sp.	67	8 (11,94%)
Aspergilus sp.	67	42 (62,69%)
Penicillium sp.	67	40 (59,70%)
Alternaria sp.	67	45 (67,16%)
Mucor sp.	67	38 (56,71%)
Fusarium sp.	67	21 (31,34%)

Percentage of non-dermatophyte fungi was very high in our examination. They were also isolated in the samples that were dermatophyte positive. The analyses point out that only 4.4% of pure dermatophyte culture was isolated, but in all other findings the presence of non-dermatophyte fungi was also determined. Their presence was in some cases dominant, so it was not possible

to read the results and determine other fungi, among which there could have also been dermatophyte. According to the data in the paper (J a n d, S. K., G u p t a, M. P. 1989) besides dermatophyte, in 10.8% of the cases, non-dermatophyte fungi were present, and they could be the cause of changes. The isolated species of non-dermatophyte fungi were similar to our findings (*Alternaria* sp., *Penicillium* sp., *Aspergillus* sp., *Mucor* sp., *Cladosporium* sp., *Fusarium* sp.). Our studies show that the incidence of saprophyte fungi on dog skin is not frequent. However, similar reports in the paper (C a b a n e s, F. J. et al., 1996) point out that the presence of non-dermatophyte in the samples from dogs is great and that, depending on the kind of the dog, *Penicillium* sp. is present in almost 90%. Findings of *Fusarium solani* on skin and submucous nodular changes, reported in the paper (K a n o, R. et al., 2002), and in lesions, warn of systemic fungi infection and its consequences. Saprophytic fungal organisms are widely spread in nature, and may be the cause of opportunistic infections of humans and animals. We can find reports (E v a n s. J. et al. 2004) on incidence of canine meningoencephalitis, caused by *Fusarium solani*. These systemic infections, caused by different saprophytic fungal organisms (*Fusarium* sp., *Aspergillus* sp.), were noticed even in other animals. The findings point out on possible consequences of infection with saprophyte fungi.

Undesirable influence of fungi on humans was noticed while examining the factors (E n r i q u e z P a l o m e c, O. et al., 1997) that provoke allergy. It was revealed that *Candida* sp. and *Fusarium* sp. present predominant etiologic agents of allergy in the dry season.

Saprophytic fungal organisms, that are widely spread in nature, are in daily contact with humans and animals. Pets carry them on their skin and increase the possibility of contaminating the homes they live in the influencing the humans too. Mycotoxins that present metabolic products of these fungi, may influence the organisms in different ways and cause damages of certain tissues and organs. This depends on toxins, quantity and time of exposure to the toxins. The consequences are damages on liver and kidney, damage of immune system and haemapoiesis organs, and carcinogen changes on organs (K a r a k a š e v i ć, B., 1989). The researchs of reproductive disorders in female dogs in Poland (G o l i n s k i, P. K., N o w a k, T., 2004) show that mycotoxins have a potential estrogen effect. It was revealed that dog food contained unsteroid mycotoxins, Zearalenone, whose activity was similar to that of an estrogen, and caused reproductive disorders in the examined female dogs, which was manifested in complex pyogenic endometritis (EPC).

CONCLUSION

Saprophytic fungal organisms are present on the changed parts of dog skin in a very high percentage (95.6%). The findings point out that larger number of different fungi are present, and that this may have health implications on both animals, and the owners.

Saprophytic fungi from the soil and the environment may be found on skin. Due to their specific features, they have negative influence on animal and

human health and cause system infection. Their products, mycotoxins, in a long run, exhibit negative effects on the health of all the beings exposed.

Pets in urban environment increase the exposure of humans and animals to saprophytic fungi organisms and cause health problems. All these facts should influence the decisions regarding pets and reducing the potential of risks.

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ПРИСУСТВО И ЗНАЧАЈ САПРОФИТНИХ ГЉИВИЦА НА КОЖИ ПАСА

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Резиме

Пси спадају међу најчешће гајене животиње у граду и здравствени проблеми везани за њих могу утицати на здравље људи. Промене на кожи паса и етиолошки фактори који их изазивају су важан сегмент болести које ремете здравље ове врсте кућних љубимаца.

Предмет нашег рада су миколошка испитивања скарификата и брисева коже паса који су имали клиничке симптоме болести. Циљ нам је да утврдимо које се врсте гљивица налазе на промењеним деловима коже пса и да ли се, поред дерматофита, и сапрофитне гљивице из околине могу сматрати узрочником промена и какав је њихов значај за домаћина. У току годину дана анализирано је 67 узорака брисева и скарификата коже паса на присуство гљивица. За миколошка испитивања узети узорци су засејавани на Сабуро декстрозни агар и инкубирани на 25°C, 10—21 дана. Израсле колоније су идентификоване на основу њиховог изгледа и боје, а микроскопски у односу на грађу њихових конидија, макроконидија и конидијофора.

Од укупно 67 анализа код 59 је утврђено присуство сапрофитних гљивица и то: *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Mucor* sp. и *Fusarium sp*. Присуство ових врста гљивица на кожи паса значи да се у домовима власника може очекивати значајно повећање ове врсте микробиолошке флоре које могу бити узрочници системских микоза и алергијских реакција људи и животиња, као и могући контаминенти хране и узрочници микотоксикоза.

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TOXIGENIC AND PATHOGENIC FUNGI IN NORWAY RAT (*RATTUS NORVEGICUS* BERK., 1769) FROM NATURAL POPULATIONS IN SEMIAGRICULTURAL HABITATS

ABSTRACT: In this study, the presence of toxigenic and pathogenic fungi was detected in individuals of natural populations of Norway rats from semiagricultural habitats. The presence of fungi was noted in 19 out of 30 (63%) individuals examined. Six fungal species were isolated and identified, of which majority belonged to *Hyphomycetes* (*Deuteromycotina*) and *Mucor racemosus* from *Zygomycotina*. All of detected species are of public health importance and some of them might influence animals health.

KEY WORDS: fungi, lung homogenates, Norway rats, SMA medium

INTRODUCTION

Norway rat is a common pest rodent species with wide distribution (Hrgović, Vukićević and Kataranovski, 1991). Rats are synantropic animals, that live near humans who provide them shelter and food. Owing to their life style (omnivorous, active whole day, feeding everywhere) wild rat populations are constantly exposed to various microbe species including viruses, helminths, bacteria and fungi. For many of these microbial agents rodents are reservoirs and vectors, serving thus as an important indicator of the epizootic and epidemic situation of their living environment (Wincewicz, 2002). While there is considerable knowledge of viral, bacterial and helminth burden in natural populations of rodents, there are virtually no data regarding presence of fungal components in animal organisms. Spores of fungi float in the air, and may be found in different environmental substrates. Fungal spores

may colonize rodent tissues in contact, establishing biological sources of these agents. In this way, rodents might act as reservoirs of these agents contributing to their spreading. Great number of fungal species cause diseases known as mycoses and mycotoxicosis. Presence of fungi and their metabolic products, mycotoxins, in foods and grains contaminated by rat excrements, presents a potential hazard to human and animal health (B u s b y and W o g a n, 1986).

Having in mind the potential of rodents to serve as fungal reservoirs and subjects of fungal components spreading, a research has been undertaken in order to examine the presence of fungi in wild rat populations.

MATERIALS AND METHODS

Wild rats (*Rattus norvegicus*) were captured by living traps in semiagricultural habitats (Omoljica, Kovin, Leštane and Ovča). Swabs from external area of nose were taken and lung tissue homogenates prepared under sterile conditions. Swabs and specimens of lung tissue homogenates were inoculated on Sabouraud Maltose Agar (SMA) medium with streptomycin to avoid bacterial infection (T a r r a n d et al., 2005). After incubation, micromycetes were identified on the basis of micro- and macrocharacteristics of fungal isolates.

RESULTS AND DISCUSSION

Of 30 individuals examined, in 19 individuals (63%) fungi were detected in lung homogenates. Six fungal species were isolated and identified, of which five belonged to classes Hyphomycetes (Deuteromycotina) and *Mucor racemosus* from Zygomycotina. *Aspergillus* and *Penicillium* species were detected in homogenates of lungs of five and six individuals, respectively. (Figure 1). *Paecilomyces varioti* was isolated from six individuals. Swabs from nasal skin of 15 (50%) out of 30 individuals were found positive for fungi (4 for *Aspergillus*, 4 for *Penicillium*, 6 for *Paecilomyces varioti* and one for *M. racemosus*) However, in only 9 cases presence of fungi on nasal skin and in lungs coincided. Some of the fungal isolates were not identified at the species level, because of the atypical fungal elements. This particularly refers to *Penicillium* species (atypical characteristics of spores with changed shape and size could be observed at elongated phialides with branched conidiophores).

A. fumigatus is the most frequent pathogenic species that causes pulmonary aspergillosis with high mortality level. *A. flavus* is a known toxigenic species, which produces Aflatoxin B1, the most carcinogenic human mycotoxin causing hepatocellular carcinoma. *A. repens* is osmophilic species well adapted to dry substrates and commonly causes farmer's lung disease and other pulmonary infections. *Paecilomyces varioti* may cause paecilomycosis, which can be manifested as endocarditis, sinusitis and pulmonary infections at rodents and humans (B y r d et al., 1992), *M. racemosus* (known as an allergenic agent causing pulmonary infections) was isolated from one sample of rat's lung's homogenate.

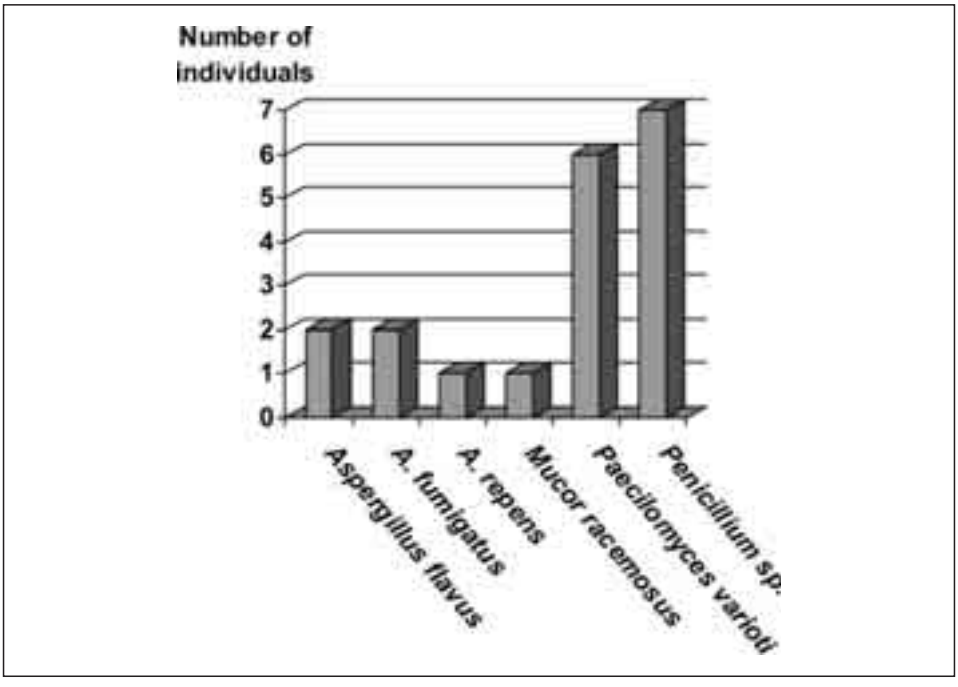

Fig. 1 — Mycological examination of lung homogenate

Of special interest is *A. fumigatus*, the most common invasive mold which is the cause of opportunistic infections in individuals with weakened immune system (patients on chemotherapy, AIDS patients, in individuals on prolonged antibiotic treatment, children and elderly people). Beside risk for human health, presence of these fungi might influence animal health as well, influencing, thus, the characteristics of rodent's natural population.

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ТОКСИГЕНЕ И ПАТОГЕНЕ ГЉИВЕ КОД СИВОГ ПАЦОВА (*RATTUS NORVEGICUS* BERK., 1769) ИЗ ПРИРОДНИХ ПОПУЛАЦИЈА СА ПОЛУПОЉОПРИВРЕДНИХ СТАНИШТА

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Резиме

У овом раду су приказана истраживања присуства токсигених и патогених гљива у јединкама сивог пацова (*Rattus norvegicus* BERK., 1769) из природних популација са полупољопривредних станишта (Омољица, Ковин, Лештане, Овча). Детекција присуства гљива у биолошком материјалу (брис коже носне шупљине и хомогенат плућа) је вршена на селективној Sabouraud Maltose Agar (SMA) подлози. Истраживање је показало присуство шест врста гљива од којих пет припада класи *Hyphomyceta (Deuteromycotina)*, а *Mucor racemosus* класи *Zygomycotina*. Врсте рода *Aspergillus* су детектоване у хомогенатима плућа шест јединки а *Penicillium* код пет јединки. *Paecilomyces varioti* је изолован из ткива шест јединки сивог пацова. Брисеви коже носне шупљине су дали позитивне резултате у 15 (50%) од 30 јединки (четири позитивна за *Aspergillus*, четири за *Penicillium*, шест за *Paecilomyces varioti* и једна за *Mucor racemosus*). Међутим, у само 9 случајева забележено је поклапање присуства гљива у кожи носне шупљине и плућа.

Налази који показују присуство патогена, изазивача тешких гљивичних обољења, указују на сивог пацова као на потенцијални резервоар ових агенаса. Добијени подаци представљају, према нашем сазнању, прве податке о присуству гљива код пацова из природне средине забележене код нас.

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EFFECT OF SALT HYPEROSMOTIC STRESS ON YEAST CELL VIABILITY

ABSTRACT: During fermentation for ethanol production, yeasts are subjected to different kinds of physico-chemical stresses such as: initially high sugar concentration and low temperature; and later, increased ethanol concentrations. Such conditions trigger a series of biological responses in an effort to maintain cell cycle progress and yeast cell viability. Regarding osmostress, many studies have been focused on transcriptional activation and gene expression in laboratory strains of *Saccharomyces cerevisiae*. The overall aim of this present work was to further our understanding of wine yeast performance during fermentations under osmotic stress conditions. Specifically, the research work focused on the evaluation of NaCl-induced stress responses of an industrial wine yeast strain *S. cerevisiae* (VIN 13), particularly with regard to yeast cell growth and viability. The hypothesis was that osmostress conditions energized specific genes to enable yeast cells to survive under stressful conditions. Experiments were designed by pretreating cells with different sodium chloride concentrations (NaCl: 4%, 6% and 10% w/v) growing in defined media containing D-glucose and evaluating the impact of this on yeast growth and viability. Subsequent fermentation cycles took place with increasing concentrations of D-glucose (20%, 30%, 40% w/v) using salt-adapted cells as inocula. We present evidence that osmostress induced by mild salt pre-treatments resulted in beneficial influences on both cell viability and fermentation performance of an industrial wine yeast strain.

KEY WORDS: *Saccharomyces cerevisiae*, wine yeast, salt stress, cell growth, cell viability

INTRODUCTION

During industrial fermentations that exploit yeast, cells are confronted with a multitude of chemical, physical and biological stresses that may impair cell function and thus fermentation progress. Cells adapt to such stresses by eliciting responses designed to maintain cell growth and survival. Yeast stress

responses can be distinguished by different stages: cellular changes that occur immediately as direct consequences of the physico-chemical forces; activation of the primary defense processes and changes in cell homeostasis. Concerning osmotic stress, a number of physiological changes take place, including: efflux of intracellular H_2O, rapid reduction in total cell volume, including the vacuole (1); transient increases in glycolytic intermediates (2); accumulation of cytosolic glycerol; and triggering of the HOG (Hyper Osmotic Glycerol) signaling pathway (3).

Microorganisms such as the yeast, *Saccharomyces cerevisiae* develop systems to counteract the effect of osmotic stress such as salt stress (NaCl). Specifically, salt-induced stress results in two different phenomena: ion toxicity and osmotic stress. Defense responses to salt stress are based on osmotic adjustments by osmolyte synthesis and cation transport systems for sodium exclusion. In osmostressed *S. cerevisiae*, polyols (glycerol in particular) are the major osmolytes produced accumulated by cells (4, 5, 6). Other products synthesized by yeast during stress 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 (7, 8), but also in response to a number of other stress conditions, and has been shown to protect cells against high temperature by stabilizing proteins and maintaining membrane integrity (9, 10, 11).

Exposing yeast cells in a hyper osmotic environment leads to a rapid initial efflux of water into the medium, which, in other words, is what is meant as cell dehydration. Dehydration is a rapid process mediated solely by water efflux through the lipid bilayer. Intracellular water is recruited from the vacuole into the cytoplasm thus partially compensating for the sudden increase in macromolecular concentration. Additionally, the cytoskeleton collapses leading to depolarization of actin patches. This cell dehydration leads to growth arrest. Under these conditions, cellular reprogramming or "adaptation" represents major defenses including accumulation of compatible solutes to balance the intracellular osmotic pressure with the external environment. The compatible solutes can be: glycerol, trehalose, amino acids, and fatty acids in cell membranes.

A commonly used osmolyte in experiments to cause hyperosmotic stress is sodium chloride. It has been established that the intracellular concentration of glycerol increases in parallel to the external concentration of sodium chloride. In general, an increase in the intracellular concentration of glycerol can be the result of increased glycerol production, 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). (12, 13, 14). Under osmotic stress, increased levels of glycerol take place due to the increase of the activity of GPD. Glycerol formation requires an equimolar amount of cytoplasmatic NADH and when cells are osmotically stressed, there is a decreased reduction of acetaldehyde to ethanol and an 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 hypo-

thesis that mild osmotic stress caused by NaCl would improve wine yeast fermentation performance due to accumulation of cellular protecting molecules. The results obtained with a wine yeast indicate that salt adaptation or "preconditioning" may have potential benefits for industrial fermentation processes.

MATERIALS AND METHODS

Microorganism: *Saccharomyces cerevisiae* (strain Vin 13 from Anchor, S. Africa) and was grown in glucose-based defined medium, without shaking at 25°C.

Medium: 200 gr glucose/lit, 1 gr of K_2HPO_4, 1 gr of $K_2H_2PO_4$, 0.2 gr of $ZnSO_4$, 0.2 gr of $MgSO_4$, 2 gr of yeast extract, 2 gr of NH_4SO_4.

Yeast count: Yeast cell number was determined using haemocytometer (Thoma type). **Glucose measurement**: Glucose was determined using the DNS method (15)

Yeast cell viability: 1 ml of yeast taken from Erlenmeyer flask diluted in 9 ml of deionized water and then 1ml of this solution dissolved with 1ml of 10% methylene blue solution. After 10 min 0.01 ml added to a haemocytometer (Thoma type). Continuously yeast total cell number and yeast living cell number took place by optical microscope (Olympus).

Fermentations: 250 ml volume conical Erlenmeyer flasks without shaking used in a temperature of 25°C.

Repeat fermentations: During the second part of the experiments repeat fermentations have been performed. After 400 hours a new medium containing 200 gr lit of D-glucose inoculated by $5x10^3$ living cells preconditioned in 6% and 10% of NaCl. After the end of the fermentation new medium containing 300 gr lit of D-glucose inoculated by $5x10^3$ living cells. Finally after the end of the fermentation new medium containing 400 gr lit of D-glucose inoculated by $5x10^3$ living cells. This experiment was to observe the tolerance of the preconditioned cells to high sugar concentrations.

RESULTS AND DISCUSSION

The overall aim of this work was to gain further understanding of yeast fermentation performance during osmotic stress conditions. Specifically, work was focused on the evaluation of NaCl-induced osmotic stress responses on industrial strains of the yeast, *Saccharomyces cerevisiae*.

Figure 1 shows that osmotic stress had a minor effect on yeast cell growth while sodium chloride concentrations increased. During the first 16 hours fermentation with glucose, no differences were observed in yeast growth rate. After this period, differences between stressed and unstressed cells became apparent, especially around 90 hours. The lag phases of the cultures treated with 4% and 6% w/v NaCl were protracted by 24 hours. For the 4% and 6% NaCl concentration, the total cell number measurements occurred to have a difference from the beginning of the fermentation compared to that of the

untreated cells. As far as the 10% sodium chloride concentration was concerned, the growth rate had shown minimum changes.

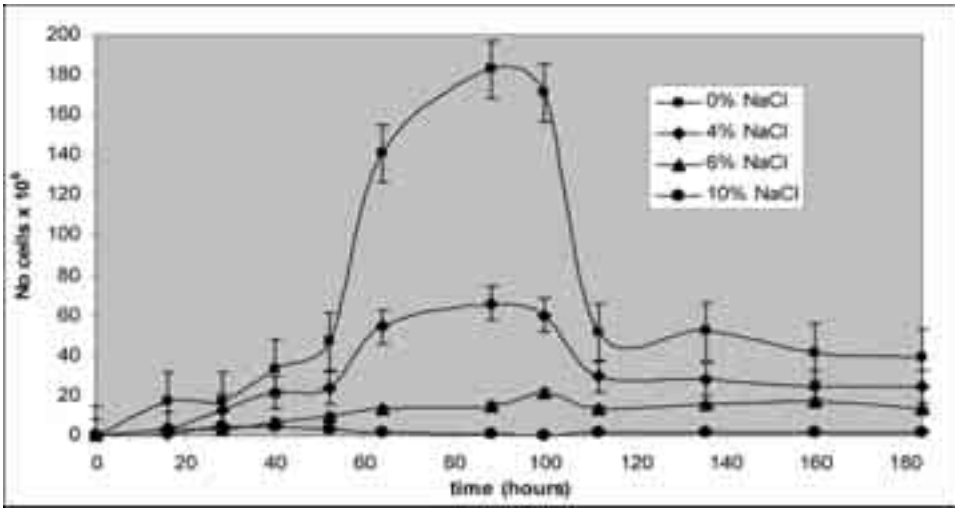

Fig. 1 — Yeast growth under NaCl stress

Growth of *S. cerevisiae* (VIN 13) was monitored in the presence of 0% 4%, 6%, 10% w/v NaCl in glucose-based defined medium without shaking at 25°C

In contrast to the total cell number and yeast growth rate, it was surprising that cells under the higher osmotic stress conditions (especially 10% NaCl) maintained a high viability over time compared with that of unstressed cells (Figure 2). After prolonged fermentation times, such differences became

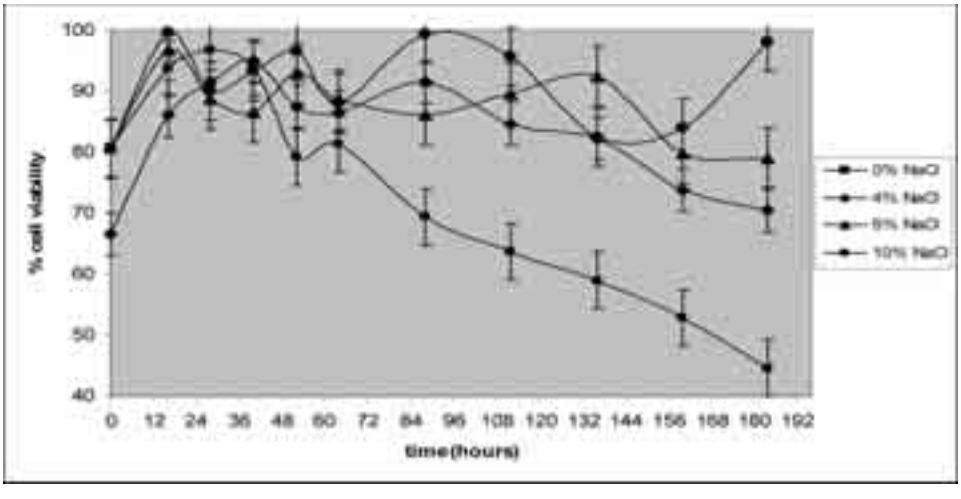

Fig. 2 — Viability under NaCl stress

more pronounced. Under physiological conditions, without sodium chloride, yeast cell viability fell to around 50%. In contrast, viability remained at high levels for salt-stressed cells, even after 180 hours of fermentation. These data clearly show that at 10%w/v NaCl concentration, whilst yeasts retained their viability, their growth rate was adversely affected.

Viability of *S. cerevisiae* (VIN 13) was monitored in the presence of 0% 4%, 6%, 10% w/v NaCl culture in glucose-based defined medium without shaking at 25°C.

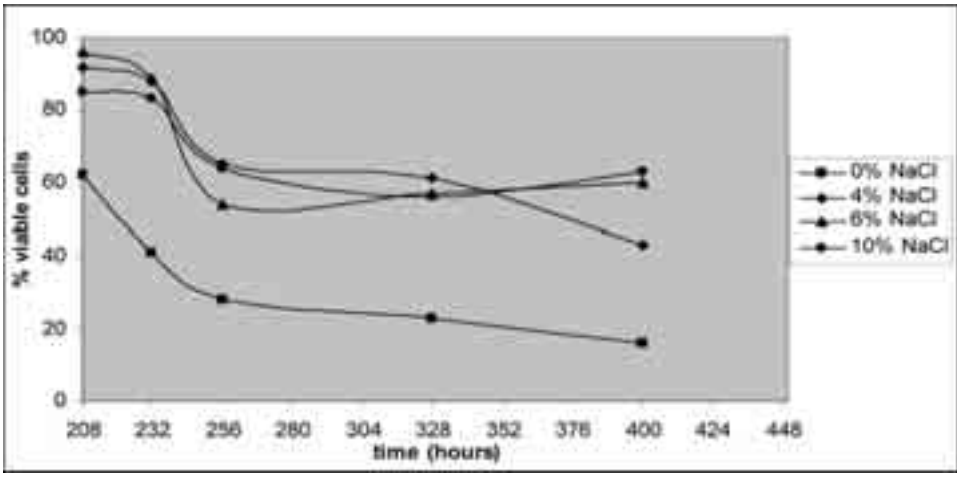

Fig. 3 — Yeast cell viability over an extended period of 192 hours after fermentation for cells with 0% 4%, 6%, 10% w/v NaCl culture in defined medium without shaking at 25°C

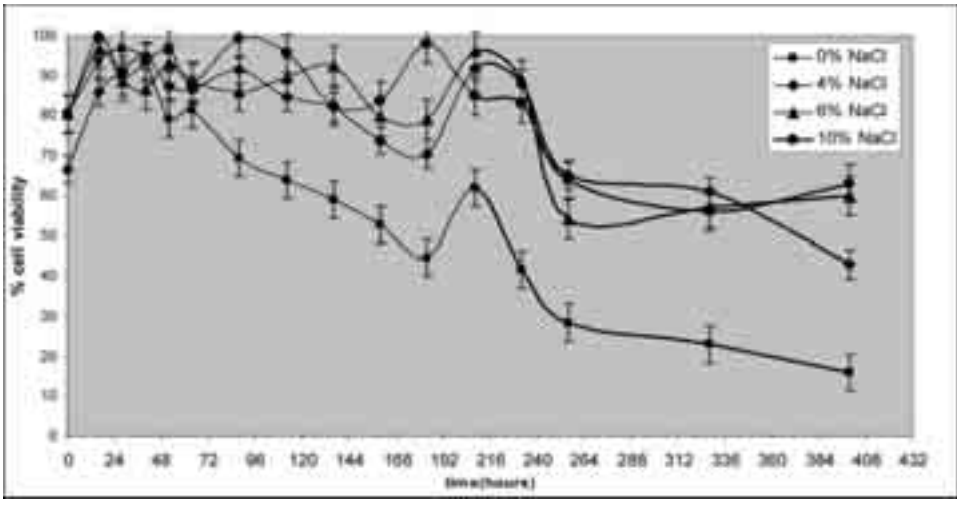

Fig. 4 — Cell viability over an extended time period of 400 hours including fermentation for cells with 0%, 4%, 6%, 10% w/v NaCl stress culture in defined medium without shaking at 25°C

Figures 3 and 4 clearly show that sodium chloride had a positive effect in maintaining yeast cell viability especially for extended fermentation periods of time. After 400 hours, cells treated with 6% and 10% NaCl had the highest viability. It is possible that maintenance of high yeast viability in presence of high salt was due to the synthesis of stress responsive compounds including trehalose and glycerol.

Continuous fermentations at increasing glucose concentrations were performed. Pre-conditioned stressed cells at 6% and 10% w/v/ NaCl used as inoculum for fermentations media containing 20, 30, and 40% of D-glucose in the absence of NaCl stress. These experiments were designed to observe the fermentability, cell growth and viability under non-stress conditions. After 400 hours, media containing 20% w/v D-glucose inoculated by $5x10^3$ living cells preconditioned in 6% and 10% w/v NaCl. Subsequently, media containing 30, then 40% w/v D-glucose were inoculated in a similar manner. Yeast cell growth and viability were monitored. These experiments aimed to observe the capability of salt-preconditioned cells to grow and ferment high sugar concentrations.

Figures 5, 6, 7 and 8 show that for 40% of D-glucose, the fermentation time was 116 hours longer and the total cell number was almost the half comparing to the other two fermentations of 20 and 30% w/v of D-glucose. Growth rate in media with 20% and 30% of D-glucose was similar. The most important observation was that for higher glucose concentrations (30% and 40%) the total cell number was close to $5x10^7$/ml for the fermentation of 40% D-glucose and close to 10 $x10^7$ for the fermentation of 30% D-glucose.

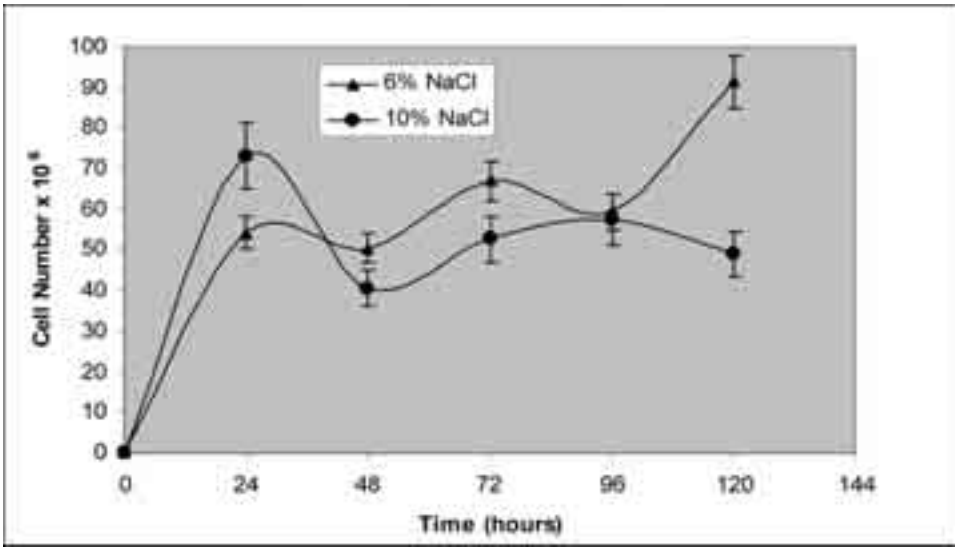

Fig. 5 — Growth for pre-conditioning cells in 6% and 10% of NaCl cultured in define medium containing 20% of D-Glucose

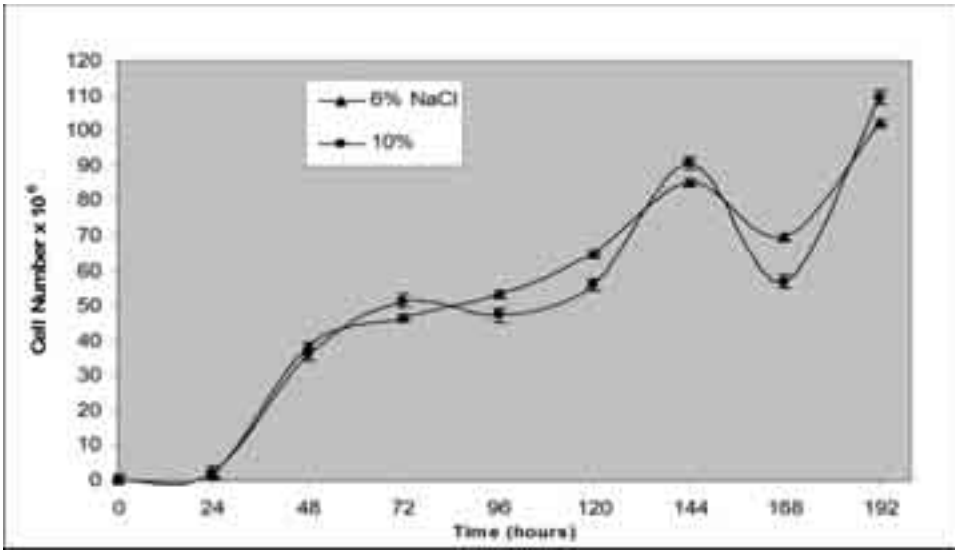

Fig. 6 — Yeast growth for pre-conditioning cells in 6% and 10% of NaCl cultured in define medium containing 30% of D-Glucose

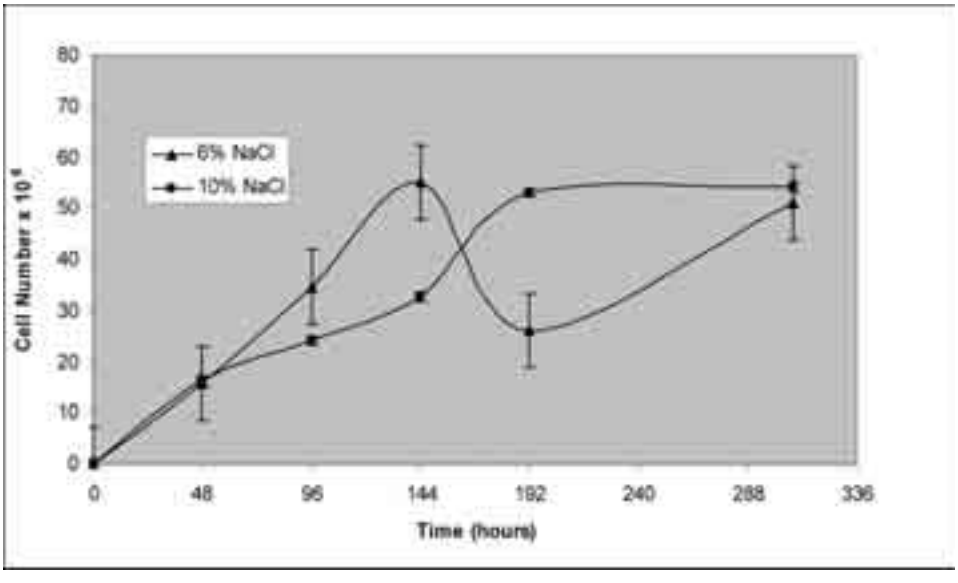

Fig. 7 — Yeast growth for pre-conditioning cells in 6% and 10% of NaCl cultured in define medium containing 40% of D-Glucose

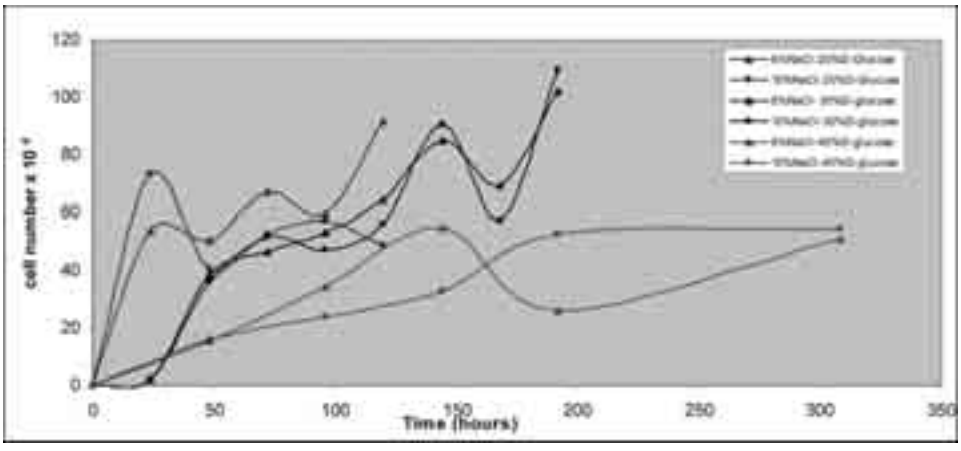

Fig. 8 — Yeast growth for pre-conditioning cells in 6% and 10% of NaCl cultured in define medium containing 20, 30 and 40% of D-Glucose

Figures 9, 10 and 11 show that for 20% D-glucose consumption was almost linear and the amount for residual sugars was the same (26 gr lit). For the fermentation in media with 30% D-glucose the sugar consumption occurred to be significant when the inoculum cells pre-treated with 10% NaCl. At very high glucose levels (40% w/v), a rapid decrease in sugar consumption was observed between 48 and 96 hours from 395 gr lit to 64 gr lit of glucose and then remains constant.

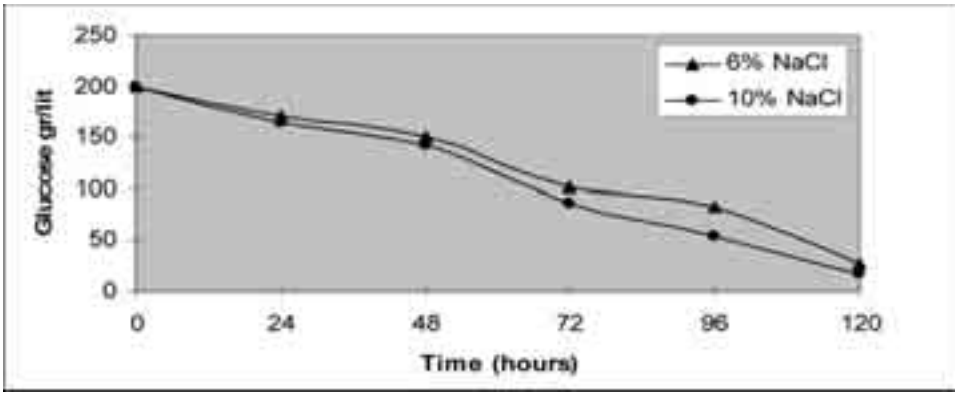

Fig. 9 — Glucose consumption in wine yeast cells pre-conditioned in 6% and 10% w/v NaCl in a medium containing 20% w/v D-glucose

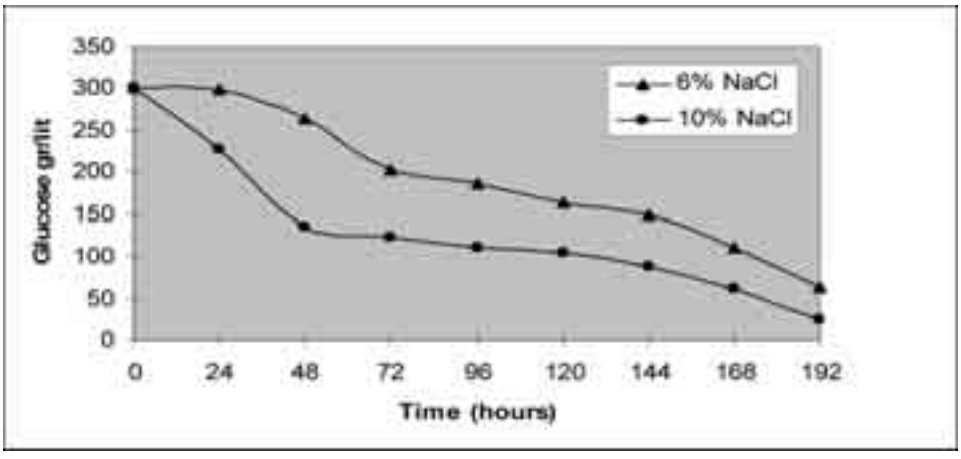

Fig. 10 — Glucose consumption for pre-conditioning cells in 6% and 10% of NaCl cultured in define medium containing 30% of D-Glucose

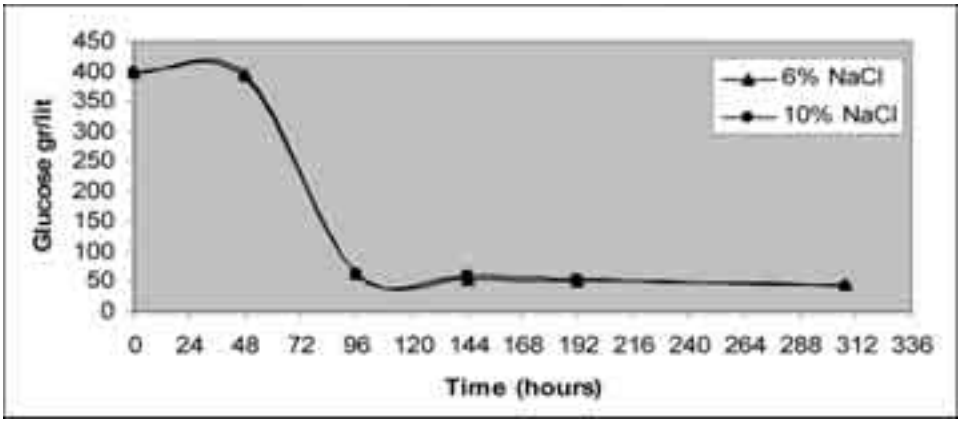

Fig. 11 — Glucose consumption for pre-conditioning yeast cells in 6% and 10% of NaCl cultured in define medium containing 40% of D-Glucose

Figures 12, 13, 14 and 15 show that yeast cell viability decreased when the sugar levels increased. For example, for 20% D-glucose yeast viability remained high even towards the end of the fermentation. However, at 30% and 40% D-glucose yeast viability remained low.

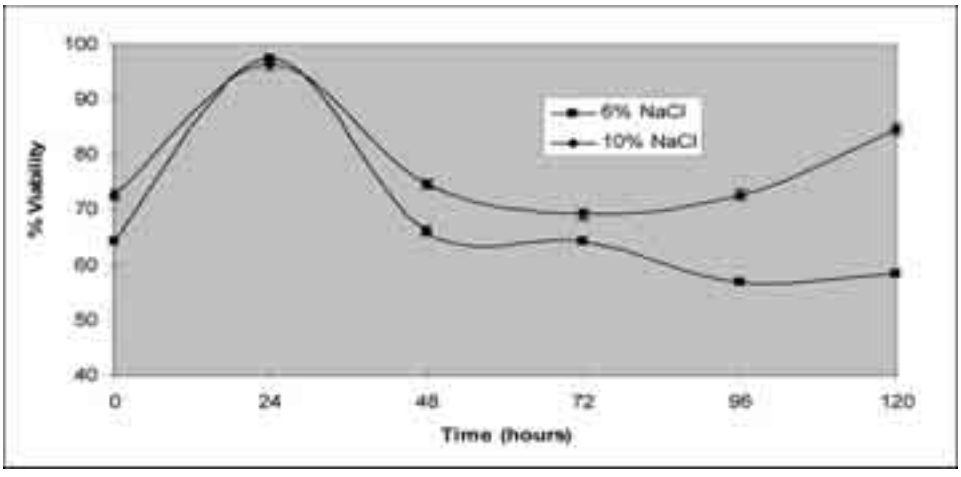

Fig. 12 — Viability for pre-conditioning cells in 6% and 10% of NaCl cultured in define medium containing 20% of D-Glucose

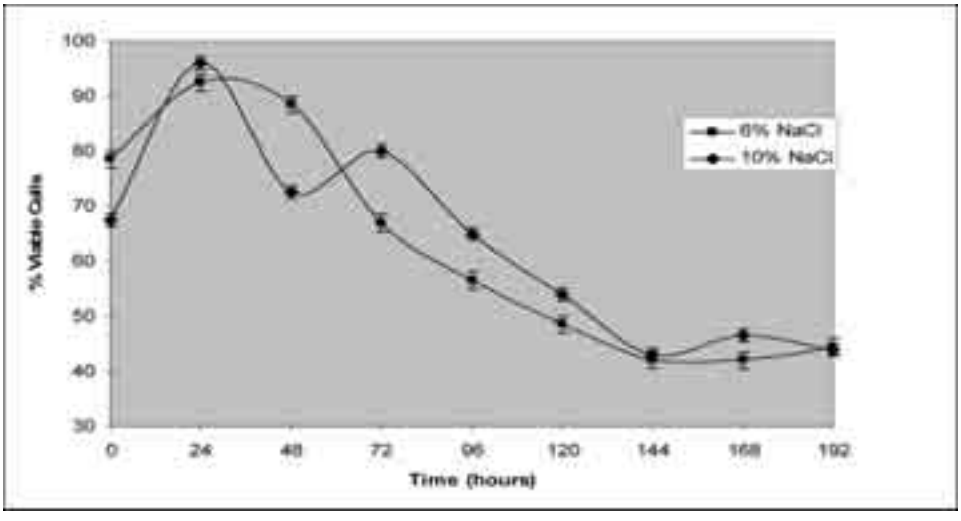

Fig. 13 — Viability for pre-conditioned cells in 6% and 10% of NaCl cultured in define medium containing 30% of D-Glucose

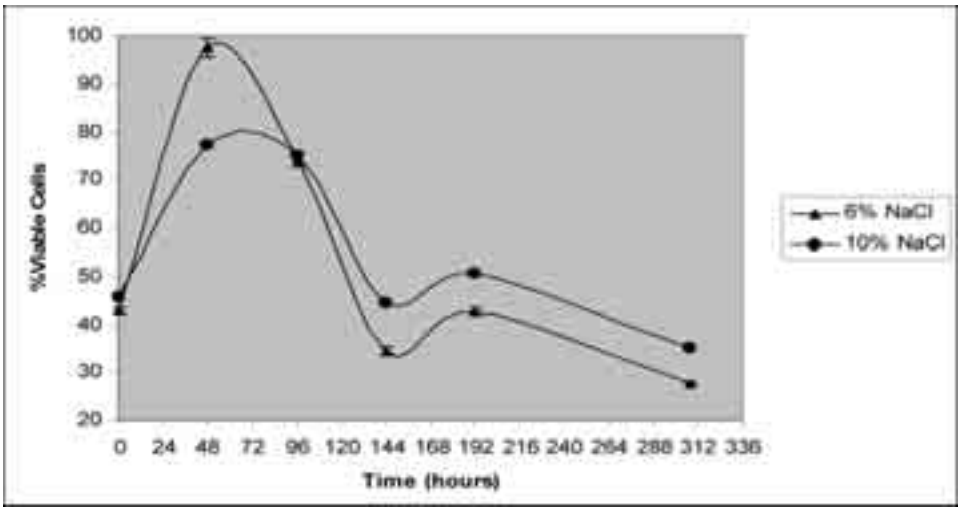

Fig. 14 — Viability for pre-conditioned cells in 6% and 10% of NaCl cultured in define medium containing 40% of D-Glucose

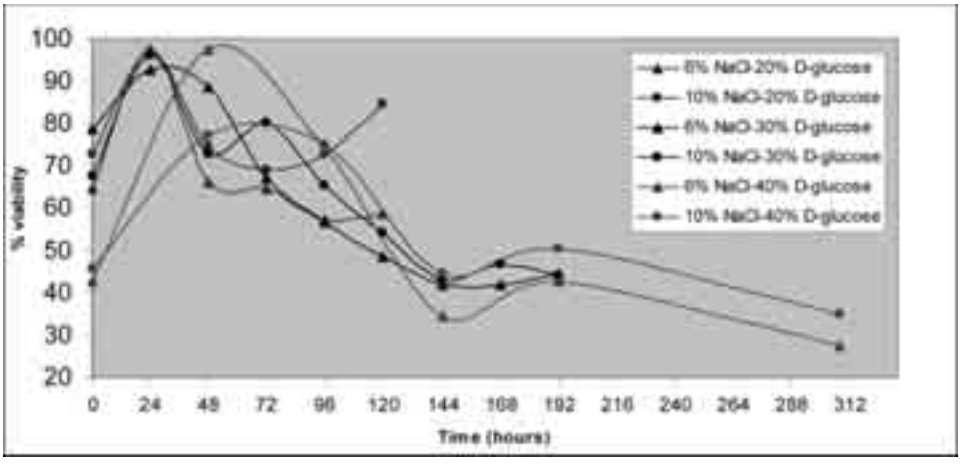

Fig. 15 — Viability for pre-conditioned cells in 6% and 10% of NaCl cultured in define medium containing 20, 30 and 40% of D-Glucose

CONCLUSIONS

Since the early seventies sodium chloride and the effects of saline stress to yeast cells have been the subject of research in many research labs. Several research studies have been contacted until today regarding the effects of salt on yeast genes expression and transcriptional response (16, 17, and 18).

Scope of this paper was to observe the effect of high osmotic stress to yeast growth and yeast viability. The main points derived from these results

are the stress of yeast cells under 6% concentration NaCl and the high percentage of yeast cell viability for higher concentrations of 10% w/v NaCl. Growth rate arrest under osmotic stress conditions was clearly showed in our experiments according to previous research work (18). Most important, was that during the experiments, when salt concentration increased, the yeast growth rate decreased almost proportionally.

The cell viability remained at high levels for all experiments series even for those under extremely stress conditions such as 10% w/v of NaCl. For the same levels of NaCl concentrations (6 and 10% w/v) it was shown that cell viability remained almost constant and at high levels for more than 400 hours of culture.

Repeated fermentations under increasing concentrations of glucose conclude that pre-conditioned yeast cells in 6 and 10% of NaCl remain viable for a long period of time and exhibit high fermentability.

According to these results future work is needed using higher amounts of sodium chloride (more than 10% w/v) in order to elucidate the effects of salt-induced stress on yeast cell viability during alcoholic fermentation and the ability of the cells to remain viable under different kind of stresses.

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УТИЦАЈ ХИПЕРОСМОТСКОГ СТРЕСА ИЗАЗВАНОГ NaCl-ом НА ВИЈАБИЛНОСТ ЋЕЛИЈА КВАСЦА

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Резиме

У производњи етанола квасци су током ферментације изложени различитим врстама физичко-хемијског стреса као што су: високе почетне концентрације шећера и ниске температуре, а касније повећане концентрације етанола. Овакви услови су покретачи серије биолошких одговора који имају за циљ одржавање ћелијског цикличног развоја и вијабилности ћелија квасца. Што се тиче осмотског стреса, многе студије су усмерене на транскрипциону активацију и

експресију гена у лабораторијским сојевима *Sacchavomyces cerevisiae*. Циљ овог рада је био да се боље сагледају и перформансе винског квасца током ферментације у условима осмотског стреса. Истраживачка активност је посебно усмерена на испитивање одзива индустријских сојева винског квасца *S. cerevisiae* (VIN 13) у условима стреса индукованог NaCl-ом, са посебним освртом на раст ћелија квасца и њихову вијабилност. Хипотеза је била да се у условима осмотског стреса енергетски активирају специфични гени који омогућују преживљавање квасних ћелија у датим условима. Оглед је изведен претходним третирањем ћелија растворима NaCl различитих концентрација (NcAl: 4%, 6% и 10% m/v), коришћењем дефинисаних подлога са D-глукозом и испитивањем њиховог утицаја на раст и вијабилност ћелија. У касније спроведеним циклусима ферментације повећаване су концентрације D-глукозе (20%, 30%, 40% m/v), а као инокулум су се користиле ћелије квасца претходно адаптиране на повишене концентрације соли. У раду је показано да осмотски стрес индукован благим третманима у растворима соли позитивно делује и на вијабилност ћелија и на ферментационе перформансе индустријских сојева винског квасца.

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PRODUCTION OF BIOETHANOL FROM TRITICALE

ABSTRACT: Triticale (*Triticosecale*) is a crop species resulting from a plant breeder's cross between wheat (*Triticum* sp.) "mother" and rye (*Secale* sp.) "father". Today, it has been cultivated in more than 50 countries worldwide. During theresearches conducted over period 2005—2006, the quality of three varieties of triticale was examined. Chemical quality parameters were the scope of the paper. The analyzed varieties of triticale showed high a-amylase activity, that was measured by falling number and amylolitic activity. While investigating thermal preparation of the samples at three different temperatures 60, 70 and 90°C, the optimum temperature was determined. Three different modes of thermal preparations were applied in the experiment: 1) without the addition of technical enzymes (a-amylase and glucoamylase), 2) with the addition of glucoamylase, and 3) with the addition of glucoamylase and a-amylase. The enzymes were dosed according to the recommendations of the manufacturer. The thermal preparation of samples, conducted at 90°C, produced the lowest content of fermentable starch. This is due to inactivation of amylolytic enzymes in triticale at 90°C. During 2006, the survey on bioethanol production from triticale was directed towards lowering the temperature regimes of the preparation step up to 60°C. During the first preparation mode (without the additional enzymes), the obtained results for the content of fermentable starch and the ethanol yield, showed that native amylolytic enzymes of triticale can degrade 80—90% of the available starch. The addition of glucoamylase, during the second preparation mode, increased the content of fermentable starch and ethanol yield. The best results were achieved applying the third mode of preparation. Comparing the preparation modes, it could be concluded that the application of both a-amylase and glucoamylase in the preparation step, increased the content of fermentable starch and ethanol yield by 7—13%. Further research should optimize the addition of a-amylase and glucoamylase. According to the results, the thermal preparation modes at 60°C are considered more suitable because of the energy savings.

KEY WORDS: amylase, enzyme, ethanol yield, fermentable starch, triticale

INTRODUCTION

Bioethanol is a strategic raw material with wide range of applications in food, pharmaceutical, cosmetic, and petrochemical industry (Baras et al., 2002). Also, it has been used as a modern biofuel, applied directly as a gasoline improver or gasoline substituent (Demirbas, 2006), or in the form of

ETBE (ethyl tertiary buthyl ether), to substitute for currently added synthetically-produced octane enhancers (R o s e n b e r g e r, 2005), and in ethanol-diesel blends with particular purpose to reduce the emissions of exhaust gasses (H a n s e n et al., 2005). Triticale (*Triticosecale*) is a crop species resulting from a plant breeder's cross between wheat (*Triticum* sp.) "mother" and rye (*Secale* sp.) "father". (O e l k e 1989). The time span from its creation to its commercialization was long (Q u a l s e t, 1996). Triticale is tolerant to climatic changes, certain diseases, extreme soil conditions, and gives relatively high yields (O e l k e, 1989). In 2005, according to the Food and Agricultural Organization (FAO), 13.5 million tons of triticale was harvested in 28 countries across the world (*Wikipedia*, 2006). Triticale has just recently been used as a raw material in the bioethanol production. Starch content in triticale is approximately 60%, and ethanol yield amounts to 38 l per 100 kg (S e n n, 2001). Triticale contains amylolitical and proteolitical enzymes, and their inhibitors, too (W e s e n b e r g, 1990). Triticale is self-polinating (similar to wheat), and not cross pollinating (like rye) (O e l k e, 1989). Triticale does not contain high amounts of pentosans like rye, therefore there are no problems during fermentation because of high viscosity. Also, triticale is characterized with high autoamylolitic activity enabling its use without the addition of commercial saccharifying enzymes. Some varieties of triticale contain surplus amylases, which can be used for saccharifying other substrates. Enzyme activity of native triticale enzymes is expressed with Autoamylolitical Quotient (AAQ). Autoamylolitical Quotient is defined as the percentage yield of ethanol obtained without the addition of technical enzymes, compared with the ethanol yield with addition of an optimum combination of technical enzymes (S e n n, 2001).

The aim of the investigation was to optimize the process of triticale thermal preparation for the production of bioethanol. Yields of ethanol from triticale samples prepared at various temperatures (60, 70, and 90°C), were determined.

MATERIAL AND METHODS

Over the period 2005—2006, the quality and the processing potential for bioethanol production of three triticale varieties: ORION, NS-TRITICALE and JUTRO were investigated. The following chemical quality parameters were determined: the content of solids, ash and protein, the falling number according to Hagberg, and the maximum peak viscosity (K a l u đ e r s k i, F i l i p o v i ć, 1998).

The preparation of the samples for fermentation comprised: stillage formation, dosage of the processing microorganism, starch saccharifying, and inoculation with working microorganism. The stillage formation of grinded samples was conducted by applying hydromodul 1:3 (material to water ratio) in a stillage tank. The pH was measured on pH-meter, and was adjusted in the range 5.4—5.5.

The following three temperature regimes of the preparation of triticale samples were used:

Thermal preparation at 60°C
— heating up to 60°C tests I, II, III. To test III+ Thermamyl SC
— keeping for 60 min at 60°C,
— cooling to 55°C. To tests II, III + SAN Super 360L,
— keeping for 30 min at 55—60°C.

Thermal preparation at 70°C
— heating up to 60°C tests I, II, III. To test III + Thermamyl SC,
— heating tests I, II, III up to 70°C,
— keeping for 60 min at 70°C,
— cooling to 55°C. To tests II, III + SAN Super 360L,
— keeping for 30 min at 55—60°C.

Thermal preparation at 90°C
— heating up to 60°C tests I, II, III. To test III + Thermamyl SC,
— heating tests I, II, III up to 90°C,
— keeping for 60 min at 90°C,
— cooling to 55°C. To tests II, III + SAN Super 360L,
— keeping for 30 min at 55—60°C.

Preparation modes of triticale samples for each temperature regime were the following:

— I test: sample without enzymes
— II test: sample + bacterial glucoamylase SAN Super 360L (Novozymes A/S)
— III test: sample + glucoamylase SAN Super 360L (Novozymes A/S) + bacterial thermostable a-amylase Termamyl SC (Novozymes A/S).

After the thermal processing, the samples were cooled to 30°C, and inoculated with *Saccharomyces cerevisiae* (Alltech-Fermin, Serbia). Batch fermentations were carried out under anaerobic conditions at constant temperature (30°C), controlling the rate of fermentation, and measuring the weight of produced CO_2 after each 24-hour period, until the fermentation ended.

On the basis of the obtained results, Autoamylolitical Quotients of the samples were determined for each temperature regime (60, 70 and 90°C) according to equation:

$$\text{AAQ } (\%) = \frac{\text{Ethanol yield (g ethanol / 100 g d.b.) without technical enzymes}}{\text{Ethanol yield (g ethanol / 100 g d.b.) with technical enzymes}} \text{ x } 100$$

RESULTS AND DISCUSSION

The chemical parameters characterizing the quality of triticale samples are presented in Table 1. The moisture contents of all the investigated samples were at levels that ensure safe and long storage of the grains. The ash content

in all samples was very high, providing necessary mineral food for the activity of yeast during fermentation. Protein contents of Orion and Jutro varieties (10.13% and 10.7%, respectively) were at lower levels than NS-triticale (11.94%). The falling numbers of the samples were lower than 250 s. These results designate the presence of active a-amylases in the samples. The amylogram peak viscosities confirmed this statement. The lowest peak viscosity (90 B.U.) was measured in the Orion sample.

Tab. 1 — Chemical quality parameters of triticale variety

SAMPLE	Moisure content (%)	Ash content (% d.b.)	Protein Content (% d.b.)	Falling number (s)	The mazimum peak viscosity (aj)
ORION	9.51	2.04	10.13	184	90
NS-TRITIKALE	9.50	2.01	11.94	223	125
JUTRO	9.46	1.87	10.69	215	160

The contents of fermentable starches and ethanol yields for triticale samples, subjected to thermal processing at various temperatures (60, 70, and 90°C) are presented in Table 2. The variety Orion produced the highest quantities of fermentable starch and ethanol in the preparation mode, without commercial enzymes at 60°C. The addition of glukoamylase SAN Super 360L increased the contents of fermentable sugar and ethanol yields for each triticale variety. When glucoamylase was added, the content of fermentable sugar in NS-triticale variety increased by 1.5%. The addition of glukoamylase and thermostable a-amylase increased the contents of fermentable sugar and ethanol in all samples.

At 70°C, the preparation mode without the addition of commercial enzymes gave the highest content of fermentable starch in NS-triticale sample (61.3%), followed by Jutro (58.4%), and Orion (53.80%). The addition of glycoamylase increased the contents of fermentable starch in all samples with the highest content achieved for NS-triticale. All samples demonstrated an increase in the yield of fermentable starch and ethanol with the addition of both commercial enzymes, with superior performance of Jutro variety. Each preparation mode conducted at 90°C, resulted in significantly lower fermentable sugar and ethanol production, compared to that obtained at 60 and 70°C, for all samples.

Tab. 2 — Contents of fermentable sugar and ethanol yields of triticale samples prepared under different temperature regimes

PREPARATION MODES OF TRITICALE SAMPLES		TRITICALE SAMPLE					
		ORION		NS-TRITIKALE		JUTRO	
		fermentable sugar (g/100 g sm)	ethanol yield (g/100 g sm)	fermentable sugar (g/100 g sm)	ethanol yield (g/100 g sm)	fermentable sugar (g/100 g sm)	ethanol yield (g/100 g sm)
Thermal preparation at 60°C	Sample without enzyme addition	57.15	32.46	56.61	32.15	55.70	31.63
	Sample + SAN Super 360 L	62.61	35.55	64.14	36.42	62.44	35.16
	Sample + SAN Super 360 L + Termamyl SC	64.69	36.73	64.88	37.26	63.47	36.05
Thermal preparation at 70°C	Sample without enzyme addition	53.80	30.60	61.3	34.8	58.40	33.20
	Sample + SAN Super 360 L	66.60	37.80	67.7	38.4	65.40	37.17
	Sample + SAN Super 360 L + Termamyl SC	67.70	38.40	68.3	38.8	69.27	39.30
Thermal preparation at 90°C	Sample without enzyme addition	50.28	28.87	52.36	29.76	52.70	29.95
	Sample + SAN Super 360 L	59.80	33.99	55.7	31.66	56.80	32.28
	Sample + SAN Super 360 L + Termamyl SC	63.32	35.37	60.26	34.8	60.49	34.69

The Autoamylolitical Quotients of triticale samples prepared on 60, 70, and 90°C are presented in Table 3. The Autoamylolitical Quotient was determined as the ratio of ethanol yield obtained without the addition of commercial enzymes, and the ethanol yield obtained with the addition of Termamyl SC. Orion and Jutro demonstrated the best performance at preparation temperature 60°C (88%). The Autoamylolitical Quotient of 88% means that the sample contains enough amylases to degrade 88% of total available starch. The highest Autoamylolitical Quotient at 70°C was obtained in the NS-triticale sample. From the data presented, it is evident that the autoamylolitic coefficients depend on the variety. Also, it could be concluded that the investigated triticale varieties are not suitable for saccharifying other starchy substrates because of the lack of native amylases. The results proved that triticale is a suitable raw material for bioethanol production. Preparation procedures conducted at 60°C are recommended because of energy savings.

Tab. 3 — Autoamylolitical Quotients of triticale samples.

Thermal preparation	The Autoamylolitical Quotient (AAQ) (%)		
	ORION	NS-TRITIKALE	JUTRO
60°C	88	86	88
70°C	80	90	84
90°C	82	86	86

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ПРОИЗВОДЊА БИОЕТАНОЛА ИЗ ТРИТИКАЛЕА

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Резиме

Тритикале (*Triticosecale*) је најстарија вештачки створена хибридна житарица настала укрштањем пшенице *Triticum* и ражи *Secale*. Данас се тритикале у свету култивише у више од 50 земаља. Током истраживања 2005. и 2006. године испитан је квалитет три сорте тритикалеа. Одређени су хемијски показатељи квалитета узорака. Анализиране сорте тритикалеа су показале изразиту активност а-амилазе, што се изражава бројем падања и амилолитском активношћу. У току истраживања испитиване су оптималне температуре термичке припреме узорака тритикале, на температурама 60°C, 70°C, 90°C. Термичке припреме извођене су на три начина: без додатка а-амилазе и глукоамилазе (техничких ензима), уз додатак глукоамилазе и уз додатак глукоамилазе и термостабилне а-амилазе. Дозирање наведених ензима извршено је према препорукама произвођача. Најнижи резултати садржаја ферментабилног скроба добијени су током припреме узорака на 90°C. Објашњење за овако ниске садржаје ферментабилног скроба може се наћи у чињеници да се на 90°C инактивирају амилолитски ензими узорака тритикалеа. Током 2006. године истраживања производње биоетанола из тритикалеа била су усмерена ка испитивању могућности да се термичка припрема узорака изводи на 60°C. Резултати садржаја ферменталног скроба и приноса етанола током првог поступка припреме (без додатака ензима) показали су да амилолитски ензими тритикалеа могу да разграде сопствени скроб у износу 90—98% (у зависности од узорка). Додатком глукоамилазе у току другог поступка припреме повећава се садржај ферментабилног скроба и принос етанола. Најбољи резултати садржаја ферментабилног скроба и приноса етанола добијени су уз додатак термостабилне а-амилазе и глукоамилазе. Упоређивањем резултата сва три поступка припреме може се закључити да се применом термостабилне а-амилазе и глукоамилазе добијају само 2—10% виши садржаји ферментабилног скроба и приноси етанола. У даљем раду треба оптимизирати додатке термостабилне α-амилазе и глукоамилазе. Добијени резултати садржаја ферментабилних шећера и приноса етанола из узорака тритикалеа недвосмислено дају предност поступку припреме узорака на 60°C због уштеде енергије.

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INFLUENCE OF DOUGH FREEZING ON *SACCHAROMYCES CEREVISIAE* METABOLISM

ABSTRACT: The need to freeze dough is increasing in bakery production. Frozen dough can be stored for a long time without quality change. The capacity of bakery production can be increased in this way, and in the same time, the night shifts can be decreased. Yeast cells can be damaged by freezing process, resulting in poor technological quality of dough after defrostation (longer fermentation of dough).

The influence of frozen storage time of dough on survival percentage of *Saccharomyces cerevisiae* was investigated. Dough samples were taken after 1, 7, 14 and 28 days of frozen storage at –20°C. After defrosting, at room temperature, samples were taken from the surface and the middle part of dough (under aseptic conditions), and the percentage of living *S. cerevisiae* cells was determined. During frozen storage of dough, the number of living *S. cerevisiae* decreased. After 28 days of frozen storage, the percentage of live cells on the surface and inside the dough was 53,1% and 54,95%, respectively. The addition of k-carragenan to dough increased the percentage of living cells in the middle part of dough up to 64,63%. Pure cultures, isolated from survived *S. cerevisia* cells in frozen dough by agar plates method (Koch's method), were multiplied in optimal liquid medium for yeasts. The content of cytochromes in *S. cerevisiae* cells was determined by spectrophotometric method. The obtained results showed that the content of cytochromes in survived *S. cerevisiae* cells was not affected by dough freezing process. Growth rate and fermentative activity (Einchor's method) were determined in multiplied cells.

KEY WORDS: cytochromes, fermentative activity, freezing, κ-carragenan, survival, *Saccharomyces cerevisiae*

INTRODUCTION

Freezing and storage in the frozen state leads to decreased viability and fermentative activity of yeast cells (Berglund et al., 1991). Mazur (1963) reported that a number of factors affect damage, and it depends on whether ice is formed intracellularly (high freezing rates), or extracellularly (lower freezing rates). It was concluded that freezing regimes affect strongly the loss of cell viability. Cells are damaged during the freezing process, while the defrosting

regime affects insignificantly the survival of yeast cells. Yeast cell metabolism decreases drastically at low temperatures; therefore, long term storage of cells is possible.

High survival level can be achieved by appropriate freezing process: survival about 95% (Janković et al., 1999), or even 100% (Pejin, 1989). Freezing of yeast suspension in water affects decreased fermentative activity. The decrease of fermentative activity is lower when yeast is frozen in starch dough (Berglund, 1988).

Mayers and Attfield (1999) consider that the loss of yeast cell viability is affected by freezing of intracellular water, resulting in damage of cytoplasmatic membrane and increase of components in the cytoplasma. This can affect decrease of pH value, increase of dry matter content, and decrease of glycolytic enzymes activity in the cytoplasma.

According to Gao and Critser (2000), if the loss of water during freezing is higher than the critical moisture content, the proteins and cytoplasmatic membrane are irreversibly denaturated, and this can cause the death of cell. The denaturation of cell parts can be caused by: concentration increase of some ions, change of pH, and hydratation decrease of macromolecules in the cytoplasma. The fermentative activity of yeast in frozen dough is lower by 30—35%, compared to the control (Mikinčić-Pešut, 1989). Yeast resistance during freezing is affected by duration of dough fermentation before the freezing process, freezing and defrosting rate of dough, frozen storage time, and temperature oscillations during storage (Giannou et al., 2003).

Several ways of decreasing the effect of freezing and frozen storage on yeast survival and fermentative activity can be found in recently presented papers, even on improving characteristics and quality of frozen dough and obtained bakery products: addition of hydrocolloids, lower water content in prepared dough, higher amount of yeast, compared to the traditional production, and shorter dough fermentation before freezing (Giannou et al., 2003), use of instant yeast (Abd El-Hady et al., 1999), use of cryotolerant and/or cryoresistant strains of bakery yeast (Takano et al., 2002), use of modified yeast strains (Tanghe et al., 2000; Van Dijck et al., 2000; Tennissen et al., 2002).

The aim of the research presented in this work was to investigate the possibility of kapa-carragenan use to protect the yeast cells during freezing of dough. κ-carragenan-hydrocolloid — is biochemically inert, and can modify the dough structure, bind the free water and control water migration in the dough (Ribotta et al., 2004; Žeželj, 2005). Binding and immobilization of water decreases the ice crystal formation, and also the damage of glutene and yeast cells (Sharadanant and Khan, 2003b). This is significant from the standpoint of production of bakery products from frozen dough.

MATERIAL AND METHODS

Average quality commercial T-500 flour was used for the production of dough, which was frozen later. Quality characteristics were analyzed according

to the Regulations on methods of physical and chemical analyses for quality control of wheat, milling and bakery products, pasta and fast frozen dough (Yugoslav Official Register, N° 74/1988).

Dough intended for freezing was prepared according to the following procedure: flour + water + bakery yeast (2,5% calculated on flour), placed in the spin kneading machine with helical agitators, and mixed for 10 min at 85 rpm (control). Aiming to investigate the effect of hydrocolloids on yeast, during freezing of dough, κ-carragenan (Fluka AG, Buch, Switzerland) was added as a component into the dough prepared according to the described procedure.

The temperature of mixed dough was 20 ± 1°C (Kenny et al., 2001). The dough was divided into portions, formed without fermentation in mass (Barconas et al., 2003), frozen at –35 ± 1°C until –12 ± 1°C was reached in the centre of the sample (freezing chamber KOMA, the Netherlands), according to producer's recommendations. The frozen dough portions were packed in PVC bags, and stored at –20 ± 1°C (chamber KOMA), for 1, 7, 14 and 28 days. The samples were defrosted at +4 ± 1°C for 12 hrs, and for additional 1,5 hr at +20 ± 1°C. The number of living *Saccharomyces cerevisiae* cells was determined according to the method given in the Rulebook on methods of performing microbiological analyses and superanalyses of food products (Yugoslav Official Register N° 25, 1980). Pure cultures were transferred into the liquid nutritive medium for yeasts (Mihajlović, 1983). Specific growth rate, and fermentative activity according to Einhorn (Reiff et al., 1960), were determined in regular time intervals, during 24 hrs. The content of cytochromes was determined by method according to Oure and Suomalainen (1970), with the aim of determining the respiration intensity.

RESULTS AND DISCUSSION

Number and percentage of survived *S. cerevisiae* cells in frozen dough are presented in Table 1. During frozen storage of dough for 28 days, the percentage of living cells from dough surface is 53,11%, and from the centre 54,95%. Comparing these results, it is quite clear that the cells in the centre of the dough are protected from low temperature, and the number of survived cells is higher. In dough samples, prepared with addition of κ-carragenan, in concentrations 0,1, 0,3 and 0,5%, the number and percentage of survived cells increases both on the surface and in the centre. In dough with 0,5% a κ-carragenan, the percentage of survived cells on dough surface is 61,23%, by 8,12% higher compared to the control sample. In the centre 64,63% cells survived, about 10% more compared to the control.

Tab. 1 — Number and percentage of survived *Saccharomyces cerevisiae* cells in frozen dough samples with different content of κ-carragenan during storage

k-karagenan content (%)	Number of living cells x 10^4 in 1g of dough					Survirval percentage cells
	Days of keeping at –20 ± 1°C (dani)					
	0	1	7	14	28	
0,0 on surface	2350	2060	1562	1438	1248	53,11
0,0 in the middle	2475	2380	2123	1520	1360	54,95
0,1 on surface	2240	2090	1525	1498	1095	48,88
0,1 in the middle	2460	2110	1677	1570	1348	54,80
0,3 on surface	2320	1990	1572	1490	1345	57,97
0,3 in the middle	2450	2050	1648	1550	1525	62,33
0,5 on surface	2270	2100	1700	1425	1390	61,23
0,5 in the middle	2460	2030	1850	1630	1590	64,63

Tab. 2 — Specific growth rate of pure *Saccharomyces cerevisiae* cultures, isolated from frozen samples: the control dough and dough containing κ-carragenan

Days of keeping at –20°C	Place of cell sampling	μ-Specific growth rate (h^{-1})			
		Without κ-karagenan		With κ-karagenan	
		0—4	0—24	0—4	0—24
0	on surface	0.6074	0.1425	0.6575	0.1430
	in the middle	0.5678	0.1452	0.6441	0.1420
1	on surface	0.5756	0.1510	0.6471	0.1390
	in the middle	0.5269	0.1540	0.6427	0.1380
7	on surface	0.5175	0.1420	0.6307	0.1370
	in the middle	0.5100	0.1300	0.5832	0.1360
14	on surface	0.5165	0.1350	0.5833	0.1300
	in the middle	0.5099	0.1300	0.5175	0.1260
28	on surface	0.5170	0.1340	0.1813	0.1300
	in the middle	0.4946	0.1300	0.0965	0.1360

Specific growth rates were calculated according to the method by P e j i n (1989), using the data on content of dry matter determined during multiplication on *liquid nutritive medium*, under intensive aeration, during 24 h, at 30°C. Analyzing the results from Table 2, it can be seen that the growth of yeast cells is very intensive during the first four hours. It is also interesting to mention that the specific growth rate of cells, isolated from dough surface, is higher than of cells isolated from the centre of sample. The specific growth rate of pure *S. cerevisiae* culture decreases with longer storage of frozen dough. In fresh state, the specific growth rate of yeast cells was 0,6047 × h^{-1}, during the first 4 hours of cultivation, and after frozen storage of dough at –20°C, for 28 days, it was 0,517 × h^{-1}. The mean specific growth rate was calculated after 24 hrs of multiplication, and the obtained values showed that the specific growth rate of pure *S. cerevisiae* cultures decreases constantly with longer fro-

zen storage of dough. The mean specific growth rate decreases by about 10%, during 28 days of storage at –20°C.

The addition of hydrocolloid κ-carragenan affects positively the specific growth rate of pure cultures isolated from frozen dough samples with addition of 0,5% of κ-carragenan. In dough samples, stored for 14 days in frozen state, no significant decrease of specific growth rate, during the first four hours of cultivation was estimated. However, the specific growth rate of pure cultures isolated from dough, kept for 28 days at –20°C, was three times lower, compared to the isolates without addition of κ-carragenan. The "lag-phase" of these samples was considerably longer, causing decrease of specific growth rate in the first four hours of cultivation. It is interesting to mention that the mean specific growth rate, determined during 24 hours is higher for isolates from frozen dough samples prepared with κ-carragenan, than for solates from frozen doughs without κ-carragenan.

The results of fermentative activity of pure *S. cerevisiae* cultures isolated from frozen dough samples, with and without κ-carragenan, are presented in Table 3.

Tab. 3 — Fermentative activity of pure *Saccharomyces cerevisiae* cultures isolated from frozen dough samples, with and without κ-carragenan

Days of keeping at –20°C	Place of cell sampling	cm^3 CO_2/1g dry matter	
		Without κ-karagenan	With κ-karagenan
0	on surface	46.41	96.29
	in the middle	41.62	87.56
1	on surface	43.80	93.15
	in the middle	42.26	67.47
7	on surface	43.90	68.68
	in the middle	41.90	49.90
14	on surface	43.00	62.22
	in the middle	39.80	49.10
28	on surface	42.04	46.88
	in the middle	34.48	34.82

The fermentative activity of pure cultures isolated from frozen dough samples, containing κ-carragenan, was about 2 times higher compared to pure cultures isolated from dough without κ-carragenan (Table 3). This can be explained by the fact that this hydrocolloid binds the water around yeast cells, and protects them from freezing, preventing the formation of large ice crystals which can damage the cell wall and cytoplasmatic cell membrane.

It is interesting to mention that the fermentative activity of cells on dough surface is higher compared to the cells from the middle part. It leads to a conclusion that yeast cells, isolated from the dough surface, are less damaged by the freezing process, than the ones isolated from the middle.

The results of fermentative activity determination for cells isolated from frozen doughs after 28 days, show that the change of this characteristic is insignificant for dough samples without κ-carragenan.

Respiratory activity e.g. content of cytochromes after multiplication for 24 h, under aerobic conditions, was also determined in pure cultures, isolated from frozen dough samples, with and without addition of κ-carragenan (Tables 4 and 5).

Tab. 4 — Influence of storage time on cytochrome content in pure culture cells isolated from dough samples

Days of keeping at –20°C	Place of cell sampling	Cytochrome moles x 10^5/kg yeast with 25% dry matter					
		aa_3		b		c	
		605 nm	444 nm	560 nm	532 nm	520 nm	550 nm
0	on surface	6.45	4.76	6.98	6.28	4.39	5.68
	in the middle	5.32	3.72	5.76	5.76	3.92	4.76
1	on surface	5.24	4.33	6.68	5.46	3.34	5.27
	in the middle	2.62	3.17	4.97	4.82	2.47	3.22
7	on surface	0.88	2.66	4.81	4.79	2.39	2.93
	in the middle	0.87	2.10	4.70	4.72	2.35	2.89
14	on surface	0.65	2.70	5.76	5.73	2.85	7.00
	in the middle	0.57	2.70	5.48	4.82	2.40	5.98
28	on surface	0.42	2.94	5.04	3.45	1.51	6.70
	in the middle	0.37	2.58	4.99	3.00	1.45	5.70

The time of storage affects negatively the content of cytochromes aa_3, b and c (Table 4). The decrease of cytochromes aa_3 content is the highest, and of cytochromes b, the lowest. Cytochrome content decrease points to lower activity of respiratory enzymes, which provide energy for the growth of cells. Due to decreased enzyme activity, the yeast cells have no energy necessary for the growth, resulting in decrease of specific cell growth activity.

Tab. 5 — Influence of storage time on cytochrome content in pure isolate cells, isolated from frozen dough samples containing κ-carragenan

Days of keeping at –20°C	Place of cell sampling	Cytochrome moles x 10^5/kg yeast with 25% dry matter					
		aa_3		b		c	
		605 nm	444 nm	560 nm	532 nm	520 nm	550 nm
0	on surface	5.76	4.79	6.76	6.25	4.47	5.47
	in the middle	4.86	4.52	5.82	5.55	3.98	4.92
1	on surface	4.25	2.86	6.31	4.90	2.47	4.05
	in the middle	3.86	2.26	4.14	4.58	3.63	5.86
7	on surface	0.99	2.29	6.29	4.72	3.53	5.78
	in the middle	0.72	1.76	5.08	3.79	1.89	4.14
14	on surface	0.00	2.26	4.67	4.66	2.37	5.70
	in the middle	0.00	1.66	3.99	3.58	1.85	3.90
28	on surface	0.00	1.29	3.89	4.66	2.33	4.76
	in the middle	0.00	1.16	3.19	3.58	1.85	3.90

κ-carragenan has no positive effect on the content of cytochromes in yeast cells (Table 5). In yeast cells isolated after 14 days from frozen dough

storage, the content of cytochromes aa_3 is about 10 times lower, the contents of cytochromes b and c are also lower, compared to the samples from frozen dough without κ-carragenan.

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УТИЦАЈ ЗАМРЗАВАЊА КВАСНИХ ТЕСТА НА МЕТАБОЛИЗАМ *SACCHAROMYCES CEREVISIAE*

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Резиме

У пекарској производњи расте потреба за замрзавањем квасних теста. Замрзнута квасна теста могу се дуго чувати у замрзнутом стању без губитка квалитета. На овај начин може се повећати капацитет пекарске производње, а на тај начин смањити потреба за ноћним радом пекара. Процесом замрзавања квасних теста могу се оштетити квасне ћелије и њихов ензимски систем тако да ће након одмрзавања квасно тесто имати лошији технолошки квалитет (продужено време ферментације теста). У раду је испитивано како период чувања замрзнутог квасног теста утиче на проценат преживљавања *Saccharomyces cerevisiae.* Период чувања квасног теста је био 28 дана, на температури „–20°C". Током овог периода узимани су узорци за испитивање након 1, 7, 14. и 28. дана. У наведеним периодима узорци су одмрзавани на собној температури и са површине и из унутрашњости теста (под асептичним условима) узимани су узорци квасног теста и у њима је одређиван проценат преживелих ћелија *Saccharomyces cerevisiae.* Током чувања квасног теста у замрзнутом стању смањивао се постепено број преживелих ћелија *Saccharomyces cerevisiae*. Након 28. дана чувања квасног теста

у замрзнутом стању проценат преживелих ћелија на површини теста је био 53,11%; а у унутрашњости теста је био 54,95%. Додатак к-карагенана у тесто повећао је проценат преживелих ћелија у унутрашњости замрзнутог теста и до 64,63%. Од преживелих ћелија *S. cerevisiae* из замрзнутих теста методом агарних плоча (Коховом методом) изоловане су чисте културе. Чисте културе умножаване су, до потребних количина, у оптималној течној подлози за квасце. У ћелијама *S. cerevisiae* одређиван је садржај цитохрома спектрофотометријском методом. Добијени резултати садржаја цитохрома су показали да процес замрзавања квасног теста није утицао негативно на саржај цитохрома у преживелим ћелијама *S. cerevisiae*. Умноженим ћелијама одређивана је брзина раста и ферментативна активност Einchor-овом методом.

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INFLUENCE OF THE CULTIVATION CONDITIONS ON LIGNINOLYTIC ENZYME PRODUCTION IN *PLEUROTUS PULMONARIUS*

ABSTRACT: The highest level of laccase activity (391 Ul^{-1}), as well as significant Mn-oxidizing peroxidases production, were found in solid-state culture with grapevine sawdust as the carbon source. After purification of extracellular crude enzyme mixture of *Pleurotus pulmonarius*, grown in the medium with the best carbon source (grapevine sawdust), three peaks of laccase activity were noted. The results obtained by purification also showed that the levels of phenol red oxidation, in absence of external Mn^{2+}, were higher than phenol red oxidation levels in presence of external Mn^{2+}. The highest laccase activity was in the medium with grapevine sawdust, as carbon source, and NH_4Cl at a nitrogen concentration of 30 mM (441 Ul^{-1}). The best nitrogen source for Mn-oxidizing peroxidase production was NH_4NO_3 at nitrogen concentration of 30 mM. The highest laccase activity was found in the presence of 5 mM Cu^{2+}, and 5 mM Mn^{2+}, respectively. The absence of Cu^{2+} and Mn^{2+}, as well as their presence at the concentration of 1 mM, led to the peaks of Mn-oxidizing peroxidases activities. Zn^{2+} and Fe^{2+} caused a decrease, and Se, in all investigated forms, an increase of laccase and peroxidases activities.

KEY WORDS: *Pleurotus pulmonarius*, laccase, Mn-oxidizing peroxidases, raw plant materials, carbon and nitrogen sources, microelements

INTRODUCTION

Pleurotus pulmonarius (Fr.) Quél. is an edible and medicinal species that belongs to the group of white-rot fungi, produces laccase (Lac), Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and versatile peroxidase (VP)], and aryl-alcohol oxidase (AAO), and has an ability to modify and degrade lignin, and other aromatic compounds (M u ñ o z et al., 1997). Due to those properties, it can be cultivated on different lignocellulolytic materials, such as most agricultural wastes, sawdust, paper products etc. (C r o a n, 2000). This species can transform raw plant materials to feed and fuel, and participates in biopulping, biobleaching of paper pulp, bioremediation of soils, and industrial

waters polluted with toxic chemicals and dyes, etc. (Reddy, 1995; Cerniglia, 1997; Ooi, 2000).

Ligninolytic enzyme production depends on cultivation conditions: submerged or solid-state fermentation; carbon and nitrogen sources and concentrations; presence of inducers and other small molecules; and presence of different microelements and their concentrations (Leontievsky et al., 1994; Mansur et al., 1997; Galhaup and Haltrich, 2001). Likewise, the significance of *P. pulmonarius* intraspecies diversity in production of these enzymes (Stajić et al., 2004).

Submerged fermentation (SF) and solid-state fermentation (SSF) are characterized by different physical and chemical features. SSF conditions are very similar to those existing in nature on the wood substrates, and under them fungi may synthesize ligninolytic enzymes, set differently from those under SF conditions.

Agricultural wastes are produced in enormous amounts worldwide, 123 x 10^6 tons per year (Smith et al., 1988). Chemical and physical degradations of raw plant materials are very expensive and inefficient processes, while biological degradation is more acceptable.

Grapevine sawdust and mandarine peels represent very common agricultural wastes in some regions, and they are prospective substrates for the bioconversion into fungal biomass and ligninolytic enzymes. Grapevine sawdust contained: 0.7—0.8% of total nitrogen, 1.6—1.8% of reducing substances, 12—13% of hemicellulose, 23—26% of cellulose, and 23—26% of lignin, while mandarin peels contained 1.1—1.3% of total nitrogen, 11—14% of reducing substances, 32—34% of soluble carbohydrates, 12—14% of cellulose and, 9—11% of lignin (Tsiklauri, 1999). Microelement concentrations in grapevine sawdust are: 107 ppm Mn, 13 ppm Cu, 30 ppm Fe, and 73 ppm Zn (Kilby, 1999), while these concentrations in mandarine fresh fruit are significantly lower: 0.4 gt^{-1} Mn; 0.6 gt^{-1} Cu; 2.6 gt^{-1} Fe, and 0.8 gt^{-1} Zn (Chapman, 1968).

The purpose of these investigations was to study the effect of different inorganic and organic carbon and nitrogen sources, as well as different microelements on production of Lac and Mn-oxidizing peroxidases in *P. pulmonarius*, under SF and SSF conditions.

MATERIALS AND METHODS

Organisms and growth conditions

Pleurotus pulmonarius HAI 572 originated from Czech Republic, and preserved in the culture collection of the Institute of Evolution, University of Haifa (HAI) was used for the investigations.

The inoculum was prepared by growing fungus at room temperature (22 ± 2°C), on a rotary shaker at 180 rpm, in 250 ml flasks containing 100 ml of synthetic medium (Stajić et al., 2004). After 7 days of cultivation, mycelial

pellets were harvested and homogenized using a laboratory homogenizer at 10,000 rpm.

The effect of different carbon sources on the production of laccase and Mn-oxidizing peroxidases

SF was carried out at room temperature, on a rotary shaker at 180 rpm, in 250 ml flasks containing 50 ml of the synthetic medium, which contained either 1% of one of the investigated carbon sources (Table 2), or 4% of dry ground mandarin peels. The initial pH of the medium with mandarin peels, as a carbon source, was adjusted to 9.4 prior to sterilization, so that the pH would be 6.0 after sterilization, while in other cases it was adjusted to 6.0. Homogenized suspensions (of 5 ml) were used for inoculation of one flask. Biomasses were separated by centrifugation after 5 and 7 days of cultivation, and clean supernatants were used to estimate enzyme activity.

SSF was carried out at 25°C in 100 ml flasks containing 4 g of grapevine sawdust, and 12 ml of modified synthetic medium (without glucose and with 4.0 gl^{-1} of NH_4NO_3) pH 6.0. Suspensions (of 3 ml) after inocula homogenization were used per flask. The extracellular enzymes were extracted after 7 and 10 days of cultivation, by sample grinding in a mortar, with 20 ml of distilled water, for 5 min on ice. This procedure was repeated 3 times, and the obtained extracts were mixed. Solids were separated by centrifugation, and supernatants were used for measurements of the Lac and Mn-oxidizing peroxidases activities.

Three replications for each investigated carbon sources were used.

The effect of different nitrogen sources and concentrations on the production of laccase and Mn-oxidizing peroxidases

One of the inorganic nitrogen sources [(NH_4Cl), (NH_4NO_3), ($NH_4H_2PO_4$), $(NH_4)_2SO_4$, and (KNO_3), at a nitrogen concentration of 30 mM], or one of the organic ones [bacteriological peptone, casein acid hydrolysate vitamin free, at a concentration of 0.5%, and corn step liquor at a concentration of 0.8%] was added to the modified synthetic medium without NH_4NO_3 and with the best carbon source for ligninolytic enzyme production.

The effect of different concentrations of the best nitrogen sources for the enzyme production was studied in a medium with 20 mM, 40 mM, and 60 mM of nitrogen.

The effect of different microelements and their concentrations on the production of laccase and Mn-oxidizing peroxidases

The effect of different concentrations of Cu^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, and Se, in the forms of: $CuSO_4$ x 7 H_2O, $MnSO_4$ x H_2O, $FeSO_4$ x 7 H_2O, $ZnSO_4$ x 7 H_2O, Na_2SeO_3, Na_2SeO_4, SeO_2, on Lac, and the peroxidase production was stu-

died in the modified synthetic medium with the grapevine sawdust, as the carbon source, and NH_4NO_3, as nitrogen source, at nitrogen concentration of 30 mM. The analyzed microelement concentrations were: *(i)* Cu^{2+} in the concentrations of: 0; 1 mM; 5 mM; 10 mM; *(ii)* Mn^{2+} in the concentrations of: 0; 1 mM; 3 mM; 5 mM; *(iii)* 1 mM of Zn^{2+}, Fe^{2+} or Se.

Enzyme activity assays

Laccase activity was assayed using syringaldazine as a substrate, and by measuring the increase in absorbance at 525 nm (ε 525 = 65000 M^{-1} cm^{-1}) for 60 seconds. The mixture contained: 0.1 M acetic buffer (pH 5.0), 1 mM syringaldazine (dissolved in 96% of ethanol), and enzyme preparation (V_{tot} = 1 ml).

Activities of Mn-oxidizing peroxidases were determined by 3 mM phenol red as the substrate, in a buffer with the following contents: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt, pH 4.5 (ε 610 = 22000 M^{-1} cm^{-1}). The mixture contained: buffer, enzyme preparation, 2 mM H_2O_2, and phenol red, with or without 2 mM $MnSO_4$ (V_{tot} = 500 µl). Reaction was ceased by 2 M NaOH.

Enzymes purification

Purifications of Lac and Mn-oxidizing peroxidases were carried out on 10-day-old solid-state culture of *P. pulmonarius*, using FPLC AKTA explorer (Pharmacia Biotech. Sweden). The supernatant from the solid-state culture, after centrifugation, was filtrated using a 0.45 µm filter. The fluid was then concentrated by ultrafiltration with a PM-10 membrane (10 kDa, Amicon). The concentrated fluid was loaded onto a HITrap Q anion-exchange column (5 ml column, Pharmacia Biotech.) previously equilibrated with 20 mM sodium acetate, pH 6.0. After washing with 6 column volumes (cv) of 20 mM sodium acetate buffer, the enzyme was eluted by two linear NaCl gradients: 0—0.3 M in 30 cv, and 0.3—1.0 M in 10 cv. 1.5 ml of each fraction was collected. Lac activity of separated fractions was performed by 50 mM ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] in phosphate buffer, pH 6.0 (ε 436 = 29300 M^{-1} cm^{-1}), and Mn-oxidizing peroxidases activities by 3 mM phenol red.

RESULTS AND DISCUSSION

Effect of different carbon sources on the production of laccase and Mn-oxidizing peroxidases

P. pulmonarius produced Lac under both submerged and solid-state conditions, while the peroxidases production was significant only in solid-state cultures (Table 1). SSF conditions of grapevine sawdust were the best for Lac production (391 Ul^{-1}, after 10 days of cultivation), in comparison to other carbon sources.

Tab. 1 — Laccase activity in *Pleurotus pulmonarius* depending on the carbon source in the medium

Species	Strain (No.)	Carbon source	Period of cultivation (days)	Lac activity (Ul^{-1})
P. pulmonarius	572	Glucose	7	2.82 ± 1.05
			10	4.80 ± 0.08
		Maltose	7	2.2 ± 0.3
			10	6.9 ± 0.4
		Mannitol	7	2.6 ± 1.1
			10	5.00 ± 1.06
		D-gluconic acid sodium salt	7	7.24 ± 0.02
			10	18.4 ± 0.5
		Xylan	7	22.7 ± 2.5
			10	12 ± 2
		Cellulose	7	7.5 ± 0.7
			10	15.0 ± 1.8
		Carboxymethyl cellulose	7	10.2 ± 0.3
			10	16.2 ± 1.1
		Dry ground mandarin peels	5	2.45 ± 0.08
			7	9 ± 4
		Grapevine sawdust	7	350 ± 45
			10	391 ± 18

SSF conditions of wheat bran were also the best for Lac production by a Brazilian strain of *P. pulmonarius*, and under these conditions, Lac was the main enzyme produced by this mushroom (Marques De Souza, C. G., 2002).

Regarding significant Mn^{2+} concentration in grapevine sawdust (Kilby, 1999), and the fact that only trace Mn^{2+} concentrations could be enough for MnP action (Martínez, 1996), in this case, it can not be spoken about MnP and VP activities, but only about activity against phenol red, in presence and absence of external Mn^{2+}, respectively. Activity against phenol red in presence of external Mn^{2+} increased during cultivation, while the activity in absence of external Mn^{2+} decreased (Table 2).

After purification of extracellular crude enzymes from 10-day-old culture of *P. pulmonarius*, grown in the medium with the most optimum carbon source (grapevine sawdust), three peaks of Lac activity (153 Ul^{-1}, 477 Ul^{-1}, 3018 Ul^{-1}), and one peak of activity against phenol red, in absence of external Mn^{2+} (11.71 Ul^{-1}), were observed, which was in accordance with results of Camarero et al. (1996), who also obtained one peroxidase peak by purification of 15-day-old SSF extracts of wheat straw.

Effect of different nitrogen sources and concentrations on the production of laccase and Mn-oxidizing peroxidases

P. pulmonarius produced Lac and peroxidases under SSF conditions with all investigated nitrogen sources and concentrations (Table 2 and 3).

Tab. 2 — Laccase activity and activity against phenol red in presence and absence of external Mn^{2+} in *Pleurotus pulmonarius* depending on the nitrogen source in the medium

Species	Strain (No.)	Nitrogen source	Period of cultivation (days)	Lac activity (Ul^{-1})	Activity against phenol red (Ul^{-1})	
					+ Mn^{2+}	– Mn^{2+}
P. pulmonarius	572	Peptone	7	352.4 ± 21.3	48.1 ± 4.4	12.4 ± 0.8
			10	101.8 ± 7.6	54 ± 28	11.3 ± 0.9
		Casein acid hydrolysate vitamin free	7	368.6 ± 22.6	29.7 ± 2.4	15.6 ± 0.8
			10	276.9 ± 16.2	44.3 ± 7.8	19 ± 3
		Corn step liquor	7	273.3 ± 16.7	56.8 ± 5.1	16.6 ± 0.9
			10	251.8 ± 0.7	37.4 ± 4.1	14.9 ± 3.7
		KNO_3	7	257 ± 22	59.0 ± 3.2	13.4 ± 0.3
			10	201.9 ± 14.5	31.9 ± 0.7	20.6 ± 2.2
		NH_4Cl	7	325.6 ± 30.2	32.2 ± 5.8	9.6 ± 0.3
			10	441.0 ± 11.6	41.0 ± 0.5	3.8 ± 0.9
		$(NH_4)_2SO_4$	7	335.8 ± 1.9	40.3 ± 2.3	10.1 ± 0.5
			10	311.7 ± 6.7	69.6 ± 3.0	4.1 ± 1.3
		$NH_4H_2PO_4$	7	240.2 ± 106.9	55.02 ± 2.05	13.1 ± 1.3
			10	310.2 ± 32.6	61.2 ± 3.8	4.6 ± 0.1
		NH_4NO_3	7	281.4 ± 24.3	71.6 ± 2.6	12.6 ± 0.5
			10	159.1 ± 10.8	75.8 ± 2.1	4.6 ± 1.6

Tab. 3 — Laccase activity and activity against phenol red in presence and absence of external Mn^{2+} in *Pleurotus pulmonarius* depending on the nitrogen concentration in the medium

Species	Strain (No.)	Nitrogen source	Nitrogen concentration	Period of cultivation (days)	Lac activity (Ul^{-1})	Activity against phenol red (Ul^{-1})	
						+ Mn^{2+}	– Mn^{2+}
P. pulmonarius	572	NH_4NO_3	20 mM of N	710	344.1 ± 1.7	32.4 ± 0.1	11.16 ± 0.003
					384 ± 2	5.13 ± 0.04	16.97 ± 0.02
			40 mM of N	710	282.7 ± 1.2	40.63 ± 0.08	10.28 ± 0.05
					177.3 ± 4.1	2.55 ± 0.01	14.3 ± 0.1
			60 mM of N	710	168.7 ± 0.8	34.27 ± 0.03	11.28 ± 0.04
					222.6 ± 1.7	4.0 ± 0.1	12.4 ± 0.1

The Lac level was the highest in the medium with NH_4Cl, after 10 days of cultivation (441.0 Ul^{-1}), (Table 2). The peak of activity against phenol red, in presence of external Mn^{2+} was found in the medium with NH_4NO_3, at nitrogen concentration of 30 mM. This activity increased during cultivation, while activity against phenol red, in absence of external Mn^{2+}, decreased in the media with almost all investigated nitrogen sources (Table 2).

These results are in accordance with the results of K a a l et al. (1995), who have also noted more significant lignin mineralization by *P. ostreatus*, in the presence of the high nitrogen concentration, in the form of glutamate, than in the nitrogen limited medium. On the contrary to those results, H a m m e l (1997) and M a s t e r and F i e l d (1998) emphasized that the ligninolytic enzymes are produced during the fungal secondary metabolism, under condi-

tions of limited nitrogen. Martinez et al. (1996) found MnP production in *P. eryngii, P. ostreatus, P. pulmonarius*, and *P. sajor-caju* in peptone medium, but it was not detected in media with other investigated organic nitrogen sources (corn-step liquor, malt extract, and ammonium tartrate).

Effect of different microelements concentrations on the production of laccase and Mn-oxidizing peroxidases

P. pulmonarius produced Lac and Mn-oxidizing peroxidases in the media with all investigated Cu^{2+} and Mn^{2+} concentrations (Table 4). Lac activity showed the highest level at Cu^{2+} and Mn^{2+} concentration of 5 mM, respectively (413.19 Ul^{-1} and 345.59 Ul^{-1}, respectively, on the 7th day of cultivation), (Table 4).

Metal ions (Cu, Mn, Cd, Hg, Pb) are an important group of modulators of ligninolytic enzyme activity (Wariishi et al., 1988; Baldrian and Gabriel, 2002). Cu^{2+} has an important role in Lac synthesis, regulating gene transcription (Palmieri et al., 2000; Galhaup and Haltrich, 2001), as well as influencing the enzyme activity and stability (Baldrian and Gabriel, 2002). Palmieri et al. (2001) explained the positive effect of Cu^{2+} on Lac synthesis by the results which showed that Cu^{2+} concentration of 1 mM decreases the activity of extracellular protease (to 77%), which is responsible for Lac degradation. Likewise, Cu^{2+} concentration of 1 mM also had a stimulatory effect on the Lac production in *Trametes pubescens*, and the optimal concentrations were 1.5—2.0 mM (Galhaup and Haltrich, 2001). Mn^{2+}, Fe^{3+}, Zn^{2+} did not show any significant effect on total Lac activity, but they influenced the production of the different Lac isoenzyme (Palmieri et al., 2000).

The highest levels of phenol red oxidation, in presence and absence of external Mn^{2+}, were obtained in the medium with 1 mM Cu^{2+} (45.00 Ul^{-1}, on the 10th day, and 65.25 Ul^{-1}, on the 7th day of cultivation, respectively). Peaks of these activities were noted in the medium without, and with 1 mM Mn^{2+}, respectively (62.8 Ul^{-1}, on the 10th day, and 67.3 Ul^{-1}, on the 7th day of cultivation) (Table 4).

Mn^{2+} is both an active mediator for MnP, and a regulator of MnP, Lac, and lignin peroxidase production (Kerem and Hadar, 1993). Bonnarme and Jeffries (1990) showed that levels of MnP production in Basidiomycetes were higher in the medium with $MnSO_4$ or $MnCl_2$ (up to 24 times), in comparison with those in the medium without Mn^{2+}. Martínez et al. (1996) and Camarero et al. (1996) showed that MnP production in *P. eryngii, P. ostreatus, P. pulmonarius*, and *P. sajor-caju*, cultivated under submerged and solid-state conditions, depended on the Mn^{2+} concentration. Martinez et al. (1996) obtained the highest level of the peroxidase activity at *P. eryngii* cultivation in the glucose/peptone/yeast extract medium, without Mn^{2+}, under SF conditions, and purified two peaks with Mn-independent activity. However, Mn^{2+} concentration of 5 μM produced significant decrease (approximately 90%), and no peroxidase activity was found at Mn^{2+} concentration of 25 μM.

Tab. 4 — Laccase activity and activity against phenol red in presence and absence of external Mn^{2+} in *Pleurotus pulmonarius* depending on the concentration of Cu^{2+} and Mn^{2+} in the medium

Species	Strain (No)	Concentration of Cu^{2+} and Mn^{2+}	Period of cultivation (days)	Lac activity (Ul^{-1})	Activity against phenol red (Ul^{-1})	
					+ Mn^{2+}	– Mn^{2+}
P. pulmonarius	572	Control (-Cu)	710	308.37 ± 9.36 216.49 ± 11.66	14.09 ± 0.31 46.73 ± 0.07	58.22 ± 3.06 58.91 ± 0.96
		1mMCu	710	355.21 ± 34.56 209.13 ± 1.30	13.79 ± 1.12 45.00 ± 1.11	65.25 ± 2.22 25.46 ± 4.95
		5mMCu	710	413.19 ± 6.72 147.92 ± 28.54	9.83 ± 0.79 15.64 ± 0.38	13.35 ± 1.34 23.69 ± 1.86
		10mMCu	710	358.54 ± 6.00 287.32 ± 61.03	3.71 ± 0.40 27.33 ± 6.64	8.66 ± 0.40 14.39 ± 1.03
		Control (-Mn)	710	308.90 ± 8.97 199.57 ± 13.63	17.41 ± 1.66 62.87 ± 9.54	37.28 ± 1.25 9.25 ± 1.00
		1mM Mn	710	295.62 ± 8.90 87.10 ± 14.47	17.14 ± 0.62 38.14 ± 2.86	67.33 ± 1.01 59.12 ± 6.67
		3mM Mn	710	309.60 ± 20.04 185.18 ± 5.45	17.10 ± 1.19 17.37 ± 1.93	58.49 ± 5.77 45.15 ± 5.14
		5mM Mn	710	345.59 ± 19.00 229.95 ± 18.71	20.06 ± 0.91 58.45 ± 1.63	61.91 ± 0.53 54.62 ± 4.11

The addition of Zn^{2+} and Fe^{2+} to the medium, at a concentration of 1 mM, led to decrease of Lac activity compared to the control. The presence of the same concentration of Se, in the forms of Na_2SeO_3 and SeO_2, caused an increase of Lac activity, while activity in Na_2SeO_4-enriched medium was similar to that in the control. The presence of Zn^{2+} or Fe^{2+} in the medium caused a decrease, and the presence of Se a slight increase of phenol red oxidation levels in both presence and absence of external Mn^{2+}. According to Baldrian and Gabriel (2002), Zn^{2+} added to liquid nitrogen-limited medium, at a concentration of 1 mM, caused a decreased Lac activity, which is contrary to these results. There is no literature data about the influence of Fe and Se on the production of Lac and Mn-oxidizing peroxidases by *P. pulmonarius*.

This study presents another attempt of finding the optimal conditions for successful cultivation of *P. pulmonarius*, and production of the ligninolytic enzymes, which have the most important role in the processes of biotransformation of raw plant materials to feeds and fuels, and paper manufacturing.

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УТИЦАЈ УСЛОВА КУЛТИВАЦИЈЕ НА ПРОДУКЦИЈУ ЛИГНИНОЛИТИЧКИХ ЕНЗИМА КОД *PLEUROTUS PULMONARIUS*

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Резиме

Највиши ниво лаказне активности (391 Ul^{-1}) и значајна продукција Mn-оксидујућих пероксидаза нађени су у чврстој култури са пиљевином винове лозе као извором угљеника. После пречишћавања екстрацелуларне сирове смеше ензима *P. pulmonarius* која је расла на подлози са најбољим извором угљеника (пиљевина винове лозе), забележена су три пика лаказне активности. Пречишћавањем смеше је такође показано да су нивои оксидације фенол реда у одсуству спољашњег Mn^{2+} били виши него нивои оксидације фенол реда у присуству спољашњег Mn^{2+}. Највиша лаказна активност је била у подлози са пиљевином винове лозе, као извором угљеника, и NH_4Cl при концентрацији азота од 30 mM (441 Ul^{-1}). Најбољи извор азота за продукцију Mn-оксидујућих пероксидаза је био NH_4NO_3 при концентрацији азота од 30 mM. Највећа лаказна активност је забележена у присуству 5 mM Cu^{2+} односно 5 mM Mn^{2+}. Mn-оксидујуће пероксидазе су показале пик активности у одсуству Cu^{2+} и Mn^{2+}, као и њиховом присуству у концентрацији од 1 mM. Присуство Zn^{2+} и Fe^{2+} су проузроковали смањење, а Se, у свим проучаваним формама, повећање и лаказне и пероксидазних активности.

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PRODUCTION OF EXO-PECTINASE BY *PENICILLIUM ROQUEORTI* USING PUMPKIN OIL CAKE

ABSTRACT: Submerged fermentation (SmF) was carried out using the pumpkin oil cake (PuOC) as a substrate for the production of exo-pectinase (exo-p) by *Penicillium roqueforti*. PuOC was used as carbon, nitrogen and mineral source for fungi growth and exo-p production. SmF was carried out in the production medium containing 0.2% KH_2PO_4, 0.1% $MgSO_4$ and 0.1% NaCl, supplemented with PuOC at 2% concentration. The obtained results show that *P. roqueforti* cultivated in a liquid medium in the presence of PuOC as a source of nitrogen, produced significant amount of exo-p, activity 161.03 U/ml.

KEY WORDS: pectinase, pumpkin oil cake, *Penicillium roqueforti*, submerged fermentation

INTRODUCTION

Agro-industrial residues are generally considered as convenient substrates for biotechnological processes (Pandey et al., 2000). Wheat bran (Sumantha et al., 2005, Taragano et al., 1997), sugar beet (Solis-Pereira et al., 1993), coffee pulp (Boccas et al., 1994), lemon peel (Siessere et al., 1989), apple pomace (Peričin et al., 1992), grape pomace (Boltella et al., 2007) and deseeded sunflower head (Patil, 2006), have been employed for the cultivation of microorganisms to produce pectinase. In recent years there has been an increasing trend towards efficient utilization and value-addition of oil cakes. Biotechnological process has contributed enormously to such reutilization. There are several reports describing the application of sunflower oil cake (SuOC), sesame oil cake (SOC), soy-bean cake (SBC), coconut oil cake (COC) and olive oil cake (OOC) for microbial production of enzymes (Ramachandran et al., 2006). In this respect, use of pumpkin oil cake (PuOC), as fermentation media, may be of an interest.

PuOC cake is a by-product obtained after oil extraction from dried pumpkin seed. Its only use is to feed animals and no other application has been found, yet. Due to their composition (soluble sugars, crude proteins, crude fi-

bers, lipids and trace amounts of minerals), PuOC could serve as a good substrate for SSF and SmF production of enzymes.

Microorganisms, especially fungi, owing to their GRAS (Generally Regarded As Safe) nature, have now become popular, especially with respect to enzyme application in the food industries (P a n d e y, 1992). *Aspergillus* and *Penicillium* sp. are the predominant fungal sources of the pectinase (B o t e l l a et al., 2007, B l a n d i n o et al., 2001).

Penicillium roqueforti is a filamentous fungus used in the dairy industry, and plays an important role in blue cheeses production. Although *P. roqueforti* is able to produce several mycotoxines, such as PR-toxin, eremofortin, roquefortin C, mycophenolic acid patulin, penicillic acid and isofumigiclavins, these toxins are unstable or of low concentration (V a l i k et al., 1999). Some strains that are considered as GRAS are used as starters, for cheese production.

There are several reports describing the use of oil cake for producing enzymes by different fungi, but there is no data about *P. roqueforti.*

The objective of this work was to investigate the potential of PuOC as a substrate for the production of exo-p using a GRAS strain of *P. roqueforti* in submerged fermentation (SmF). To the best of our knowledge this is the first report on pectinase production using PuOC.

MATERIAL AND METHODS

Microorganisms and maintenance of culture

Penicillium roquefroti was used in this study. The fungi was grown and maintained on potato dextrose agar (PDA) slants. Spore suspension of 10^8 spores/ml in 0.1% of Tween 80, produced from one-week-old fully sporulated slant, was used for inoculation.

Substrates

Substrates used in the study were PuOC. Chemical composition is shown in Table 1.

Tab. 1 — Chemical composition of substrate (all values expressed as percent)

Constituent	Value
Dry weight	97,62
Crude protein	63.52
Crude fiber	4.50
Crude fat	8.66
Soluble sugar	1.10
Ash	8.80

Media and fermentation procedure

SmF was carried out by taking 50 ml of the production medium, containing 0.2% KH_2PO_4, 0.1% $MgSO_4$ and 0.1% NaCl in 300 ml Erlenmeyer flask. This was supplemented with PuOC at 2% of the concentration. All the flasks were autoclaved at 121.5°C for 20 min and after cooling were inoculated with 1 ml of spore suspension and incubated on a rotary shaker at 180 rpm at 26°C for a desired period.

During the fermentation, samples (inoculated and without inoculate) were collected from the shaker at regular intervals during 24 h (every day from the beginning of the process) for further analysis. Samples were filtrated, and filtrate was used for measuring the pH, reducing sugars, proteins and exo-pectinase activity. Solids were used for measuring the cell dry weight.

Biomass production measurement

Biomass production was measured as dry weight (DW). DW measurement was based on differences between weight of filter paper measured before and filter paper with biomass, measured after drying.

After filtration, filtrate was removed and solids on the filter papers were dried at 40°C for about 2 days, until a constant weight was obtained.

Enzyme assay

Exo-pectinase (exo-p) activity was measured according to the literature (Aguilar et al., 1990). One unit was defined as the amount of enzyme that catalyzed the formation of 1 μmol of galacturonic acid per hour at pH 5.0.

Analytical methods

Protein content was determined by the method of Lowry (1951), with BSA as a standard.

Concentration of reducing sugars was examined by DNS method by Miller (1959).

RESULTS

As it can be seen in Fig. 1, it was possible to grow *P. roqueforti* using PuOC as the sole nutrient source in the culture medium. *P. roqueforti* biomass was determined by measuring DW. Growth of *P. roqueforti* was low during the first five days, but later an increase was obtained. This trend can be explained considering both the rate of suspended solids consumption and the rate of microorganism growth. Moreover, it is necessary to consider that DW measurements from the bulk liquor include all solids present in the fermenta-

tion broth. In this respect, we decided to measure both the inoculated medium and the same one, but without inoculate (WI). The obtained results are in correlation with previous works (B l a n d i n o et al., 2001, W e b b et al., 1997).

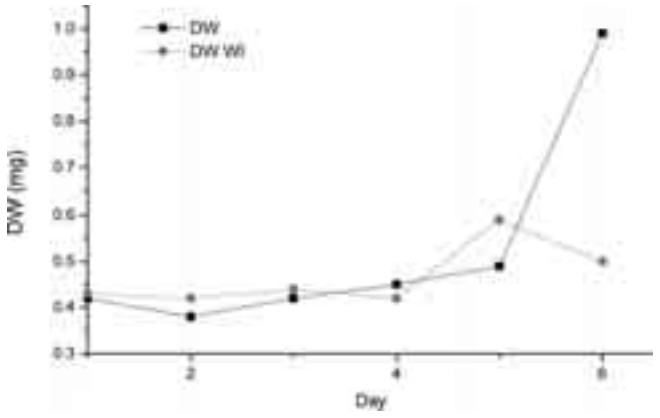

Fig. 1 — Dry weight time course in inoculated medium and in one without inoculate

Fig. 2 showed that the production of exo-p was significant, under these experimental conditions.

As it can be seen, exo-p showed two-phase kinetics. In the first stage, a continuous increase in exo-p activity was observed, obtaining the highest value on the 3rd day. This phase is in correlation with lag phase of growth of *P. roquefotri* (Fig. 1). In the second stage, 6th day of fermentation, second maximum was reached. In the beginning, fungi used for its growth the fermentable sugars and synthesed basal activity of exo-p, which was followed by the maximum activity of 111.75 U/ml. When the easy-degradable sugars were exhausted from the medium, and basal activity of exo-p were secreted, the

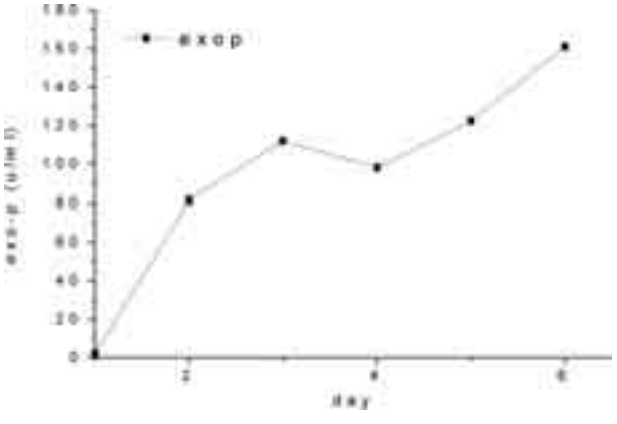

Fig. 2 — Time course of exo-pectinase production during cultivation in shake flasks

fungi started to degrade more complex compounds and exo-p activity started increasing more, after the fourth day of the inoculation. New obtained maximum was 161.03 U/ml. Hydrolysing polysaccharides, the amount of reduced sugar increased, although the fungi were using them for their growth (Fig. 3).

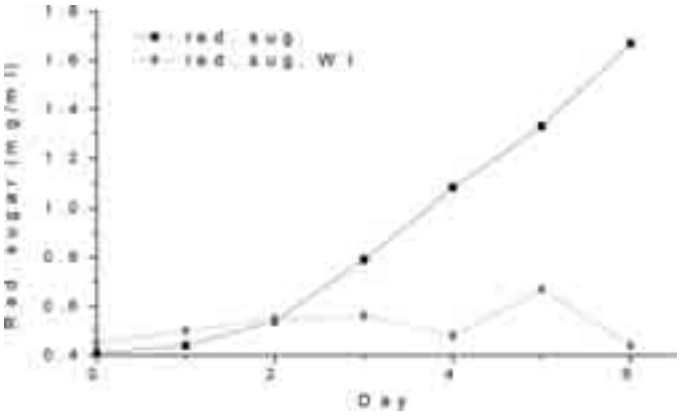

Fig. 3 — Time course of reducing sugar during submerged fermentation of *P. roqueforti*

Fig. 4 illustrated the comparison of time course of protein concentration during the fungi cultivation, in fermentation medium and in medium without inoculums (WI). The obtained results show an increase of protein amount, in inoculated medium, which is in correlation with the production of enzyme secretion, shown in Fig. 2.

The crude extract of the pectinase *P. roqueforti* showed high activity after five days of cultivation, as it was shown in Fig. 1, which was followed with the increase of pH in fermentation medium (Fig. 5).

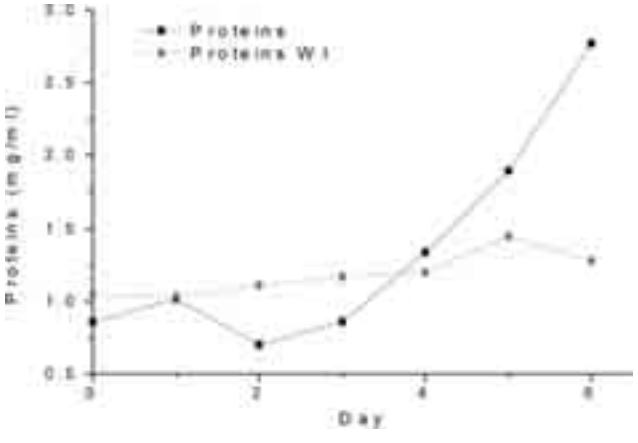

Fig. 4 — Time course of soluble protein during submerged fermentation of *P. roqueforti*

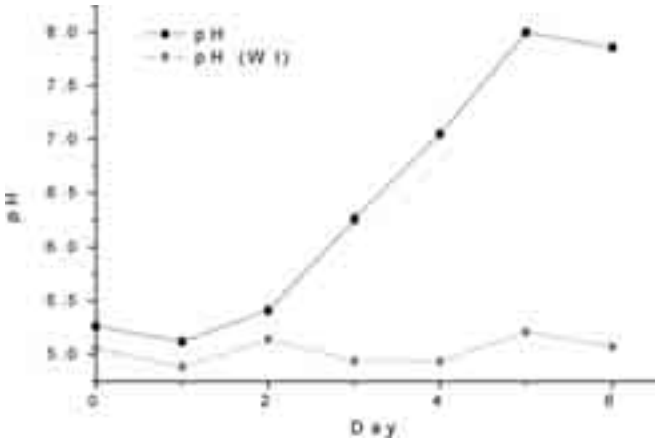

Fig. 5 — Time course of pH during submerged fermentation of *P. roqueforti*

DISCUSSION

Based on the results, we found that *P. roqueforti* cultivated in a liquid medium in the presence of PuOC as a source of nitrogen, produced significant amount of exo-p. Firstly, fungi used the fermentable sugars from the cake for their growth, and later they started consuming more complex compounds, which led to higher concentration of reducing sugars in the cultivation medium. After six days of fermentation the amount of reducing sugars in medium with and without inoculation, were 1.67 and 0.44 mg/ml, respectively. It should be also, pointed out, that with enzyme production, pH increases, which is not in agreement with the findings of Friderich et al., 1989, who stated that the growth of *A. niger* was followed with acidification and decreasing of pH.

ACKNOWLEDGEMENTS

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ПРОДУКЦИЈА ЕГЗО-ПЕКТИНАЗА ИЗ *PENICILLIUM ROQUEFORTI* НА БУНДЕВИНОЈ УЉАНОЈ ПОГАЧИ

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Резиме

Циљ овог рада је био истраживање микробиолошке конверзије и ревалоризације бундевине уљане погаче (PuOC) помоћу биотехнолошких поступака. Изведена је субмерзна култивација помоћу плесни *Penicillium roqueforti* (ГРАС статус), а у циљу добијања егзо-пектиназа (егзо-п).

Субмерзна ферментација (СмФ) изведена је у течној подлози са 0.2% KH_2PO_4, 0.1% $MgSO_4$ и 0.1% NaCl, у коју је додато 2% PuOC. Добијени разултати показују да је продукција егзо-п помоћу *P. roqueforti* у оваквој подлози могућа, да се ензим покорева двостепеној кинетици и да има први максимум активности, вредности 111.75 U/ml, трећег дана култивације и други максимум, чија је вредност 161.03 U/ml, шестог дана процеса.

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EFFECTS OF *LACTOBACILLUS* SPP. ON MOULD AND YEAST GROWTH IN THE ENSILING PROCESS

ABSTRACT: The paper includes the results of effect of preparations Biomax SI (*Lactobacillus plantarum*) and Biomax WS (*L. plantarum* and *Pediococcus pentosaceus*) on mould and yeast growth in the ensiling process. The following has been analyzed: total number of mould and yeast, specific rate of mould and yeast growth, pH, content acid (lactic, acetic and butyric acid), moisture and temperature in bales during 30 days of lucerne ensiling.

KEY WORDS: bacterial inoculates, Biomax SI, Biomax WS, ensilage of lucerne, lactic acid bacteria, *Lactobacillus plantarum*, lucerne

INTRODUCTION

In the case of intensive animal production concept of feed production should be based on the maximum use of natural resources. The aim of this concept is the produce enough quantity of ponderous feed during the whole year. Ponderous feed is mostly made from hay, silage and ensilage of lucerne, grass leguminoze mixture, silage of corn etc. There is a need for higher quality of lucerne ensilage during the whole year. In order to provide the best quality ensilage, bacterial inoculates, Biomax SI and Biomax WS, were used.

Using bacterial inoculates as additives, which main metabolic production is lactic acid, is a natural way to intensify the process of fermentation in silage, and decrease the pH value in order to provide efficient conserving of nutritive components. The importance of using bacteria as additives for ensiling is in numerous advantages they have over chemical additives: easy use, safe action, they are not corrosive for machines, and they do not pollute the environment (Antov et al., 2004).

According to Bolsen (1999) and Bolsen et al. (2000), in USA 150 additives are used, of which 80 are bacterial additives. There are 203 additives available on the market of the European Union, of which 87 are bacterial additives.

The main aim of this research was to investigate the effects of Biomax SI and Biomax WS preparations on moulds and yeasts during the ensilage process of lucerne.

MATERIAL AND METHODS

In these experiments, ensilage of lucerne, harvested in 2005 in Vojvodina, on the parcels of company "PIK-Bečej" A.D. "Poljoprivreda" was used. In the first experiment, harvested lucerne was treated with Biomax SI. In 100g of Biomax SI 100L water without chlorine was added, and well homogenized. Two litres of this solution were sprayed on one tone of lucerne sheeted to 20—30 cm layer. Then, lucerne baling, using six layered of polyethylene foil was performed by Krone press machine and bales of 500kg weight were formed. In the second experiment, 60g of Biomax WS was diluted with 200L of water without chlorine and well homogenized. Two litres of this solution were sprayed on one tone of lucerne, and bales of 500kg weight were formed in the same way as in the first experiment. The samples were taken from the bales treated with Biomax SI and Biomax WS, during the first six days and then on the 12^{th}, 18^{th}, 24^{th} and 30^{th} day of ensiling.

Biomax SI is an additive for silage of grass, clover, fresh lucerne, corn and silage of whole plant. It is produced by CHR Hansen Denmark and it contains *Lactobacillus plantarum* (50 billions CFU/g).

Biomax WS is an additive for silage of corn and whole plant. It is produced by CHR Hansen Denmark and it contains *Lactobacillus plantarum* and *Pediococcus pentosaceus* (50 billions CFU/g).

All used materials were sampled according to the feed sampling methods regulated by the "Pravilnik o metodama uzimanja uzoraka i metodama fizičkih, hemijskih i mikrobioloških analiza — Sl. list SFRJ br. 15/1987".

Investigation of the effects of Biomax SI and Biomax WS preparations on moulds and yeasts, during the ensilage process of lucerne, was performed in two experiments. In the first experiment, the samples were taken from bales treated with Biomax SI, during the first six days, and then on the 12^{th}, 18^{th}, 24^{th} and 30^{th} day of ensiling for performing microbiological and chemical analyses. Microbiological investigation included the determination of total number of moulds and yeasts, and specific rate of mould and yeast growth. The following analyses were done: measurements of pH value, acid content (lactic, acetic and butyric acid), moisture and temperature in bales of lucerne during 30 days of ensiling. In the second experiment, the samples were taken from bales treated with Biomax WS, in the same manner as described above.

The evaluation of total number of moulds and yeasts was performed according to the indirect Koch's method (Škrinjar, 2001).

The evaluation of specific rate of mould and yeast growth was achieved using the equation (Pejin, 2003): $\mu = (\ln N(t) - \ln N(o))/t$ (μ — specific rate of microorganism growth, N (t)-number of microorganisms in time t, N (o)-number of microorganisms in time zero, i.e. at the beginning, t-time).

Chemical analyses (measurements of pH value, acid content (lactic, acetic and butyric acid content by destilation), moisture and temperature in bales of

lucerne) were performed according to the methods of Kolarski and Pavličević (1970).

RESULTS

The effects of Biomax SI and Biomax WS preparations on the total number of moulds are presented in Table 1 and Figure 1.

Tab. 1 — Effects of preparations Biomax SI and Biomax WS preparations on total number of moulds during 30 days of lucerne ensiling.

NM	1st day	2nd day	3rd day	4th day	5th day	6th day	12th day	18th day	24th day	30th day
SI	1.280	3.450	3.650	3.200	2.580	1.580	0	0	0	0
WS	1.950	4.550	5.500	5.450	4.200	2.900	0	0	0	0

NM — number of moulds; SI — Biomax SI; WS — Biomax WS

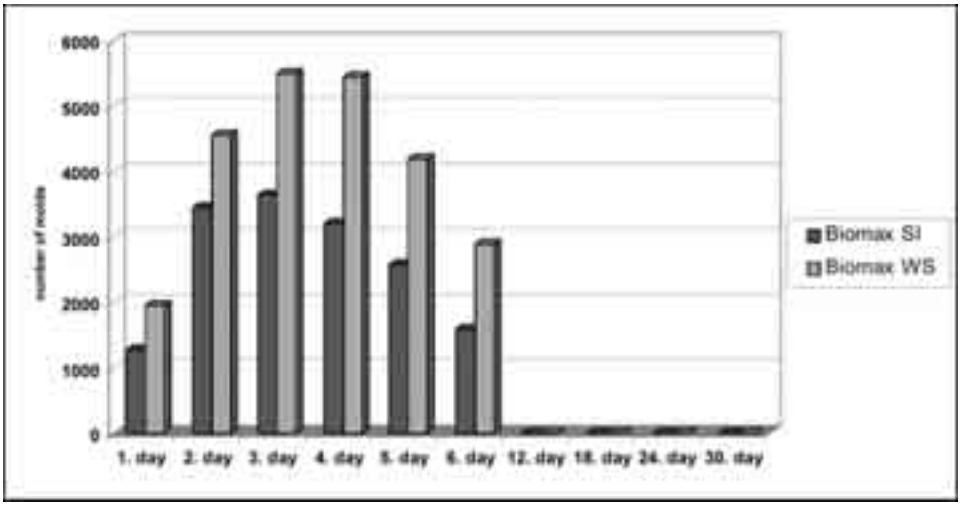

Fig. 1 — Effects of Biomax SI and Miomax WS preparations on total number of moulds during 30 days of lucerne ensilage

The effects of preparations Biomax SI and Biomax WS preparations on the specific rate of mould growth are presented in Figure 2.

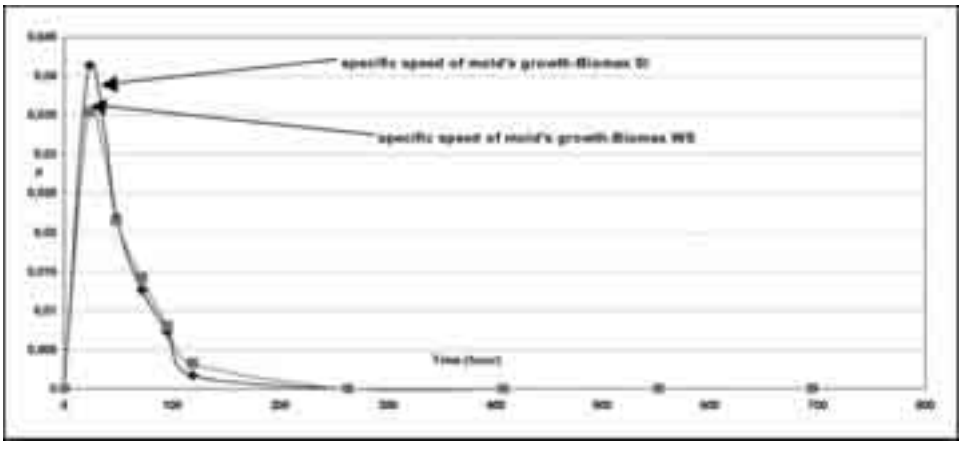

Fig. 2 — Specific speed of mould growth during 30 days of ensiling lucerne treated with Biomax SI and Biomax WS

The effects of Biomax SI and Biomax WS preparations on total number of yeasts are presented in Table 2. and Figure 3.

Tab. 2 — Effects of Biomax SI and Biomax WS preparations on total number of yeasts during 30 days of lucerne ensiling

NY	1st day	2nd day	3rd day	4th day	5th day	6th day	12th day	18th day	24th day	30th day
SI	180	650	1.000	1.110	1.130	1.120	120	110	0	0
WS	260	710	1.250	1.350	1.400	1.390	150	120	0	0

NY — number of yeasts (x 10^6); SI — Biomax SI; WS — Biomax WS

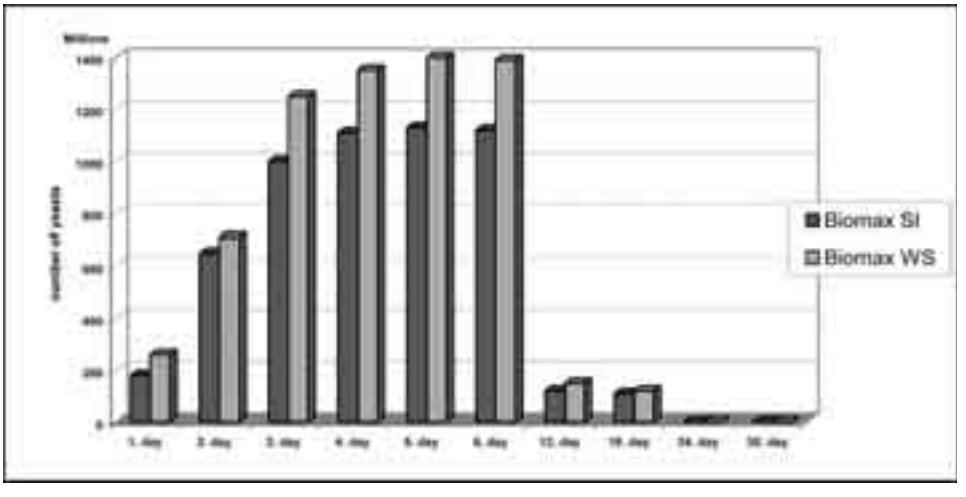

Fig. 3 — Effects of Biomax SI and Biomax WS preparations on total number of yeasts during 30 days of lucerne ensilage

The effects of Biomax SI and Biomax WS preparations on the specific rate of yeast growth are presented in Figure 4.

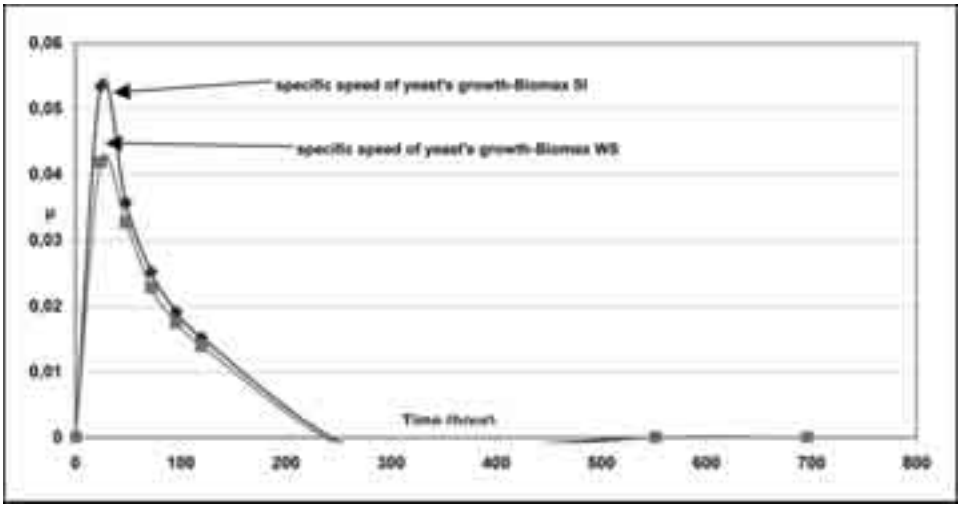

Fig. 4. — Specific speed of yeast growth during 30 days of ensiling lucerne treated with Biomax SI and Biomax WS

Figure 5. represents the changes in pH value of lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling.

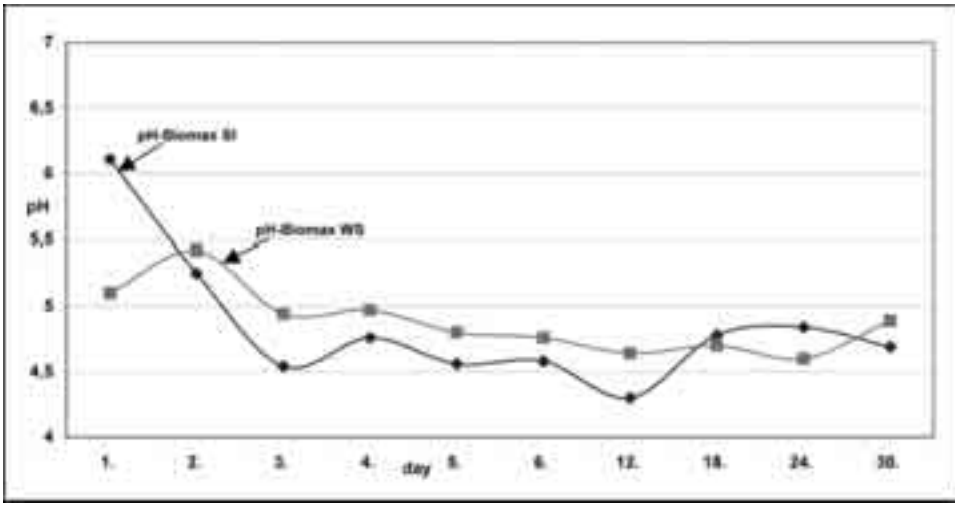

Fig. 5 — pH value in lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling

Figure 6. represents the percentage of lactic acid in lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling, and Figure 7.

represents the percentage of free and total acetic acid in lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling.

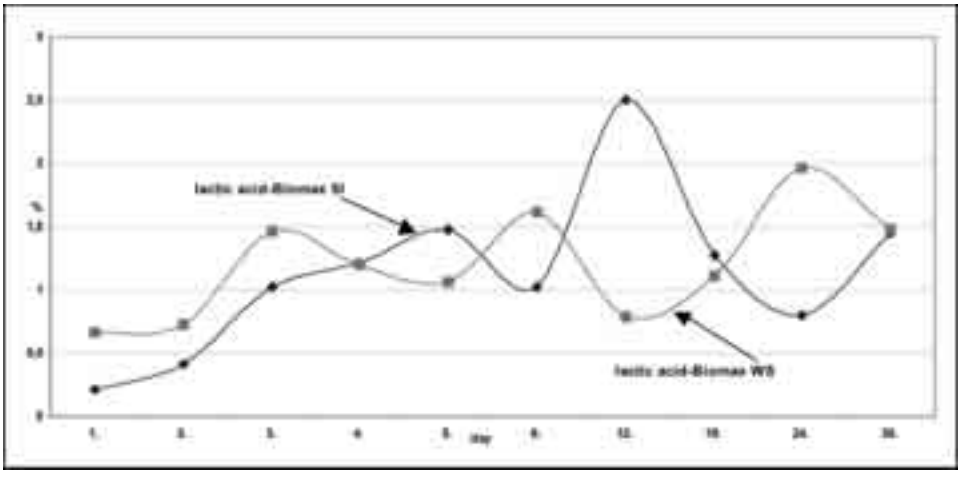

Fig. 6 — The percentage of lactic acid in lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling

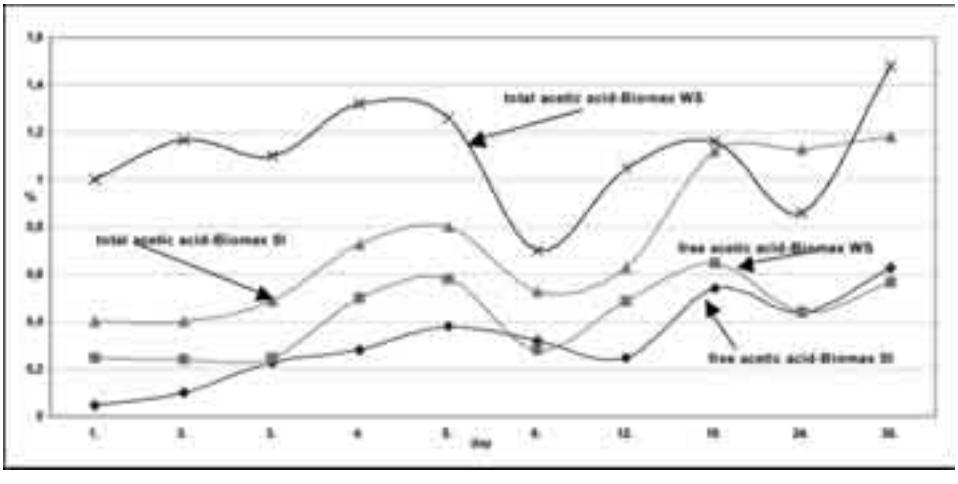

Fig. 7 — The percentage of free and total acetic acid in lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling

In Table 3. and Table 4. contents of free and total butyric acid in lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling are displayed.

Tab. 3 — Effects of Biomax SI and Biomax WS preparations on free butyric acid during 30 days of lucerne ensiling

FBA	1st day	2nd day	3rd day	4th day	5th day	6th day	12th day	18th day	24th day	30th day
SI	0	0	0	0	0	0	0	0	0	0
WS	0	0	0	0	0	0	0	0	0	0

FBA — free butyric acid; SI — Biomax SI; WS — Biomax WS

Tab. 4 — Effects of preparations Biomax SI and Biomax WS preparations on total butyric acid during 30 days of lucerne ensiling

TBA	1st day	2nd day	3rd day	4th day	5th day	6th day	12th day	18th day	24th day	30th day
SI	0	0	0	0	0	0	0	0	0,01	0
WS	0	0	0	0	0,01	0	0	0	0	0

TBA — total butyric acid; SI — Biomax SI; WS — Biomax WS

In Figure 8. and Fig. 9. moisture in lucerne, and temperature in bales of lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling are shown.

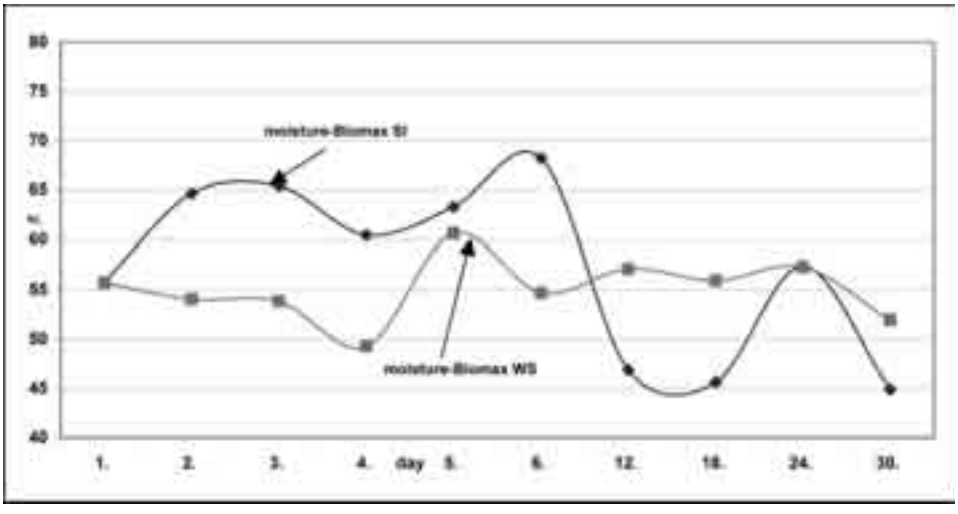

Fig. 8 — Moisture in lucerne during 30 days of ensiling treated with Biomax SI and Biomax WS

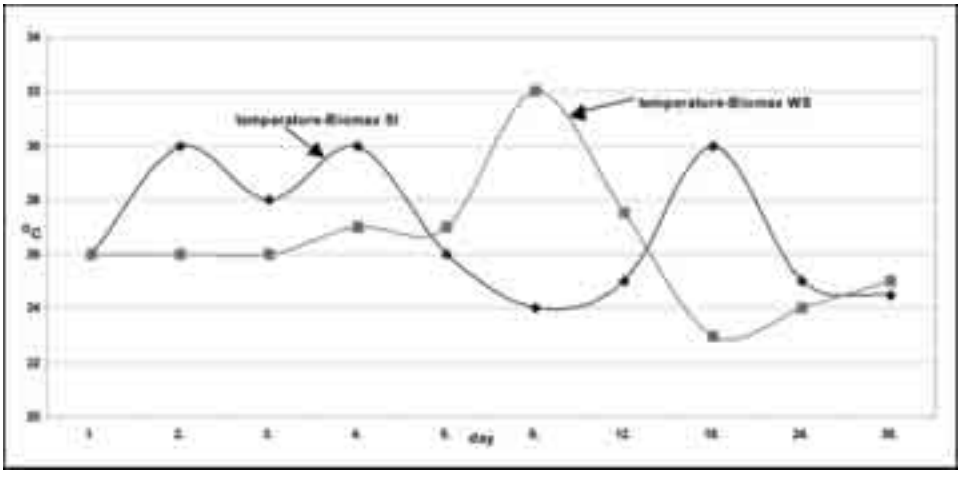

Fig. 9 — Temperature in bales of lucerne ensilage treated with Biomax SI and Biomax WS during 30 days of ensiling

DISCUSSION

From the obtained results, it can be seen that both preparations, Biomax SI and Biomax WS, completely supressed the growth of moulds on the 12th day of ensiling (Table 1, Fig. 1. and Fig. 2).

The presence of moulds in silage can change its savor, and nutritive value and decrease the content of carbohidrates and proteins in silage. Yet, the most important is that moulds in silage can disintegrate proteins and produce mycotoxins. The presence of moulds does not always mean the production of mycotoxins, but under certain conditions, the production of mycotoxins can be very fast and toxins can be very stable for a long period of time after the stunt of moulds. *Aspergillus fumigatus* is capable of producing toxic alkaloid klavin (E d w a r d s and M c D o n a l d, 1989).

The conditions that favour the growth of moulds are (A n t o v et al., 2004): moisture above 13%, relative moisture above 70%, temperature over 13°C, easily acceptable nutritive ingredients, pH value above 5, presence of oxygen.

The growth of moulds in silage will be inhibited by low pH value, however it can be supported by the presence of non fermentable carbohidrates.

Toxigenic strains of moulds can provoke intensive intoxications in humans and animals. Toxigenic strains belong to genera *Aspergillus*, *Fusarium*, *Penicillium*, *Stachybotris*, *Claviceps*, *Ustilago* and *Puccinia*. The strains grow and produce mycotoxins in food and feed only under appropriate conditions of moisture and temperature. One should bear this mind during storing and preserving feed, particularly grains (R a d a n o v - P e l a g i ć, 2000).

The number and types of filospherical microorganisms depend on: plant type, composition and amount of exudates, pH value, temperature and mois-

ture. According to Govedarica and Jarak (2001) there are 10^6 and 10^8 CFU/g of bacteria and yeasts on plants which are used for feed preparation. These findings are in accordance with the results gained in this paper. The initial number of yeasts was 180×10^6 in the ensilage of lucerne treated with Biomax SI (Table 1), and in the ensilage of lucerne treated with Biomax WS was 260×10^6 (Table 2).

From the results shown in Table 2. and Figure 3. it can be seen that both preparations (Biomax SI and Biomax WS) reduced the number of yeasts totally on the 24th day of ensiling.

According to Antov et al. (2004), the majority of yeasts grow on temperature between 0 and 37°C, but several of them are adapted to temperatures above 43°C. The number of yeasts increases if the temperature is higher than 40°C during the aerobic spoilage. They are more sensitive on higher temperatures than clostridiums. It has been noticed that the number of yeasts usually grow during wilting of sow, that is obvious for fermentative yeasts and yeasts that are using lactate. That explains why aerobic spoilage is more frequent when ensiling drier mass with low compactness. Since soil is a reservoir of yeasts, the possibilities of contamination during wilting are higher, especially after the mechanical turning over of wilting mass. Silages with yeast population that counts 10^5CFU/g are considered especially susceptible to aerobic spoilage. After two or three days of aerobic exposure the total number of yeasts can increase to 10^{12}CFU/g. Diffusion of oxygen is very important for population of yeasts that assimilate lactate. Yeasts usually grow in the range of pH values from 3 to 8, while the optimal level for the most of them ranges from 3,5 to 6,5. Under aerobic conditions, they tolerate the presence organic acids (lactic, acetic, citric, malatic, propionic, fumaric acid) better than other microorganisms.

It can be concluded that both preparations have the same effects on the specific rate of yeast growth during 30 days of lucerne ensiling (Figure 4).

Lucerne, grass, small grain corn with high moisture content, usually react well to microbiological inoculation with homofermentative lactic acid bacteria. Recapitulating the results of the researches that were published in the period 1985—1990, Muck and Bolsen (1990) concluded that in more than 2/3 in inoculated silages was a significant decrease in pH value and increase in lactic acid content, which is similar to the results obtained in this paper (Figure 5). In the experiment of lucerne ensiling with *Lactobacillus plantarum* (Antov and Čobić, 1991), the obtained lactic acid content varied from 8,94 to 10,95% in the silage with 30% of dry matter (d.m.) and in the silage with 50% d.m., the lactic acid content ranged from 4,59 to 6,91%, which is not in agreement with the results achieved in this paper, where the lactic acid content ranged from 0,80 to 2,50% for lucerne ensilage (44,20% d.m.), treated with Biomax SI and from 0,66 to 1,96% for lucerne ensilage (44,30% d.m.), treated with Biomax WS (Figure 6).

Regarding the effects of Biomax SI and Biomax WS preparations on acetic acid content (Figure 7) in lucerne ensilage (44,20% d.m.) treated with Biomax SI, the acetic acid content ranged from 0,40 to 1,13%, and in lucerne ensilage (44,30% d.m.) treated with Biomax WS, the acetic acid content ran-

ged from 0,86 to 1,48%, which is in conflict with the findings of Antov and Čobić (1991), where acetic acid content continuously decreased from 2,14 to 0,81% (30% d.m.) and from 0,98 to 0,51% (50% d.m.).

Percentage of free butyric acid was 0 in both treatments, while very low content of total butyric acid (0,01%) was found in lucerne ensilage treated with Biomax SI on the 24th day of ensiling, and on the 5th day of ensiling in lucerne ensilage treated with Biomax WS. These results prove the good technological procedure during the ensiling as well as positive effects of Biomax SI and Biomax WS on the ensiling process (Table 3. and Table 4).

At the end, it should be emphasized that bacterial additives as well as enzymes, are not so omnipotent to produce qualitative ensilage from low quality material, using inapropriate technological procedure (Antov et. al., 2004).

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УТИЦАЈ *LACTOBACILLUS* SPP. НА ПЛЕСНИ И КВАСЦЕ У ПРОЦЕСУ СЕНАЖИРАЊА ЛУЦЕРКЕ

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Резиме

Рад садржи резултате испитивања утицаја два препарата Biomax SI (*Lactobacillus plantarum*) и Biomax WS (*Lactobacillus plantarum* i *Pediococcus pentosaceus*) на раст плесни и квасаца у процесу сенажирања луцерке. Рађене су следеће анализе: укупан број плесни и квасаца, специфична брзина раста плесни и квасаца, pH, садржај киселина (млечна, сирћетна и бутерна киселина), затим влага и температура под утицајем оба препарата у процесу сенажирања током 30 дана.