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## EFFECT OF ACTIVATED CLINOPTILOLITE IN AFLATOXIN B1 CONTAMINATED LAYING HEN DIETS ON AFLATOXIN B1 RESIDUES AND QUALITY OF EGGS

**ABSTRACT:** This study was carried out to determine the effect of a high level of aflatoxin B1 in laying hen diets, supplemented with deactivated and activated clinoptilolite, on inner and outer quality and aflatoxin B1 residues in eggs. Two experimental groups were formed and fed high aflatoxin B1 diets (965 ppb) containing deactivated and activated (450 °C for 60 minutes) clinoptilolite (2% of diet) for 49 days. In the experiment, a total of 960 55-week-old Lohmann LSL (white) laying hens were used. Each group had 8 replicates and 480 hens. Egg weight, inner and outer egg quality parameters and egg aflatoxin B1 levels were determined in a total of 90 eggs collected on the 15<sup>th</sup>, 30<sup>th</sup> and 49<sup>th</sup> days of the experiment. Diets containing deactivated or activated clinoptilolite decreased aflatoxin B1 production in laying hen diets after incubation period of 15 days. Activation of clinoptilolite by heat treatment significantly reduced aflatoxin B1 level in eggs ( $p < 0.05$ ). In addition, the use of clinoptilolite as an antifungal agent in the presence of high aflatoxin B1 level in layer hen diets significantly increased the weight of eggs and significantly reduced the ratio of broken-cracked and dirty eggs ( $p < 0.05$ ). Chicken blood albumin, creatinine and calcium levels were higher in hens fed diet containing activated clinoptilolite ( $p < 0.05$ ). However, triglyceride and VLDL levels decreased significantly in the blood of these animals ( $p < 0.05$ ). In conclusion, the supplementation of hen diets containing high aflatoxin B1 with activated clinoptilolite improves production performance, egg quality and decreases aflatoxin B1 residue in the egg.

**KEYWORDS:** aflatoxin B1, clinoptilolite, egg, laying hens

## INTRODUCTION

Egg has a great importance in meeting animal protein needs of mankind due to high biological value of its protein. However, it is mandatory that egg is

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produced through safe food production process and feed used in its production is formulized in such a way that it does not create any mycotoxin in egg. In recent years, the use of natural and synthetic zeolites as feed additives in order to prevent feed mycotoxins from passing to egg, utilize adsorbent feature and improve eggshell quality has been brought to agenda. A group of minerals are called zeolites. It has been explored that nine zeolite minerals are located in nature in great amounts. Clinoptilolite (CLP) zeolite has wide inner and outer surface areas for ion-exchange reactions thanks to its cage-like structure. Pores present there amount to about 50% of the volume. They are negatively-charged and have capacity of high ion exchange. However, CLP should be produced and processed using appropriate methods in appropriate conditions in order to utilize these features. CLP is a natural material which can hold water, gases and metal ions in itself in exchangeable situation, does not include hazardous elements, it is resistant for temperatures up to 750 °C and acid-bases (pH 1.5-11) (Baran and Kutay, 1999; Melenova *et al.* 2003).

CLP, a natural zeolite, has been approved by European Union in 1999 as a product which can be used in organic animal production. CLP remains active making ion exchange permanently, beginning from the moment it gets involved in digestive system to the moment it is removed. CLP does not interact with other ration nutrients (vitamins, minerals, etc.) therefore it can be included in complex feed additives safely. CLP can hold moisture and fat to a great extent, therefore it prevents degenerations in feeds caused by moisture during storage and it also prevents mycotoxin formation in feed (Pond *et al.* 1988). It is possible to activate zeolite and increase its porosity and adsorption capacity by acid-washing and heating. In this way, acid-resistant zeolites are advantageous in holding gases such as SO<sub>2</sub>, HS and CO<sub>2</sub>, as well as drying acidic gases such as Cl (Tsithisvili *et al.* 1992; Ragnarsdottir *et al.* 1996). Silicate structure of zeolite is degenerated by acid treatment. The increase of Si/Al ratio in this way is called dealumination process (Kuhl, 1999). The advantages of dealumination are removal of cations in zeolites, increase in heat-sensitivity, extension of pore size and increase in adsorbent feature of zeolite (Gottardi, 1986). It has been determined that heat treatment performed by anaerobic heating method in zeolite subjected to acid-treatment contributes to the increase in porosity and toxic substances holding. It has been indicated that adsorption capacity of zeolites can also increase because of crystallized spaces left by evaporated water heated at temperature of 350–400 °C. Treatment of zeolite with acids such as HCL, H<sub>3</sub>BO<sub>3</sub> and H<sub>3</sub>PO<sub>4</sub>, and the fact that it is subjected to heat-treatment with various acid concentrations in various periods and temperatures, can make significant changes and improvements in adsorption features (Lee *et al.* 2000; Hwang *et al.* 2002; Rožić *et al.* 2002; Ackley *et al.* 2003; Cheng *et al.* 2005; Campos and Büchler, 2007).

In feeds and foods, there are more than seven mycotoxins that have natural toxicity. They are: aflatoxin, zearalenone, ochratoxin A, citrinine, tirchothecenes, patulin, penicillic acid and ergot alkaloids. Aflatoxins make hepatotoxic, mutagenic and hepatocarcinogenic effect on liver. It has also been determined that 0.5% of aflatoxin taken through feed can pass to egg (Denli *et al.* 2005).

It decreases egg productivity and quality when aflatoxin B1 is included in laying hen diets (Herzallah, 2013). Zeolites added in poultry diets adsorb toxic substances and reduce effect of their accumulation by inhibiting their absorption through digestive tract (Çelebi and Kaya, 2012). Moreover, adding zeolite in diets reduces feed passing velocity through digestive system and causes nutrients to get more exposed to digestive enzymes (Khambualai *et al.* 2009). The objective of this study is to analyze the effect of addition of deactivated CLP of 2% (de-CLP) and activated CLP (a-CLP) periods just after heat-treatment application in laying hen diets on egg quality parameters, aflatoxin B1 levels in eggs and some blood parameters.

## MATERIALS AND METHODS

In this study, two experimental groups were formed and laying hens were fed laying hen diets containing de-CLP and a-CLP (Table 1). Experimental diets were supplemented with 2% de-CLP and a-CLP. CLP obtained from Bigadiç was activated by heating at 450 °C for 60 minutes. De-CLP and a-CLP samples were subjected to physical and chemical analyses to determine their characteristics related to adsorption, as well as other chemical contents (Table 1) in MTA laboratories in Ankara. A Total of 960 55-week-old Lohmann LSL (white) hens were divided into two experimental groups and fed diets containing de-CLP and a-CLP during 49 days. Triple laying hen cages were used in the experiment for housing. Hens were organized and placed in cages in such a way that hen house was triple-tier having 4 cage sections in each floor and 5 hens included in each cage section. Thus, 8 replicates including 480 hens for each experimental group using 8 cage blocks were used. Diets containing 965 ppb aflatoxin in the beginning formed the basal diet. After 15 days of basal diet, hens were fed basal diets supplemented with 2% of de-CLP and 2% of a-CLP. Two diets were kept under same storage conditions after adding de-CLP and a-CLP, and the samples were taken from the diets on the 15<sup>th</sup> day of incubation to analyse mycotoxins.

General growth and production performances were monitored in this research. Egg weight, inner and outer quality parameters of 90 eggs collected from each group on the 15<sup>th</sup>, 30<sup>th</sup> and 49<sup>th</sup> day of the experiment were determined by using egg quality measuring device (DET 6000). Egg yolk color was determined using the device in accordance with Roche colour scale. In the same way, aflatoxin B1 (AFB1) levels in eggs were detected in additional 90 eggs collected on the 15<sup>th</sup>, 30<sup>th</sup> and 49<sup>th</sup> day of the experiment. Blood samples were taken from 24 hens from each group and albumin, ALT, ALP, total bilirubin, creatinine, GGT, LDH, cholesterol, triglycerides, VLDL, and calcium were determined by using autoanalyzer.

Data obtained in the experiment conducted in accordance with randomized blocks experiment plan were analyzed by t-test with the usage of SPSS 15.0 program. Tukey multiple comparison test was used for detection of differences in means of groups.

Table 1. Composition of experimental diet and chemical and physical characteristics of CLP

Contents and analytical composition of basal diet	de-CLP	a-CLP
Corn (g/kg)	545	545
Soybean meal (g/kg)	130	130
Sunflower meal (g/kg)	114	114
Limestone (g/kg)	100	100
Guar meal (g/kg)	20	20
Soybean oil (g/kg)	30	30
Meat-bone meal (g/kg)	25	25
DCP (g/kg)	2.5	2.5
Vitamin-mineral premix (g/kg)	2.5	2.5
Salt (g/kg)	2.7	2.7
DL-Methionine (g/kg)	1.8	1.8
Sodiumbicarbonate (g/kg)	1.6	1.6
L-Lysine (g/kg)      Multienzyme+Phytase (g/kg)	1.3	1.3
Clinoptilolite (g/kg)	1.6	1.6
Metabolizable energy (kcal/kg of diet)	20	20
CP (%)	2,800	2,800
Ca, (%)	17.5	17.5
Available P (%)	4.1	4.1
Na, %	0.39	0.39
	0.16	0.16
<i>Chemical/physical composition of CLP</i>		
Oil absorption capacity, ml/100g	38.00	45
Water absorption capacity, ml/100g	18.63	21
Apparent porosity, %	19.10	40
Water absorption, %	17.75	19
Cadmium, mg/kg	1.30	<0.1
Lead, mg/kg	50.90	69.73
Arsenic, mg/kg	58.37	65.76
Mercury, mg/kg	<0.5	<0.5
Cation Exchange Capacity (CEC), meq/g	1.6	2.7
Dioxin (ng/kg)	0.2	0.44

Table 2. Effect of CLP activation on aflatoxin level in diets, performance parameters, egg quality, AFB1 levels in eggs, and some blood parameters

Parameters	de-CLP	a-CLP	P-Value	Sign.
Diet total aflatoxin, ppb (initial)	965±13.2	965±13.2	0.998	NS
Diet total aflatoxin, ppb (15 d)	362±14.4	317±12.2	0.742	NS
Diet AFB1, ppb (15d)	362±14.4	317±12.2	0.742	NS
Egg AFB1 content, ppb	0.246±0.0326	0.202±0.0165	0.0007	p<0.05
Feed intake, g/hen	5,325±17.5	5,066±31.5	0.000	p<0.05
Initial body weight, g	1,845±60	1,839±46	0.934	NS
Final body weight, g	1,899±62	1,888±48	0.969	NS
Liver weight, g/100 g BW	2.473±0.084	2.546±0.11	0.599	NS
Heart weight, g/100 g BW	0.492±0.022	0.486±0.021	0.835	NS
Spleen weight, g/100 g BW	0.123±0.0080	0.137±0.0092	0.266	NS
Egg production ratio, %	85.66±0.38	86.08±0.0032	0.398	NS

Egg weight, g	68.02±0.061	68.23±0.049	0.011	p<0.05
Broken-cracked egg ratio, %	1.995±0.15	1.345±0.11	0.001	p<0.05
Dirty egg ratio, %	3.53±0.32	2.270±0.095	0.000	p<0,05
Haugh Unit, HU	84.85±0.54	83.98±0.55	0.261	NS
Albumin height, mm	7.715±0.085	7.586±0.077	0.263	NS
Egg breakage resistance, STR	4.017±0.098	3.880±0.076	0.275	NS
Shell thickness, mm	0.416±0.004	0.427±0.004	0.064	NS
Shell weight, g	9.428±0.072	9.418±0.069	0.917	NS
Egg yolk colour	12.69±0.088	12.77±0.074	0.473	NS
Albumin, g/dl	2.167±0.062	2.371±0.075	0.041	p<0.05
ALP, U/L	895±75	1,006±115	0.427	NS
ALT, U/L	34.1±9.5	27.4±8.9	0.608	NS
Total bilirubin, mg/dl	0.250±0.026	0.304±0.021	0.110	NS
Creatinine, mg/dl	0.195±0.018	0.292±0.028	0.006	p<0.05
GGT, U/L	42.5±11	32.4±7.3	0.436	NS
LDH, U/L	1,480±254	2,197±285	0.067	NS
Cholesterol, mg/dl	137.3±11	129.6±10	0.607	NS
Triglyceride, mg/dl	1,427±107	1,148±85	0.047	p<0.05
VLDL, mg/dl	285±21	229.5±17	0.047	p<0.05
Calcium, mg/dl	15.73±1.1	17.10±0.74	0.299	NS

## RESULTS AND DISCUSSION

The effects of addition of a-CLP, heated at 450 °C for 60 minutes, in laying hen diets containing high levels (965 ppb) of total aflatoxin were examined. Total aflatoxin amount in diets was 362 ppb in the diet containing de-CLP and 317 ppb in the diet containing a-CLP, although at the beginning, diet total aflatoxin level was 965 ppb because of lack of any antifungals in the diet. Effects of treatments on body weight gain, feed intake, feed efficiency and internal organ weights are presented in Table 2. The usage of a-CLP in the diet led to a significant decrease in feed consumption (p<0.05). On the other hand, its effect on final live weight, weights of liver, heart and spleen was insignificant. A-CLP significantly (p<0.05) reduced aflatoxin B1 levels in eggs. Additionally, a-CLP significantly (p<0.05) increased egg weight and reduced rates of broken-cracked and dirty eggs. Haugh unit (HU), albumin height, egg breakage resistance, shell thickness, shell weight and egg yolk colour were not affected by the treatments (Table 2). Albumin, creatinine and calcium levels in blood increased in hens fed diet containing a-CLP (p<0.05). On the other hand, triglyceride and VLDL levels in blood of these animals decreased significantly (p<0.05).

The decrease in feed intake by usage of a-CLP in the diet makes a remarkable result for production economics. If the level of AFB1 in diet increased, a larger amount of AFB1 would pass to egg and leave residue. In this study, the usage of a-CLP in the diet significantly reduced the AFB1 amount in egg. Similarly, Mizrak *et al.* (2014) reported that no aflatoxin was encountered when

sepiolite was added into laying hen diets in 1.5% and 3% rates. However, it should be taken into consideration that rates of aflatoxins in diets used in these studies were various. Using a-CLP, AFB1 level in egg was significantly reduced ( $p<0.05$ ). Furthermore, it also significantly increased the egg weight and reduced the rate of broken-cracked and dirty egg. HU, albumin height, egg breakage resistance, shell thickness, shell weight and egg yolk color were not affected by dietary treatments. Despite the fact that a statistically significant effect on shell thickness was not observed, the rate of broken-cracked egg decreased significantly due to the usage of a-CLP. Similarly, Bozkurt *et al.* (2001) reported that zeolite usage decreased the rates of broken-cracked egg without affecting shell resistance and egg shell thickness. Çelebi and Kaya (2012) reported that a-CLP significantly increased egg weight and reduced the rate of damaged egg and also improved the egg shell thickness, especially if added during the late period. Mizrak *et al.* (2014) indicated that positive effect of zeolites on eggshell formation and bone development reduced phosphorus utility by forming insoluble aluminium silicate compounds with phosphorus ion in blood plasma of aluminium and silicon ions in its structure. Thus, the absorption of plasma calcium and calcium mobilization from bones were accelerated and the shell quality improved. Likewise, zeolites enhance the usage of vitamin D<sub>3</sub> regulating calcium and phosphorus metabolism and thus have positive effect on shell quality and bone structure. Zeolites perform this effect by binding mycotoxins, significant vitamin D<sub>3</sub> binders, and inhibiting their activity to bind vitamin D<sub>3</sub>. It was observed in studies, that there was a decrease in eggshell quality when phosphorus was not included in diet in high amounts (Çelebi and Kaya, 2012). Addition of de-CLP or a-CLP affected egg yield. Similarly, Balevi *et al.* (1998) reported that the addition of zeolite in laying hen diets did not affect egg yield. It was considered that the used dose can be effective as zeolite did not have any effect on egg yield. As a matter of fact, Yalçın *et al.* (1987) reported that the addition of zeolite at 2% rate in laying hen diets did not affect egg yield. However, egg yield was increased by the addition of zeolite at 4% rate. It was observed that the addition of a-CLP in laying hen diets reduced feed intake without affecting egg yield. Similarly, Balevi *et al.* (1998) also reported that addition of zeolite in 2.5% and 3.5% rates in laying hen diets reduced feed intake without affecting egg yield. Despite the findings of Miles *et al.* (1986), reporting that there was a decrease in egg weight by addition of zeolite in 1.5% level in diet for egg weight, it was observed in this study that the egg weight can be also significantly increased, especially by the activation of CLP. On the other hand, Oğuz *et al.* (2017) added perlite, expanded 10–30 times more than its normal volume by heating at temperature of 700–1,000 °C, into laying hen diets in 1.2% and 3% levels and they reported that addition of perlite reduced egg weight. It was considered that zeolite source could have effect if used within this variety. Gezen *et al.* (2004) considered that natural CLP extracted from Manisa region can affect egg weight positively due to its structural difference. It was considered in this study that CLP extracted from Bigadiç region and activated separately put forward this variety. Besides, Machaček *et al.* (2010) reported that the egg weight was increased by the addition of CLP at 2% rate and decreased by its

addition at 4% rate, and thus this situation made scientists conclude that the used dose can also have effect on weight. Among criteria assessed in terms of egg quality, the results found for HU, albumin height, egg breakage resistance, shell thickness, and yolk colour exhibit similarities to the results presented by Öztürk *et al.* (1998), Kralik *et al.* (2006), and Mizrak *et al.* (2014). However, Oğuz *et al.* (2017) found that albumen index was decreased by the addition of perlite. Vogt (1991) reported that the addition of CLP improved yolk color.

Albumin, creatinine and calcium levels were found at higher levels in blood of hens fed diets containing a-CLP ( $p < 0.05$ ). On the other hand, triglyceride and VLDL levels decreased significantly in blood of these animals ( $p < 0.05$ ). Serum ALP, ALT, total bilirubin, GGT, LDH, and cholesterol levels were not affected by the activation of CLP. Similarly, Mizrak *et al.* (2014) reported the similar results by adding sepiolite. Kralkik *et al.* (2006) added a commercial product called Nanofeed, activated tribomechanically and containing CLP, in laying hen diets, and on the 14<sup>th</sup> day detected that levels of creatinine, total bilirubin, total protein, globulin and ferritin increased. Denli and Okan (2006) reported that the hydrated sodium calcium aluminosilicate HSCAS in diets containing 80 µg/kg AFB1 inhibited the increase of serum AST in broilers. Increase in AST and ALT in serum is one of well-known effects of aflatoxicosis. It is attributed to protective effect of CLP binding aflatoxin that in this study ALP and ALT levels were not affected.

## CONCLUSION

It has been observed that utilization of de-CLP or a-CLP at 2% level in laying hen diets has positive effects on egg quality, yield performance and some blood parameters. It can be said that the increase in cation exchange capacity of CLP by activation also increased the adsorption capacity of CLP for aflatoxin.

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## УТИЦАЈ АКТИВИРАНОГ КЛИНОПТИЛОЛИТА НА ОСТАТКЕ АФЛАТОКСИНА Б1 И КВАЛИТЕТ ЈАЈА КАДА ЈЕ ХРАНА КОКА НОСИЉА КОНТАМИНИРАНА АФЛАТОКСИНОМ Б1

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**РЕЗИМЕ:** Ова студија спроведена је како би се одредио ефекат деактивираних и активираних клиноптилолита код високог нивоа афлатоксина Б1 у храни кока носиља на унутрашњи и спољашњи квалитет јајета, као и на остатке афлатоксина Б1 у јајету. Формиране су две експерименталне групе које су током 49 дана храњене храном с високим нивоом афлатоксина Б1 (965 ppb), а која је садржавала и деактивирани и активирани (грејањем 60 минута на 450 °C) клиноптилолит (2% у храни). У експерименту је испитано укупно 960 јединки 55-онедељних Ломан ЛСЛ (белих) кока носиља. Свака група имала је осам реплика са 480 кокошака. Тежина јајета, параметри унутрашњег и спољашњег квалитета јајета, као и ниво афлатоксина Б1 у јајету утврђени су код укупно 90 јаја сакупљених 15, 30, и 49. дана експеримента. Деактивирани или активирани клиноптилолит смањио је производњу афлатоксина Б1 у храни кока носиља након инкубације од 15 дана. Активација клиноптилолита грејањем значајно је смањила ниво афлатоксина Б1 у јајима ( $p < 0,05$ ). Поред тога, употребом клиноптилолита као антифунгалног агенса у присуству високог нивоа афлатоксина Б1 у храни кока носиља значајно

је повећана тежина јајета а значајно смањен однос поломљених и запрљаних јаја ( $p < 0,05$ ). Ниво албумина, креатинина и калцијума био је већи код кокошака хранених храном која садржи активиран клиноптилолит ( $p < 0,05$ ). Међутим, нивои триглицерида и липопротеина (VLDL) у крви ових животиња значајно су се смањили ( $p < 0,05$ ). Може се закључити да суплементација хране кока носиља која садржи висок ниво афлатоксина Б1 активираним клиноптилолитом побољшава производне перформансе, као и квалитет јаја, те смањује остатак афлатоксина Б1 у јајету.

КЉУЧНЕ РЕЧИ: афлатоксин Б1, клиноптилолит, јаје, коке носиље

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## DETERMINATION OF THE PRESENCE OF AFLATOXIN B1 IN FOOD AND FEED IN REPUBLIC OF SRPSKA (BOSNIA AND HERZEGOVINA) IN THE PERIOD BETWEEN 2014 AND 2016

**ABSTRACT:** Aflatoxins are a mixture of related chemical compounds of bisfokumarine type. They are synthesized in a variety of agricultural and food products such as oilseeds, spices, cereals and other products. In the period of 2014–2016, 418 samples were analyzed for the presence of aflatoxin B1, using the ELISA method. The analysis included samples of silage (75 samples), concentrated feed (272 samples), dry fruits (16 samples), nuts (15 samples), flours (5 samples) and other types of samples (35 samples). The presence of aflatoxin B1 was determined in all of the analyzed samples. The highest detected concentration of aflatoxin B1 was 4 µg/kg in the silage samples, 30 µg/kg in the concentrated feed samples, 0.40 µg/kg in samples of dried fruit, 0.81 µg/kg in nuts, 0.5 µg/kg in flour samples and 0.5 µg/kg in the other analyzed samples. Higher concentration of aflatoxin B1 from maximum residual level specified in Regulation was detected in samples of concentrated feed (2.57%).

**KEYWORDS:** Aflatoxin B1, ELISA, food, feed

## INTRODUCTION

Mycotoxins in food and feed are recognized as a public health problem. Many researchers dealt with fungal toxins establishing their carcinogenicity. Aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2) are potential teratogenic and carcinogenic metabolic products of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Kurtzman *et al.* 1987). Fungi are commonly found on the grains, almonds, walnuts, peanuts.

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Aflatoxins are a mixture of related chemical compounds. Series of aflatoxin B has the structure of molecules, where a cyclopentane ring is replaced by a G in the series of the lactones. Three structural variations give 18 molecules of aflatoxin, 8 of which are toxic and so far known aflatoxin B1 is the most toxic (Sinovec *at al.* 2006). Clinical signs of an acute aflatoxicosis include loss of appetite, lethargy, weight loss, neurological disorders, jaundice mucous membranes and cramps. High doses of aflatoxins are acutely toxic, causing substantial damage to the liver and intestinal and peritoneal bleeding, which can be lethal (Marriott and Gravani, 2006).

## MATERIAL AND METHODS

As the material in our testing, we used a feed: 103 samples in 2014; 155 samples in 2015; 86 samples (silage, maize, concentrate feed) and 71 samples (dried fruit, nuts, flour and other foods (pasta, etc)) in 2016. Analyses were performed by ELISA method, using test kit produced by manufacturer Bioscintific, Max signal (Austin USA).

### *Test protocol*

5.0 g of representative and ground samples were extracted with 25 mL of 70% methanol. Extraction was performed by combination of shaking, vortex (10 min.) and centrifuge (4,000 rpm during 10 min.) of the samples. The obtained supernatants were diluted with solution C and vortexed. 50 µL of the diluted samples was used for the analysis.

## RESULTS OF ANALYSIS

### *Feed samples*

The obtained results for feed samples are shown in Table 1

*Table 1.* Presence of aflatoxin B1 in feed materials collected in period 2014–2016

	2014			2015			2016		
	<5 µg /kg	5 µg /kg – 20 µg /kg	>20 µg /kg	<5 µg /kg	5 µg /kg – 20 µg /kg	>20 µg /kg	<5 µg /kg	5 µg /kg – 20 µg /kg	>20 µg /kg
Concentrated feed	90	10	1	73	8	4	78	6	2
Silage	2	nd	nd	73	Nd	nd	nd	nd	nd

Table 1 shows review for period 2014–2016 where we analyzed a total of 418 samples. 2.57% (7 samples) were contaminated by aflatoxin B1 at concen-

trations higher than allowed by valid regulation ( $>20\mu\text{g/kg}$ ). The greatest number of samples (316) had a concentration of aflatoxin B1 less than  $5\mu\text{g/kg}$  and 20 samples in the concentration range from  $5\mu\text{g/kg}$  –  $20\mu\text{g/kg}$ . The highest detected concentration of aflatoxin B1 in silage was  $4\mu\text{g/kg}$  and at concentrate feed it was  $30\mu\text{g/kg}$ .

### *Food samples*

In all analyzed samples of food obtained concentrations were below  $2\mu\text{g/kg}$ .

The largest number of analyzed samples (35 samples) was food like pasta, spices and grains. There were also 16 samples of dry fruits, 15 samples of nuts and 5 samples of flour. The highest recorded concentration was  $0.81\mu\text{g/kg}$  detected in samples of nuts.

All analyzed samples of food were within range of maximum residual level of valid regulation.

## DISCUSSION

Results of our studies are compared to results of available literature. Kos *et al.* (2013) in their studies found that of the 78 analyzed samples, presence of aflatoxin B1 was detected in 44. The most contaminated samples had a concentration (17.9%) of 1–10 ppb. Škrinjar *et al.* (2013) reported that in 12 of the tested samples the concentration aflatoxin B1 in feed material was from  $6\mu\text{g/kg}$  to  $145.8\mu\text{g/kg}$ , while we in our studies recorded a highest concentration of  $30\mu\text{g/kg}$ . Nedić *et al.* (2014) in their work detected 7.21% of positive samples in concentrate feed analyzed in 2013, which is slightly higher than the results of our examination. Increased values of concentration of aflatoxin B1 (51%) were found by Škrinjar *et al.* (2013) in their studies. Almeida *et al.* (2013) in thier research reported that the largest number of analyzed samples had a concentration of aflatoxin less than  $5\mu\text{g/kg}$ , which would correspond to our test results. Results of the analysis by Nedić *et al.* (2014) and Škrinjar *et al.* (2013) indicate an increased concentration of aflatoxin B1 in feed in 2012 which is direct consequence of drought that had happened that year in this region. Results of our examination show that in the following period (2014, 2015 and 2016), concentration of aflatoxin B1 was smaller than in 2012.

According to official data (FAO 1995), the average content of aflatoxin B1 varied between 4 and  $8\mu\text{g/kg}$  with a maximum value of  $30\mu\text{g/kg}$ . In world-wide scale in the period 1986–1997 from 2,460 tested samples of grain, 1,273 of the samples contained B1 at concentrations of 7–44  $\mu\text{g/kg}$ . In Brazil, from 2,546 samples of corn, 51% were contaminated with aflatoxin B1, a maximum quantity amounted to  $2,440\mu\text{g/kg}$  (Sinovec *et al.* 2006), which is more than what we have found in our tests. Higher concentrations of aflatoxin B1 ( $251\mu\text{g/kg}$ ) are recorded in researches in China.

Researchers in Spain, by analyzing the rice from different areas, have found that the rice is contaminated by aflatoxin B1 in the range from 0.8 µg/kg to 91.7 µg/kg. The same researchers examined rice in Mexico where they found concentration of aflatoxin B1 in range from 4.5 µg/kg to 8.1 µg/kg (Bonnet *at al.* 2013). The mean concentration of aflatoxin B1 in peanut samples, in season 2014/2015 amounted to 38.24 µg/kg (Villers, 2017), which is greater than the value that we have got we in our tests.

### Conclusion

Based on the results obtained in this experiment, it is possible to conclude that it is necessary to do monitoring of concentration of aflatoxin B1. Concentration of aflatoxin B1 depends on climate changes. Thus, it is necessary to apply agro-technical measures and make selection of corn species and other food and feed, which are more resistant to contamination of aflatoxin B1.

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ОДРЕЂИВАЊЕ ПРИСУСТВА АФЛАТОКСИНА Б1 У ХРАНИ И  
ХРАНИ ЗА ЖИВОТИЊЕ У РЕПУБЛИЦИ СРПСКОЈ (БИХ)  
У ПЕРИОДУ 2014–2016

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**РЕЗИМЕ:** Афлатоксини су мјешавина повезаног хемијских једињења типа бисфокумарине. Синтетисани су у различитим пољопривредним и прехранбеним производима, као што су уљарице, зачини, житарице и други, производима с ниском активношћу воде. У периоду од 2014. до 2016. године, методом Елиса анализирано је 418 узорака на присуство афлатоксина Б1. Анализа је обухватила узорке силаже (75 узорака), концентроване хране (272), сувог воћа (16), језгру ораха (15), брашна (5) и других врста узорака (35). Присуство афлатоксина Б1 утврђено је у свим анализираним узорцима. Највећа детектована концентрација афлатоксина Б1 била је 4 ppb у узорцима силаже, 30 ppb у узорцима концентрисане хране, 0,40 ppb у узорцима сувог воћа, 0,81 ppb у језгру ораха, 0,5 ppb у узорцима брашна, и 0,5 ppb у осталим анализираним узорцима. Већа концентрација у односу на максимално дозвољену концентрацију афлатоксина Б1 прописана важећим правилником детектована је у узорцима концентрата.

**КЉУЧНЕ РЕЧИ:** афлатоксин Б1, ЕЛИСА тест, храна, храна



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## THE FREQUENCY OF PRESENCE OF AFLATOXIN B1 IN FOODSTUFFS OF VEGETABLE ORIGIN

**ABSTRACT:** Cereals, nuts and spices are foods that are used in the daily human diet. According to FAO the average consumption of foods of vegetable origin in people's diet is increasing. Due to inadequate conditions during storage of foods of vegetable origin, there is possibility of contamination by mold that produces mycotoxins. Since the intake of these products in organism has been increased, there is a risk of exposure to mycotoxins and their harmful effect on the consumers' health. The aim of this study was to determine the presence of aflatoxin B1 in products of vegetable origin (cereals, nuts and spices). Aflatoxin B1 was determined by enzyme-immunochemical method (*ELISA*), using commercial kit. 38 samples were tested. In 25 analyzed samples, the content of aflatoxin B1 was higher than 1 µg/kg (1 µg/kg is limit of detection). Out of the total number of tested samples, in 18 samples the content of aflatoxin B1 was determined higher than the allowed amount for this product group by the current regulations (2 µg/kg for cereals, 2 µg/kg for nuts and 5 µg/kg for spices).

**KEYWORDS:** aflatoxin B1, foodstuffs of vegetable origin, ELISA, safety

## INTRODUCTION

Cereals, nuts and spices are foods that are used in the daily human diet. According to FAO (Food and Agricultural Organization) the average consumption of foods of vegetable origin in people's diet is increasing (Food and Agricultural Organization, 2003). Although it is recommended to be used in people's diet for its nutritional composition, this food can cause adverse effects on human health. Mold can be developed in seed products even before they get on the market due

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to inadequate conditions during the storage or inadequate treatment of products. Mycotoxins are secondary metabolic products of mold. The most important molds, which secrete mycotoxins, belong to the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria species* (Kabak, 2009). The most important mycotoxins are aflatoxins, ochratoxin A, fumonisin, deoxynivalenol, zearalenone, patulin (Kabak, 2009).

According to FAO (Food and Agricultural Organization, 2003) 30% of the world cereal production is more or less contaminated with mycotoxins. Mycotoxins can occur in all phases of food processing, before the harvest, during the harvest and storage. Food damaged by insects, slow drying and storage of seeds in wet conditions are suitable for mold development (Egmond *et al.* 2007). Food contamination by mycotoxins can be direct and indirect. In the direct contamination, mycotoxins get into the food as a result of mold growth on food, and in the indirect, contaminated ingredients are used in the food processing or food contains mycotoxin residues (Sinovec *et al.* 2006). Grain contamination depends on the environmental conditions (moisture content, the relative humidity, temperature, pH value). High soil moisture and grain damage are suitable for mold development (Sinovec *et al.* 2006).

As a result of consuming food contaminated with mycotoxins, there are serious health problems, which can sometimes be fatal (Boutrif, 1995). Mycotoxins cause a variety of harmful effects on human and animal health, such as hemorrhage, hepatotoxicity, nephrotoxicity, neurotoxicity. In addition, mycotoxins can have teratogenic, mutagenic and carcinogenic effects on the body (Chen *et al.* 2010). Due to harmful effects on human and animal health, the European Commission (EC) has prescribed the maximum allowed content of several mycotoxins in foods. Maximum allowed content of aflatoxin B1 in foods ranges from 2.0–8.0 µg/kg (EC, 2006).

The best way to prevent mycotoxins development is to prevent mold growth in all phases of production, collecting and transporting, treatment, storage and processing of food. In order to achieve this, it is necessary to control the presence of mycotoxins and mold throughout the food chain.

B1, B2, G1, G2, M1 and M2 aflatoxins represent the highest danger to human health. They are produced by molds of *Aspergillus flavus* and *Aspergillus parasiticus* genus. Under the influence of ultraviolet light, aflatoxins B1 and B2 fluoresce blue and aflatoxins G1 and G2 fluoresce green-yellow (Beltran *et al.* 2009, Groopman and Kensler, 2005; Malir *et al.* 2006). Aflatoxin B1 is the most toxic and it is always present in products that contain mycotoxins B2, G1 and G2 (Ilić *et al.* 2010).

The most commonly used methods for the determination of mycotoxins content in foodstuffs are: reversed phase high pressure-liquid chromatography (RP-HPLC) with UV or fluorescence detector, liquid chromatography (LC) and gas chromatography (GC) with mass spectrometry and enzyme-immunochemical method (ELISA) (Meneely *et al.* 2011; Sulyok *et al.* 2010). The most widely used method is the immuno-affinity chromatography with HPLC and the screening method that is used for mycotoxins determination is ELISA method (Krska and Molinelli, 2009).

The aim of this study was to check the frequency of presence of aflatoxin B1 in products of vegetable origin and to check whether content of aflatoxin B1 was present in these products in legally prescribed amounts.

## MATERIAL AND METHODS

The aflatoxin B1 content was determined in products of vegetable origin. Samples were purchased on the market of the Republic of Srpska/Bosnia and Herzegovina. They were divided into three groups: cereals (corn, wheat, barley) – 13 samples, nuts (walnut, hazelnut, pistachios) – 19 samples and spices (curcuma, white mustard, pepper) – 6 samples. The samples differed according to their type and the producer. They were prepared according to the instructions of the kit manufacturer. The procedure was the same, only two types of solvents were used. To extract cereals and spices, 70% methanol was used and to extract nuts, 60% methanol was used (Tecna, 2016).

For the determination of aflatoxin B1 content in products of vegetable origin, a commercial kit (Celer AFLA B1, Tecna, Trieste, Italy) was used. The kit contains a set of prepared chemicals. These are standard solutions of following concentrations: 0, 1, 5, 20 and 40 µg/kg, conjugate, a wash solution, solution for color development, a stop solution and 96 wells. The kit is stored at 2–6 °C, according to manufacturer's instructions (Tecna, 2016).

Aflatoxin B1 content in samples of vegetable origin was determined by ELISA method, measuring the color intensity of the product which appeared in the reaction between the enzyme and added substrate (Šimat, 2010).

Softver Excel spreadsheet for Celer Afla B1 (MA220) was used to measure the content of aflatoxin B1 in the products of vegetable origin.

## RESULTS AND DISCUSSION

Table 1 shows the measured absorbance of standard solutions (solutions of well-known concentration) of aflatoxin B1, using an ELISA reader at 450 nm. Aflatoxin B1 standard solutions have the following concentrations: 0, 1, 5, 20 and 40 µg/kg.

Table 2 shows how many samples (cereals, nuts and spices) have the content of aflatoxin B1 ≤1 µg/kg, 1–40 µg/kg and ≥40 µg/kg.

*Table 1.* The measured absorbance of aflatoxin standard solutions

Standard concentration (µg/kg)	0	1	5	20	40
The absorbance (450 nm)	2.4060	2.0380	0.9890	0.5440	0.4390

Table 2. Aflatoxin B1 content in the analyzed samples

Samples	Number of samples	Aflatoxin B <sub>1</sub> content		
		(≤1 µg/kg)	(1-40 µg/kg)	(≥ 40 µg/kg)
Cereals	13	5 (samples)	4 (samples)	4 (samples)
Nuts	19	8 (samples)	3 (samples)	8 (samples)
Spices	6	–	4 (samples)	2 (samples)

According to producer, limit of detection for ELISA Celer Afla B1 (code MA220) is 1 µg/kg for corn, nuts and pistachios and 2 µg/kg for dried fruit and figs (Tecna, 2016).

Out of total 38 tested samples, 13 samples contained less than 1 µg/kg of aflatoxin B1 (limit of detection is 1 µg/kg). Fourteen samples contained more than 40 µg/kg of aflatoxin B1. The content of B1 aflatoxin ranged between 1–40 µg/kg in 11 samples. For samples where content of aflatoxin B1 was ≥ 40 µg/kg, sample extract was diluted (5x) and again determined by enzyme immunochemical method (ELISA).

The highest content of aflatoxin B1 in cereals was 88.29 µg/kg. According to the Regulation of maximum prescribed amounts for certain contaminants in food from Bosnia and Herzegovina, the maximum allowed content of aflatoxin B1 in cereals is 2 µg/kg (*Službeni glasnik*, 2014). Eight cereals samples contained more aflatoxin B1, than the maximum allowed concentration.

The highest content of aflatoxin B1 in nuts was 94.87 µg/kg. According to the Regulation of maximum allowed amounts for certain contaminants in food from Bosnia and Herzegovina, the maximum allowed content of aflatoxin B1 in nuts is 2 µg/kg. 8 samples contained more aflatoxin B1 than it is allowed by the Regulation (*Službeni glasnik*, 2014).

The highest content of aflatoxin B1 in spices was 99.64 µg/kg. According to the Regulation of the maximum prescribed content for certain contaminants in food from Bosnia and Herzegovina, the maximum prescribed aflatoxin B1 content in spices is 5 µg/kg (*Službeni glasnik*, 2014). Two samples contained more aflatoxin B1 than it is allowed by the Regulation.

Czerwiecki *et al.* (2006) determined mycotoxins content in foodstuffs (cereals and their products, nuts, culinary spices, coffee and dried fruit) available on the Polish market. Mycotoxins content was determined by high pressure liquid chromatography (HPLC) with fluorescence detector. The average content of aflatoxin B1 in the nuts samples was 0.13 µg/kg. The highest content of aflatoxin B1 in the analyzed samples was 7.8 µg/kg (this concentration exceeded the permissible aflatoxin B1 content for nuts, as the European Union recommended, 2 µg/kg. In the analyzed samples of cereals and spices, aflatoxin B1 ranged from 0.02 to 0.4 µg/kg, and an average content was 0.12 µg/kg).

Pluyer *et al.* (1987) treated peanuts by roasting them in an oven at a temperature of 150 °C, for a period of 30 minutes, and then they monitored what would happen next. Based on the obtained results they concluded that the aflatoxin B1 content decreased for 30–45%. Yazdanpanah *et al.* (2005) treated pistachios by frying them at a temperature of 90, 120 and 150 °C, for 30, 60 and

120 minutes. It could be noticed that the aflatoxin B1 content decreased for 17–63%, depending on the time and temperature of frying. Ogunsanwo *et al.* (2004) conducted studies, based on the process of drying seeds at 140 °C for a period of 40 minutes. On that occasion, the content of aflatoxin B1 decreased by 58.8%. Drying at a temperature of 150 °C for a period of 25 minutes, aflatoxin B1 content decreased by 68.5%.

Number of tested samples in this study was small (38), therefore it was expected to get the high percentage (47.37%) of contaminated samples. Considering that the main source of mycotoxins are cereals in the human and animal food chain, it is possible to prevent mold growth and the formation of mycotoxins by applying the measures, good manufacturing practices and the application of HACCP principles. These measures include the selection of varieties resistant to mold, weed control, drying of the grain reducing mechanical damage to a minimum during the harvest, as well as proper drying and storage. In order to protect consumers, it is very important to know the stability of different mycotoxins during thermal processing.

## CONCLUSION

In the group of tested samples of cereals, 8 samples contained more aflatoxin B1 than it was allowed. In the group of tested samples of nuts, 8 samples contained more aflatoxin B1 than it was allowed. In tested samples of spices, 2 samples contained more aflatoxin B1 than it was allowed. In 25 analyzed samples, the content of aflatoxin B1 was higher than 1 µg/kg (1 µg/kg is limit of detection).

Taking into consideration the small number of tested samples, with a prior suspicion of the presence of mycotoxins, the authors are reserved about the high percentage of contaminated samples in relation to the tested ones.

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## УЧЕСТАЛОСТ ПОЈАВЕ АФЛАТОКСИНА Б1 У НАМИРНИЦАМА БИЉНОГ ПОРИЈЕКЛА

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**РЕЗИМЕ:** Житарице, језграсто воће и зачини представљају намирнице које се користе у свакодневној исхрани људи. Према подацима ФАО-а просјечна потрошња намирница биљног поријекла повећава се у исхрани становништва. Услед неадекватних услова током складиштења намирница биљног поријекла, постоји могућност да дође до њихове контаминације плијеснима, које производе микотоксине. С обзиром на пораст уноса ових производа у организам, постоји ризик уноса микотоксина и њиховог штетног дјеловања на здравље потрошача. Циљ овог рада био је да се утврди присуство афлатоксина Б1 у производима биљног поријекла (житарице, језграсто воће и зачини). Одређивање садржаја афлатоксина Б1 вршено је ензимско-имунохемијском методом (*ЕЛИСА*), коришћењем комерцијалног кита. Испитивано је 38 узорак. Код 25 испитиваних узорак садржај афлатоксина Б1 био је већи од 1 µg/kg (1 µg/kg представља лимит детекције). Од укупно тестираних узорак, у 18 је утврђен садржај афлатоксина Б1 већи од количине дозвољене за ове групе производа у важећим прописима.

**КЉУЧНЕ РИЈЕЧИ:** афлатоксин Б1, намирнице биљног поријекла, ЕЛИСА, безбједност



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## DEOXYNIVALENOL OCCURRENCE IN SERBIAN MAIZE UNDER DIFFERENT WEATHER CONDITIONS

**ABSTRACT:** The aim of this paper was to investigate deoxynivalenol (DON) occurrence in maize samples originating from two harvest seasons in Serbia. The key differences between harvest seasons were weather conditions, specifically the humidity. The samples were analyzed using high performance liquid chromatography with DAD detection, after clean-up on SPE columns. In samples from 2014, DON was found in 82 (100.0%) samples with the average content of 2.517 mg/kg (ranged from 0.368 to 11.343 mg/kg). Two samples exceeded maximum level permitted by EU regulations. However, analyzing larger number of samples (163) from 2015 harvest season, DON was present in 51 (31.3%) samples in significantly lower concentrations (average of 0.662 mg/kg, ranged from 0.106 to 2.628 mg/kg). None of the samples from 2015 exceeded maximum level permitted by EU regulations. The data on DON presence in Serbian maize were in relation to the different weather conditions that prevailed during the two harvest seasons.

**KEYWORDS:** deoxynivalenol, HPLC, maize, Serbia, weather

## INTRODUCTION

Deoxynivalenol (DON) is the most widely spread mycotoxin from the trichotecene group. It is most commonly produced by *Fusarium graminearum* and *F. culmorum* molds. Maize is one of the most susceptible crops to *Fusarium* infection. A disease of maize caused by *Fusarium* molds is known as *Gibberella* ear rot (JECFA, 2001).

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Animal's susceptibility to DON exposure differs among species. Pigs and poultry are extremely susceptible, while ruminants are relatively unaffected (Pestka, 2007). Most common symptoms of chronic DON intake are decreased food intake and growth, as well as altered immune function. Acute poisoning with high doses of DON causes acute gastroenteritis with vomiting (Pestka, 2007).

The European Union regulated DON content in cereals and cereal products with the exception of maize by-products at 8 mg/kg (European regulation, 2006). In Serbia, the maximum permitted level for this mycotoxin has been changed recently. Until 2014, the maximum permitted level for DON was regulated only in complete and supplementary diets for pigs (0.5 mg/kg) (Serbian regulation, 2010). Since April 2014, the Serbian legislation has been harmonized with the European legislation (Serbian regulation, 2014).

In Serbia, arable land covers about 74.3% of utilized agricultural land. In 2015, in the structure of sown arable land areas, cereals participated with 68.8%, industrial crops with 14.5%, vegetables with 2.5% and fodder crops with 9.6% (Statistical Office of the Republic of Serbia, 2016). However, cereals were grown on 1,782,010 ha in 2015, which is less compared to 2014 (1,819,188 ha). During 2015, maize was harvested from 1,010,227 ha, with total production of 5,454,841 t. The average yield in 2015 was 5.4 t/ha, which is lower in comparison with 2014 (7.5 t/ha) and 2013 (6.0 t/ha) (Statistical Office of the Republic of Serbia, 2016). Furthermore, in 2016 Serbia was ranked among top ten maize exporters (Index Mundi, 2016), and among top twenty maize producers in the world (Index Mundi, 2016a).

RASFF (2017) reported five notifications regarding DON presence in maize and maize products originating from Serbia for the years 2014–2016. The highest notified level (16.18 mg/kg) was detected in corn flour in 2015. However, no notifications have been made in 2017 so far.

The aim of this research was to determine DON content in maize samples from 2014 and 2015 harvests, collected in Serbia. Also, the presence of DON was investigated in terms of weather conditions recorded during the period of investigation.

## MATERIALS AND METHODS

### *Chemicals*

Acetonitrile and water (all HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DON crystalline substance (D0156) was also purchased from Sigma-Aldrich (St. Louis, MO, USA). Calibration solution was prepared in ethyl-acetate:methanol (19:1, v/v) at the concentration of 0.1 mg/ml from crystalline substance according to AOAC method 986.17. Stock solution was prepared by measuring 1.00 ml of calibration solution of DON into a 10 ml volumetric flask and diluting to volume with ethyl-acetate:methanol (19:1, v/v). Working calibration solutions were prepared by evaporating the appropriate

volume of the stock solution and diluting with 1.00 ml of mobile phase. Standard solutions were stored at 4 °C.

### *Samples and sample preparation*

In total, 245 (82 from 2014 and 163 from 2015 harvest) samples of maize were randomly collected from different locations in Serbia. Each sample was immediately transported to the laboratory and was stored in a freezer at -20 °C until analysis. Prior to each analysis, the samples were allowed to reach room temperature. All samples were milled on a laboratory mill in such a way that >93% passed through a sieve with pore diameter of 0.8 mm and a portion was taken for analysis.

Twenty-five grams of maize sample were extracted with 100 ml acetonitrile: water (84:16, v/v) by high speed blending on an Ultra Turrax (Ultra Turrax T18, IKA, Staufen, Germany). The extract was filtered through slow filtration filter paper (Filtros Anioia, Barcelona, Spain) and 3 ml was cleaned up on Myco-sep™ 225 (Trich) columns (Romer Labs. Inc., Union, MO, USA). The cleaned-up extract was evaporated just to dryness on a Reacti-Therm™ Heating/Stirring Module (Pierce, Rockford, IL, USA) in gentle steam of nitrogen delivered by Reacti-Vap™ Evaporator (Pierce, Rockford, IL, USA).

### *HPLC analysis*

The HPLC analysis was carried out by slightly modified chromatography conditions proposed by Abramović *et al.* (2005).

The equipment consisted of an Agilent Technologies 1260 series HPLC system (Agilent Technologies, USA) with a DAD detector and a column Hypersil ODS (150 x 4.6 mm ID, particle size 5 µm, Agilent Technologies, USA).

The DON analysis was performed after evaporation. The residue was redissolved in 300 µl of mobile phase and filtered through a 0.22 µm nylon membrane filter (Agilent Technologies, USA). A 15 µl aliquot of the solution was injected into the HPLC system. The mobile phase consisted of an isocratic mixture of water:acetonitrile (84:16, v/v), with a flow rate of 0.8 ml/min. The detection of DON was performed at 220 nm. The mobile phase was filtered through a 0.45 µm regenerated cellulose membrane filter (Agilent Technologies, USA). Identification of DON was done by comparing the retention times and spectra of DON from samples with those of the standards.

## RESULTS AND DISCUSSION

Samples of maize collected during two different harvests (2014 and 2015), were analyzed for the presence of DON and the results are presented in Table 1. As can be seen, there was a significant difference in DON presence between

production years. In 2014, DON was found in 100% samples, while in 2015 its content was above the LOQ in 31.3% of analyzed samples. The average DON content in 82 analyzed samples from 2014 was 2.517 mg/kg with the median value of 2.100 mg/kg. In a large number of samples from 2015 (163), significantly smaller average DON content (0.662 mg/kg) and median value (0.458 mg/kg) were found in comparison with samples from 2014 harvest. Despite high presence of DON in maize from 2014 harvest season, only 2 (2.4%) samples contained DON above maximum permitted level (8 mg/kg) regulated by Serbian regulation (2014) and European regulation (2006). On the other hand, no samples from 2015 harvest exceeded mentioned regulations.

Table 1. DON content in maize samples.

Harvest year	2014	2015	Total
Number of samples	82	163	245
Positive samples, %	100.0	31.3	54.3
Average, mg/kg	2.517	0.662	1.806
Median, mg/kg	2.100	0.458	1.467
Standard deviation, mg/kg	1.904	0.649	1.790
Range, mg/kg	0.368–11.343	0.106–2.628	0.106–11.343
Number of samples above EU regulation (%)	2 (2.4)	0 (0.0)	2 (0.8)

Sutton (1982) described that in case of maize, *Fusarium* infection most commonly takes place through the tip of the ear, when the fungi penetrate through the silk in the phase of maize flowering. 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 Serbia takes place during the month of July and the first half of August.

According to the reports of the Republic Hydrometeorological Service of Serbia (2014), in the territory of Serbia was recorded the most humid weather in the past 45 years. During the vegetation period of 2014, an average of 700 mm of rainfall was noted, which was 2 to 3 times higher in comparison with the multi-annual average. Standardized precipitation index (SPI-6) showed extreme moisture conditions in most parts of Serbia during this period (April–September). Regarding the critical period for *Fusarium* infection of maize (July–August), it was characterized as moderately warm with very humid and rainy weather. The most frequent precipitation was recorded during July and early August (Figure 1). Maximum mid-day air temperatures during the summer were below average, while mornings were warm with minimum temperatures above the average. Also, during the summer, a frequent occurrence of extreme weather with hail storms was recorded, that caused damage to crops and probably

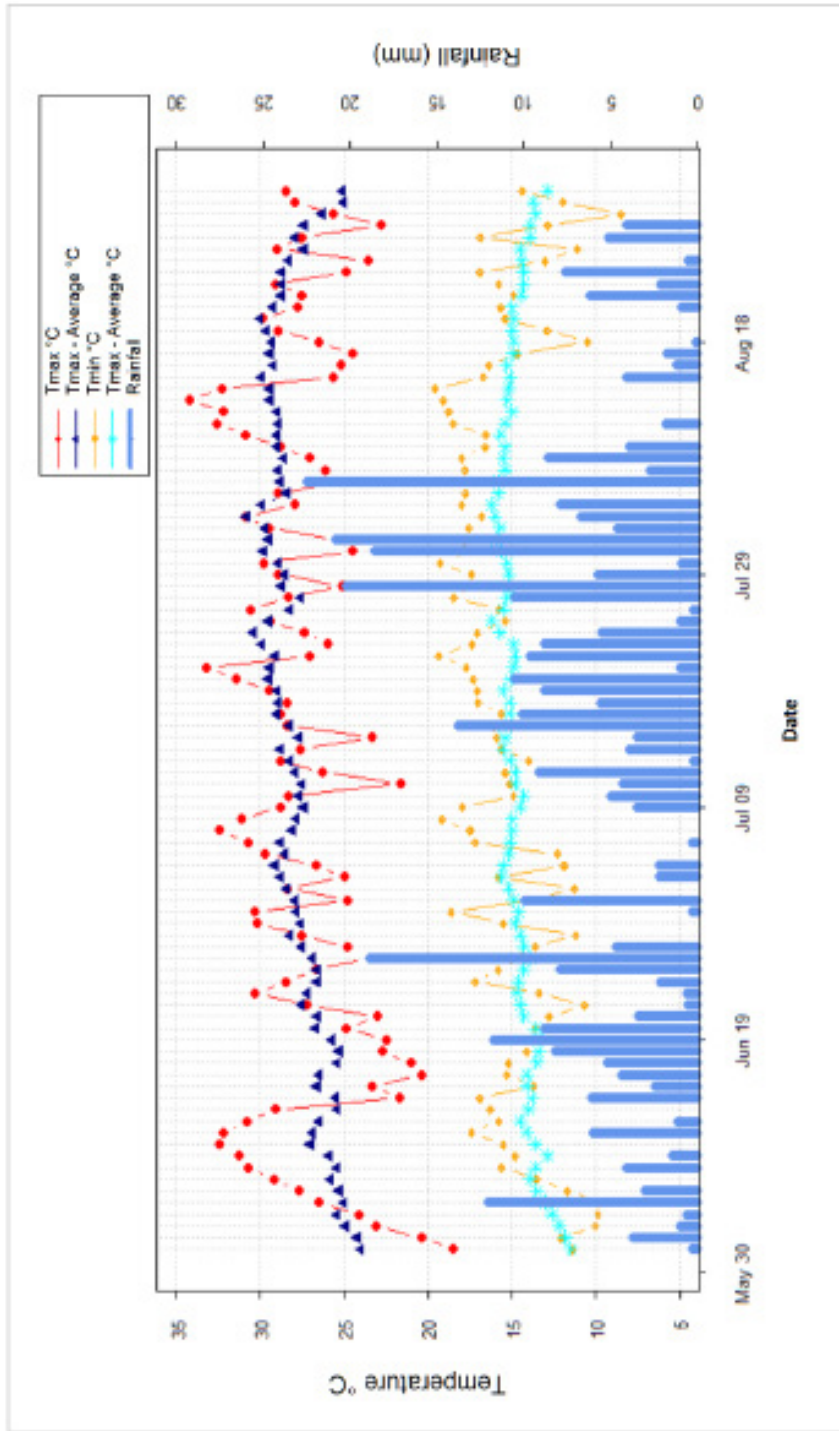


Figure 1. Weather conditions in Serbia during June–August 2014  
(Republic Hydrometeorological Service of Serbia, 2014)

contributed to *Fusarium* infection of maize. Additional factor for high DON occurrence in 2014 was the period of harvest. The maize harvest in 2014 lasted unpredictably long, from mid-September through mid-December, due to wet weather in autumn (United States Department of Agriculture, 2015). Furthermore, maize moisture content was above 20% at the beginning of the harvest and most possibly contributed to the development of *Fusarium* mold and substantial DON production.

Summer of 2015 (June–August) was warmer compared to a long-term average, with significantly less amount of rainfall. Besides the rainfall quantity, rainfall distribution during the year was also unfavorable for crops (Republic Hydrometeorological Service of Serbia, 2015). Based on the standardized precipitation index (SPI-3), the humidity conditions in 2015 were unfavorable in main agricultural areas of Serbia. The most unfavorable period for most agricultural crops was from the end of the first decade of July to mid-August (Figure 2), and the most affected were spring crops, especially maize (Republic Hydrometeorological Service of Serbia, 2015). Although dry weather usually leads to a plant stress (Zandalinas *et al.* 2017), it is not quite favorable for *Fusarium* molds growth as well. But then again, during the second half of August some precipitation was registered and this may have led to *Fusarium* growth and DON production afterwards. In addition, during September 2015 the weather was warmer, compared to long-term average, with higher amount of rainfall.

Harvest season of 2014 was undoubtedly extremely favorable for the growth of *Fusarium* molds due to the extremely humid and relatively warm weather. On the other hand, most of the vegetation period in 2015 was probably unfavorable for *Fusarium* molds growth, except maybe in September, when the amount of precipitation was above long-term average precipitation.

In Serbia, the importance of DON occurrence in maize and other cereals has not been properly perceived until recently. Jajić *et al.* (2008) gave the first indications of the presence of DON in most commonly grown cereals in Serbia. The authors examined 139 samples of different cereals from 2004 and 2005 harvests for the presence of this mycotoxin. DON occurrence in maize was 44.7% in range from 0.04 to 2.46 mg/kg. The authors also noted that during 2004 and 2005 occurred favorable weather conditions for the development of *F. graminearum*. Later, Kos *et al.* (2014) analyzed 90 samples of maize from Autonomous Province of Vojvodina for the presence of *Fusarium* mycotoxins. Samples were collected after 2012 harvest, when drought conditions prevailed. The authors found no samples containing DON, while some other *Fusarium* toxins occurred. More recently, Kos *et al.* (2017) presented the results of a three-year DON monitoring in Serbian maize. The authors analyzed a highly representative number of samples (1,800) and came up with conclusions that amount of precipitation represented a climatic factor with the strongest influence on the DON occurrence in maize.

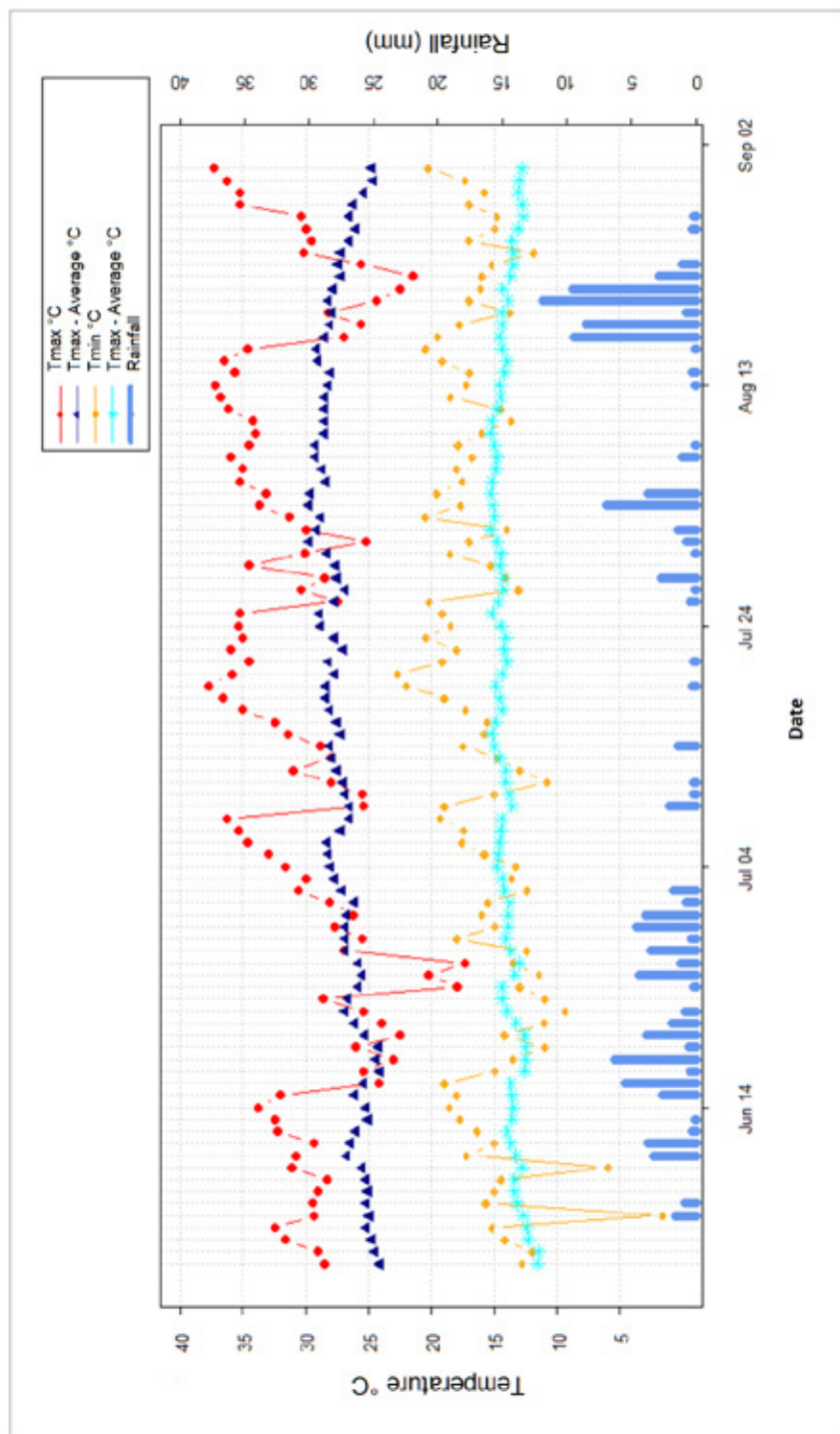


Figure 2. Weather conditions in Serbia during June–August 2015  
(Republic Hydrometeorological Service of Serbia, 2015)

## CONCLUSION

In conclusion, it can be said that the high DON presence and its content during 2014 was a consequence of rather favorable weather conditions for *Fusarium* mold growth that prevailed in almost entire territory of Serbia during vegetation period (April–September), particularly during maize silking period (July–August). The results may also be the consequence of the extremely humid conditions during fall months and high moisture content of maize, which resulted in maize harvest delay up to mid-December. It is significant to emphasize that development of *Fusarium* mold and DON production in 2014 was similar to extreme *Aspergillus* infection and aflatoxin contamination in maize during 2012. However, since aflatoxin is known as carcinogenic substance, its occurrence involved more public attention than DON. In contrast, samples from 2015 showed significantly lower presence of DON, along with its lower levels. Less favorable weather conditions for *Fusarium* growth were the reason for this, since the humidity conditions were predominantly important for their growth and DON production.

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## ПОЈАВА ДЕОКСИНИВАЛЕНОЛА У КУКУРУЗУ ИЗ СРБИЈЕ ПРИ РАЗЛИЧИТИМ ВРЕМЕНСКИМ УСЛОВИМА

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**РЕЗИМЕ:** Циљ овог рада био је да се испита појава деоксиниваленола у узорцима кукуруза који потичу из Србије из два жетвена периода. Кључну разлику између два жетвена периода су чинили временски услови, а нарочито услови влажности. Узорци су анализирани техником течне хроматографије високих перформанси (HPLC) с детектором с низом диода (DAD), након пречишћавања на SPE колонама. Када су у питању узорци из 2014. године, присуство деоксиниваленола је утврђено у 82 (100,0%) узорка са средњим садржајем овог микотоксина од 2,517 mg/kg (опсег од 0,368 до 11,343 mg/kg). Два узорка су превазилазила максимални ниво који је дозвољен европском законском регулативом. С друге стране, анализирајући већи број узорака (163) из 2015. године, деоксиниваленол је био присутан у 51 (31,3%) узорку у значајно нижим концентрацијама (просечна вредност од 0,662 mg/kg, у опсегу од 0,106 до 2,628 mg/kg). Ниједан од узорака из 2015. није превазилазио максимални садржај прописан европском регулативом. Подаци о присуству деоксиниваленола у кукурузу из Србије могли су се повезати са значајно другачијим временским условима који су преовлађивали на територији Србије током ове две жетвене године.

**КЉУЧНЕ РЕЧИ:** деоксиниваленол, HPLC, кукуруз, Србија, временски услови

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## APPLICATION OF ATR-FTIR ANALYSIS FOR DETERMINATION OF FUMONISINS IN CORN

**ABSTRACT:** In order to develop rapid, inexpensive and, at the same time, reliable method for the analysis of molds of the genus *Fusarium* as an indicator of the presence of fumonisins in corn samples, possible application of Fourier transform infrared spectroscopy (FTIR) with attenuated total reflection (ATR) technique was examined. The content of fumonisins in contaminated corn samples had previously been quantified by ELISA method. At the spectrum of the sample contaminated with a high concentration of mycotoxins, there was a lack of the peak at  $1,743\text{ cm}^{-1}$ , but the peak was observed at  $1709\text{ cm}^{-1}$ . To the purpose of result classification the principal component analysis (PCA) and cluster analysis were applied. Conclusions of the two methods were similar both when applying ATR technique in the whole region of the spectrum ( $1,150\text{--}1,770\text{ cm}^{-1}$ ) and when the whole spectrum was divided into two regions:  $1,150\text{--}1,450$  and  $1,450\text{--}1,770\text{ cm}^{-1}$ . However, classification of samples was somewhat better in the ranges  $1,150\text{--}1,770$  and  $1,450\text{--}1,770\text{ cm}^{-1}$ . Of the 16 analyzed corn samples, only very contaminated corn sample with  $190\text{ mg/kg}$  was correctly classified as compared to the other samples with the content of less than  $10\text{ mg/kg}$ . Also, it was found that evaluation of fumonisins in corn by this technique requires further investigation encompassing recording of spectra of contaminated corn of the same genotype in order to avoid the possible impact of different hybrids on the spectrum.

**KEYWORDS:** ATR-FTIR, fumonisins, corn

## INTRODUCTION

Thanks to its numerous advantages such as: easy to use, fast, non-invasive and non-destructive, infrared spectroscopy has been widely applied in the field

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of plant protection as well as analysis of the quality and safety of food and animal feed (Dale *et al.* 2012) and particularly in quality control and production process monitoring in food industry (Pojić and Mastilović, 2013). To this end, spectra recorded at various wavelength ranges and different methods for recording and processing of the spectra are used. However, the obtained spectra contain information about the overall physical-chemical characteristics of the analyzed sample, thus, qualitative and quantitative chemometric methods are indispensable for developing a calibration model. Calibration model defines the relationship between the obtained spectral data and compound content or feature of interest. Among others, qualitative methods encompass the analysis of principal components (PCA) (Kos *et al.* 2002; Abramović *et al.* 2007) and cluster analysis (Kos *et al.* 2003; 2004; Abramović *et al.* 2007) and artificial neural network (Gordon *et al.* 1998). Quantitative methods include multiple linear regression (MLR) (Abramović *et al.* 2007), partial least squares (PLS) regression (Della Riccia and Del Zotto, 2013) and others.

In recent times, fundamental or mid-infrared (IR) (Gordon *et al.* 1998; 1999; Kos *et al.* 2002; 2003; 2004; Abramović *et al.* 2007) and near-infrared spectroscopy (NIR) (Dowel *et al.* 2002; Berardo *et al.* 2005; Gaspardo *et al.* 2012; Della Riccia and Del Zotto, 2013) have been applied for detection of fungi and to predict the occurrence of mycotoxins. Considering very low levels of mycotoxins in cereals, this technique relies on prediction of the presence of mycotoxins based on the identification of fungal-damaged kernels (Pojić and Mastilović, 2013). The detection of fusarium fungi as an indicator for the presence of deoxynivalenol (min 310 µg/kg) according to Kos *et al.* (2002; 2003) requires the application of Fourier transform infrared spectroscopy (FTIR). Multivariate data analysis allows for more than one wavelength to be taken into account for statistical treatment of spectra. Therefore, selection of a suitable set of wavelengths can give a good representation of the spectral measurement minimizing the impact of noise and eliminating irrelevant information (Kos *et al.* 2003). Moreover, ATR technique proved advantageous over diffuse reflection (DR) due to better classification and quantification performance as well as simple usage and easier interpretation of the results (Kos *et al.* 2004; Abramović *et al.* 2007).

Apart from the methods of the mid-infrared spectrum, detection of fungi and prediction of mycotoxin contamination were frequently performed using NIR spectroscopy. Measurements in near-infrared region proved appropriate for quantitative evaluation of visible changes in the kernel and estimation of the levels of DON (Dowell *et al.* 1999; Delwiche, 2003; Delwiche and Hareland, 2004; Siuda *et al.* 2006), fumonisin (Dowell *et al.* 2002; Berardo *et al.* 2005) and aflatoxin (Pearson *et al.* 2004). The application of Fourier transform instruments (FT-NIR) has been showing increasing tendency because of a high signal-noise ratio, accuracy in wavelength defining, rapid spectral recording and high resolution (De Girolamo *et al.* 2009; Gaspardo *et al.* 2012; Della Riccia and Del Zotto, 2013).

Taking into consideration permanent and increasing need for more rapid and cost-effective analysis methods, this study is aimed at investigating potential application of FTIR method as a screening approach in the determination of fumonisins.

## MATERIAL AND METHODS

Corn samples (13) artificially inoculated with fungus *Fusarium verticillioides* were used to investigate the possibility of determining fumonisin content using FTIR spectroscopy. In all samples, the contents of total fumonisin were determined by ELISA method utilizing *ELISA Ridascreen® Fumonisin R3401* test kit according to manufacturer's instruction (R-biopharm, [www.r-biopharm.de](http://www.r-biopharm.de)).

Prior to recording FTIR spectra, each sample was prepared by grinding in a laboratory mill in such a way that 93% passed through a sieve with pore diameter of 0.8 mm. Spectra were recorded using a Thermo Nicolet Nexus 670 spectrometer with deuterated triglycine sulfate (DTGS) as detector which requires no maintenance. The interferometer was operated at scan rate of 0.47 cm/s. All spectra were acquired at 4 cm<sup>-1</sup> resolution between 650 and 4,000 cm<sup>-1</sup>, as described by Abramović *et al.* (2007). Unbiased raw data, without smoothing, baseline corrections or other manipulations, were collected. The spectra were acquired using software provided with the spectrometer. All spectra (used for statistical treatment) were averaged from four repeated ATR measurements, each recorded from a new sub-sample. These averaged spectra were normalized. Several spectral windows were chosen for data analysis. Further data analysis was performed with data analysis software system PAST (version 2.12, Oslo, Norway). The number of data points varied from 300 to 620 for each spectrum before PCA calculations. PCA was based on covariance matrix of active variable. Results were displayed as score/score plots. Cluster analysis of relevant principal components for classification purposes was performed using Chords distance and average linkage method (Miller and Miller, 2010).

## RESULTS AND DISCUSSION

Having in mind the results obtained by Abramović *et al.* (2007), we applied only ATR technique with previous assessment of the effects of particle size on the reproducibility of measurement in given experimental conditions. The results revealed satisfactory reproducibility of spectral measurements provided that 90% of particles were ≤ 0.8 mm in size. Even though we recorded the entire spectrum in the range 600–4,000 cm<sup>-1</sup>, higher wave number regions of 1,170 cm<sup>-1</sup> were not displayed since they are irrelevant for this research. Namely, spectral changes at ~3,300 cm<sup>-1</sup> were excluded from the calculations because the intensity of the OH-stretching vibration had a rather large variation, mainly because of the drying process, which was not very reproducible. Valence oscillations of C-H bonds in the CH<sub>2</sub> group were observed in the ranges 2,925 and 2,855 cm<sup>-1</sup>, which were also removed due to their non-specificity. The changes in atmospheric concentration of CO<sub>2</sub> result in spectral alteration in the range between 2,250 and 2,400 cm<sup>-1</sup>, so this spectral region was excluded from further analysis.

As visible in Figure 1A, in spite of only slight differences between the spectra of corn samples, visual inspection reveals changes in the spectrum of the sample contaminated with higher fumonisin content (1) as compared with

the sample with lower concentration (2) in the range 1,700–1,750  $\text{cm}^{-1}$ . Namely, in the spectrum of the sample contaminated with higher mycotoxin concentration there is apparent lack of the peak in 1,743  $\text{cm}^{-1}$ , whereas peak is visible in 1,709  $\text{cm}^{-1}$  (Figure 1B).

Similar spectral changes in wheat were observed in a study of DON-contamination of wheat (Abramović *et al.* 2007). Quantification of DON using PLS and MLR method showed good correlation with the reference HPLC method. The authors also reported certain advantages of ATR over DR technique reflected in better differentiation between contaminated and non-contaminated samples, as well as its simple and easy usage. It was also established that the assessment of DON-contents in wheat is feasible by measuring absorbance at only two wave numbers (1,709 and 1,743  $\text{cm}^{-1}$ ). Namely, for the majority of spectra of contaminated wheat the carbonyl peak in 1,743  $\text{cm}^{-1}$  (ATR) was lower than that of the non-contaminated wheat. The correlation between shoulder height in 1,709  $\text{cm}^{-1}$  (ATR) and occurrence of fungi on wheat has also been established. Similar results were reported by Gordon *et al.* (1998) after investigating

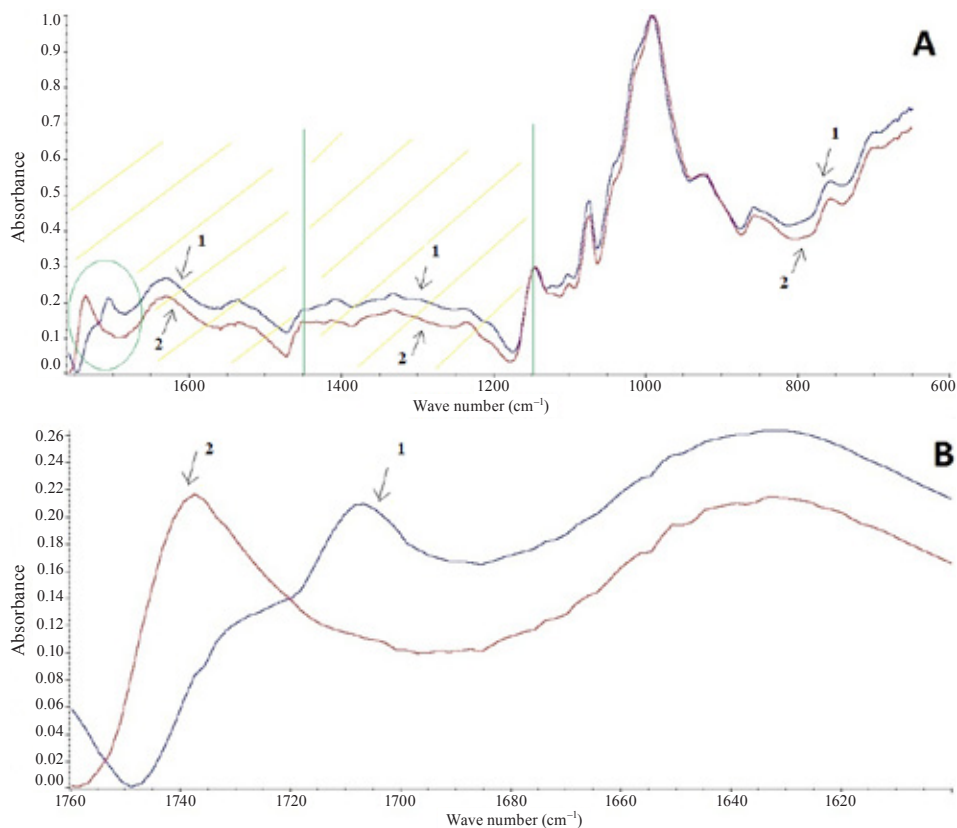


Figure 1. FTIR spectra of corn with 190 mg/kg (1) and 0.325 mg/kg fumonisins (2) (A); an enlarged part of the spectrum in the range 1,600–1,780  $\text{cm}^{-1}$  (B).

applicability of FTIR spectra for detection of corn kernels infected with mycotoxigenic molds. Shoulder height is an empirical feature for which no biochemical rationale has been proposed so far (Gordon *et al.* 1998). However, our research did not include sufficient number of such highly contaminated samples to determine the correlation of fumonisin concentration and the height of the aforementioned peak.

In order to establish relevant information that could enable differentiation of other, less contaminated samples, potential application of multivariate method was investigated. Sample numbers on Figures 2–4 represent fumonisin concentration (mg/kg) determined using ELISA. We attempted to enable satisfactory classification by narrowing spectral range to the optimal one. It was established that classification of the results obtained using PCA and cluster analysis was similar, irrelevant whether ATR technique was applied in the entire aforementioned spectral region (1,150–1,770  $\text{cm}^{-1}$ ) or the same spectral region was divided into two regions: 1,150–1,450 and 1,450–1,770  $\text{cm}^{-1}$ .

Major components of PC1 and PC2 calculated from the average ATR spectra in different spectral ranges are shown in Figures 2–4. Data treatment was kept to a minimum. All data represented the average of four consecutive measurements and then normalized. As obvious from the Figures 2–4, quite good differentiation between the less contaminated and highly contaminated samples was obtained. Two clusters of less contaminated and highly contaminated corn were clearly separated.

These two clusters are also reflected in the resulting dendrogram after performing cluster analysis with the first two principal components, which enables an accurate classification of samples with fumonisin contents of less than 10 mg/kg and those with 190 mg/kg (Figure 5).

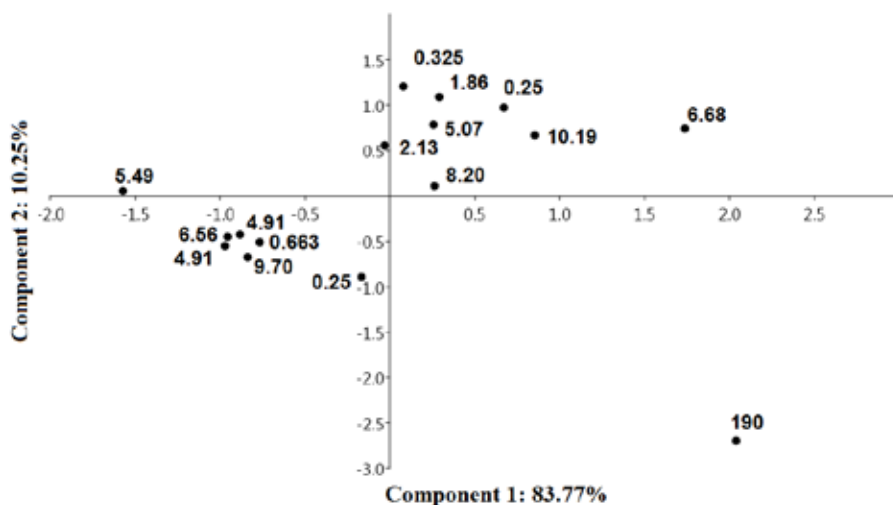


Figure 2. PCA of averaged and normalized FTIR spectra of corn with different fumonisin content. Sample numbers in the plot represent fumonisin concentration expressed in mg/kg. Analyzed spectral range 1,150–1,770  $\text{cm}^{-1}$

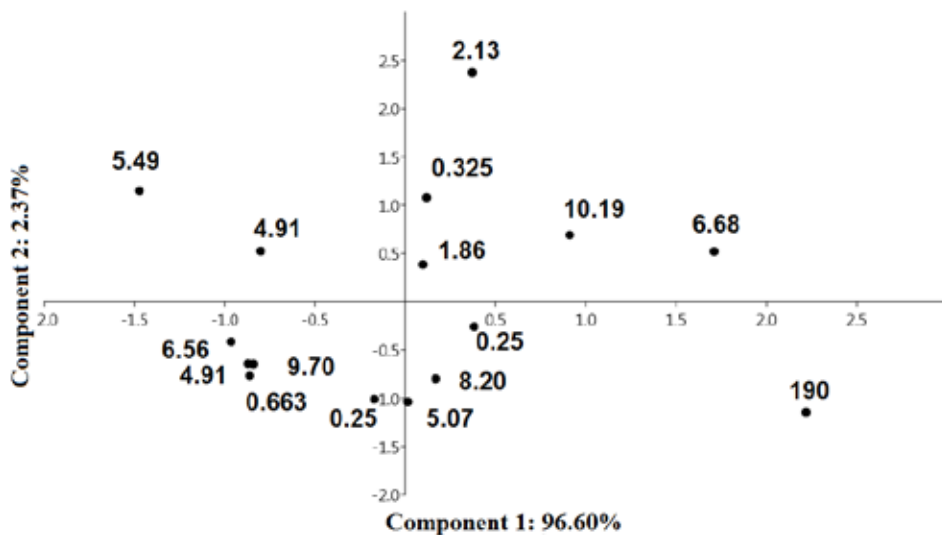


Figure 3. PCA of averaged and normalized FTIR spectra of corn with different fumonisin content. Sample numbers in the plot represent fumonisin concentration expressed in mg/kg. Analyzed spectral range 1,150–1,450  $\text{cm}^{-1}$

Samples with low fumonisin contents were impossible to classify, which is most likely due to the differences in the structure of various corn hybrids (Abramović *et al.* 2007).

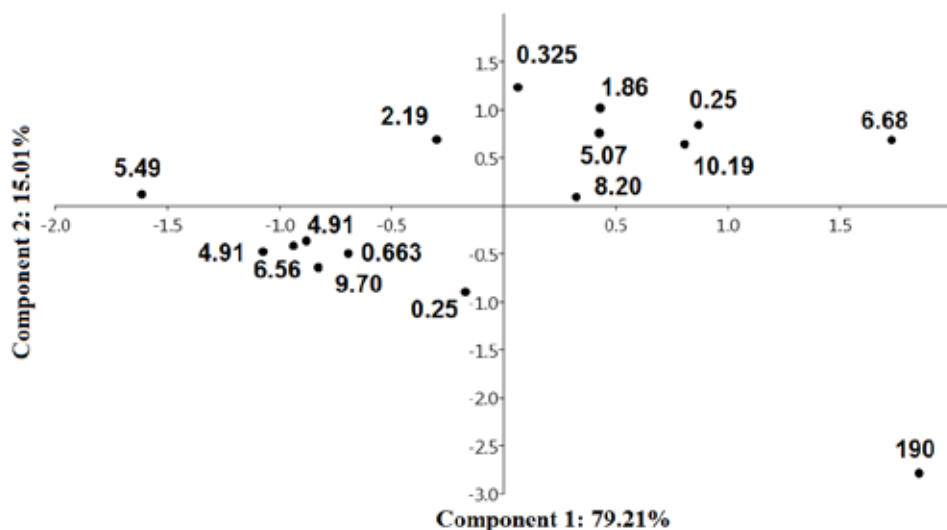
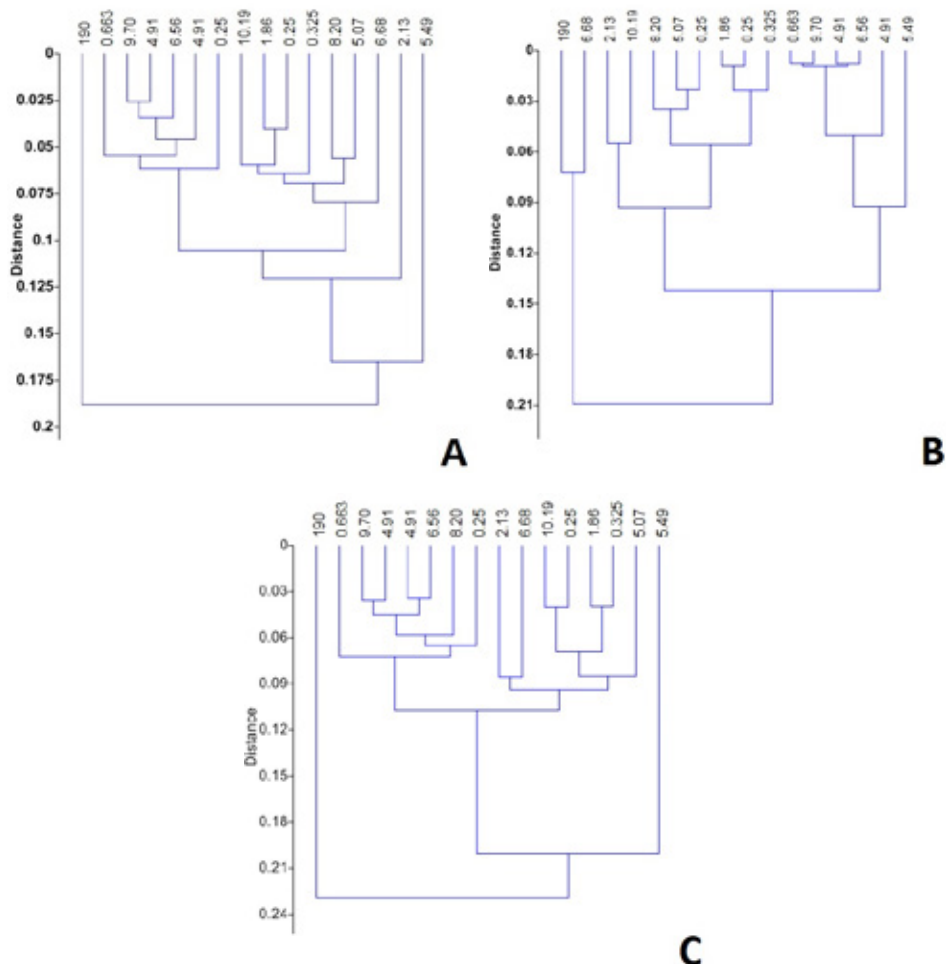


Figure 4. PCA of averaged and normalized FTIR spectra of corn with different fumonisin content. Sample numbers in the plot represent fumonisin concentration expressed in mg/kg. Analyzed spectral range 1,450–1,770  $\text{cm}^{-1}$



*Figure 5.* Cluster analysis of average and normalized FTIR spectra (A: 1,150–1,770  $\text{cm}^{-1}$ ; B: 1,150–1,450  $\text{cm}^{-1}$ ; C: 1,450–1,770  $\text{cm}^{-1}$ ) of corn with different content of fumonisin. Dendrograms obtained by Chord's average distances. The numbers of samples represent the concentrations of fumonisins in mg/kg.

Namely, only highly contaminated sample was clearly separated from other contaminated samples. Clear separation and linking of sub-clusters of contaminated samples was not adequate. When spectra were analyzed in the ranges 1,150–1,770 and 1,450–1,770  $\text{cm}^{-1}$ , besides the separation of the sample with fumonisin content 190 mg/kg, two clusters of corn with fumonisin contents 2.13 to 10.19 mg/kg separated according to the similar pattern. However, in the ranges 1,150–1,450  $\text{cm}^{-1}$ , the sample with 190 mg/kg fumonisin was not clearly separated from other samples.

## CONCLUSION

According to the obtained results of recordings of ATR-FTIR spectra, the 16 analyzed corn samples were classified into two clusters – the first one with highly contaminated corn sample (fumonisin content 190 mg/kg) and the second one with fumonisin content less than 10 mg/kg. Namely, with fumonisin content of less than 10 mg/kg classification of corn samples into separate clusters was not possible using the aforementioned method. This is most probably due to the fact that different corn hybrids were analyzed. To that end, investigation of the spectra of contaminated corn of the same genotype is planned to eliminate potential effects of different hybrids on spectral appearance.

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## ПРИМЕНА ATR-FTIR АНАЛИЗЕ ЗА ОДРЕЂИВАЊЕ ФУМОНИЗИНА У КУКУРУЗУ

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**РЕЗИМЕ:** У циљу развоја брзе, јефтине, а у исто време поуздане методе анализе плесни рода *Fusarium* као индикатора присуства фумонизина у узорцима кукуруза, испитана је могућност примене инфрацрвене спектроскопије с Фуријеовим трансформом (FTIR) применом техника умањене укупне рефлексije (ATR). Садржај фумонизина у узорцима контаминираног кукуруза претходно је квантификован ELISA методом. Код спектра узорка контаминираног с високом концентрацијом микотоксина уочен је недостатак пика на  $1.743\text{ cm}^{-1}$ , али је уочена појава пика на  $1.709\text{ cm}^{-1}$ . За класификацију резултата коришћене су анализа главних компонента (PCA) и кластер анализа, а закључци обе методе су били слични када је ATR техника примењивана у целом региону спектра ( $1.150\text{--}1.770\text{ cm}^{-1}$ ), као и када је исти део спектра подељен у два региона:  $1.150\text{--}1.450$  и  $1.450\text{--}1.770\text{ cm}^{-1}$ . Међутим, класификација узорака је нешто боља када се анализира спектар у опсезима  $1.150\text{--}1.770$  и  $1.450\text{--}1.770\text{ cm}^{-1}$ . Од 16 анализираних узорака кукуруза коректно је класификован једино јако контаминиран кукуруз са  $190\text{ mg/kg}$  у односу на остале са садржајем мањим од  $10\text{ mg/kg}$ . Такође, утврђено је да је за процену садржаја фумонизина у кукурузу овом техником потребно даље испитивање снимањем спектра контаминираног кукуруза али истог генотипа, како би се избегао могући утицај различитих хибрида на изглед спектра.

**КЉУЧНЕ РЕЧИ:** ATR-FTIR, фумонизини, кукуруз

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## PRESENCE OF DEOXYNIVALENOL IN WHEAT MILLING PRODUCTS IN SERBIA DURING 2016–2017

**ABSTRACT:** Deoxynivalenol (DON) is one of several mycotoxins produced by certain *Fusarium* species that frequently infect wheat, corn, rice, oats, barley and other grains in the field or during storage. DON affects animal and human health causing vomiting, acute temporary nausea, diarrhea, abdominal pain, headache, dizziness and fever. The objective of this study was to evaluate the natural occurrence of deoxynivalenol (DON) in white wheat flour, whole wheat flour and wheat bran. In this study, a total of 75 white wheat flour, whole wheat flour and wheat bran samples were collected in the period of 2016–2017. All samples were analyzed for DON by enzyme-linked immunosorbent assay. DON was detected in 23 out of 45 white wheat flour samples (51.11%), at levels ranging from 99 µg/kg to 440 µg/kg. Out of 15 whole wheat flour samples, 14 were contaminated by DON (93.33%), at levels ranging from 98 µg/kg to 479 µg/kg. The maximum contamination level of DON (2,790 µg/kg) in this study was found in wheat bran. Presence of DON was detected in all 15 samples of wheat bran (100%). These results suggest a high percentage of contaminated samples, especially among wheat bran samples, which raises a risk for consumers of wheat bran and the need to monitor final products before consumption.

**KEYWORDS:** Deoxynivalenol, wheat flour, wheat bran, ELISA

## INTRODUCTION

Wheat and wheat-based products are considered to be staple food for the majority of the world population (Škrbić *et al.* 2012). Unfortunately, wheat like many other cereals is susceptible to fungal attack, therefore to possible mycotoxin contamination. The occurrence of mycotoxins in cereals is of great concern worldwide, because their presence is often associated with chronic or acute mycotoxicoses. Approximately 25% of cereals produced in the world are contaminated with mycotoxins (Charmley *et al.* 1995).

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A great variety of fungi can produce mycotoxins; however several *Fusarium* species, a widespread pathogens on cereals in both temperate and semitropical areas, are a major concern for all European cereal growing areas (Bottalico, 1998). The percentage of contamination on the worldwide level for some *Fusarium* toxins, such is DON, is considered to be much higher than 25% (Bullermann, 1996).

Deoxinivalenol (DON, vomitoxin) is a natural-occurring mycotoxin, type B-trichothecenes produced mainly by strains of *F. graminearum*, a food-borne fungi widely spread in crops. DON is considered to be one of the most important mycotoxins in wheat and wheat based products. It affects both animal and human health by causing gastro-intestinal problems followed by diarrhea and vomiting (Kushiro, 2008).

Approximately 600 million tons of wheat are produced per year and most of it is converted to wheat flour for human consumption (Kushiro, 2008). This makes DON contamination of wheat a great concern for human health. Wheat-based products, such is wheat flour, hold an essential place in Serbian diet, as well. Wheat flour and wheat flour-based products, such are bread, pasta, pastry and cookies represent approximately 26% of Serbian market basket (Škrbić *et al.* 2012). Although, occurrence and prevention of DON have been intensively studied, there are only a few of studies conducted in Serbia on retention of DON after harvest and during processing. The study on retention of DON during primary processing (milling) is important for the risk assessment and management for majority of world population (Kushiro, 2008).

The objective of this study was the examination and determination of the presence of DON in wheat flour and wheat bran collected from Serbian producers in order to determine the levels of contamination in different wheat milling products.

## MATERIALS AND METHODS

### *Reagents and chemicals*

RIDASCREEN FAST DON SC (R-Biopharm), a competitive enzyme immunoassay for quantitative analysis of DON in cereals, malt and feed was used according to manufacturer's instruction (RIDASCREEN FAST DON SC Art. No.: R5905). Distilled water was used for the extraction.

### *Collection of samples*

From October 2016 until April 2017, 75 samples of white wheat flour, whole wheat flour and wheat bran were collected from 9 Serbian producers, as a part of the food safety control. Samples were collected in packs of 1 kg, according to European regulation on methods for sampling (EC 401/2006). Out of total number of samples, 45 were white wheat flour, type T 400 (25 samples) and T 500 (20 samples). 15 samples of whole wheat flour and 15 samples of wheat

bran were collected, as well. Ash content, calculated on dry matter for T 400 flour is up to 0.45%, for T 500 it ranges from 0.46 to 0.60%, for whole wheat flour it is up to 2.2%, while for wheat bran it is up to 7.0% (*Službeni glasnik Republike Srbije*, 68/16)\*. Before analysis, the samples were stored at 4–6 °C and were protected from light.

### *Sample preparation*

All samples were thoroughly mixed in order to homogenize. Namely, 5 g of each sample of white wheat flour, whole wheat flour and wheat bran were extracted by shaking with 100 mL of distilled water manually for 5 minutes. After shaking, sample extracts were filtered through Whatman No.1 filter. 50 µL of the filtrate was used for further analysis according to RIDASCREEN FAST DON SC manual.

### *Instrumental conditions*

The measurement was performed photometrically at 450 nm. UT-2100C microplate reader with absorbance range 0–3,500 A was used. Continuous reading mode was used with reading speed  $t < 5$  s. The absorbance is inversely proportional to the DON concentration in the sample. Using method was validated (LoD = 75 µg/kg, Recovery = 92%).

## RESULTS AND DISCUSSION

The results on occurrence of DON in white wheat flour, whole wheat flour and wheat bran are given in Table 1.

*Table 1.* Occurrence of deoxynivalenol (DON) in white wheat flour, whole wheat flour and wheat bran. a) Arithmetic mean. Values below the detection limit (75 µg/kg) are set to have concentration of half of detection limit

Commodity	DON (µg/kg)				
	No. of positives/total	Average value*	Median value	max value	Interval of concentration (contaminated samples)
white wheat flour	23/45	142	81	440	99–440
whole wheat flour	14/15	307	354	479	98–479
wheat bran	15/15	1,074	961	2,790	161–2,790

\* Official gazette of the Republic of Serbia, translator's comment

DON was detected in 23 out of 45 white wheat flour samples (51.11%), at levels ranging from 99 to 440  $\mu\text{g/kg}$ . The average and median values obtained for DON in white wheat flour were 142  $\mu\text{g/kg}$  and 81  $\mu\text{g/kg}$ , respectively. Out of 15 whole wheat flour samples, 14 were contaminated by DON (93.33%), at levels ranging from 98  $\mu\text{g/kg}$  to 479  $\mu\text{g/kg}$ . The average and median values obtained for DON in whole wheat flour were 307  $\mu\text{g/kg}$  and 354  $\mu\text{g/kg}$ , respectively. None of the white wheat flour nor whole wheat flour samples exceeded the limit of 750  $\mu\text{g/kg}$  set by Serbian regulative for allowed presence of DON in cereal flour (*Službeni glasnik Republike Srbije*, 29/2014, 37/2014 – isp. 39/2014, 72/2014, 80/2015, 84/2015, 35/2016 and 81/2016). The maximum contamination level of DON (2,790  $\mu\text{g/kg}$ ) in this study was found in wheat bran. Presence of DON was detected in all 15 samples of wheat bran (100%). The average and median values obtained for DON in wheat bran were 1,074  $\mu\text{g/kg}$  and 961  $\mu\text{g/kg}$ , respectively. Nine of the 15 contaminated samples of wheat bran exceeded the limit of 750  $\mu\text{g/kg}$  set by Serbian regulative for allowed presence of DON in wheat bran intended for human consumption (*Službeni glasnik Republike Srbije*, br. 29/2014, 37/2014 – isp., 39/2014, 72/2014, 80/2015, 84/2015, 35/2016 and 81/2016). However, wheat bran is mostly used as an animal feed. The limit of 8000  $\mu\text{g/kg}$  set by Serbian regulative for allowed presence of DON in wheat bran intended for animal feed was not exceeded in this case (*Službeni glasnik Republike Srbije*, 27/14).

The obtained results are in compliance with the conclusions of the study conducted by Abbas *et al.* (1985) which shows that the distribution of DON is not uniform in the milling fractions. They also found that the highest concentration of DON was in bran, followed by reduction flour and break flour, which proves that the invasion of fungus into the wheat is not uniform, as well. Trigo-Stockli *et al.* (1996) reported in the similar study that DON levels were the highest in the bran (3.4 mg/kg) and the lowest in the flour (1.5 mg/kg), as well. This could be due to the fact that after milling most of the concentration remains in outer layers (Tanaka *et al.* 1986). Others have also reported various concentrations of DON in different milling fractions. For example, Hart and Braselton (1983) reported concentrations of DON of 5.2 mg/kg and 4.5 mg/kg in bran and straight grade flour, respectively. The correlation of DON levels with ash concentration was also reported by Abbas *et al.* (1985). However, this still remains to be studied for application.

## CONCLUSION

The presence of DON was detected in 52 out of 75 analyzed samples of white wheat flour, whole wheat flour and wheat bran. The highest percentage of contaminated samples was detected among wheat bran samples (100%), followed by whole wheat flour (93.33%) and white wheat flour (51.11%). The maximum contamination level of DON (2,790  $\mu\text{g/kg}$ ) in this study was found in wheat bran. All of the wheat flour samples are in compliance with Serbian regulative (*Službeni glasnik Republike Srbije* 29/2014, 37/2014 – isp. 39/2014,

72/2014, 80/2015, 84/2015, 35/2016 and 81/2016). All of the wheat bran samples are in compliance with Serbian regulative for allowed presence of DON in wheat bran intended for animal feed (*Službeni glasnik Republike Srbije*, 27/14). However, 60% of wheat bran samples could not be used for human consumption due to exceeding levels set by Serbian regulative for allowed presence of DON in wheat bran intended for human consumption (*Službeni glasnik Republike Srbije*, 29/2014, 37/2014 – isp. 39/2014, 72/2014, 80/2015, 84/2015, 35/2016 and 81/2016).

These results suggest a high percentage of contaminated samples, especially among wheat bran samples, which raises a risk for wheat bran consumers. Additionally, this study indicates the need for continuous monitoring of final wheat based products before consumption.

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## ПОЈАВА ДЕОКСИНИВАЛЕНОЛА У МЛИНСКИМ ПРОИЗВОДИМА ОД ПШЕНИЦЕ У ПЕРИОДУ 2016–2017. У СРБИЈИ

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**РЕЗИМЕ:** Деоксиниваленол (DON) један је од неколико микотоксина које про-  
дукују одређене врсте рода *Fusarium*, које често контаминирају пшеницу, куку-  
руз, пиринач, овас, јечам и остале житарице, како у пољу, тако и током периода  
складиштења. DON утиче на здравље људи и животиња изазивајући повраћање,  
акутне мучнине, дијареју, абдоминални бол, главобоље, вртоглавице и грозницу.  
Циљ овог истраживања био је да се испита и процени природна појава деоксини-  
валенола (DON) у белом пшеничном брашну, интегралном пшеничном брашну и  
пшеничним мекињама. Укупно 75 узорак белог пшеничног брашна, интегралног  
пшеничног брашна и пшеничних мекиња узорковано је у периоду 2016–2017. Сви  
узорци су анализирани на присуство DON-а имуноензимским ELISA тестовима.  
Присуство DON-а је детектовано у 23 од 45 узорак белог пшеничног брашна  
(51,11%), у концентрационом опсегу од 99 до 440 µg/kg. Од 15 испитаних узорак  
интегралног пшеничног брашна 14 је било контаминирано (93,33%), а концен-  
трација DON-а кретала се у опсегу од 98 до 479 µg/kg. Максимална концентрација  
DON-а (2.790 µg/kg) у овом истраживању забележена је код пшеничних мекиња.  
Присуство DON-а детектовано је у свих 15 испитиваних узорак пшеничних ме-  
киња (100%). Ови резултати указују на висок проценат контаминираних узорак,  
поготово код узорак пшеничних мекиња, што представља ризик по потрошаче  
и изискује потребу за мониторингом финалних производа пре пуштања у промет.

**КЉУЧНЕ РЕЧИ:** деоксиниваленол, пшенично брашно, пшеничне мекиње,  
ELISA

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## MYCOTOXINS IN MAIZE: ANNUAL VARIATIONS AND THE IMPACT OF CLIMATE CHANGE

**ABSTRACT:** The presence of aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), and fumonisins (FUMs) was examined in maize samples from the Republic of Serbia. The maize samples were collected during the period 2012–2016, and analyzed every year after harvest using validated Enzyme Linked Immunosorbent Assay (ELISA) method. The obtained results were considered regarding the weather conditions parameters recorded during the investigated maize growing seasons. Significant differences in weather-related parameters recorded in the five-year period resulted in different mycotoxin profiles between the investigated years. Obtained results indicate that the presence of ZEA and DON in maize is characteristic of years with abundant precipitation, while AFs and OTA mainly occur in maize during hot and dry years. Furthermore, FUMs were detected with different contamination frequency in maize samples from every year. Based on the findings obtained in this study, as well as on noted changes in weather conditions in the recent years it could be assumed that maize from Serbia may become susceptible to problems concerning mycotoxins. Therefore, there is a necessity for monitoring and research related to the mycotoxins occurrence in maize from Serbia.

**KEYWORDS:** climate changes, maize, mycotoxins

## INTRODUCTION

Maize is one of the major crops cultivated throughout the world (Bitocchi *et al.* 2009). Due to its nutrient composition, maize has great usage value in both human and animal diet. It is also one of the major crops grown in the Republic of Serbia (Maslac, 2015). The total area of maize production in Serbia in the recent years has been around 1,150,000 ha with an average production of 6,650,000 Mt (IndexMundi 2012–2016; [www.indexmundi.com/agriculture](http://www.indexmundi.com/agriculture)). Furthermore, maize represents one of the most important export items of Serbia,

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which classified Serbia as a leader in terms of maize export in Europe as well as in the whole world (Maslac, 2015). Unfortunately, maize is constantly exposed to the risk of fungal development and mycotoxins synthesis (Pleadin *et al.* 2012). Furthermore, maize production in Serbia is largely rainfed and highly dependent on climate parameters (Kresović *et al.* 2014). Based on everything stated above, the presence of mycotoxins in maize should be recognized as significant concern in Serbia since these contaminants may have numerous different implications for human and animal health (IARC, 2012) and also could be a cause of great economical losses (Kresović *et al.* 2014).

Many published papers confirmed that maize could be contaminated by different mycotoxins. However, aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON) and fumonisins (FUMs) are recognized as main potential contaminants of maize (Trung *et al.* 2008). AFs represent one of the best known groups of mycotoxins mainly produced by *Aspergillus* species. OTA is mainly produced by *Aspergillus ochraceus* and *Penicillium verrucosum*. Furthermore, FUMs, ZEA and DON are mainly produced by *Fusarium sporotrichioides*, *F. verticillioides*, *F. proliferatum*, *F. culmorum*, and *F. graminearum* (Placinta *et al.* 1999). *Aspergillus* species generally occur in agricultural products in tropical and subtropical regions where there are optimum conditions of temperature and humidity for growth of fungi and production of toxins (Rustom, 1997). On the other hand, *Fusarium* species require different conditions for their growth and mycotoxins synthesis, and their presence in maize is mainly associated with moderate climate. Hence, *Fusarium* toxins are commonly found in the temperate regions of America, Europe, and Asia (Creppy, 2002). However, the presence of mycotoxin-producing fungi in maize depends on annual meteorological conditions related to the changes in weather conditions recorded in the past decades (Battilani *et al.* 2008). Furthermore, many authors indicate that weather conditions (mainly air temperature and amount of precipitation) represent factors with the strongest influence on the growth of toxigenic fungi in maize and the production of mycotoxins (Sanchis and Magan, 2004; Van der Fels-Klerx *et al.* 2013). Beside weather conditions, the presence of mycotoxins in maize can depend on several factors as follows: agronomic factors (type of hybrid, soil, tillage and previous crop), storage conditions (temperature, humidity, handling, presence of insects, rodents, and birds), as well as storage time (Hell *et al.* 2000).

AFs, OTA, ZEA, DON, and FUMs can have immunotoxic, hepatotoxic, pneumotoxic, nephrotoxic, teratogenic, mutagenic and/or carcinogenic effect (IARC, 1993; IARC, 2002). The International Agency for Research on Cancer included AFs in the first group as primary carcinogenic compounds. Furthermore, OTA and FB1 were classified in group 2B as possible carcinogenic compound to humans (IARC, 2002). Based on limited data and evidence in humans and experimental animals, DON and ZEA were classified in group 3 (IARC, 1993).

Since the presence of mycotoxins in maize may potentially affect human and animal health, maximum levels (ML) have been established in numerous countries over the world. The Regulation of Serbia (*Official Gazette RS* 28/2011)

on the control of mycotoxins in food was harmonized with the Regulation of European Union (EU) and adopted in 2011 (EC 1881/2006). According to these regulations, concentrations of MLs for AFs, OTA, ZEA, DON, and FUMs in unprocessed maize intended for human consumption are 10, 5, 350, 1,750, and 4,000 µg/kg, respectively. Furthermore, if maize is intended for animal feed, AFs, OTA, ZEA, and DON cannot be greater than 30, 250, 4,000, and 8,000 µg/kg, respectively (*Official Gazette RS* 27/2014). According to this Regulation (2014) ML for FUMs in maize for animal consumption is not declared. This Regulation is not completely harmonized with EC 576/2006. The main differences are observed in MLs for AFB1 (EC 100/2003), and guidance values for ZEA and FUMs (EC 576/2006) which are 30, 2,000, and 60,000 µg/kg, respectively.

Based on everything stated above, the aim of the present study was to provide a representative picture of the dependence of maize contamination by AFs, OTA, ZEA, DON, and FUMs and weather conditions over the 5-years investigation period.

## MATERIAL AND METHODS

### SAMPLES

A total of 50 maize samples were collected every year after harvest in the period 2012-2016. Sampling was performed according to EU requirements (EC 401/2006) in order to overcome irregular mycotoxins distribution.

### MYCOTOXINS ANALYSIS BY ELISA

Determination of AFs (AFB1, AFB2, AFG1 and AFG2), OTA, ZEA, DON, and FUMs (FB1, FB2 and FB3) was performed by Enzyme Linked Immunosorbent Assay (ELISA) method using test kits produced by Neogen Corporation (Neogen Veratox®, Lansing, USA). The analyses were performed according to the test kits instructions in the accredited laboratory of the Institute of Food Technology, University of Novi Sad. Laboratory is accredited in agreement with standard SCS ISO/IEC 17025 (2006).

Limits of quantification (LOQ) for AFs, OTA, ZEA, DON, and FUMs were 1, 2, 25, 250, and 500 µg/kg, respectively.

### METEOROLOGICAL DATA

The meteorological data related to the deviation of average air temperature (°C), number of days with precipitation, sum of precipitation (mm), and deviation of sum of precipitation (%) were provided from the Republic Hydrometeorological Service of Serbia (2012–2016). Listed data were recorded between the 1st April and the 30th September in the period 2012–2016. Deviations were determined by making comparisons with the data recorded in the long-term period (1981–2010).

## RESULTS AND DISCUSSION

The obtained results for AFs, OTA, ZEA, DON and FUMs contamination in maize samples are presented in Figure 1.

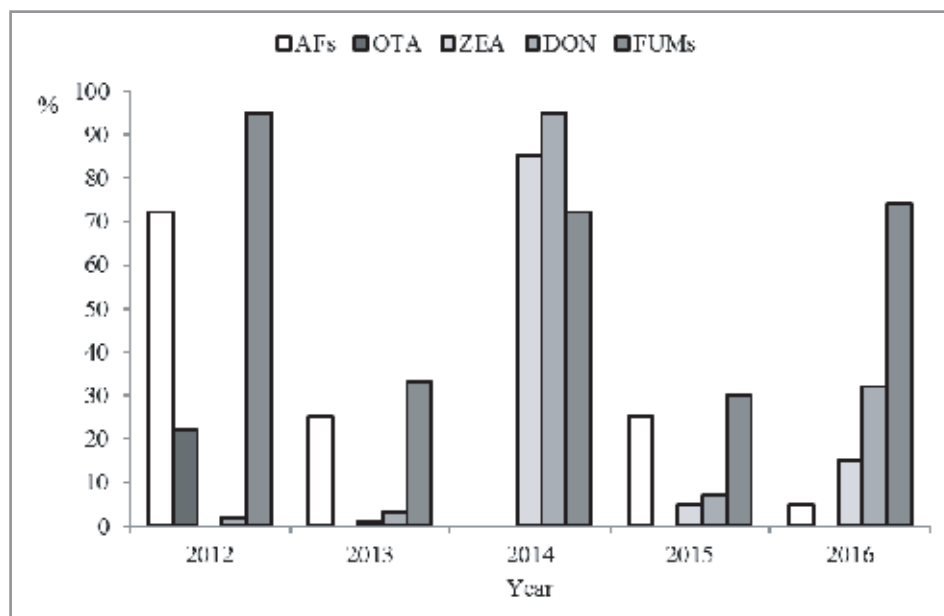


Figure 1. The presence of AFs, OTA, ZEA, DON, and FUMs in maize samples harvested in the Republic of Serbia in the period 2012–2016

As can be seen, the obtained data indicate significant differences in the occurrence of examined mycotoxins in the samples collected in the period 2012–2016. Due to the fact that weather conditions (mainly air temperature and amount of precipitation) can have a great influence on the growth of toxigenic fungi and production of mycotoxins (Sanchis and Magan, 2004), weather-related parameters for the period of maize planting, growing and harvesting (April–September, 2012–2016) were examined and shown in Table 1.

As can be seen in Table 1, maize growing season in 2012 was characterized by extremely hot and dry conditions. During the period from April to September 2012 the deviation of average air temperature was 2.4 °C. Besides that, during the same period, a significantly lower amount of precipitation (269 mm) was recorded in comparison to other investigated years (2013–2016) as well as to long term period (1981–2010). The recorded drought conditions were favorable for the growth of certain *Aspergillus* and *Fusarium* species, which resulted in the presence of AFs and FUMs in 72% and 95% of examined maize samples, respectively. Furthermore, OTA was determined in 22% of maize samples, while DON was determined in only 2% of maize samples. None of the analyzed maize samples from 2012 was contaminated with ZEA.

Table 1. Weather-related parameters for maize growing seasons in the Republic of Serbia (April–September, 2012–2016).

Year	Deviation T average (°C)	N precipitation	Σ P (mm)	Σ P (%)
2012	2.4 <sup>d</sup>	39 <sup>a</sup>	269 <sup>a</sup>	77 <sup>a</sup>
2013	1.2 <sup>b</sup>	46 <sup>a</sup>	305 <sup>b</sup>	84 <sup>a</sup>
2014	0.7 <sup>a</sup>	70 <sup>b</sup>	698 <sup>d</sup>	190 <sup>c</sup>
2015	1.9 <sup>c</sup>	43 <sup>a</sup>	297 <sup>b</sup>	79 <sup>a</sup>
2016	1.0 <sup>b</sup>	49 <sup>a</sup>	428 <sup>c</sup>	117 <sup>b</sup>

Different letters in the same column indicate significant differences ( $P < 0.05$ ) between values according to the Duncan's multiple range test.

T: temperature; N: numbers of days; P: precipitation; Σ: sum.

The maize growing season of 2013 was also hotter in comparison to long term period (1981–2010). However, deviation of average air temperature (1.2 °C) was lower, while the amount of precipitation (305 mm) was higher in comparison to the values recorded in 2012. FUMs, AFs, DON, and ZEA were detected in 33%, 25%, 3%, and 1% of examined maize samples, respectively, while none of the examined samples from 2013 was contaminated with OTA. Contrary to hot and dry maize growing seasons of 2012 and 2013, maize growing season of 2014 was extremely wet and rainy. The rainiest period in the recent years resulted in the presence of DON, ZEA, and FUMs in 98%, 85%, and 72% of examined samples, respectively. Furthermore, the outcome of rainy conditions in 2014 was that none of the maize samples from that year was contaminated with AFs and OTA.

The weather conditions recorded during maize growing season of 2015 were similar to weather conditions in 2013. FUMs, AFs, DON, and ZEA were determined in 30%, 25%, 7%, and 5% of maize samples from 2015, respectively. Higher amount of precipitation in maize growing season of 2016 in comparison to the long-term period (1981–2010) in combination with temperature around average values resulted in the presence of FUMs and DON in 74% and 32% of maize samples, while ZEA and AFs were determined in 15% and 5%, respectively. As well as in the previous years, the presence of OTA was not determined.

According to several previous studies (Van der Fels-Klerx *et al.* 2013; Baranyi *et al.* 2015), the obtained results in this study confirmed that the presence of AFs and OTA are characteristic for hot and dry years, while ZEA and DON mainly occur in years with abundant precipitation. Even though the significant differences between weather conditions were recorded in the investigated period, it could be noted that FUMs were detected in maize samples from every year. However, it should be emphasized that in maize from different years FUMs were detected with different contamination frequency.

## CONCLUSION

The findings obtained in this study indicate significant differences in the occurrence of AFs, OTA, ZEA, DON, and FUMs in the maize samples harvested

during five maize growing seasons. Regarding the changes in weather conditions in the recent years, it could be assumed that maize from Serbia may become susceptible to problems concerning mycotoxins. Therefore, there is a need for future constant monitoring of mycotoxins occurrence in maize from Serbia in order to protect human and animal health and to avoid economic losses.

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## МИКОТОКСИНИ У КУКУРУЗУ: ГОДИШЊЕ ВАРИЈАЦИЈЕ И УТИЦАЈ КЛИМАТСКИХ ПРОМЕНА

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**РЕЗИМЕ:** Појава афлатоксина (AF), охратоксина А (ОТА), зearаленона (ZEA), деоксиниваленона (DON) и фумонизина (FUM) испитана је у узорцима кукуруза сакупљених на територији Републике Србије. Узорци кукуруза сакупљени су током периода од 2012. до 2016. године, и анализирани су сваке године након жетве применом валидоване ELISA методе. Добијени резултати у вези са појавом микотоксина у кукурузу тумачени су у односу на забележене временске услове

током испитиваних производних година. Значајне забележене разлике у временским условима, током испитиваног периода од пет година, условиле су и присуство односно одсуство појединих микотоксина. Добијени резултати указују да је појава ZEA и DON карактеристична за године с великим количинама падавина, док је контаминација кукуруза са AF и OTA уочена у годинама у којима су временски услови окарактерисани као суви и топли. Надаље, FUM су детектовани у узорцима кукуруза из свих пет производних година, али с различитом фреквенцијом појаве. На основу добијених резултата у овом раду, као и на основу уочених промена у временским условима током испитиваног периода 2012–2016. оправдано је очекивати да ће и у будућности кукуруз из Србије бити подложен контаминацији микотоксинима. Стога, постоји константна потреба и тежња за континуираним мониторинзима и истраживањима у вези с појавом микотоксина у кукурузу из Србије, како би се заштитило здравље људи и животиња, а уједно и смањили економски губици.

КЉУЧНЕ РЕЧИ: климатске промене, кукуруз, микотоксини

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## FUMONISINS PRODUCTION POTENTIAL OF *Fusarium verticillioides* ISOLATED FROM SERBIAN MAIZE AND WHEAT KERNELS

**ABSTRACT:** The production of fumonisins by potentially toxigenic *Fusarium verticillioides* isolates originating from Serbian maize and wheat kernels was tested *in vitro*. A total of six *F. verticillioides* isolates were incubated on yeast extract sucrose medium (YESA) for 4 weeks at 25 °C in the dark. Their toxin production potential was tested by applying a modified HPLC method for determination of fumonisins in cereals, since the TLC method gave no results. Analyses were performed on a HPLC-FLD system after sample extraction from YESA and extract clean-up on a SPE column.

Although the isolates were tested for fumonisin B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>, only fumonisin B<sub>1</sub> was detected. The results showed that all tested isolates had toxigenic potential for fumonisin B<sub>1</sub> production. The average fumonisin B<sub>1</sub> production of the isolates ranged from 7 to 289 µg/kg, thus indicating a highly variable toxigenic potential among the isolates. Isolate 1282 expressed the highest toxigenic potential for fumonisin B<sub>1</sub> production (289 µg/kg), while isolate 2533/A showed a questionable potential for fumonisin production (7 µg/kg).

**KEYWORDS:** fumonisin, *Fusarium verticillioides*, cereals, toxigenic potential

## INTRODUCTION

Cereals are commonly invaded by *Fusarium* spp. and often contaminated by their secondary metabolites that have a major impact on human and animal

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health, welfare and productivity. Among *Fusarium* toxins, zearalenone, fumonisins (FUMs) and trichothecenes are the most toxicologically important and occur most frequently (Cetin and Bullerman, 2005). The most important fumonisins (FUMs) are FB<sub>1</sub>, fumonisin B<sub>2</sub> (FB<sub>2</sub>), and fumonisin B<sub>3</sub> (FB<sub>3</sub>), which are predominantly produced by *F. verticillioides*, *F. proliferatum*, *F. anthophilum*, and *F. nygamai* (Yazar and Omurtag, 2008). FUMs are usually present in maize and maize products, but they also can be found in wheat and non-corn based foodstuffs (Cirillo *et al.* 2003; Kushihiro *et al.* 2009).

FUMs are structurally similar to sphingosine, a component of sphingolipids. Sphingolipids are typically found as myelin components. It is believed that FUMs toxicity is a result of sphingolipid biosynthesis blockage. It has been known that FUMs cause leucoencephalomalacia in horses, pulmonary edema in swine, and hepatotoxicity in rats (Gelderblom *et al.* 2001). There is a report about an outbreak of FUMs toxicosis in Serbia (Jovanović *et al.* 2015). In feed samples from the premises, fumonisin B<sub>1</sub> and B<sub>2</sub> were found in the following concentrations: 6.0 mg/kg FB<sub>1</sub> and 2.4 mg/kg FB<sub>2</sub> in the milled maize samples, and 6.05 mg/kg FB<sub>1</sub> and 1.68 mg/kg FB<sub>2</sub> in maize grain, respectively. IARC classified FB<sub>1</sub> as a group 2B carcinogen that is possibly carcinogenic to humans (Bray *et al.* 2002).

The occurrence of *F. verticillioides* and other toxigenic *Fusarium* spp. as well as the production of their secondary metabolites (FUMs and other fusariotoxins) are determined by environmental factors in the field, and also by transportation and storage. In wheat harvested in southern Brazil, Mendes *et al.* (2015) found that 54% of the samples were contaminated with FB<sub>1</sub> at levels ranging from 958 to 4,906 µg/kg. Natural FUMs presence in common wheat grains in Argentina was even higher (Cendoya *et al.* 2014). Out of the total number of samples collected during the 2011 harvest, 93% showed FUM contamination with levels ranging from 0.15 to 1,304.39 µg/kg. In 2015, there were five notifications (three in 2014) related to the presence of FUMs in maize and maize products (one of which combined with a high level of aflatoxins) (RASFF, 2016).

Studies in Serbia have shown different results of FB<sub>1</sub> occurrence in wheat among different production years. Stanković *et al.* (2012) reported high incidence (82.1% and 92.0%) and levels (up to 5,400 µg/kg) of FB<sub>1</sub> in wheat kernels from the 2005 and 2007 harvests, while Jakšić *et al.* (2012) indicated significant presence of FB<sub>1</sub> (50.7%), but at lower levels (27–614 µg/kg), in the samples from the 2010 harvest. *F. verticillioides* and *F. proliferatum* isolates originated from wheat kernels had a high FB<sub>1</sub> production potential (Stanković *et al.* 2012).

Maize infections by *Fusarium* species and its contamination with FUMs in Serbia are not so rare. Stanković *et al.* (2011) found a very high occurrence of FB<sub>1</sub> (70.7%) in 203 maize samples. However, Krnjaja *et al.* (2011) reported about not so high occurrence of *Fusarium* spp. in two hybrids of maize grains for silage (3.89% and 42.00%). These authors also found that *F. verticillioides* was the dominant mould from *Fusarium* genera. The toxigenic potential of some maize isolates has also been confirmed. Namely, Tančić *et al.* (2012) investigated 16 maize isolates of *F. verticillioides* for the ability to produce FB<sub>1</sub>. It was reported that all isolates produced FB<sub>1</sub> in concentrations ranging from 88.60 to 1,300.60 mg/kg.

Hence, the aim of this research was to investigate *in vitro* production of FUMs by *F. verticillioides* isolates that originated from Serbian maize and wheat kernels, by using screening (TLC) and confirmatory (HPLC) analytical methods.

## MATERIALS AND METHODS

### Isolation of *Fusarium verticillioides*

Potentially toxigenic *F. verticillioides* cultures were isolated from Serbian maize and wheat kernels collected in the Province of Vojvodina (northern part of Serbia) (Table 1). Coarsely ground kernel samples were placed on DG 18 (dichloran 18% glycerol agar) and incubated at 25 °C for 7 days in the dark. *Fusarium* spp. isolates obtained from growing colonies on DG 18 were transferred individually on PDA (potato dextrose agar) for further purification and identification. Fragments of the colonies developed on PDA were transferred to SNA (synthetic nutrition agar) (Nirenberg and O' Donnell, 1998). Determinations of the fusaria were done according to Nelson *et al.* (1983). The stock cultures of *F. verticillioides* were maintained on PDA at 4–6 °C as a part of the Maize Research Institute “Zemun Polje” collection.

Table 1. Origin of the cereal samples

Ord. No.	Cereal	Locality	Sample designation
1.	Wheat	Despotovac	825
2.	Corn	Zemun Polje	914
3.	Corn	Bačka Topola	1282
4.	Corn	Zemun Polje	2533

### Production and TLC analysis of fumonisins

*F. verticillioides* isolates were cultivated on yeast extract-sucrose agar (YESA – 2% yeast extract, 15% sucrose, and 2% agar, pH 6.5) (Samson and van Reenen-Hoekstra, 1988). The capacity for toxin production of the tested isolates was determined after 28 days of cultivation at 25 °C in the dark according to a rapid screening method (Bočarov-Stančić *et al.* 2009). The agar plugs (diameter 5 mm) were cut out from the colony center with a sterile metal borer, removed from the agar plate and placed with sterile tweezers in a sterilized Petri dish with the mycelial side up. The circular plugs were wetted with 10–20 µl of chloroform/methanol (2:1 v/v) and after few minutes the rapidly extracted mycelial side was gently applied against the TLC plate (Alugram SIL G/UV 254, Macherey-Nagel). After drying the application spot, another agar plug of the same colony was applied nearby, together with 30 µl of the working FB1 standard (100 µg/ml). The thin-layer chromatography was performed in tanks with a saturated toluene-ethyl acetate-formic acid developing solvent

(50+40+10, v/v). After developing plates and air drying in a dark fume extractor, the plates were sprayed with 20%  $\text{AlCl}_3$ , dried in the oven (120 °C) and examined under long wave UV light (366 nm). Three replicates were performed for all analyses.

### Extraction of fumonisins from YESA

#### Reagents

HPLC gradient grade methanol (MeOH) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Reagent solution o-phthalaldehyde (OPA) for derivatization was prepared as follows: 40 mg of OPA (Sigma-Aldrich, Darmstadt, German) was dissolved in 1 ml of MeOH, diluted with 5 ml of 0.1 mol/l  $\text{Na}_2\text{B}_4\text{O}_7$ , and finally 50  $\mu\text{L}$  of 2-mercaptoethanol (Serva Electrophoresis, Heidelberg, Germany) was added.

#### Extraction and Clean-up

A 20 g sample of YESA containing 4-week incubated *F. verticillioides* was extracted using 50 ml of chloroform–methanol (2+1, v/v) mixture. After 30 min extraction on a magnetic stirrer (Automatic Science Instrument, China), the extract was filtered through slow running filter paper (Filtros Anioia, Barcelona, Spain) and evaporated to dryness. The residue was dissolved in 25 ml of solvent MeOH–water (75+25, v/v). Clean-up was done with MycoSep 231 Fum columns (RomerLabs, USA), using 5 ml of the dissolved extract (pH adjusted to 8–9 using 12.5% ammonia solution) purified in the column and 3 ml evaporated to dryness and reconstituted in 200  $\mu\text{L}$  of ACN– $\text{H}_2\text{O}$  (50+50, v/v). The reconstituted extract was derivatized with OPA at a ratio of 1:1 (v/v), and 5  $\mu\text{L}$  was injected into the HPLC system after 1 min.

### Quantification of fumonisins by the modified HPLC method

#### Analytical Standards

Fumonisin mix solution OEKANAL®, analytical standard 50  $\mu\text{g}/\text{ml}$  FB1 and FB2 in ACN–water (50+50, v/v), Sigma-Aldrich Article/Product 34143, and FB3 solution OEKANAL®, analytical standard 50  $\mu\text{g}/\text{ml}$  in ACN–water (50+50, v/v), Sigma-Aldrich Article/Product 32606, were used to prepare calibrant solutions in the concentrations of 0.25 to 4.0  $\mu\text{g}/\text{ml}$  in ACN–water (50+50, v/v) for each toxin.

#### HPLC–FLD Analysis

The researchers used HPLC Dionex UltiMate 3000 Series system with FLD 3100 (Thermo Scientific, Germany), consisting of an auto sampler WPS-3000, degasser, quaternary pump, and SupelcosilTMLC-18-DB column (250×4.6 mm, particle size 5  $\mu\text{m}$ ; Supelco, USA). The system was controlled by Chromeleon® 7 software (Thermo Scientific). The mobile phase was 0.1 mol/l MeOH –

NaH<sub>2</sub>PO<sub>4</sub> (80+20, v/v) adjusted to the pH 3.40 with H<sub>3</sub>PO<sub>4</sub>, filtered through 0.22 µm membrane filter, at a flow rate of 1.0 ml/min. Wavelength of excitation radiation was 335 nm and emission 440 nm.

## RESULTS AND DISCUSSION

TLC screening analysis showed that none of the investigated *F. verticillioides* isolates produced FB<sub>1</sub>. Therefore, it can be said that this method was not suitable for testing the potential for the biosynthesis of FUMs by *F. verticillioides* isolates under laboratory conditions, although it has given good results in the case of other fusaria and fusariotoxins (deoxynivalenol, zearalenon, diacetoxyscirpenol and T-2 toxin) (Bočarov-Stančić *et al.* 2009).

However, the HPLC method showed that all the isolates possessed a toxigenic potential for FUM production (Table 2). It is important to emphasize that only FB<sub>1</sub> was detected, although FB<sub>2</sub> and FB<sub>3</sub> were also analyzed.

The average FB<sub>1</sub> production in two wheat isolates were 29 and 61 µg/kg, while in four maize isolates the average FB<sub>1</sub> levels ranged from 7 to 289 µg/kg.

Table 2. Fumonisin production (µg/kg) by *Fusarium verticillioides* isolates

Ord. No.	Isolate designation	Fumonisin B1 (µg/kg)
1.	825/A	29
2.	825/B	61
3.	914	99
4.	1282	289
5.	2533/A	7
6.	2533/B	44

Many isolates of *F. verticillioides* could produce FUMs. It was found by Nelson *et al.* (1991) that Australian isolates produced only trace amounts of FB<sub>1</sub>. Nevertheless, Miller *et al.* (1993) discovered medium level (147 mg/kg) producing isolates in Southeast Asia. One of the greatest FB<sub>1</sub> yields (17,900 mg/kg) was reported in South Africa. This toxin yield was obtained from whole maize kernels as a culture material, with *F. verticillioides* MRC 826 as inoculum, incubated at 20 °C in the dark for 13 weeks (Alberts *et al.* 1990).

In Europe, the isolate of *F. proliferatum* from maize investigated for FUMs production biosynthesized much larger quantities of this fusariotoxin (31,000 mg/kg) (Castellá *et al.* 1999).

According to Nelson *et al.* (1991), strains producing less than 50 mg/kg of FB<sub>1</sub> are low producers, those producing 50–500 mg/kg are intermediate producers and those producing more than 500 mg/kg are high producers. Regarding our results, it can be concluded that all our investigated isolates of *F. verticillioides* are low FB<sub>1</sub> producers.

Having in mind some previous researches in Serbia, it can be said that Serbian isolates generally showed a significantly less capacity for toxin production than South African or Spanish strains. Namely, maize isolates reported by Tančić *et al.* (2012) produced 88.60–1,300.60 mg/kg of FB<sub>1</sub> on maize grain media, which is a larger magnitude when compared to our results.

## CONCLUSION

The fast screening TLC method with cultivation of mycobiota on a YESA medium was not suitable for testing the potential for the biosynthesis of FUMs by *F. verticillioides* isolates under laboratory conditions, probably due to high detection limit.

However, a modified screening method that consisted of the extraction of FUMs from YESA, clean-up of the extract on a SPE column followed by the HPLC-FLD analysis was suitable for detection of FUMs production *in vitro*.

All the tested *F. verticillioides* isolates from maize and wheat kernels biosynthesized FB<sub>1</sub> *in vitro*, although rather low yields of toxin were obtained (7–289 µg/kg).

Future investigations should show whether such low levels of FB<sub>1</sub> are the result of the cultivation procedure or poor extraction efficacy of the modified analytical method.

## ACKNOWLEDGEMENT

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## ПОТЕНЦИЈАЛ ЗА ПРОИЗВОДЊУ ФУМОНИЗИНА КОД ИЗОЛАТА *Fusarium verticillioides* СА ЗРНА КУКУРУЗА И ПШЕНИЦЕ ИЗ СРБИЈЕ

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**РЕЗИМЕ:** Производња фумонизина код потенцијално токсикогених *Fusarium verticillioides* изолата са зрна кукуруза и пшенице пореклом из Србије тестиран је *in vitro*. Укупно шест изолата *F. verticillioides* инкубирано је на агаризованој подлози са екстрактом квасца и сахарозом (YESA) током четири недеље, у мраку на 25 °C. Капацитети одабраних изолата за производњу токсина детектовани су применом модификоване HPLC методе за одређивање фумонизина у зрну житарица, јер брза тријажна TLC метода није дала позитивне резултате. Анализа је изведена на HPLC-FLD систему након екстракције узорка из YESA култура изолата и пречишћавања екстракта на SPE колони. Све анализе су урађене у три понављања. Иако су изолати *F. verticillioides* тестирани на присуство фумонизина B<sub>1</sub>, B<sub>2</sub> и B<sub>3</sub>, само је фумонизин B<sub>1</sub> био детектован. Резултати су показали да сви тестирани изолати имају потенцијал у синтези фумонизина B<sub>1</sub>. Просечна вредност произведеног фумонизина B<sub>1</sub> код изолата *F. verticillioides* кретала се од 7 до 289 µg/kg, што указује на изузетно варијабилан токсигени потенцијал истих изолата. Изолат означен са 1282 показао је највећи потенцијал за биосинтезу фумонизина B<sub>1</sub> (289 µg/kg), док је изолат 2533/A испољио дискутабилан потенцијал за производњу истог фумонизина (7 µg/kg).

**КЉУЧНЕ РЕЧИ:** фумонизин, *Fusarium verticillioides*, житарице, токсикогени потенцијал

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## COMPARISON OF *ELISA* AND *HPLC* METHODS FOR THE DETECTION OF MYCOTOXINS BY ANALYSING PROFICIENCY TEST RESULTS

**ABSTRACT:** Different analytical techniques for the detection of mycotoxins have been developed in order to control the levels of mycotoxins in food and feed. Conventional analytical methods for mycotoxin determination are involving techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Also, rapid methods for mycotoxin analysis have become increasingly important. Enzyme-linked immuno-sorbent assay (ELISA) is one of the most common rapid methods for determination of these natural contaminants. The aim of this study was to provide a comparison between two different methods of analysis (HPLC and ELISA) for the detection of different mycotoxins using data that originate from commercial proficiency tests. Based on the statistical evaluation of the results for both methods, in three proficiency tests for various mycotoxins (aflatoxins, ochratoxin and zearalenone), it could be concluded that both techniques can equally be used, although ELISA is considered to be the screening one.

**KEYWORDS:** ELISA, HPLC, food and feed, mycotoxins

## INTRODUCTION

Mycotoxins are a major analytical challenge due to the range of chemical compounds that they represent and the vast array of feed matrices in which they are found. Analysis is essential for determining the extent of mycotoxin contamination, for risk analysis, confirming the diagnosis of a mycotoxicosis and for monitoring mycotoxin mitigation strategies (Nesic *et al.* 2013). In order to control the levels of these natural contaminants in food and feed, different analytical tools for their detection have been developed. The most common

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are chromatographic techniques, but also rapid immunochemical methods have become increasingly important.

Chromatographic techniques are based on the physical interaction between a mobile phase and a stationary phase. The components to be separated are distributed between these two phases. The mobile phase is usually a fluid that penetrates through or along the stationary bed (liquid or solid). Liquid, gas and supercritical fluids are currently used as mobile phase and chromatographic techniques derive their names from the nature of the mobile phase: liquid chromatography, gas chromatography and supercritical fluid chromatography, respectively. In practice, the sample to be analyzed is dissolved in the mobile phase and applied as a spot on the stationary phase. The analyte or sample is carried along by the mobile phase and partitions between the solid and liquid stationary phase are called the sorbent. The various constituents in the analytes travel at different speeds resulting in differential partitioning of the constituents between the mobile and the stationary phases. The most commonly used chromatography techniques for analysis of mycotoxins are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Although many of the chromatographic techniques are very sensitive, they require trained technician, cumbersome pretreatment of sample and expensive apparatus/equipment (Wacoo *et al.* 2014).

High-performance liquid chromatography (HPLC) is the most popular chromatographic technique for separation and determination of organic compounds. It is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. All chromatographic separations, including HPLC, operate under the same basic principle – separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation. HPLC instrumentation includes a pump, an injector, a column, a detector and an integrator or an acquisition and a display system. The heart of the system is the column where separation occurs (Brown and DeAntonois, 1997).

High-performance liquid chromatography provides fast and accurate mycotoxin detection results within a short time. A sensitivity of detection as low as 0.1 ng/kg has been reported. However, the disadvantage of using HPLC for this purpose is the requirement of rigorous sample purification using immunoaffinity columns. In addition, HPLC requires tedious pre- and post column derivatization processes to improve the detection limits. Therefore, to overcome the challenges associated with derivatization processes in mycotoxin analysis, a modification of the HPLC method, whereby the HPLC is coupled to mass spectroscopy, has been made and is currently employed in the determination of mycotoxins (Wacoo *et al.* 2014). Since the mass spectrometer requires neither use of UV fluorescence nor the absorbance of an analyte, the need for chemical derivatization of compounds is eliminated. The HPLC-MS/MS uses small amounts of sample to generate structural information and exhibits low detection limits (Rahmani *et al.* 2009). However, HPLC-MS/MS is bulky and very expensive equipment which

can only be operated by trained and skilled personnel. Besides, this also limits its use to laboratory environment only and not field conditions.

Immunochemical detection methods vary from simple immunoassay to highly sophisticated immunosensors, but they all rely on the specificity of binding between antibodies and antigens. The immunochemical reaction, i.e., the binding of antibody and antigen, in an assay is not visible and therefore several means to detect the reaction product, the immune complex, have been developed based on signal-generating components and appropriate measuring devices. The various immunoassays are named based on the signal-generating component or tracer: Radioimmunoassay (RIA), Enzyme-Linked Immunosorbent assay (ELISA), Chemiluminiscent Immunoassay (CL-IA), Fluorescent Immunoassay (FIA), Lateral Flow Immunoassay (LF-IA) and immunosensors (Meulenbergh, 2012). The popularity of the antibody-antigen based techniques, since their development in the late 1970s, is due to their high level of specificity and sensitivity even in the presence of contaminating materials. Besides, immunochemical methods do not require skilled and highly trained personnel to troubleshoot in case of any problems during separation; they are less labor intensive and consume less time, in which respects they are preferable to both chromatographic and spectrophotometric techniques.

ELISA test kits are well favored as high through-put assays with low sample volume requirements and often less sample clean-up procedures compared to conventional methods such as TLC and HPLC. They are rapid, simple, specific, sensitive and have become the most common quick methods for the detection of mycotoxins in foods and feeds. However, although the antibodies have the advantage of high specificity and sensitivity, because the target compounds are mycotoxins but not the antigens, compounds with similar chemical groups would also interact with the antibodies. This so-called matrix effect or matrix interference commonly occurs in ELISA methods, which can give rise to underestimates or overestimates in mycotoxin concentrations in commodity samples. Additionally, insufficient validation in ELISA methods causes the methods to be limited in the range of matrices examined. Therefore, an extensive study on the accuracy and precision of the ELISA method over a wide range of commodities is still needed and a full validation for an ELISA method is essential and critical (Mohamadi *et al.* 2012). The conventional wisdom is that ELISA kits should be used routinely only for the analysis of matrices that are extensively tested. Confirmatory analyses by more robust methods, e.g. HPLC with IAC clean-up or LC-MS, are required for the contamination levels that approach the legal limit (Pascale, 2009).

When monitoring for the presence of mycotoxins either in raw and derived agricultural products, there is a large choice of methods. Depending on the purpose, either rapid detection or validation according to the regulations, one can use quantitative and qualitative methods. Among the available conventional methods, HPLC has been traditionally applied, and among the immunochemical methods, ELISA has also been used. Having all that on our mind, the aim of this paper is to provide a comparison between two most used methods for the detection of different mycotoxins (HPLC and ELISA) using data that originate from commercial proficiency tests.

## MATERIAL AND METHODS

The data were obtained from the official reports of three commercial international proficiency tests (PT):

- Aflatoxin PT in 2015. The sample was naturally contaminated corn. The material was sent to 222 laboratories and the results returned from 160 laboratory (72%). The following methods were reported to be used: ELISA in 93 labs, HPLC with a variety of detection systems (FLD, MS, MS/MS) in 58 labs, fluorimeter in 1 lab, LFD (Lateral Flow Device – strips for rapid determination) in 4 laboratories and 4 laboratories did not declare the method they used.
- Zearalenone PT in 2014. The test material was naturally contaminated wheat. Sample was sent to 170 laboratories, while 124 of them (73%) sent the feedback. Among them ELISA method was used in 75 laboratories, HPLC with a variety of detection systems (FLD, MS, MS/MS) in 45 laboratories, LFD (Lateral Flow Device – strips for rapid determination) in 2 laboratories, TLC in 1 laboratory and 1 laboratory did not express the method.
- Ochratoxin PT in 2013. Sample was naturally contaminated wheat. The material was sent to 98 laboratories and the results were reported by 78 laboratories (80%), where: 30 laboratories used ELISA method and 48 laboratories used HPLC with a variety of detection systems (FLD, MS, MS/MS).

In order to compare the most used methods (HPLC and ELISA) for statistical analysis of data in all three proficiency tests, for calculations and graphs, MS Excel 2002 was applied (Schmuller, 2009), as well as Grubbs' test on-line calculator on the GraphPad portal [www.graphpad.com/quickcalcs/grubbs2/](http://www.graphpad.com/quickcalcs/grubbs2/) (Motulsky, 1999). For the calculation of robust parameters of data distribution free Excel add-in Robust Statistics Toolkit was used, which can be downloaded from the portal of Royal Society of Chemistry, United Kingdom, Analytical Methods Committee (AMC, 2001). Also, GraphPad Prism v4.0 for Mann-Whitney U test, which is considered a non-parametric t-test, was applied. In the analyzed PT schemes target standard deviation  $SD_p$  is calculated, depending on the target concentration, by the first of three forms of modified Horwitz equation (Thompson, 2000; FAPAS, 2016):

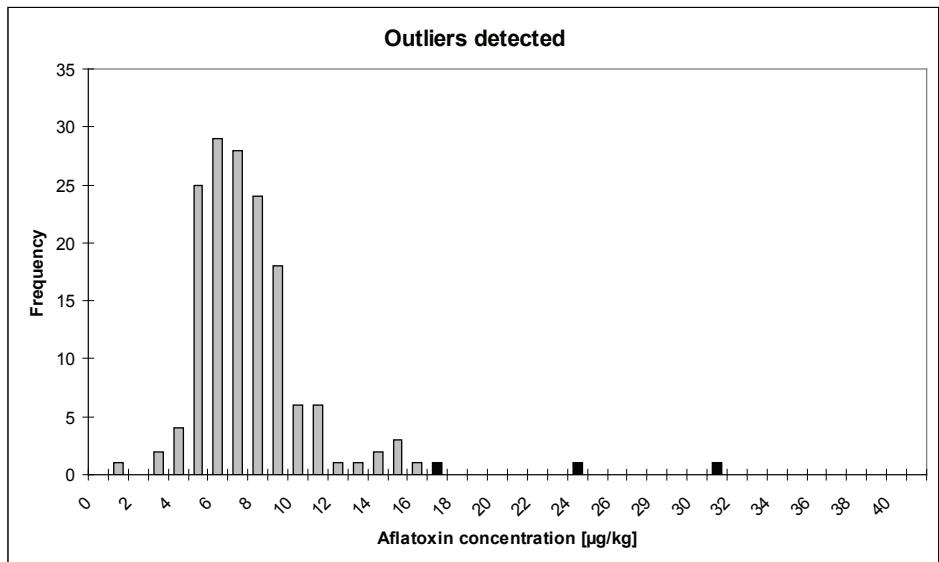
$$SD_p = 0,22 \times \text{assigned value} \quad \text{If assigned value} < 120 \mu\text{g/kg (ppb)}$$

## RESULTS AND DISCUSSION

### *Aflatoxin*

According to the report of the PT provider, the assigned value for total aflatoxins in the proficiency test 2015 was  $6.4 \mu\text{g/kg}$  (ppb). The assigned value was calculated as the robust mean by Huber's H15 method. Target standard

deviation  $SD_p$  calculated by modified Horwitz equation was  $SD_p = 1.4$  ppb. This means that the target accuracy was (CV%) 22%. Satisfactory results ( $z < 2$ ) were achieved by 78.5% of laboratories that used ELISA method and 82.8% who used HPLC.

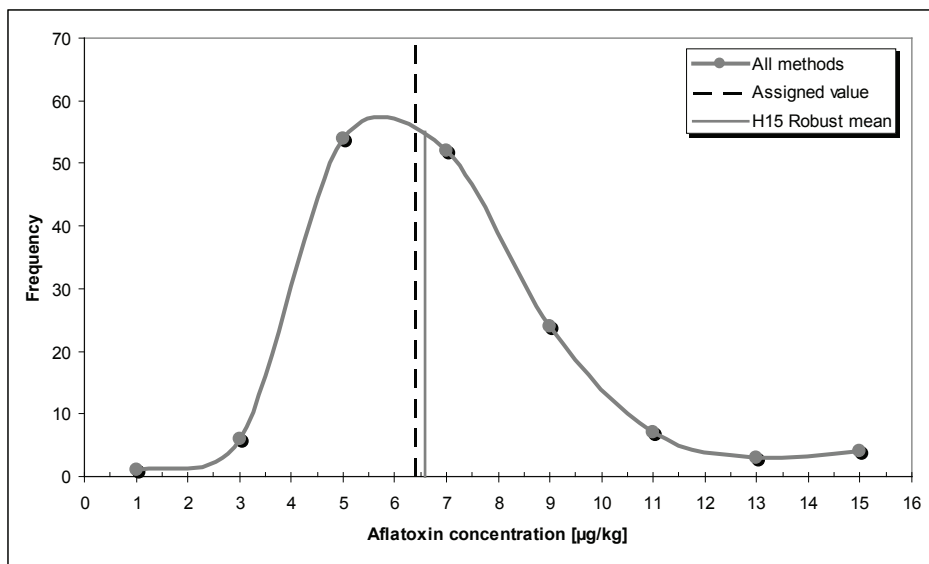


Graph 1. Outliers in the overall aflatoxin PT results

First of all, in analysis of data available in the PT report, results in non-numerical form (given as e.g. “< 2 ppb”) were removed. Outliers were excluded using Grubbs’ test on-line calculator. On the Graph 1, with the data sorted into the class intervals of 1 ppb, outliers are colored in black. From a total of 160 results 9 of them were excluded from further analysis.

For the rest of results it was calculated: Mean value = 6.89 ppb (higher than assigned value 6.4 ppb); SD = 2.41 ppb (higher than  $SD_p = 1.40$  ppb); CV% = 34.9% (the accuracy of all methods, the real inter-laboratory reproducibility); Median = 6.50 ppb;  $MAD_e = 2.05$  ppb (Median of Absolute Deviations); Huber’s H15 Robust Mean = 6.59 ppb; Huber’s H15 Robust SD = 2.03 ppb; Trimmed Mean 25% = 6.61 ppb; Modus = 5.92 ppb, calculated over the data sorted into class intervals width of 2 ppb.

The asymmetry of data distribution – Skewness = 1.103 (where 0 means there is no asymmetry, while a positive number means that there is a right-sided asymmetry distribution). Asymmetry is visible on the chart with the data sorted into class intervals width of 2 ppb, but we estimate that it is not too big and in with further calculations it can be considered that the data have a normal distribution.

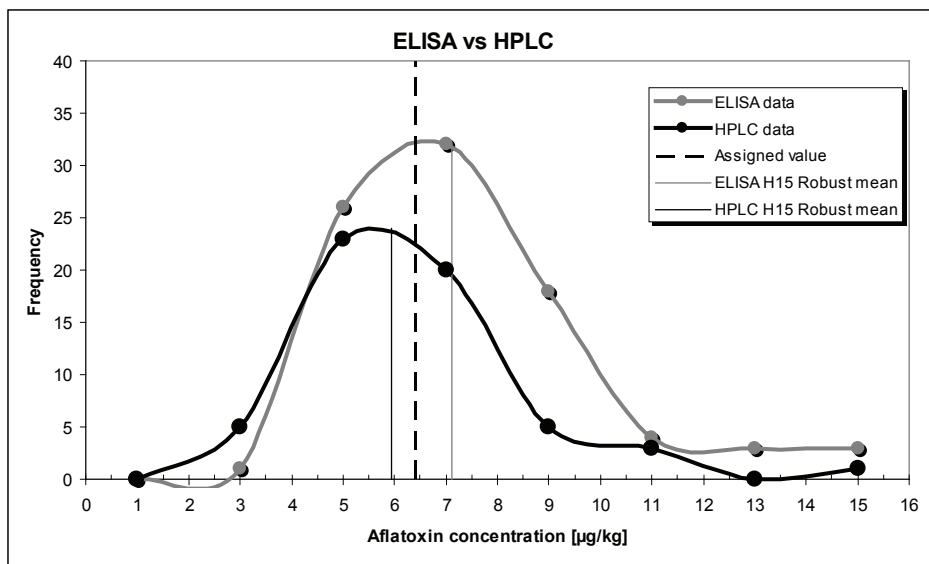


Graph 2. Frequency distribution of aflatoxin PT participants results

For each method following parameters have been separately calculated and showed in Table 1:

Table 1. Statistical parameters for ELISA and HPLC aflatoxin detection

	ELISA	HPLC
Mean [ppb]	7.47	6.23
SD [ppb]	2.38	2.22
CV%	31.8%	35.6%
Median [ppb]	7.10	6.03
MAD <sub>e</sub> [ppb]	1.85	1.97
H15 Robust Mean [ppb]	7.11	5.94
H15 Robust SD [ppb]	1.93	1.78
Trimmed Mean 25% [ppb]	7.15	5.97
Modus [ppb]	6.60	5.71
n	87	57
Skewness	1.297 right-skewed	1.267 right-skewed



Graph 3. Visual comparison of ELISA and HPLC frequency distributions of aflatoxin results

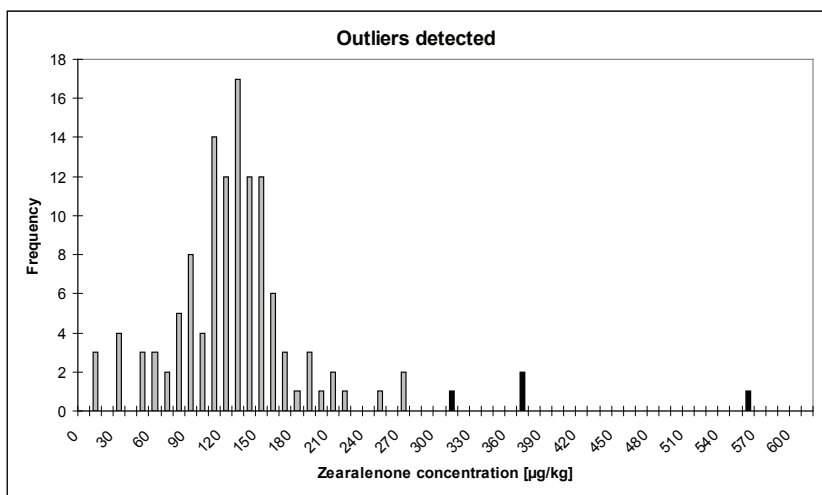
F test Two-Sample for Variances showed that there was no statistically significant difference in the variation coefficient (interlaboratory reproducibility – precision) between the ELISA and HPLC methods ( $p = 0.29$ ).

Non-parametric (robust) Mann-Whitney U-test and parametric t-test two-tailed, equal variances, showed that there was a statistically highly significant difference between median and mean values of these two methods ( $p = 0.004$  and  $p = 0.002$ ), but as they are on opposite sides almost equally distant from the assigned value, we believe that there is no difference in accuracy between them.

### *Zearalenone*

The PT assigned value for zearalenone was 119 µg/kg (ppb). The assigned value was calculated as the robust mean by Huber's H15 method. Calculated target standard deviation was  $SD_p = 26$  ppb. This means that the target accuracy was (CV%) 22%. According to the analysis done by PT provider, satisfactory results ( $z < 2$ ) were shown by 64.0% of the laboratory that used ELISA method and 97.8% which used HPLC.

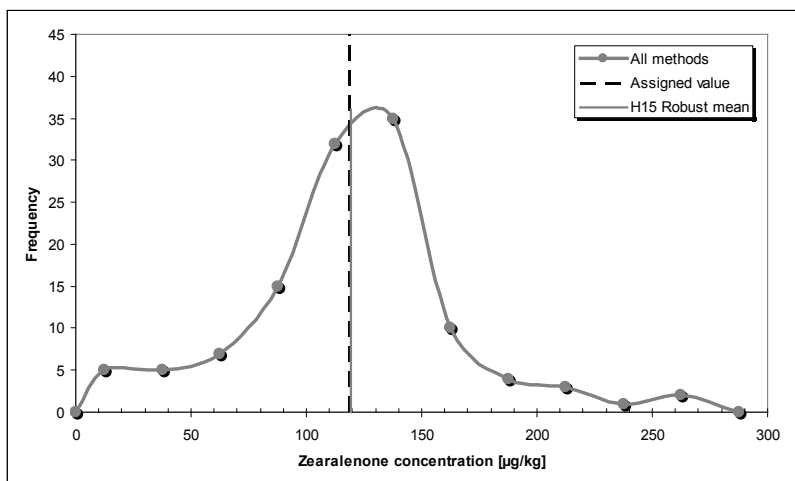
At the beginning, from the reported quantifications all non-numerical results in the form "<2 ppb" were removed. Removing outliers was done by Grubbs' test on-line calculator. On the Graph 4 data are sorted into class intervals width of 10 ppb and outliers are colored in black. Totally 5 of 124 results were removed.



Graph 4. Outliers in the overall zearalenone PT results

Other data were used to calculate: Mean = 118.4 ppb (assigned value 119 ppb); SD = 47.5 ppb (higher than  $SD_p = 26$  ppb); CV% = 40.1% (the accuracy of all methods, the real inter-laboratory reproducibility); Median = 123 ppb;  $MAD_e = 32.5$  ppb (Median of Absolute Deviations); Huber's H15 Robust Mean = 119.5 ppb; Huber's H15 Robust SD = 35.0 ppb; Trimmed Mean 25% = 118.8 ppb; Modus = 127.7 ppb, calculated over the data sorted into the class interval width of 25 ppb.

Asymmetry of data distribution – Skewness = 0.185 (mild right-sided asymmetry), which means that it can be considered that the data have a normal distribution.

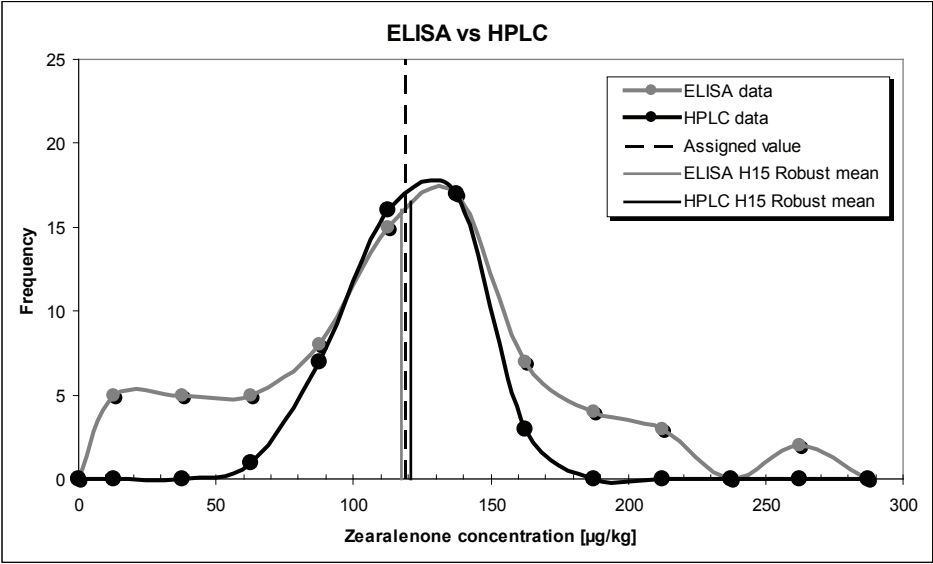


Graph 5. Frequency distribution of zearalenone PT participants results

The following parameters were calculated for each method separately and showed in Table 2:

Table 2. Statistical parameters for ELISA and HPLC zearalenone detection

	ELISA	HPLC
Mean [ppb]	116.6	119.9
SD [ppb]	56.6	21.5
CV%	48.6%	17.9%
Median [ppb]	117.0	124.6
MAD <sub>e</sub> [ppb]	49.8	22.4
H15 Robust Mean [ppb]	117.4	120.9
H15 Robust SD [ppb]	49.5	20.2
Trimmed Mean 25% [ppb]	116.4	120.5
Modus [ppb]	129.2	126.7
n	71	44
Skewness	0.124 right-skewed	-0.234 left-skewed



Graph 6. Visual comparison of ELISA and HPLC frequency distributions of zearalenone results

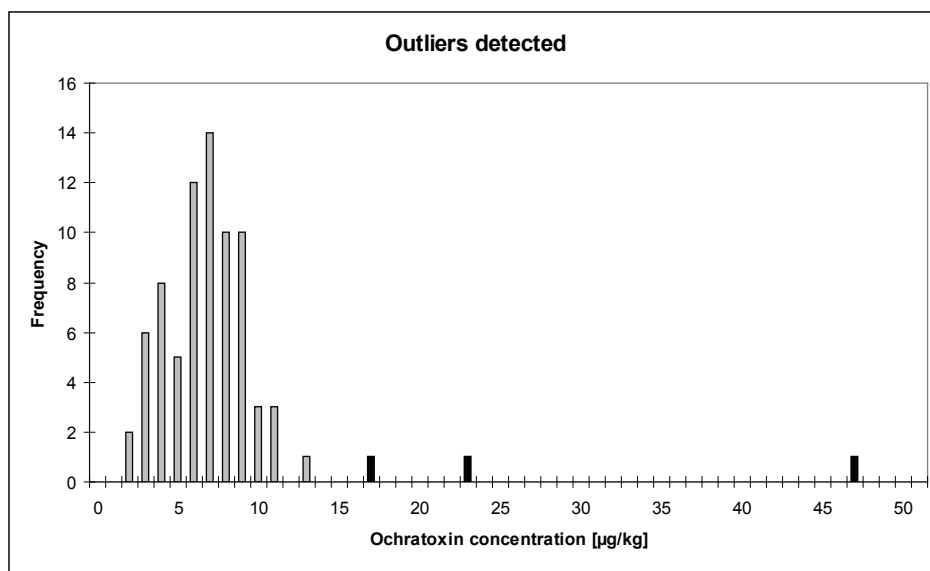
F test Two-Sample for Variances showed that there was statistically significant difference in the variation of results (in interlaboratory reproducibility – precision) between the ELISA and HPLC methods ( $p = 3.4 \cdot 10^{-10}$ ) and that in this case, as it could be seen in the Graph 6, an ELISA method showed lower precision.

Non-parametric (robust) Mann-Whitney U-test and parametric t-test two-tailed, unequal variances, showed that there was no statistically significant difference between median and mean values of two methods ( $p = 0.97$  and  $p = 0.65$ ), which means that there was no statistically significant difference in accuracy between these two methods.

### *Ochratoxin*

The assigned value for ochratoxin in the proficiency test 2013 was  $6.8 \mu\text{g/kg}$  (ppb). It was calculated as the robust mean by Huber's H15 method and target standard deviation  $SD_p$  was  $SD_p = 1.5$  ppb. This means that the target accuracy was (CV%) 22%. According to the report of the PT provider, 63.3% of the laboratory that had used ELISA demonstrated satisfactory results ( $z < 2$ ), as well as 79.2% who had used HPLC.

Analyzing the data available in the PT report, all results in non-numerical form (given as e.g. " $< 2$  ppb") were removed. Outliers were excluded using Grubbs' test on-line calculator. On the Graph 7, with the data sorted into the class intervals of 1 ppb, outliers are colored in black. From a total of 78 results 4 of them were removed.

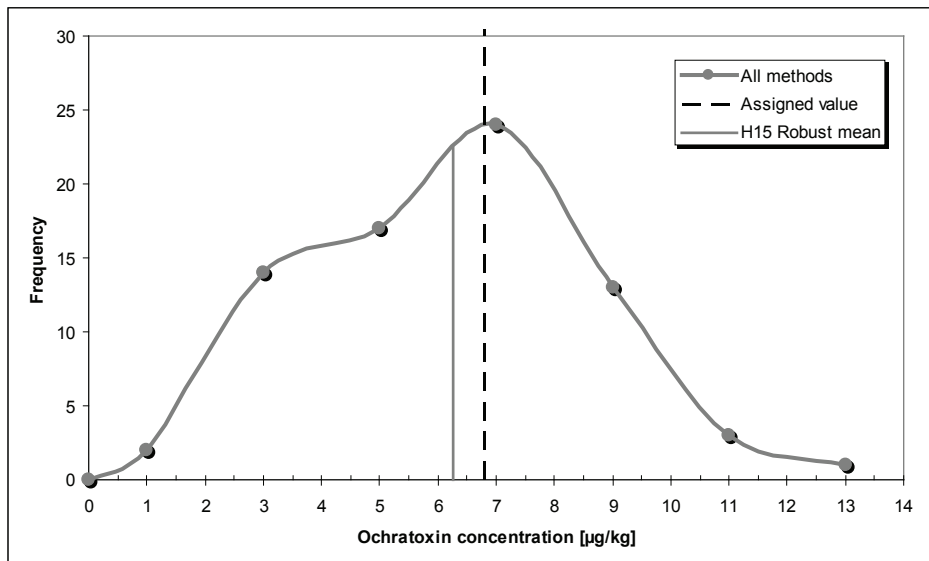


Graph 7. Outliers in the overall ochratoxin PT results

The rest of the laboratory results were used to calculate: Mean = 6.22 ppb (assigned value 6.8 ppb);  $SD = 2.33$  ppb (higher than  $SD_p = 1.5$  ppb); CV% = 37.4% (the accuracy of all methods, the real inter-laboratory reproducibility); Median

= 6.35 ppb;  $MAD_e = 2.21$  ppb (Median of Absolute Deviations); Huber's H15 Robust Mean = 6.26 ppb; Huber's H15 Robust SD = 2.43 ppb; Trimmed Mean 25% = 6.23 ppb; Modus = 6.78 ppb, calculated over the data sorted into the class interval width of 2 ppb.

Asymmetry of data distribution – Skewness = 0.07 (almost no asymmetry).

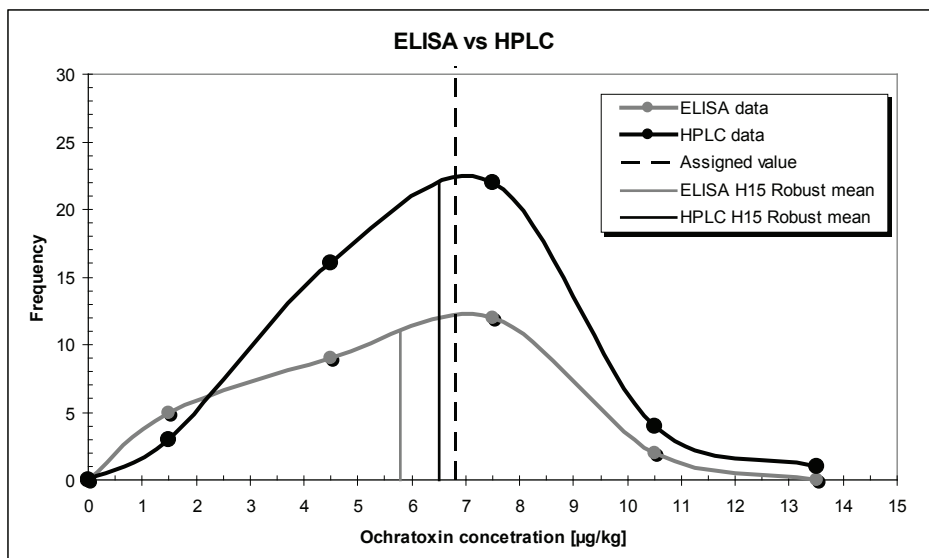


Graph 8. Frequency distribution of ochratoxin PT participants results

Statistical parameters showed in Table 3 have been separately calculated for each method:

Table 3. Statistical parameters for ELISA and HPLC ochratoxin detection

	ELISA	HPLC
Mean [ppb]	5.80	6.47
SD [ppb]	2.53	2.18
CV%	43.7%	33.7%
Median [ppb]	5.85	6.44
$MAD_e$ [ppb]	3.31	1.85
H15 Robust Mean [ppb]	5.78	6.51
H15 Robust SD [ppb]	3.00	2.04
Trimmed Mean 25% [ppb]	5.75	6.48
Modus [ppb]	6.69	6.75
n	28	46



Graph 9. Visual comparison of ELISA and HPLC frequency distributions of ochratoxin results

F test Two-Sample for Variances showed that there was no statistically significant difference in the variation of results (in interlaboratory reproducibility – precision) between the ELISA and HPLC methods ( $p = 0.18$ ).

Non-parametric (robust) Mann-Whitney U-test and parametric t-test two-tailed, equal variances showed that there was no statistically significant difference between median and mean values of two methods ( $p = 0.27$  and  $p = 0.23$ ), which means that there was no statistically significant difference in accuracy between these two methods.

In all proficiency tests the estimated standard deviation of PT ( $SD_P$ ), an important parameter for the calculation of z-score and ranking of laboratories, was always much smaller than the actual variability of the reported results. It is now clear that the  $SD_P$  was calculated only on the basis of the assigned value and that it was an estimation of the provider as variability of results should be, not what they were actually. Therefore, we express certain doubts into the usefulness of such a parameter that is not based on reality.

Taking into account all results mentioned above and in spite of some literature data (Pascale, 2009; Mohamadi *et al.* 2012), we abandoned the strict statement that one method is more useful than the other. Although such comparison of methods in this case is rather crude, because of different detection systems in HPLC and various kits for ELISA method that were used, we noted well performance of both techniques and we consider information from proficiency tests extremely beneficial for the analysis. It appeared that the use of ELISA method is entirely appropriate for the determination of mycotoxins,

especially in animal feed where the legal limits are higher. Due to its simplicity and accessibility, analysts of different profiles and little training can apply it. This may also be the cause of less accurate determination of zearalenone in PT2014. In the Graph 6 it could be seen that there was a core of 64% of laboratories (which had satisfactory results of z-score <2) whose distribution curve resulted in a sharp peak and almost exactly matched the results of the HPLC method. It seems like good proof of usability of ELISA method also for zearalenone detection, but that reliable results could be achieved with good training and enough experience.

In laboratory practice, HPLC is the “number one” technique for the measurement of main mycotoxins occurring in cereals and cereal-based products and LC-MS/MS is the most promising technique to be used in the future for multi-mycotoxins analysis. Also, the common perception is that various immunological methods, ELISA and other rapid antibody-based tests should generally be used for screening purposes only and that these methods often require confirmatory analyses with more robust methods. Some authors (Pascale, 2009; Mohamadi *et al.* 2012) emphasize that ELISA kits should be used routinely only for the analysis of matrices that are extensively tested and that confirmatory analyses by e.g. HPLC with IAC clean-up or LC-MS, are required for the contamination levels that approach the legal limit. Despite of general acceptance of this view, the results obtained by ELISA method in analyzed proficiency tests showed that it is very reliable and that the importance of this technique can not be neglected, even for forensic purposes.

## CONCLUSION

Final reports of proficiency tests contain important information for the general scientific and professional community and upon our experience it would be good to have them more available. Based on the analysis of more than 360 laboratory results of the tests presented here, in which our laboratory took part, we came to conclusions useful not only for direct participants, but for the wider society. Considering that parts of these reports are useful as a scientific and technical literature, we analyzed them in order to check whether the HPLC method has such clear advantages in comparison to the ELISA. Finally, it was concluded that both techniques could be successfully used for mycotoxin control, especially of animal feed where legal limits are higher than in food.

## ACKNOWLEDGEMENT

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## ПОРЕЂЕЊЕ *ELISA* И *HPLC* МЕТОДЕ ЗА ДЕТЕКЦИЈУ МИКОТОКСИНА АНАЛИЗОМ РЕЗУЛТАТА *PROFICIENCY* ТЕСТОВА

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**РЕЗИМЕ:** У циљу контроле присуства микотоксина и нивоа контаминације хране и хране за животиње развијене су различите аналитичке технике за детекцију ових природних контаминената. Конвенционалне аналитичке методе за утврђивање микотоксина су танкослојна хроматографија (ТЛЦ), течна хроматографија високих перформанси (ХПЛЦ) и гасна хроматографија (ГЦ). Такође, брзе

методе за микотоксиколошке анализе постају све важније, међу којима је ЕЛИСА (*Enzyme linked immuno-sorbent assay*) једна од најприменљивијих. Циљ овог рада био је да се две различите и најчешће коришћене лабораторијске методе за утврђивање различитих микотоксина (ХПЛЦ и ЕЛИСА) упореде анализом података који потичу из комерцијалних тестова оспособљености (*proficiency* тестова). На основу детаљне статистичке процене резултата добијених применом ових метода за квантификацију афлатоксина, охратоксина и зеараленона, у три комерцијална *proficiency* теста, може се закључити да обе технике могу равноправно да се користе с великом поузданошћу, иако се често наводи да је ЕЛИСА погодна само за почетну тријажу узорка.

KEYWORDS: ELISA, HPLC, food and feed, mycotoxins



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## COMPARISON OF METHODS FOR DETERMINATION OF THE TOXIGENIC POTENTIAL OF *Aspergillus parasiticus* Sp. AND *Aspergillus flavus* L. ISOLATED FROM MAIZE

**ABSTRACT:** Maize is considered one of the most susceptible crops to mycotoxins worldwide. Compared to other mycotoxins, the greatest attention has been paid to aflatoxins, due to their potential carcinogenicity and due to significant and longstanding problems they can cause in humans and animals. *A. flavus* and *A. parasiticus* produce aflatoxins in many economically significant crops in both fields and storages. Because of the potential aflatoxin contamination of maize grain, the toxigenic potential of *A. flavus* and *A. parasiticus* isolates, originating from Serbia, was tested in the present study. Furthermore, various applied methods for detection of these mycotoxins were compared in the study. Cultural, serological and analytical methods for the detection of mycotoxins were compared in the course of the experiment by the direct extraction of aflatoxins from the nutrient medium. The cultural methods for the detection of aflatoxin production were applied to 20 isolates of *A. flavus* (MRIZP Af18-20) and *A. parasiticus* (MRIZP Ap1-17). These methods are based on the yellow pigment formation in mycelia and nutrition media, occurrence of fluorescence on PDA (potato dextrose agar), agar containing  $\beta$ -cyclodextrine (CD-PDA), as well as on the red pigment formation after adding ammonium hydroxide to the existing medium. The ELISA was used to check quantitative and qualitative analyses of total aflatoxins (B1, B2, G1, G2) while the HPLC method was applied to establish ability of isolates to synthesize aflatoxins B1, B2, G1, G2. The yellow pigment formation, fluorescence and colony color changes of isolates into red, as a proof of toxigenicity of isolates, were confirmed in all cases by ELISA. A high potential of total aflatoxin production was determined in the majority of observed isolates. The ability of *A. parasiticus* isolates to synthesize aflatoxins G1 and G2 was confirmed by the HPLC method. This was essential for a better understanding of the key role of the suitability of cultural methods for preliminary evaluation of a large number of isolates. Our goal was to employ rapid biochemical approaches to prevent aflatoxin contamination of crops, and to reduce human and animal exposure to foodborne mycotoxins.

**KEYWORDS:** *A. flavus*, *A. parasiticus*, maize, toxigenic potential

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## INTRODUCTION

The majority of aflatoxins producing fungi are members of the genus *Aspergillus* classified into the section *Flavi* (Frisvad *et al.* 2004). These fungi are often isolated from warm-humid areas. Among 22 closely related species in *Aspergillus* section *Flavi*, *Aspergillus flavus* and *A. parasiticus* are commonly encountered in a variety of agricultural products. The two species are responsible for the majority of aflatoxin contaminations, but *A. flavus* is the most common one (Varga *et al.* 2011).

Numerous studies have shown that the mycotoxigenic potential and profile of *A. flavus* is far more variable, with some isolates producing little or no aflatoxins. *A. parasiticus* typically produces more consistent and higher concentrations of aflatoxins. Razzaghi-Abyaneh *et al.* (2006) reported 100%, i.e. 27.5% of aflatoxigenic *A. parasiticus*, i.e. aflatoxigenic *A. flavus* strains, respectively. Similarly, Rodrigues *et al.* (2009) detected 77% of atoxigenic isolates in *A. flavus*, while all *A. parasiticus* isolates were found to be aflatoxigenic. *A. flavus* typically produces AFB and can be most frequently isolated from the above-ground plant parts (leaves, flowers), while *A. parasiticus* produces AFG1 and AFG2, as well as AFB1 and AFB2 and is more adapted to soil environments (EFSA, 2007). Due to their high toxicity and carcinogenic potential, they are of high concern for the safety of food worldwide (Ellis *et al.* 1991).

There are many highly specialised and sensitive methods that can be applied to the determination of the aflatoxin concentration in commodities or in cultures, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), and fluorescence polarisation assay (Seitz, 1975; Trucksess *et al.* 1994; Whitaker *et al.* 1996; Maragos and Thompson, 1999; Stroka and Anklam, 2000; Nasir and Jolley, 2002; Sobolev and Dorner, 2002; Abbas *et al.* 2004). These methods are usually expensive and time-consuming. Commercially available ELISA kits provide a relatively simple assay for quantification of the total aflatoxin concentration, but do not provide the identification of individual aflatoxins present in the sample (Abbas *et al.* 2004).

Considering the significance of the problems caused by aflatoxins, simple, rapid, and reliable cultivation methods were necessary to determine aflatoxins tested in this study. Several screening methods have been developed for the direct visual determination. Aflatoxin production by *Aspergillus* isolates can be visualised under long-wave UV light (365 nm) when grown on media suitable for aflatoxin production, such as potato dextrose agar and coconut agar (Gupta and Gopal, 2002). The fluorescence emission of AFB1 and AFG1 is substantially improved when treated with enhancer agents, such as cyclodextrins (CDs). Fente *et al.* (2001) showed that adding  $\beta$ -CD to a suitable agar medium enhanced detection of aflatoxin production by *A. flavus* and *A. parasiticus* in the blue fluorescent zone under 365 nm. A second cultural method involves yellow pigment production by *A. flavus* colonies that correlates with aflatoxin production (Lin and Dianese, 1976). Aflatoxin production can be

detected with ammonia vapour as it changes the colour of toxigenic colonies from yellow to pink upon exposure (Saito and Machida, 1999).

The major objective of this study was to screen aflatoxin producing fungi in the maize samples based on the different cultural methods and to compare them with analytical methods. Because of the potential aflatoxin contamination of maize grain, the toxigenic potential of *A. flavus* and *A. parasiticus* isolates, originating from Serbia, was tested in the present study. Furthermore, various methods applied to detect these mycotoxins were compared in the study. Cultural, serological and analytical methods for the detection of mycotoxins were compared in the course of the experiment by the direct extraction of aflatoxins from the nutrient medium.

## MATERIALS AND METHODS

### *Aspergillus strains*

The trial with a total of 20 isolates of *A. flavus* and *A. parasiticus*, isolated from Serbian maize, was carried out in the period 2013–2015. In order to identify fungi and to perform morphological studies, fungi were cultured on Czapek yeast agar (CYA) and malt extract agar (MEA). *A. flavus* and *A. parasiticus* were identified according to the standard identification keys (Raper *et al.* 1973; Klich and Pitt 1988; Samson *et al.* 2007; Samson *et al.* 2014). The obtained pure cultures of *A. flavus* and *A. parasiticus* were maintained in the fungal collection of the Maize Research Institute “Zemun Polje” and were then used to obtain monospore cultures. Twenty isolates were selected for further toxicological analyses: 17 *A. parasiticus* isolates and three isolates of *A. flavus*.

### *Sample preparation for the mycotoxin assessment*

The point inoculation of isolates was done in the center of the plate by using a dense conidial biomass. Isolates were grown on potato dextrose agar (PDA) and PDA enriched with 0.3%  $\beta$ -CD (CD-PDA) (Acros, Organics, China) as single colonies in the 9-cm Petri dishes. Cultures were incubated for 5 days at  $28 \pm 1$  °C in the dark (Abbas *et al.* 2004). All strains were tested for aflatoxin production according to the following methods:

### *Cultivation methods and observation of fluorescence*

Rapid techniques for the detection of aflatoxigenic and non-aflatoxigenic *Aspergillus* were investigated in this study using a yellow pigment formation in mycelium and media (Lin and Dianese, 1976; Gupta and Gopal, 2002) ammonia vapour (Saito and Machida, 1999; Kumar *et al.* 2007) and methylated  $\beta$ -cyclodextrine (Fente *et al.* 2001) as indicators of *Aspergillus* colonies grown

on PDA and CD-PDA. In the course of the application of these methods, aflatoxigenic strains were developed on the reverse side of colonies of purple reddish colour, when subjected to ammonium vapour and fluorescence under UV light.

### *Yellow pigmentation*

The yellow pigment formation in mycelia and media is a basis for the diagnostic determination of aflatoxigenic isolates (Abbas *et al.* 2004a; Shier *et al.* 2005; Odhiambo *et al.* 2014). The degree of yellow pigmentation is proportional to blue fluorescence in culture media (Lin and Dianese, 1976).

### *Methyl- $\beta$ -cyclodextrin test*

The presence or absence of fluorescence in the agar surrounding the colonies grown on PDA and CD-PDA was determined under UV radiation (365 nm) and assayed as positive or negative. All strains were analysed with ELISA and HPLC to confirm the correlation between fluorescence and aflatoxin production.

### *Ammonia vapour test*

After five-day growth of colonies, Petri dishes were inverted over 3 drops of 28–30% ammonium hydroxide (Acros Organics, USA). Toxicity of isolates was determined on the basis of a change in colour of the nutrient medium. The color change occurred after the colony was in contact with ammonia vapour. In the non-toxic isolates there was no change of colour.

### *Determination of aflatoxins by ELISA and HPLC*

The contents of PDA and CD-PDA plates were scraped into a tube to collect fungal biomass. Fungal biomass (mycelia, conidia heads, conidia) was placed in glass scintillation vials (20 mL) and fresh weights were recorded (typically 0.5–1 g). Methanol-water (70:30, v/v) was added (10:1, v/m) to vials, and the vials were shaken for 30 min at high speed with a reciprocal shaker. A 1-mL aliquot of extract was removed and centrifuged at 12 000 g for 10 min with a MicroSpin 12S centrifuge. The supernatant was assayed for the presence of total aflatoxins (B1, B2, G1, and G2), using ELISA kits (Elabsience Biotechnology Co., Ltd). The obtained strains were analysed by high-pressure liquid chromatography (HPLC) with fluorescence detection to confirm the presence of aflatoxins B1, B2, G1, and G2. Prior to the separation by HPLC, fungal biomass had to be extracted with a solvent mixture acetonitrile-dd H<sub>2</sub>O

(90:10, v/v) and was added (100:1, v/m) to vials (Abbas *et al.* 2004). All experiments were repeated three times.

## RESULTS AND DISCUSSION

A large number of fungal colonies showing positive results after 5 days of growth on PDA and CD-PDA were determined with one of the methods stated below. Agreements and minor divergences are shown in Table 1.

### *Yellow pigmentation*

*A. parasiticus* cultures producing aflatoxin formed yellow pigmentation in almost all strains used in our experiments. The isolate MRIZP – AP17 was an exception as it did not produce yellow pigment in mycelium, but it produced toxins. The degree of yellow pigmentation was in concordance with fluorescence in all isolates producing toxins. Non-aflatoxigenic isolates did not produce yellow pigments (MRIZP – AF1, MRIZP – AF2, and MRIZP – AF3). This is in agreement with results obtained by Lin and Dianese (1976) who indicated that the degree of yellow pigmentation was proportional to fluorescence in all media tested.

### *Ammonium hydroxide vapour test*

*A. parasiticus* isolates MRIZP – AP1, MRIZP – AP2, MRIZP – AP10, and MRIZP – AP14 produced purple reddish pigmentation, while *A. parasiticus* isolates MRIZP – AP3, MRIZP – AP4, MRIZP – AP5, MRIZP – AP6, MRIZP – AP7, MRIZP – AP8, MRIZP – AP9, MRIZP – AP11, MRIZP – AP12, MRIZP – AP13, MRIZP – AP15, and MRIZP – AP16 produced moderate purple reddish pigmentation on the reverse side of colonies with the ammonium hydroxide test. *A. flavus* isolates MRIZP – AF1, MRIZP – AF2, and MRIZP – AF3 did not show color change in any of the experiments, because they are non-aflatoxigenic strains. The isolate MRIZP – AP17, which did not produce yellow pigment in mycelium, showed red pigmentation, which was unusual. There was toxin confirmation when this isolate was tested with ELISA and HPLC. Nevertheless, it can be concluded that the ammonium hydroxide test was a reliable test for detection of aflatoxins. Saito and Machida (1999) reported that the ammonium hydroxide vapour test gave 11% false positive and 6% false negative results for aflatoxigenicity. Kumar *et al.* (2007) reported 92% efficacy for the ammonium vapour test having 8% false negatives. Abbas *et al.* (2004) used yellow pigmentation combined with the ammonium hydroxide vapour test and thus false negatives were reduced to 7%.

The number of positive colonies detected using this methodology also followed a similar pattern as exhibited by the results obtained from colony fluorescence.

### *Methyl- $\beta$ -cyclodextrin test*

Results gained with the methyl- $\beta$ -cyclodextrin test are in accordance with the findings discovered with the ammonium hydroxide vapour test. The only exception was the isolate MRIZP – AP17 as toxins were produced in it, which was indicated by results of ELISA and HPLC. Moreover, false positives were obtained by the absence of blue fluorescence in the CD-PDA media under UV. Our findings showed that the methyl- $\beta$ -cyclodextrin test was sensitive enough to detect aflatoxins.

### *ELISA and HPLC*

Aflatoxin production abilities tested previously by fluorescence under UV light of strains cultivated on potato dextrose agar were in concordance

*Table 1.* Agreement between three methods for culturing, with ELISA and HPLC quantities from *A. flavus* and *A. parasiticus*

Isolates	Yellow pigmentation		Fluorescence		Ammonia vapour		ELISA (total aflatoxins) ng/g	HPLC G1/G2 (ng/g)
	PDA	CD-PDA	PDA	CD-PDA	PDA	CD-PDA		
MRIZP Ap 1	+	+	+	+	+	+	2,110	3.123,01/ ND
MRIZP Ap 2	+	+	+	+	+	+	4,140	5.059,26/114,57
MRIZP Ap 3	+	+	+	+	±	±	4,970	5.746,97/217,83
MRIZP Ap 4	±	+	±	±	±	±	5,595	6.449,07/92,02
MRIZP Ap 5	±	+	±	±	±	±	2,350	3.437,81/306,65
MRIZP Ap 6	+	+	+	+	±	±	2,525	3.461,35/260,47
MRIZP Ap 7	±	+	±	+	±	±	3,215	7.122,59/395,18
MRIZP Ap 8	±	±	±	±	±	±	2,300	8,03/ ND
MRIZP Ap 9	±	±	±	±	±	±	2,695	554,22/47,69
MRIZP Ap 10	+	+	+	+	+	+	6,655	7.421,58/374,93
MRIZP Ap 11	±	±	±	±	±	±	2,510	3.285,56/188,75
MRIZP Ap 12	±	+	±	+	±	±	4,595	634,80/ ND
MRIZP Ap 13	±	+	±	+	±	±	2,175	4.976,12/109,58
MRIZP Ap 14	+	+	+	+	+	+	7,260	7.095,32/102,61
MRIZP Ap 15	±	±	±	±	±	±	5,075	5.124,99/128,02
MRIZP Ap 16	±	±	±	±	±	±	7,490	6.240,54/201,47
MRIZP Ap 17	–	–	–	–	+	+	2,435	6.518,88/90,52
MRIZP Af 1	–	–	–	–	–	–	ND	ND
MRIZP Af 2	–	–	–	–	–	–	ND	ND
MRIZP Af3	–	–	–	–	–	–	ND	ND

ND – not detected

with those obtained by ELISA and HPLC determination. A high potential of total aflatoxin production was established in the majority of tested isolates. In our study, all *A. parasiticus* strains produced aflatoxins. On the other hand, none of *A. flavus* strains produced aflatoxins. Their ability to synthesize a high concentration of aflatoxins G1 and G2 was confirmed by the HPLC method. *A. parasiticus* isolates showed a high production potential of B- and G-type aflatoxins in previous researches (Vaamonde *et al.* 2003; Rodrigues *et al.* 2009; Baquião *et al.* 2013).

The fluorescence on CD-PDA had the highest degree of conformity for the identification of toxigenic isolates with the ELISA assay. Abbas *et al.* (2004) achieved similar results with the strongest correlation between the cyclodextrin-enhanced fluorescence test and ELISA among the tested methods of culturing and they showed that these methods were reliable in screening aflatoxin production by *Aspergillus* strains.

## CONCLUSIONS

The present study shows the adequacy of the culturing method for the preliminary isolate evaluation. In case of limited resources, this method is suitable for the detection of aflatoxins due to the cost-effectiveness of application.

Although all culturing methods applied in this study could be used as preliminary indicators, the ELISA test, i.e. HPLC is confirmed to be a reliable method for the toxin detection in *A. flavus* and *A. parasiticus*. Having in mind that aflatoxin contamination, as a major threat to human health and to the world's food supply, will be an issue in the foreseeable future, further insight might lead to the development and improvement of methods for its continuous analysis.

As global climate changes continue to affect regions of Europe, the potential impact of aflatoxin contamination may increase beyond regions with tropical climate, which is already happening. The ability to estimate climate changes and the relation between these changes and fungal infection and subsequent aflatoxin contamination will help in predicting and dealing with this emerging risk.

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## КОМПАРАЦИЈА МЕТОДА ЗА УТВРЂИВАЊЕ ТОКСИГЕНОГ ПОТЕНЦИЈАЛА *Aspergillus parasiticus* Speare И *Aspergillus flavus* Link ИЗОЛОВАНИХ СА КУКУРУЗА

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**РЕЗИМЕ:** Кукуруз се широм света сматра једним од усева најподложнијих за контаминацију микотоксинима. Афлатоксинима се, у поређењу с другим микотоксинима, придаје највећа пажња због њихове потенцијалне канцерогености, значајних и дугорочних проблема које изазивају код људи и животиња. Врсте *A. flavus* и *A. parasiticus* могу продуковати афлатоксине код многих економски значајних култура у пољима и складиштима. Због потенцијалне контаминације зрна кукуруза афлатоксинима у овом раду је испитан токсигени потенцијал изолата управо ове две врсте пореклом из Србије и упоређене су различите методе детекције ових микотоксина. Током експеримента упоређене су одгајивачке, серолошке и аналитичке методе детекције микотоксина, директном екстракцијом афлатоксина из хранљиве подлоге. Истраживања су базирана на примени одгајивачке методе за одређивање продукције афлатоксина код 20 изолата *A. parasiticus* (MRIZP Ap1-17) и *A. flavus* (MRIZP Af18-20) пореклом из Србије. Одгајивачке методе су биле засноване на формирању жутог пигмента у мицелији и хранљивој

подлози, на појави флуоресценције на PDA (кромпир декстрозни агар) и подлози која садржи  $\beta$ -циклодекстрин (CD-PDA), као и на образовању црвеног пигмента у подлози након додавања амонијум хидроксида. ELISA тест је коришћен за проверу квантитативних и квалитативних садржаја укупних афлатоксина B1, B2, G1 и G2, док је HPLC методом утврђена концентрација појединачних афлатоксина B1, B2, G1 и G2. Образовање жутог пигмента, флуоресценција и промена боје колоније изолата у црвену, као доказ токсигености изолата, потврђена је у свим случајевима и ELISA тестом. Код већине изолата установљен је висок потенцијал продукције укупних афлатоксина. HPLC методом потврђена је и способност синтезе афлатоксина G1 и G2 од стране изолата *A. parasiticus*. Циљ експеримента био је да се испита ефикасност употребе брзих тестова за детекцију афлатоксина, како би се спречила контаминација усева и изложеност људи и животиња афлатоксинима.

КЉУЧНЕ РЕЧИ: *Aspergillus parasiticus*, *Aspergillus flavus*, кукуруз, токсигени потенцијал

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## IMPACT OF *Fusarium* MYCOTOXINS ON SWINE HEALTH – FIELD OBSERVATIONS

**ABSTRACT:** Mycotoxins are structurally diverse fungal metabolites that can contaminate a variety of dietary components consumed by animals and humans. The aim of this paper was to present the field observations of clinical and pathological consequences on swine health in the cases when *Fusarium* mycotoxins were detected in swine feed. The material for research included the samples from swine farms located in the region of Vojvodina, where health disorders resembling intestinal problems in different swine categories were detected. The applied research methods included: epidemiological and clinical evaluation, gross pathology examination, bacteriological tissue testing originating from diseased dead animals. The presence of deoxynivalenol (DON), T-2 toxin and zearalenone (ZEA) in thirteen complete swine feed mixtures were analyzed by enzyme-linked immunosorbent assay methods, using Ridascreen®FAST DON, Ridascreen®FAST T2, and Ridascreen®FAST Zearalenon test kits (R-Biopharm, Germany). By clinical and pathological examination, the lesions predominantly located in digestive tract were observed in different swine categories. The problem of persistent enteric infections in suckling piglets and alteration of growth performance were notified in weaners and fatteners. In adult categories, reduced feed consumption, sometimes distinct feed refusal and vomiting were observed. In all examined samples of complete feed mixtures for different swine categories the concentration of DON exceeded the maximum permitted levels, but also the presence of other *Fusarium* mycotoxins was detected. The obtained results indicate the existence of feed mixtures contamination with low levels of *Fusarium* mycotoxins and their possible positive interaction with etiological agents present in swine farms.

**KEYWORDS:** swine health, *Fusarium* mycotoxins, Vojvodina

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## INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by certain fungi, which can cause a variety of adverse effects on both humans and animals (Osweiler, 2006; Kabak, 2012). Depending on classification, 300–400 mycotoxins are known today (Streit *et al.* 2012; Weaver *et al.* 2013). In many parts of the world, mold-contaminated feedstuffs and resultant mycotoxin levels in the diet constitute a considerable health hazard (Jackson and Cockcroft, 2007; Streit *et al.* 2012). It is estimated that 25% of the world's crop production is contaminated by mycotoxins during the pre-harvest period, transport, processing or storage (Greinier and Applegate, 2013; Weaver *et al.* 2013). The major mycotoxin-producing fungal genera are *Aspergillus*, *Fusarium* and *Penicillium*, mainly producing aflatoxins (AF), zearalenone (ZEN), trichothecenes (TCT), fumonisins, ochratoxins and ergot alkaloids (Piotrowska *et al.* 2014). Among the mycotoxins produced by *Fusarium* genus, the broad family of TCT is extremely prevalent (Pinton and Oswald, 2014) as well as ZEN (Wache *et al.* 2009; Burel *et al.* 2013).

A major problem associated with animal feed contaminated with mycotoxins is not acute disease, but rather the ingestion of low levels of toxins, which may cause an array of metabolic, physiologic and immunologic disturbances (Prodanov-Radulović *et al.* 2014; Waśkiewicz *et al.* 2014). Consumption of fungal toxins in swine as a species may decrease resistance to infectious diseases (Prodanov-Radulović *et al.* 2011; Prodanov-Radulović *et al.* 2014). Also, chronic exposure can lead to anorexia, reduced weight gain, as well as nutritional efficiency and neuroendocrine changes (Pestka, 2007; Prodanov-Radulović *et al.* 2013). The aim of this paper was to present the field observations of clinical and pathological consequences on the swine health, in the cases when *Fusarium* mycotoxins were detected in complete swine feed mixtures.

## MATERIAL AND METHODS

The material for research included the samples from swine farms located in the region of Vojvodina, where health disorders i.e. clinical and gross pathological signs resembling the problems with permanent intestinal infectious diseases in different swine categories were detected. The applied research methods included: epidemiological and clinical evaluation, gross pathology examination and laboratory testing. The following details were determined by inspecting farm records: number and category of pigs, type of production (farrow-to-finish, fattening farm), disease status, current veterinary health plan (vaccination, medication). The clinical inspection of live animals was followed by the necropsy of dead pigs for gross pathological diagnosis and tissue sampling for further bacteriological testing. Isolation of bacteria from tissue samples deriving from dead pigs was performed by standard aerobic and microaerophilic cultivation (Quinn *et al.* 2011). Beside this, the molecular diagnostic method, a multiplex

RT-PCR for detection of *Brachyspira hyodysenteriae* and *Lawsonia intracellularis* (DNA extracted from feces) (La *et al.* 2006) was applied. The presence of deoxynivalenol (DON), T-2 toxin, and ZEA in thirteen complete swine feed mixtures were analyzed by enzyme-linked immunosorbent assay methods, using Ridascreen®FAST DON (Art. No. R5901), Ridascreen®FAST T2 (Art. No. R5302), and Ridascreen®FAST Zearalenon (Art. No. R5502) test kits (R-Biopharm, Germany).

## RESULTS AND DISCUSSION

The first examined farm represents the modern commercial, farrow-to-finish production system with the following production capacity: 750 sows, 7 boars, 120 growing gilts, 290 breeding gilts, 1,260 suckling piglets, 4,550 weaned piglets and 6,030 fatteners. The farm organizes its own veterinary services, and swine health control program includes vaccination according to the Law Regulations (*Classical Swine Fever*) and against most frequently diagnosed diseases in the region (*Porcine parvovirus*, *Mycoplasma hyopneumoniae*, *Circovirus – PCV2*, *Erysipelas*) and vaccination against *Clostridium perfringens* and *Escherichia coli*. The last mentioned vaccination of dams is applied twice during late gestation (30–42 and 15–20 days prior to farrowing date) with the aim to prevent enteric disease in piglets in the first days of life. In the case of disease outbreak, the affected categories are therapeutically treated with antimicrobials (parenteral injection for clinically diseased animals and water and/or feed medication for in-contacts). Recently, the following health disturbances in the female breeding categories in the farm were registered: different levels of decreased feed consumption and lethargy in sows and gilts, while in some animals even complete feed refusal was notified (anorexia). Vomiting in sows was also detected. Clinically, the diarrhea in weaned piglets around weaning (28–32 day of age) was notified. However, therapeutic treatment of piglets by antimicrobials did not improve health problems. The gross pathological examination of the dead weaned piglets revealed lesions predominantly in the digestive tract (*Haemorrhagiae mucosae ventriculi*, *Gastritis ulcerosa multiplex*, *Enteritis catharralis acuta et haemorrhagica*). By bacteriological testing of tissue samples deriving from dead animals the following bacteria were detected: *E. coli*, *E. coli haemolytica*, *Cl. perfringens*, and *Salmonella typhimurium*. Microbiological testing of complete feed mixture for piglets (Grover) revealed significant increase in the number of fungi genera *Fusarium* (200,000 CFU/g), as compared to the level set by the regulation (<50,000 CFU/g) (*Official Gazette RS*, 2010). Applying further laboratory testing of complete mixtures for breeding categories, the presence of DON in the feed for pregnant and lactating sows was detected. Additionally, in complete feed mixtures for weaned piglets (body weight 15–25 kg) the presence of elevated values of ZEA and DON was discovered (Table 1).

Table 1. The results of mycotoxicological testing of swine feed samples from four examined farms

Swine Farm	Complete feed mixture for category:	Detected level of investigated mycotoxins (µg/kg)		Reference value (µg/kg)*
Number 1	Lactating sows	DON	3,890	< 900
		ZEA	282.90	< 500
		T-2	< 33	/
	Pregnant gilts and sows	DON	3,140	< 900
		T-2	< 33	/
		ZEA	500.13	< 500
	Weaned piglets (15–25 kg body weight)	DON	4,249	< 900
		ZEA	> 400	< 200
		T-2	< 33	/
Number 2	Fatteners	DON	2,940	< 900
		T-2	< 33	/
		ZEA	197.01	< 500
Number 3	Fatteners	DON	4,240	< 900
		ZEA	287.01	< 500
Number 4	Weaned piglets (20–30 kg body weight)	DON	2,000	< 900
		T-2	36.99	/
		ZEA	> 400	< 200
	Weaned piglets (20–30 kg body weight)	DON	5,620	< 900
		T-2	< 33	/
		ZEA	> 400	< 200
	Breeding animals (30–60 kg body weight)	DON	1,770	< 900
		T-2	< 33	/
		ZEA	> 400	< 200
	Breeding animals (60–100 kg body weight)	DON	2,500	< 900
		T-2	41.18	/
		ZEA	500.23	< 500
	Fatteners	DON	4,340	< 900
		T-2	< 33	/
		ZEA	551.84	< 500
	Fatteners	DON	3,890	< 900
		T-2	< 33	/
		ZEA	628.27	< 500
	Lactating sows	DON	2,590	< 900
		T-2	< 33	/
		ZEA	442.52	< 500
	Pregnant sows	DON	> 6,000	< 900
		T-2	37.56	/
		ZEA	> 800	< 500

\* maximum permissible level according to Serbian regulations (*Official Gazette RS*, 2014)

In the second and third evaluated swine farms, the presence of DON in the feed for fatteners was detected (Table 1). These two farms represent one-site production system (fatteners) and have the capacity of 2,000–3,000 animals. Anamnestically and clinically, the health problems included increased incidence of gastrointestinal diseases. Analyzing the available data on the farms, the high incidence of morbidity in fatteners was noticed (intermittent diarrhea), which did not decrease after medical treatment. Therapeutic treatment of the diseased animals was intensive and multiple (antibiotics were given through feed, water and in some cases, parenterally). Clinically, the bloody diarrhea and perineal staining in most of the fatteners was notified. In some cases, the diarrhea was greyish black, with blood and mucus flecks. A reduced feed consumption, loss of weight and insufficient weight gain were also present. Therapeutic treatment with antibiotics only temporarily improved health problems. On post-mortem examination, all dead pigs were in poor condition. Applying gross pathological examination on the dead fatteners, the prominent changes on the digestive tract (*Gastroenteritis haemorrhagica*, *Typhlocolitis haemorrhagica*, *Ulcus oesophagogastricum*) were detected. The large bowel was full of liquid feces and blood. The surface of mucosa was diphtheric, serosal, dark purple and edematous. In some cases, the mucosal proliferation causing erosions in the ileum was discovered. Some animals died suddenly because of blood clot in the lumen of the large intestine. By bacteriological testing from the tissue samples deriving from the dead fatteners, only *E. coli* and *E. coli haemolytica* were isolated. However, applying molecular diagnostic method (multiplex RT-PCR) on the fecal samples derived from finishers, *B. hyodysenteriae* and *L. intracellularis* were detected.

The last examined farm represents the commercial swine farm, with one-site production system (farrow-to-finish), located in the South Bačka District in Vojvodina. At the time of examination, farm had the following production capacity: 1,250 sows, 25 boars, 205 breeding gilts, 2,365 suckling piglets, 6,073 weaned piglets, and 5,150 fatteners. The farm organizes its own veterinary services and swine health control program includes vaccination against the same diseases as first presented farm. In the case of health disturbance, the animals are therapeutically treated (when necessary parenteral injections, but mostly in-feed and in-water medication are applied). By clinical examination, in neonatal piglets the clinical signs of vulvovaginitis and diarrhea almost immediately after farrowing (first days of life) were detected. Applying gross pathological examination of dead suckling piglets, prominent lesions predominantly in the upper part of digestive tract were discovered (*Gastritis ulcerosa multiplex*, *Haemorrhagiae mucosae ventriculi*, *Enteritis haemorrhagica*). By bacteriological testing, only *E. coli* and *E. coli haemolytica* were detected. Similarly, enteric infections and alteration of growth performance were notified in weaners and a number of fatteners. In breeding swine categories, reduced feed consumption, sometimes distinct feed refusal and vomiting were periodically observed. In all examined samples of complete feed mixtures for different swine categories the concentration of DON and ZEA exceeded the maximum permitted levels (Table 1). According to Serbian Regulation (*Official Gazette RS*,

2014), there is no maximum permitted level for T2 toxin. Indicative levels for the sum of T-2 and HT-2 in compound feed according to Commission recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products (EC, 2013) is 250 µg/kg.

The results of examined complete mixture samples showed that the exposure of pigs to DON and ZEN occurred at different levels. A research investigating the influence of mycotoxins on the animal susceptibility to infectious diseases focuses mainly on exposure to single major mycotoxins. However, limited information is available on the interaction between low levels of mycotoxins and causative agents of swine infectious diseases (Osweiler, 2006; Prodanov-Radulović *et al.* 2011). The continuous intake of small amounts of mycotoxins can lead to chronic intoxication which is clinically characterized by the loss of weight, insufficient weight gain and increased susceptibility for infectious diseases (Wache *et al.* 2009; Prodanov-Radulović *et al.* 2014). The reduction in weight gain as a consequence of reduced feed consumption is strongly associated with the exposure of farm animals to DON, with pigs being one of the most sensitive species (Piotrowska *et al.* 2014; Weaver *et al.* 2013). Congestion and erosions of the gastric and intestinal mucosae have been described following chronic DON exposure in pigs. However, reporting of intestinal lesions has been inconsistent and not systematically correlated with the clinical signs (Greiner and Applegate, 2013; Pinton and Oswald, 2014). The intestinal mucosa is the first biological barrier encountered by natural toxins, and consequently, it could be exposed to high amounts of dietary toxins. The mycotoxins may induce intestinal pathologies, including necrosis of the intestinal epithelium. They also disturb the barrier function, potentially leading to the increased translocation of pathogens and an increased susceptibility to enteric infectious diseases (Pinton and Oswald, 2014). Unfortunately, the toxicity of combinations of mycotoxins cannot always be predicted based upon their individual toxicities (Wache *et al.* 2009; Burel *et al.* 2013). Clinically observed vulvovaginitis (swelling and reddening of the vulva) in newborn piglets is a consequence of the presence of an oestrogenic mycotoxin (ZEA), produced by *Fusarium* fungus (Osweiler, 2006; Jackson and Crackcroft, 2007). It is characteristic that the clinical signs appear within a few days of pigs being exposed to the mycotoxin and disappear within a few days of the toxin being absent from the food (Jackson and Cockcroft, 2007). Female piglets on the sow are most frequently affected: toxins pass into the milk and hence to suckling piglets (McOrist, 2014). This condition is called the perinatal hyperestrogenic syndrome and represents the consequence of ZEA presence in feed for pregnant sows and its excreted metabolite in their milk (Prodanov-Radulović *et al.* 2011; Prodanov-Radulović *et al.* 2013).

During the last decade, the occurrence of mycotoxins in feed materials increased, and this may be a result of changes in agricultural practice but also the consequence of climate changes (Stojanov *et al.* 2013; Weaver *et al.* 2013). Most of the known mycotoxins have a short biological half-life and do not accumulate in animal tissues. They are, therefore, not a hazard for consumers of pig meat (McOrist, 2014). Our results are in agreement with other studies, showing a transient strong effect of DON on feed intake in pigs and occurrence of clinical

signs of gastrointestinal disturbances (vomiting, anorexia, diarrhea) (Greiner and Applegate, 2013; Wache *et al.* 2009). In the investigated swine farms, we noticed the presence of various persistent infections of intestinal tract of different etiology (enterotoxigenic, salmonellosis, swine dysentery, proliferative ileitis), which react poorly or do not react to the applied antimicrobial therapy.

## CONCLUSION

In the investigated swine farms, the existence of possible positive interactions between *Fusarium* mycotoxins and causative agents of intestinal swine diseases may be suggested. A more comprehensive research is needed to understand the impact of mycotoxin combinations and to determine when synergistic interactions occur.

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## УТИЦАЈ РАЗЛИЧИТИХ МИКОТОКСИНА НА ЗДРАВЉЕ СВИЊА – ЗАПАЖАЊА С ТЕРЕНА

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**РЕЗИМЕ:** Микотоксини су структурно различити метаболити плесни који могу да контаминирају компоненте које се користе у исхрани животиња и људи. Циљ рада био је да се прикажу теренска запажања клиничких и патолошких последица по здравље свиња у случајевима када су *Fusarium* микотоксини утврђени у храни за ову врсту. Материјал за испитивање је обухватао узорке пореклом с фарми свиња у Војводини, на којима су регистровани здравствени проблеми интестиналног тракта код различитих категорија. Примењене методе испитивања су обухватале: епизоотиолошка и клиничка испитивања, макрoпатоморфолошки преглед уинутих јединки, бактериолошко и молекуларно испитивање узорака органа и ткива пореклом од оболелих, уинутих јединки и микробиолошко испитивање хране у циљу контроле присуства плесни. Присуство деоксиниваленола, Т-2 токсина и зеараленона је испитивано у 14 узорака различитих комплетних смеша за свиње применом имуноензимске технике (Ridascreen®FAST DON, Ridascreen®FAST T2, Ridascreen®FAST Zearalenon, R-Biopharm, Germany). Клиничким и патолошким прегледом, код различитих категорија свиња, утврђене су лезије доминантно у дигестивном тракту. Проблем упорних ентералних инфекција код прасади на сиси и промене у порасту су забележене код залучене прасади и товљеника. Код одраслих категорија свиња, утврђена је умањена конзумација хране, понекад изражено одбијање хране и повраћање. У свим испитаним узорцима комплетних смеша за различите категорије свиња концентрација микотоксина DON била је већа од максимално дозвољених вредности. У испитаним узорцима хране утврђено је и присуство других *Fusarium* микотоксина. Постигнути резултати указују на контаминацију хранива с ниским вредностима *Fusarium* микотоксина и на њихову могућу позитивну интерреакцију с узрочницима болести присутним на фармама свиња.

**КЉУЧНЕ РЕЧИ:** здравље свиња, *Fusarium* микотоксини, Војводина



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## LABORATORY COMPETENCE EVALUATION THROUGH PROFICIENCY TESTING – MYCOTOXINS IN FOOD

**ABSTRACT:** Laboratory for analysis of mycotoxins in food at the Institute of Public Health of Vojvodina (Novi Sad, Serbia) participated in 15 proficiency testing schemes in period 2012–2016, comprising 22 determinations of regulated mycotoxins: aflatoxins B1, B2, G1, G2 and M1, ochratoxin A, deoxynivalenone, zearalenone, fumonisins and patulin, in different food commodities: wheat, corn, barley, breakfast cereals, infant food, milk, wine and fruit juice. Analyses were carried out by high performance liquid chromatography with ultraviolet (patulin, deoxynivalenol) or fluorescence detection (aflatoxin M1, ochratoxin A, zearalenone) using *o*-phthalaldehyde precolumn derivatization (fumonisins) or UV postcolumn derivatization (aflatoxins B1, B2, G1, G2), following clean-up on immunoaffinity columns with specific antibodies, except in case of patulin when solvent extraction and solid-phase C-18 clean-up were used. Laboratory performance assessed in terms of *z* scores showed all satisfactory results. In depth evaluation revealed following distribution of *z* scores (absolute values): 59.1% up to 0.5, 36.4% between 0.5 and 1.0, and 4.5% above 1.0. Analysis of trends performed for multiple determinations of individual mycotoxins showed several changes of *z* score to better or worse rank. Overall assessment of the performance in proficiency testing demonstrated laboratory competence for analysis of mycotoxins in food.

**KEYWORDS:** quality assurance, proficiency testing, food, mycotoxins

## INTRODUCTION

Participation in proficiency testing (PT) is a powerful element of quality assurance plan for the analytical laboratories, required for the ones seeking recognition of competence through accreditation against the standard ISO/IEC 17025 (ISO, 2005). A laboratory needs to establish an appropriate PT strategy considering relevance of PT schemes and frequency of participation. To achieve

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that, a laboratory has to collect comprehensive information on the availability and scope of PT schemes in the areas of its work. A number of key principles need to be considered: PT scheme should resemble the laboratory's routine samples, analytes and concentration levels; PT items should be treated as routine samples; evaluation of the results should take into account the measurement uncertainty; unsatisfactory or repeated questionable results must be subjected to thorough root cause investigation followed by corrective actions; analysis of trends over several PT rounds should be performed and interpreted in order to improve the performance (Eurachem, 2011). PT schemes have to be organized according to principles defined in standard ISO/IEC 17043 (ISO, 2010). A laboratory should understand the basic statistics and performance scoring used by the PT providers.

In a laboratory monitoring chemical food safety, analyses need to be carried out to investigate occurrence of numerous chemicals in wide variety of food commodities. Implementation of public health protection policies in many countries led to regulation of the maximum level of chemicals in food with potential adverse health effects. Serbian legislation regarding this issue (Ministry of agriculture, 2014) is harmonized with the European Union regulation (European Commission, 2006). Selection of contaminants, based on the severity of potential health effects and the extent of exposure through food, included several mycotoxins: patulin (PAT), aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON) and fumonisins (FUM). Mycotoxins are naturally occurring toxic substances, secondary metabolites of filamentous fungi.

Patulin production is connected to the fungi belonging to *Penicillium*, *Aspergillus* and *Bysochlamys* species, growing on fruits, especially apple. Apple juice is considered a major source of patulin in human diet. Toxicological profile of patulin could be briefly summarized by provisional maximum tolerable daily intake (PMTDI) of 0.4 µg/kg bw/day (JECFA, 1995) and International Association for Research on Cancer (IARC) classification in group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 1986).

Aflatoxins are secondary metabolites of the fungi *Aspergillus flavus* and *A. parasiticus*, and less frequently other *Aspergillus* species. AFs are prevalent in food crops, particularly corn, peanuts (groundnuts), oilseeds and tree nuts. AFM1 is hydroxylated metabolite of AFB1, excreted in milk. AFB1, the most toxic aflatoxin, is extremely potent carcinogen (IARC group 1) (IARC, 2012) and therefore a health based guidance value has not been established. AFM1 is classified in IARC group 2B (IARC, 1993).

Ochratoxin A is produced by the fungi representing *Aspergillus* and *Penicillium* genera. It is most often found in cereals, grape and wine. OTA exhibits renal toxicity. Tolerable weekly intake is 120 ng/kg bw (European Food Safety Authority, 2006); IARC group 2B (potential carcinogen to humans) (IARC, 1993).

Zearalenone is biosynthesized by a large range of *Fusarium* fungi on cereal crops, especially corn. The critical effects result from its estrogenic activity leading to hyperestrogenism. Tolerable daily intake is 0.25 µg/kg bw (European Food Safety Authority, 2011); IARC group 3 (IARC, 1993).

Deoxynivalenol is type B trichotecene primarily associated with *Fusarium graminearum* fungi. It co-occurs in cereal-based food together with its acetylated derivatives. The primary toxic effect is inhibition of protein synthesis. PMTDI 1 µg/kg bw DON and its acetylated derivatives (3-Ac-DON and 15-Ac-DON) (JECFA, 2011); IARC group 3 (IARC, 1993).

Fumonisin is a group of structurally related mycotoxins primarily produced by *Fusarium verticillioides* and *Fusarium proliferatum*. Fumonisin B1 (FB1) and B2 (FB2) are the most abundant and often found as contaminants in corn products. FB1 is the most toxic fumonisin, related to an inhibition of sphingolipid synthesis and increased risk of esophageal cancer in humans. PMTDI 2 µg/kg bw (FB1, FB2 and FB3, independently or combined) (JECFA, 2002); IARC group 2B (IARC, 2002).

The reliability of routine analyses of chemical contaminants in food, reflected in laboratory PT performance, is of paramount importance for food safety evaluation, as well as for health risk characterization taking into account that population exposure assessments are based on the contaminants occurrence data. With regard to that, this report presents technical performance of the Institute of Public Health of Vojvodina – Laboratory for analysis of organic contaminants, in PT schemes for analysis of mycotoxins in food.

## MATERIAL AND METHODS

Standard solutions and reagents: Standard solutions of PAT and AFM1 were obtained from Supelco (Bellefonte, PA, USA), whereas standard solutions of AFs, OTA, ZEA, DON and FUM were from LCG Standards (Wesel, Germany). Solvents (ethylacetate, hexane, acetonitrile, methanol) were purchased from LGC Promochem (Wesel, Germany); acetic and hydrochloric acid from Carl Roth (Karlsruhe, Germany). Ultrapure water was produced by GenPure Water Purification System (Thermo Scientific, Thermoelectron LED, Langenselbold, Germany).

Samples: The Laboratory ordered 15 PT samples over four years (2012-2016). The PT providers, Fera Science (FAPAS – Food Analysis Performance Assessment Scheme, UK) and Romer Labs (Austria), distributed homogeneous and stable PT samples for analysis (details provided in Table 1 in Results and discussion session).

Sample preparation: Analyses were carried out by single analyte methods. Each mycotoxin was extracted from the food matrix and purified using immunoaffinity columns with specific antibodies (LCTech, Dorfen, Germany / Vicam, Waters, US) following producers' instructions. The exception was PAT, for which solvent extraction and solid-phase C-18 (Supelco, Bellefonte, PA, US) clean-up were used, according to Arranz *et al.* (2005).

HPLC analysis: Analyses were performed on Agilent Series 1100 HPLC system (Agilent Technologies, Wilmington, DE, USA) consisting of degasser (G1322A), quaternary pump (G1311A), autosampler (G1329A), thermostated column compartment (G1316A), UV detector (G1314A), fluorescence detector

(G1321A), and a photochemical post column derivatizer UV 254 (LCTech, Dorfen, Germany). Separations were achieved on a reverse phase analytical columns Zorbax SB-C18 (5  $\mu$ m, 4.6x250 mm; Agilent Technologies, USA) (PAT, AFM1, OTA, DON) and Eclipse XDB-C18 (5  $\mu$ m, 4.6x150 mm; Agilent Technologies, USA) (AFs, ZEA, FUM). Mobile phases were as follows: water:acetonitrile:perchloric acid (99:4:0.1, 1 ml/min) (PAT); water:methanol:acetonitrile (6:3:1.5, 1.2 ml/min) (AFs); water:acetonitrile (3:1, gradient A:B 25%→70%, 1ml/min) (AFM1); acetonitrile:2%aq.acetic acid (3:2, 1 ml/min) (OTA); acetonitrile:water (53:47, 1 ml/min) (ZEA); methanol:water:acetic acid (A 15:85:0.1, B 1:1:0, gradient A:B 100%→0%, 1ml/min) (DON); water:acetonitrile:acetic acid (A 30:69:1, B 60:39:1, gradient A:B 3:2→0:1, 1ml/min) (FUM). Mycotoxins were detected by ultraviolet (PAT 276nm, DON 220nm) or fluorescence detector (AFM1  $\lambda_{exc}$  365nm,  $\lambda_{em}$  435nm; OTA  $\lambda_{exc}$  333nm,  $\lambda_{em}$  460nm; ZEA  $\lambda_{exc}$  275nm,  $\lambda_{em}$  448nm) using *o*-phthalaldehyde precolumn derivatization (FB1 and FB2  $\lambda_{exc}$  335nm,  $\lambda_{em}$  440nm) or UV postcolumn derivatization (AFs  $\lambda_{exc}$  365nm,  $\lambda_{em}$  430nm). According to the scheme propositions, all analytical results were corrected for recovery as determined by the Laboratory in method validation studies.

Laboratory performance was assessed in terms of *z* scores, as given by the proficiency test provider. The scores were interpreted in the following way:  $|z| \leq 2$  “satisfactory”,  $2 < |z| < 3$  “questionable”,  $|z| > 3$  “unsatisfactory”.

## RESULTS AND DISCUSSION

Food commodities and mycotoxins analyzed as proficiency test samples are presented in Table 1, as well as PT providers and test codes. Performance of the Laboratory in proficiency testing is presented in Table 2, including data on mycotoxin level assigned by the PT provider, Laboratory result and obtained *z* scores.

*Table 1.* Proficiency tests – mycotoxins in food, performed over 2012–2016

PT title (PT sample)	Reference (Provider, year/test code)
Patulin in apple juice	FAPAS, 2012/1647; 2016/1656
Mycotoxin contamination in infant food	FAPAS, 2012/04199
Aflatoxins in corn	Romer Labs, 2015/M15421A; 2016/ M16411A
Aflatoxin M1 in milk	FAPAS, 2013/04224
Ochratoxin A in wine	FAPAS, 2013/17117
Ochratoxin A in barley flour	FAPAS, 2016/17163
Deoxynivalenol in breakfast cereals	FAPAS, 2014/22107
Deoxynivalenol in wheat	Romer Labs, 2014/ M14161D
Deoxynivalenol in corn	Romer Labs, 2016/16161D
Zearalenone in breakfast cereals	FAPAS, 2014/22106
Zearalenone in wheat	Romer labs, 2014/ M14421Z
Fumonisin in maize flour	FAPAS, 2013/2297; 2016/22133

Laboratory performance assessed in terms of  $z$  scores showed all satisfactory results. In depth evaluation revealed following distribution of  $z$  scores (absolute values): 13 (59.1%) up to 0.5, 8 (36.4%) between 0.5 and 1.0, 1 (4.5%) above 1.0. Analysis of trends performed for multiple determinations of individual mycotoxins showed several changes of  $z$  score to better or worse rank: PAT improved, AFB1 and DON improved and worsened, FUM slightly worsened. PAT and FUM were analyzed in the same type of food commodity, as well as AFs in last two PT rounds, while in case of other mycotoxins it should be noticed that different food commodities could have substantial influence on laboratory performance. Longer time periods and substantial financial resources are needed to obtain enough data to enable analysis of trends over several PT rounds. However, the Laboratory successfully fulfilled the requirements for the four year accreditation cycle according to the rules of Accreditation body of Serbia (2014). Analytical methods for determination of all legally regulated mycotoxins in selected food commodities are in the scope of accreditation of the Laboratory.

Table 2. Performance of the Laboratory in proficiency testing – mycotoxins in food

Mycotoxin	Food commodities and results					
PAT	Apple juice			Apple juice		
Assigned / Lab <sup>a</sup>	39.3 / 46.2			45.7 / 48.8		
z score	0.8			0.3		
AFs	Infant food		Corn <sup>b</sup>		Corn <sup>b</sup>	
	AFB1	AFB2	AFB1	AFB2	AFB1	AFB2
Assigned / Lab <sup>a</sup>	0.12 / 0.10	n.p./<LOD	6.0 / 6.2	0.37 / 0.4	6.58 / 5.27	0.72 / 0.64
z score	-0.7	—	0.1	0.3	-0.9	-0.5
AFM1	Milk powder					
Assigned / Lab <sup>a</sup>	0.181 / 0.172					
z score	-0.2					
OTA	Infant food		Wine		Barley flour	
Assigned / Lab <sup>a</sup>	0.39 / 0.40		1.94 / 2.3		3.02 / 2.95	
z score	0.1		0.8		-0.1	
ZEA	Breakfast cereals			Wheat		
Assigned / Lab <sup>a</sup>	99.6 / 88.8			119 / 125.8		
z score	-0.5			0.3		
DON	Breakfast cereals		Wheat		Corn	
Assigned / Lab <sup>a</sup>	764 / 934		915 / 955		824.73 / 907	
z score	1.3		0.3		0.6	
FUM	Maize flour			Maize flour		
	FB1	FB2	FB1+FB2	FB1	FB2	FB1+FB2
Assigned / Lab <sup>a</sup>	765 / 618	350 / 359	1123 / 977	731 / 826	144 / 132	874 / 958
z score	-0.8	0.1	-0.5	0.8	-0.4	0.6

<sup>a</sup> results in  $\mu\text{g/kg}$ .

<sup>b</sup> AFG1 and AFG2 were not present in test material and not found by laboratory

One of the main objectives of a PT scheme is to help the laboratory to assess the accuracy of its measurements. With regard to this criterion, performance of the Laboratory was also fit for purpose: PAT 107–118%, AFB1 80–103%, AFB2 89–108%, AFM1 95%, OTA 98–119%, ZEA 89–106%, DON 104–122%, FB1 81–113%, FB2 92–103%, FB1+FB2 87–110%.

## CONCLUSION

Participation in PT plays a highly valuable role providing an objective evidence of the competence of a laboratory. It is worth noticing that, apart from analytical laboratory, customers of laboratory services, accreditation bodies and regulatory authorities also have an interest in PT schemes as a means to independently monitor the validity of measurements.

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## ОЦЕНА КОМПЕТЕНТНОСТИ ЛАБОРАТОРИЈЕ КРОЗ ТЕСТИРАЊЕ ОСПОСОБЉЕНОСТИ – МИКОТОКСИНИ У ХРАНИ

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**РЕЗИМЕ:** Лабораторија за анализу микотоксина у храни Института за јавно здравље Војводине (Нови Сад, Србија) учествовала је у 15 шема за тестирање оспособљености у периоду од 2012. до 2016. године, које су обухватиле 22 одређивања законом регулисаних микотоксина: афлатоксини Б<sub>1</sub>, Б<sub>2</sub>, Г<sub>1</sub>, Г<sub>2</sub> и М<sub>1</sub>, охратоксин А, деоксиниваленол, зеараленон, фумонизини и патулин, у различитим намирницама: пшеница, кукуруз, житарице за доручак, храна за одојчад, млеко, вино и воћни сок. Анализе су урађене методом течне хроматографије високих перформанси с ултраљубичастом (патулин, деоксиниваленол) или флуоресцентном детекцијом (афлатоксин М<sub>1</sub>, охратоксин А, зеараленон) коришћењем преколоне дериватизације с *o*-фталалдехидом (фумонизини) или ултраљубичасте постколоне дериватизације (афлатоксини Б<sub>1</sub>, Б<sub>2</sub>, Г<sub>1</sub>, Г<sub>2</sub>), након пречишћавања применом имуноафинитетне хроматографије са специфичним антителима, изузев у случају патулина, за чије одређивање је примењена екстракција растварачем и пречишћавање на чврстој фази (С18). Учинак лабораторије оцењен је на основу постигнутих з скорова, при чему су сви резултати били задовољавајући. Детаљнија анализа показала је следећу расподелу з скорова: 59,1% до вредности од 0,5; 36,4% између 0,5 и 1,0; 4,5% изнад 1,0 (као апсолутне вредности). За вишеструка одређивања појединачних микотоксина анализиран је тренд, при чему је уочено неколико промена ранка з скорa ка бољем или лошијем. Учешћем у тестирању оспособљености лабораторија је потврдила компетентност за анализу микотоксина у храни.

**КЉУЧНЕ РЕЧИ:** осигурање квалитета, тестирање оспособљености, храна, микотоксини

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## DETERMINATION OF MULTIPLE MYCOTOXINS IN MAIZE USING QuEChERS SAMPLE PREPARATION AND LC-MS/MS DETECTION

**ABSTRACT:** A reliable and easy method has been used for the multiple mycotoxins determination of AFs, DON, ZEA and FBs in maize samples. Liquid chromatography coupled mass spectrometry (LC-MS/MS) was used. Mycotoxins have been extracted from maize using a QuEChERS-based extraction procedure. All validation parameters were in accordance with Reg. (EC) No 401/2006. The analyses of eight maize seed samples showed the AFs, DON, ZEA and FBs contamination with the values below the state limit standards.

**KEYWORDS:** AFs, DON, ZEA, FBs, QuEChERS, maize, LC-MS/MS

## INTRODUCTION

Mycotoxins are toxic secondary metabolites that are naturally produced by several species of fungi on agricultural products, particularly grain-based products. Mycotoxins are chemically stable and cannot be destroyed during food processing and heat treatment. Therefore, they may occur in the field, in raw materials during storage and in processed foods (Bardsley and Oliver, 2014). To date, approximately 400 compounds have been identified as mycotoxins such as aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), nivalenol (NIV), deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin (T-2), and HT-2 toxin (HT-2) (Kim *et al.* 2017). Mycotoxins present a wide range of adverse effects

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on consumer health, including carcinogenic, mutagenic, estrogenic and immunosuppressive effects (Serrano *et al.* 2015).

Monitoring and inspecting the contamination levels of mycotoxins in foods and feeds has gained major national and international attention over the years. (Comm. Reg. (EC) No 1881/2006, Directive 2002/32/EC, EFISC Code 4.4.3 Inspection, sampling and analysis).

The current mycotoxin extractions have comprised: a liquid-liquid extraction (LLE), supercritical fluid extraction (SFE); solid phase extraction (SPE), pressure liquid extraction (PLE), matrix solid-phase dispersion (MSPD), ultrasound and homogenizing extraction with various mixtures of organic solvents which are only some of the techniques in the stream of those which can be applied in the extraction of mycotoxins from various matrices (Bursić *et al.* 2013). “Green Analytical Chemistry” is a rapidly developing trend that is rooted in the desire to make chemical analyses environmentally friendlier (Breidbach, 2017). That is the reason why QuEChERS (Quick Easy Cheap Effective Rugged Safe) is recognized as the most modern procedure of extraction and extract purification (Cunha and Fernandes, 2010; Capriotti *et al.* 2012). QuEChERS has attracted increasing attention in the research field of mycotoxins due to its simplicity and effectiveness for isolating mycotoxins from complex matrices, but also it is an environment-friendly method.

Mycotoxins can be analyzed by various methods, including thin layer chromatography (TLC), enzyme-linked immunosorbent assay, gas chromatography, and immunoaffinity column/high-performance liquid chromatography with fluorescence and diode array detection (Sun *et al.* 2016). The use of LC coupled to mass spectrometry (MS) for the confirmation of polar contaminants, such as mycotoxins, has become common nowadays, in the systems designed for the control of food quality (Vuković *et al.* 2016).

The analysis of mycotoxins is challenging due to the large number of compounds to be detected and the wide physicochemical properties they possess. In the paper, the QuEChERS extraction of AFs, FBs, DON and ZEA from maize samples was used with the mycotoxins determination by LC-MS/MS.

## MATERIAL AND METHOD

**Analytical Standards** Aflatoxins, zearalenone and deoxanivalenol standards of the highest possible purity were purchased from Sigma-Aldrich and fumonisin standard (B1+B2) was purchased from R-Biopharm Rhone Ltd. The individual solutions of the solid standards were prepared using appropriate solvent solutions. A working standard solution containing all eight mycotoxins was prepared by mixing appropriate volumes of the stock solutions in a 10 mL volumetric flask and diluted to volume with methanol.

**Sample Preparation** Sample extraction: 1. Place 5 g of sample into a 50 mL centrifuge tube. 2. Add 10 mL of water. 3. Vortex briefly and allow to hydrate for at least 15 minutes. 4. Add 10 mL acetonitrile containing 2% acetic acid. 5. Vortex (5 min) samples for 30 minutes to extract mycotoxins. 6. Add 4g

MgSO<sub>4</sub> and 1g NaCl. 7. Immediately vortex for 1 minute. 8. Centrifuge for 5 min at 4,000 rpm. **Sample cleanup:** 1. Transfer 6 mL of supernatant to a dSPE tube of 15 mL (containing 1,200 mg MgSO<sub>4</sub>, 400 mg PSA and 400 mg C18). 2. Vortex for 1 min. 3. Centrifuge for 5 min at 4,000 rpm. 4. Transfer 1 mL of supernatant to a 5 mL test tube. 5. Evaporate the acetonitrile extract to dryness and reconstitute with 1 mL of mobile phase. 6. Filter the extract, using a 0.2 µm syringe filter, directly into an autosampler vial.

**Preparation of matrix-matched calibration (MMC)** A five-point matrix-matched calibration curve was prepared using the sample extracts obtained from blank samples. The blank extract was evaporated and reconstituted with calibration standards in mobile phase so that final concentrations were 1.25, 2.5, 5.0, 10 and 20 ng/mL for AB1, AB2, AG1 and AG2; 150, 250, 500, 1,000 and 2,000 ng/mL for DON; 25, 50, 100, 200 and 400 ng/mL for ZEA; 150, 250, 500, 1,000 and 2,000 ng/mL for FB1 and 43.75, 87.5, 175, 330 and 660 ng/mL for FB2.

**Instrumentation and chromatographic conditions for LC-MS/MS** LC was performed with an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump, a G1367D autosampler and a G1316B column oven (Agilent Technologies, USA). The chromatography separation was achieved by Zorbax Eclipse XDB C18 column (50 x 4.6 mm, 1.8 µm) (Agilent, USA) maintained at 30 °C. The analytical separation was performed using methanol as mobile phase A and water as mobile phase B, both containing 0.1% formic acid with gradient mode. The flow rate was maintained at 0.4 mL/min. The mass analysis was carried out with an Agilent 6410B Triple Quadrupole mass spectrometer equipped with multi-mode ion source (MMI, Agilent Technologies, Palo Alto, CA, USA). The data acquisition and quantification were conducted using MassHunter Workstation software B.04.01 (Agilent Technologies, 2010). The following ionization conditions were used: electrospray ionization (+ESI) positive ion mode, drying gas (nitrogen) temperature 325 °C, vaporizer 220 °C, drying gas flow rate 5 L/min, nebulizer pressure 40 psi and capillary voltage 2,500 V. The dwell time was 50 ms. External standard method was used for the quantification of mycotoxins.

**Validation parameters** The method was validated according to Commission Regulation (EC) No 401/2006.

The limit of detection – LOD was determined as the lowest concentration giving a response of three times the average baseline. The ratio signal/noise in the obtained chromatograms for the LOD was calculated by MassHunter Qualitative Software.

The limit of detection – LOQ is estimated as the lowest spiking level (LL) that allows reliable detection of all five replicates meeting the performance criteria of RSD < 20% and mean recovery of 60–120% at both MRM transitions.

The linearity was checked using MMS at the concentrations of 1.25: 20 ng/mL for AFs, 150: 2,000 ng/mL for DON; 25: 400 ng/mL for ZEA; 150: 2,000 ng/mL for FB1 and 43.75: 660 ng/mL for FB2.

The recovery was checked by enriching a blank sample with the mixture of mycotoxins, to get the final mass concentration of 5 and 10 µg/kg for AFs, 500 and 1,000 µg/kg for DON, 100 and 200 µg/kg for ZEA and 500 and 1,000 µg/kg for FB1.

The precision of the method in terms of repeatability (r) (intra-day precision) and reproducibility (R) (inter-day precision) was evaluated calculating the relative standard deviation (%RSD) of spiked samples analyzed in five replicates on the same and different days.

## RESULTS AND DISCUSSION

For the quantification, the ion with the best signal sensitivity (Q) was preferred and for the confirmation the second transition (q) and the ratio of abundances between both ion transitions (Q/q) were used. The cone voltages were selected according to the sensitivity of the precursor ions and the collision energies were chosen to give the maximum intensity of the fragment ions obtained. The product-ion spectra obtained on triple quadrupole instrument generally provide fragments which are of diagnostic value for structural elucidation and confirmation (Vuković *et al.* 2016). The MRM transitions of all analyzed mycotoxins are given in Table 1.

Table 1. SRM transitions

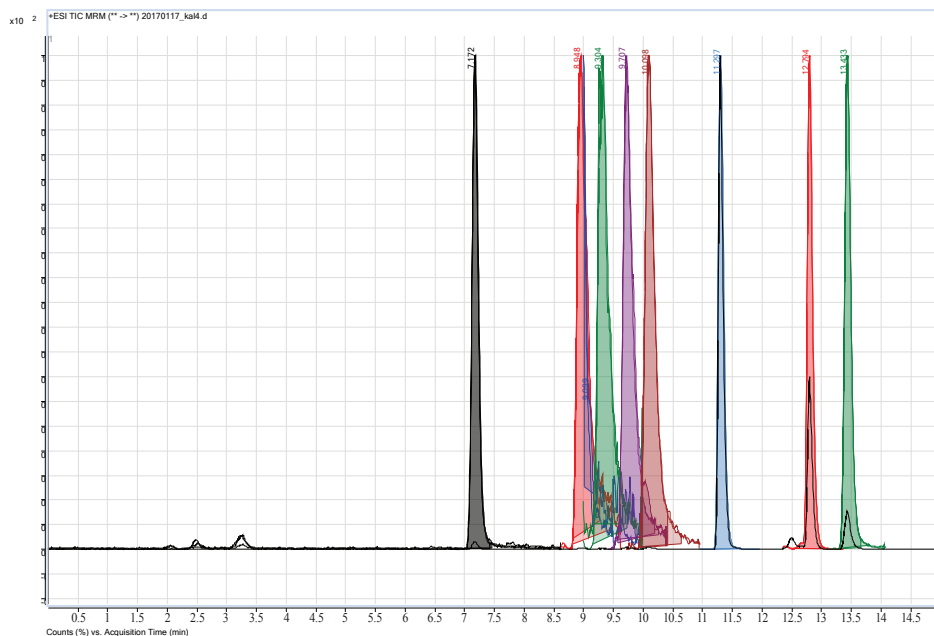
Analyte	T <sub>R</sub> (min)	Precursor ion	Product ion 1	CE 1 (V)	Product ion 2	CE 2 (V)	Frag (V)
AFB1	10.39	313	241	35	269	35	100
AFB2	10.00	315	259	35	287	35	100
AFG1	9.68	329	243	35	311	35	100
AFG2	9.00	331	313	30	245	35	100
DON	7.38	297	203	15	267	8	70
FB1	11.56	722.5	352.3	40	334.4	40	140
FB2	12.98	706.4	318.0	35	336.3	35	140
ZEN	13.63	319.2	283.3	10	185.4	25	100

The obtained validation parameters (LOD, LOQ, linearity (R<sup>2</sup>), recoveries and precision (RSD)) are shown in Table 2.

Table 2. Validation parameters

Analyte	LOQ (µg/kg)	R <sup>2</sup>	Recoveries (%)	RSD (%)
AFB1	1.25	0.9923	126.3	3.56
AFB2	1.25	0.9953	64.5	24.7
AFG1	1.25	0.9952	46.8	22.40
AFG2	1.25	0.9974	29.5	27.30
DON	150	0.9964	84.6	24.60
FB1	150	0.9903	62.8	1.240
FB2	50	0.9961	58.2	17.00
ZEN	25	0.9981	94.7	25.90

For the quantification of detected mycotoxins the matrix-matched calibration (MMS) was used (Figure 1).



*Figure 1.* Overlap MRM chromatograms of matrix match calibration of DON, AFBs, ZEN, and FBs.

**Application of developed method on real samples** The method was applied for the analysis of eight maize samples. The detected mycotoxins were DON, FBs and ZEN. The other mycotoxins (AFB1, AFB2, AFG1 and AFG2) were not detected.

LC-MS/MS chromatogram of maize samples number seven containing FB1 and FB2 mycotoxins are shown in Figure 2.

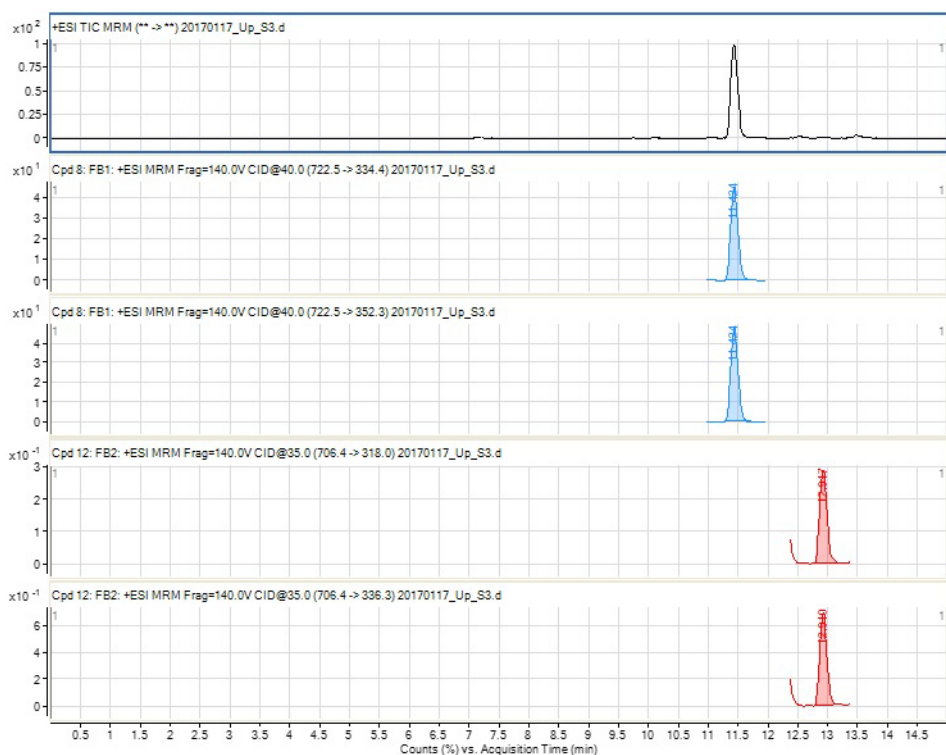


Figure 2. LC-MS/MS chromatogram of maize containing FB1 and FB2

In contaminated maize samples the DON was within the concentration ranging from 278.2 to 890.9  $\mu\text{g/kg}$ , but the values are still below the state limit standards. The contamination of ZEA was in the range from 25.8 to 91  $\mu\text{g/kg}$ , while the FBs were detected only in one sample with the concentration of FB1 of 754  $\mu\text{g/kg}$  and FB2 of 215  $\mu\text{g/kg}$  (Table 3).

Table 3. Distribution of mycotoxin contaminants in maize samples ( $\mu\text{g/kg}$ )

Analyte	Sample							
	1	2	3	4	5	6	7	8
AFB1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
AFB2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
AFG1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
AFG2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DON	778.8	890.9	<LOQ	<LOQ	384.9	<LOQ	<LOQ	278.2
FB1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	754.0	<LOQ
FB2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	215.0	<LOQ
ZEN	91.0	78.5	<LOQ	<LOQ	54.0	<LOQ	<LOQ	25.8

## CONCLUSION

A modified QuEChERS was developed with the combination of LC-MS/MS method for the quantitation of 8 common mycotoxins in maize. The developed method was applied for the determination of mycotoxins in 8 maize samples collected from Serbian markets. A total of 5 maize samples (62%) were contaminated with at least one of these mycotoxins. The results demonstrated that the procedure was suitable for the determination of mycotoxins in cereals and could be implemented for the routine analysis.

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### ОДРЕЂИВАЊЕ ВИШЕ МИКОТОКСИНА У КУКУРУЗУ QuEChERS ПРИПРЕМОМ УЗОРКА И LC-MS/MS ДЕТЕКЦИЈОМ

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**РЕЗИМЕ:** Поуздан и једноставан метод коришћен је приликом одређивања микотоксина АФс, ДОН, ЗЕА и ФБс, у узорцима кукуруза. За детекцију и квантификацију је коришћена течна хроматографија с тандем масеном спектрометријом (LC-MS/MS). Микотоксини су екстраховани из кукуруза применом поступка QuEChERS екстракције. Сви параметри валидације су у складу са Регулацивом Европске уније број 401/2006. Анализирано је осам узорака семена кукуруза у којима су садржаји афлатоксина, ДОН, ЗЕА и фумонизина били испод дефинисаних максималних граница.

**КЉУЧНЕ РЕЧИ:** АФс, ДОН, ЗЕА, ФМс, QuEChERS, кукуруз, LC-MS/MS

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## DATA ACQUISITION OF TRIPLE QUADRUPOLE LC/MS FOR THE CITRININ DETERMINATION

**ABSTRACT:** The analysis of citrinin is challenging because it needs to be detected in low concentrations in complex sample matrices. Before citrinin quantification, the data acquisition of LC-MS/MS must be performed, which includes the determination of ion monitoring reaction (SRM), finding fragmentation energies (Frag.) and collision cell energies (CE) for which the response of citrinin will be the highest for the given conditions. The best response of citrinin is obtained for Frag. of 66 V and CE of 17 and 29 V.

**KEYWORDS:** CIN, data acquisition, LC-MS/MS

## INTRODUCTION

Mycotoxins are a group of natural contaminants in raw agricultural materials, foods, and feeds, mainly produced by filamentous fungi as a series of secondary metabolites (Ji *et al.* 2015). The most predominant mycotoxins are the aflatoxins (AFs – AFB1, AFG1, AFB2 and AFG2) produced by *Aspergillus* species, ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium* species, and toxins from *Fusarium* fungi, deoxynivalenol (DON), zearalenone (ZON), T-2 and HT-2 toxins, and fumonisins (FBs – FB1 and FB2) (Škrbić *et al.* 2011). To date, approximately 400 compounds have been identified as mycotoxins (Kim *et al.* 2017), one of which is citrinin (CIN).

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Citrinin is a polyketide mycotoxin with the molecular formula  $C_{13}H_{14}O_5$ , (IUPAC: (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid). Citrinin was first isolated as a pure compound from a culture of *Penicillium citrinum* by Hetherington and Raistrick in 1931 (Flajs and Peraica, 2009). Ostry *et al.* (2013) tabulated the mold that can produce this mycotoxin (Table 1), but Doughari (2015) indicated that out of main *Aspergillus* (*A. niger*, *A. ostianus*, *A. fumigates*, *A. niveus*, *A. awamori*, and *A. parasiticus*) *A. niger* is the most potent producer of citrinin. CIN is formed after harvest under storage conditions and it occurs in cereals and cereal products, rice, apples, fruit juices, black olive, almonds, peanuts, hazelnuts, pistachio nuts, sunflower seeds, spices (turmeric, coriander, fennel, black pepper, cardamom, and cumin) and food supplements based on rice fermented with red microfungus *Monascus purpureus* (Ostry *et al.* 2013). The strains of *Monascus* are traditionally used in China to produce red and yellow pigments for food. Western countries limit the use of synthetic food colorants due to their toxicity and mutagenicity. The natural food pigments obtained from *Monascus* were good candidates for their substitution because the reports on their toxic effects have been scarce for more than 1,000 years (Flajs and Peraica, 2009). Compared with other *Monascus* metabolites, CIN can be present in products in the range of concentrations from 0.1 to 500 mg/kg (Li *et al.* 2012). The European Food Safety Authority has also reported contamination of cheese by citrinin where toxigenic strains directly grow in the cheese mass.

Table 1. *Penicillium* and *Monascus* species as citrinin producers

Genera	Subgenus	Series	Species
<i>Penicillium</i>	Furcatum		<i>P. citrinum</i>
	<i>Penicilium</i>	<i>Expansa</i>	<i>P. expansum</i>
	<i>Penicilium</i>	<i>Carymbifera</i>	<i>P. radicolola</i>
	<i>Penicilium</i>	<i>Verrucosa</i>	<i>P. verrucosum</i>
<i>Monascus</i>	<i>M. purpureus</i>	Food supplement with fermented red rice	
	<i>M. ruber</i>	Soya bean, sorgum, rice, oat	

CIN is decomposed at 175 °C by dry heating, but decomposition temperature decreases to 140 °C in the presence of a small amount of water. The decomposition products obtained by heating CIN with water at 140 °C to 150 °C are CIN H1 and CIN H2 (Figure 1). CIN H2 shows no significant cytotoxicity, while CIN H1 shows increased cytotoxicity as compared to the parent compound (Doughari, 2015). Toxicity studies indicated that citrinin had cytotoxic, genotoxic, mutagenic and immunotoxic effects on humans and animals, and the most susceptible organ is kidney (Ji *et al.* 2015).

According to Commission Regulation (EC) 1881/2006, as amended by Regulation (EU) 212/2014, the maximum level of 2,000 µg/kg of citrinin in food supplements based on rice fermented with red yeast *Monascus purpureus* was to be reviewed before 1 January 2016 in the light of information on the exposure to citrinin from other foodstuffs and updated information on the toxicity of citrinin in particular as regards carcinogenicity and genotoxicity.

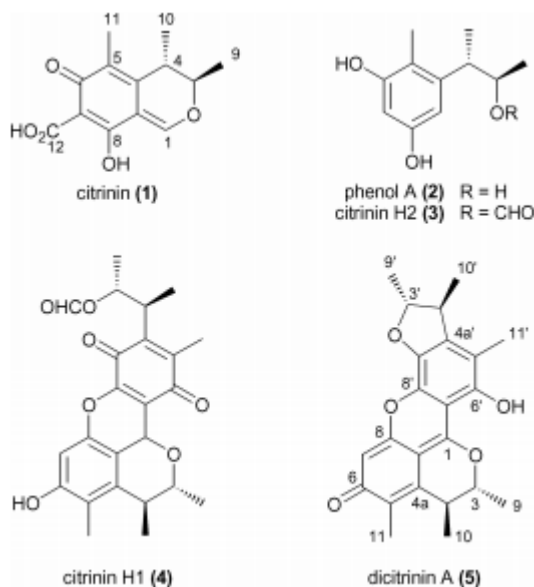


Figure 1. CIN decomposition products (Clark *et al.* 2006)

Thanks to the planar structure CIN has natural fluorescence, which indicates its detection by high-performance liquid chromatography with fluorescent detection (HPLC-FLD), but in recent years a rapid and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used (Li *et al.* 2011).

The analysis of CIN is challenging because it needs to be detected in low concentrations in complex sample matrices. Before CIN quantification, the data acquisition of LC-MS/MS must be performed.

## MATERIAL AND METHODS

**Analytical Standards.** Citrinin of the highest possible purity was purchased from Biopure, Romer Labs as a solution of 100.4 µg/mL in acetonitrile. Working standard solutions were in the concentration of 1.0 and 10 µg/mL in acetonitrile.

**Acquisition procedure.** Before performing the calibration or quantification of CIN it is necessary to establish the acquisition parameters of mass spectrometry: to determine the reaction to ion monitoring (SRM), find fragmentation energies (Frag.) and collision cell energies (CE) for which the response of the studied mycotoxin will be the highest for set conditions. SRM was determined using MassHunter Optimizer Software Version B03.01 (Agilent Technologies, 2010), as well as the data from scientific papers. Then, experimentally, the needed optimum fragmentation energy and CE for each SRM were determined by introducing the solution standard of CIN (1.0 µg/mL) into ion source. In the

process, the chromatographic column from the system can, but not necessarily, be removed. In case that MassHunter Optimizer does not detect SRM, the precursor and product ion have to be detected gradually. CIN is recorded in SCAN mode where the molecular ion is detected from mass spectre. Then, based on the familiar molecular ion in Product Ion mode, by the application of various fragmentations energy, the molecular ion is fragmented and based on the obtained mass spectres the most intensive ions are found which are supposed to be formed during the fragmentation. For each formed ion in the mode Precursor Ion the source ion is confirmed. By combining these assumptions, at least two SRMs for the given analyte are confirmed.

### **Instrumentation and chromatographic conditions for LC-MS/MS.**

LC was performed with an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump, a G1367D autosampler, and a G1316B column oven (Agilent Technologies, USA). Chromatography separation was achieved by Zorbax Eclipse XDB C18 column (50 x 4.6 mm, 1.8  $\mu$ m) (Agilent, USA) maintained at 30 °C. The analytical separation was performed using methanol as mobile phase A, and water as mobile phase B, both containing 0.1% formic acid with gradient mode (0 min – 40% B, 10 min – 5% B, 15 min – 5% B, stop time – 17 min, post time – 5 min). The flow rate was maintained at 0.5 mL/min. The mass analysis was carried out with an Agilent 6410B Triple Quadrupole mass spectrometer equipped with multi-mode ion source (MMI, Agilent Technologies, Palo Alto, CA, USA). The data acquisition and quantification were conducted using MassHunter Workstation software B.06.00 (Agilent Technologies, 2012). The following ionization conditions were used: electrospray ionization (+ESI) in positive ion mode, drying gas (nitrogen) at the temperature of 325 °C, vaporizer at 200 °C, drying gas flow rate 5 L/min, nebulizer pressure of 40 psi and capillary voltage of 2,500 V. The dwell time was 50 ms.

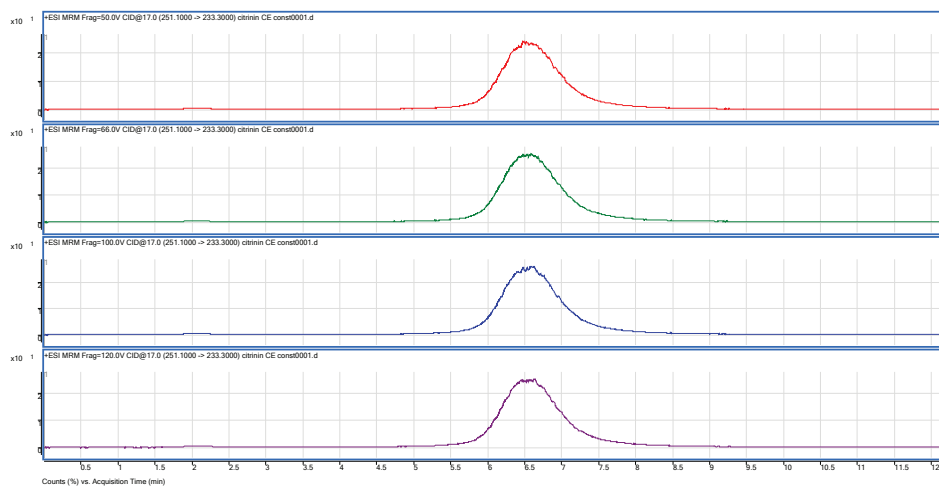
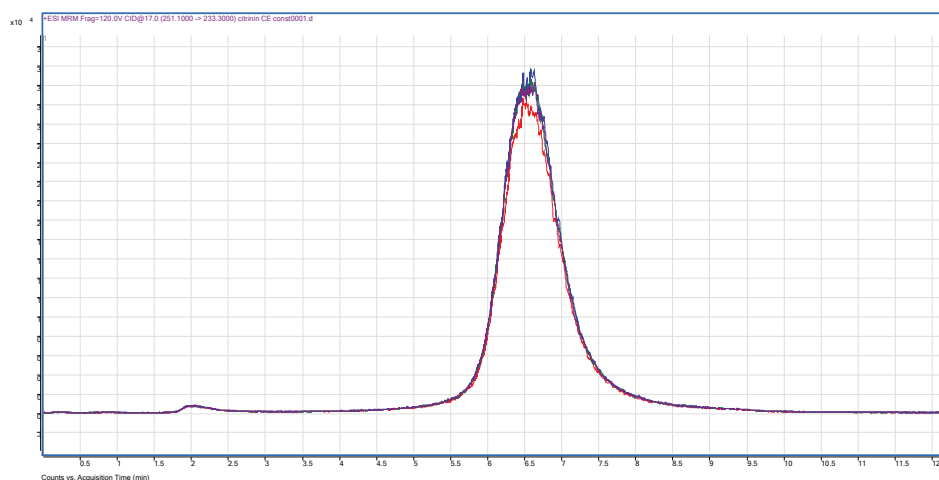
## **RESULTS AND DISCUSSION**

The first adjustments implied the constant collision cell energy, with various fragmentation energies (Table 2).

*Table 2.* Constant collision cell energy, with various fragmentation energies

Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc. Voltage	Polarity
251.1	Wide	233.3	Unit	50	100	17	7	Positive
251.1	Wide	233.3	Unit	50	66	17	7	Positive
251.1	Wide	233.3	Unit	50	50	17	7	Positive
251.1	Wide	233.3	Unit	50	120	17	7	Positive

The effect of the fragmentation energy at the same collision energy values is shown in Figure 2.

**a****b**

*Figure 2. The effect of various fragmentation energy at constant CE (15 V)*

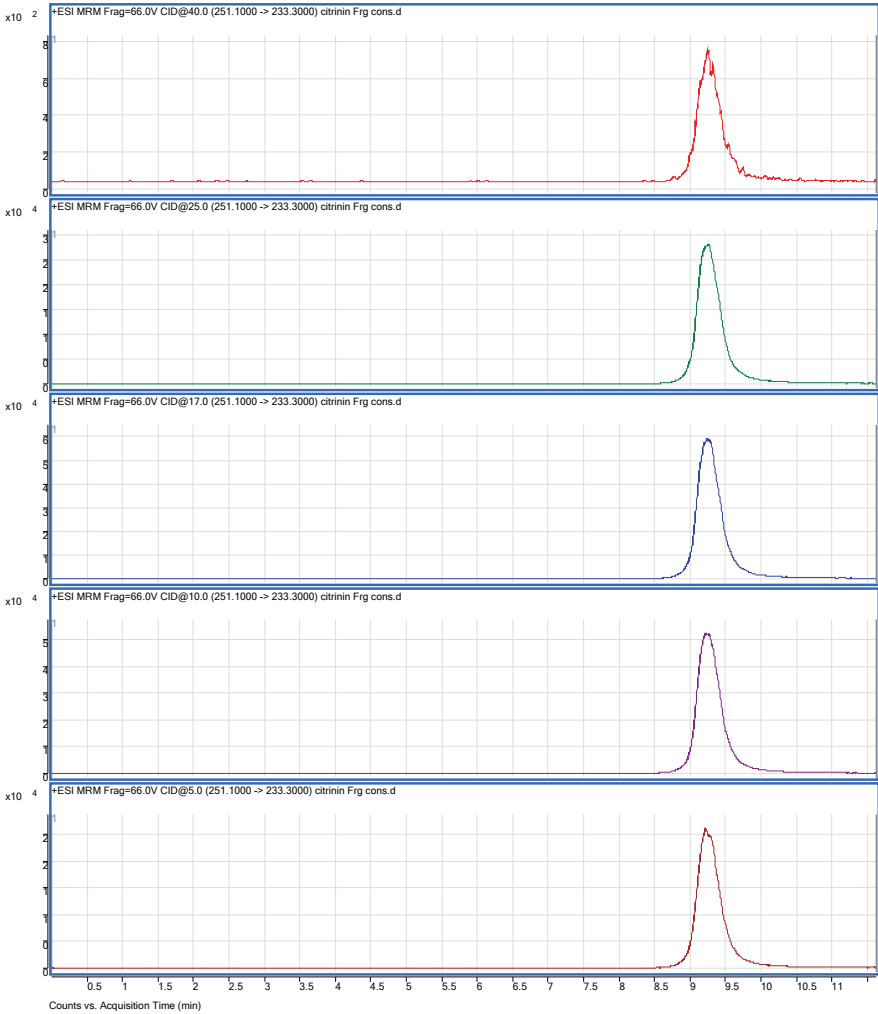
In the overlapped chromatogram (Figure 2b) it is shown that the change of collision cell energy does not significantly effect the citrinin response. Still, the best response is obtained at the collision cell energy of 66 V.

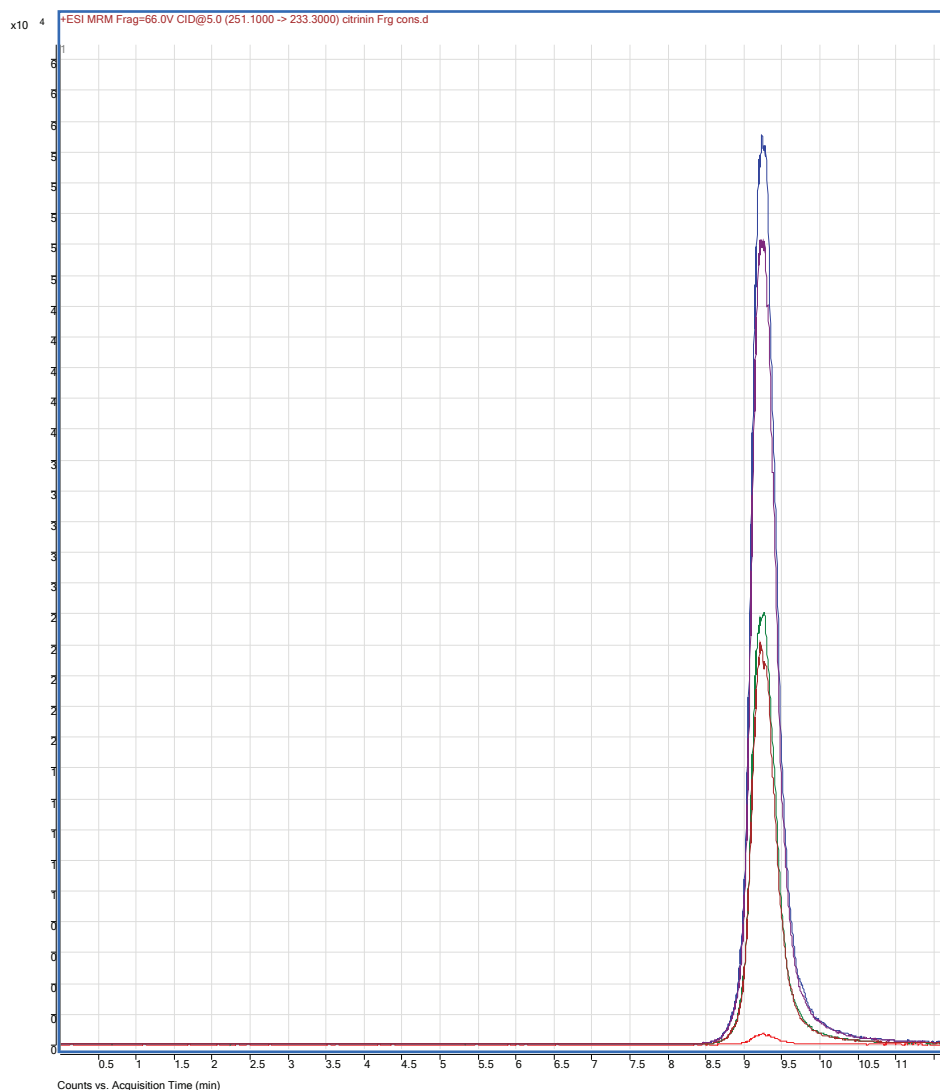
When the fragmentation energy is determined (in our case 66 V) for which the signal is the most intensive, then the fragmentation energy is kept constant and collision cell energy is changed (Table 3).

Table 3. Constant Frag. with the different CE

Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc. Voltage	Polarity
251.1	Wide	233.3	Unit	50	66	10	7	Positive
251.1	Wide	233.3	Unit	50	66	17	7	Positive
251.1	Wide	233.3	Unit	50	66	25	7	Positive
251.1	Wide	233.3	Unit	50	66	40	7	Positive
251.1	Wide	233.3	Unit	50	66	5	7	Positive

At the constant Frag. energy of 66 V, by changing the collision energy (Figure 3) the response of the signal of studied mycotoxin in the given conditions was monitored.





*Figure 3.* The effect of energy change in collision cell at the constant fragmentation energy (Frag. of 66 V)

CE of 17 V gives the best CIN signal, followed by energy of 10 V. The collision cell energy of 40 V gives the weakest citrinin response.

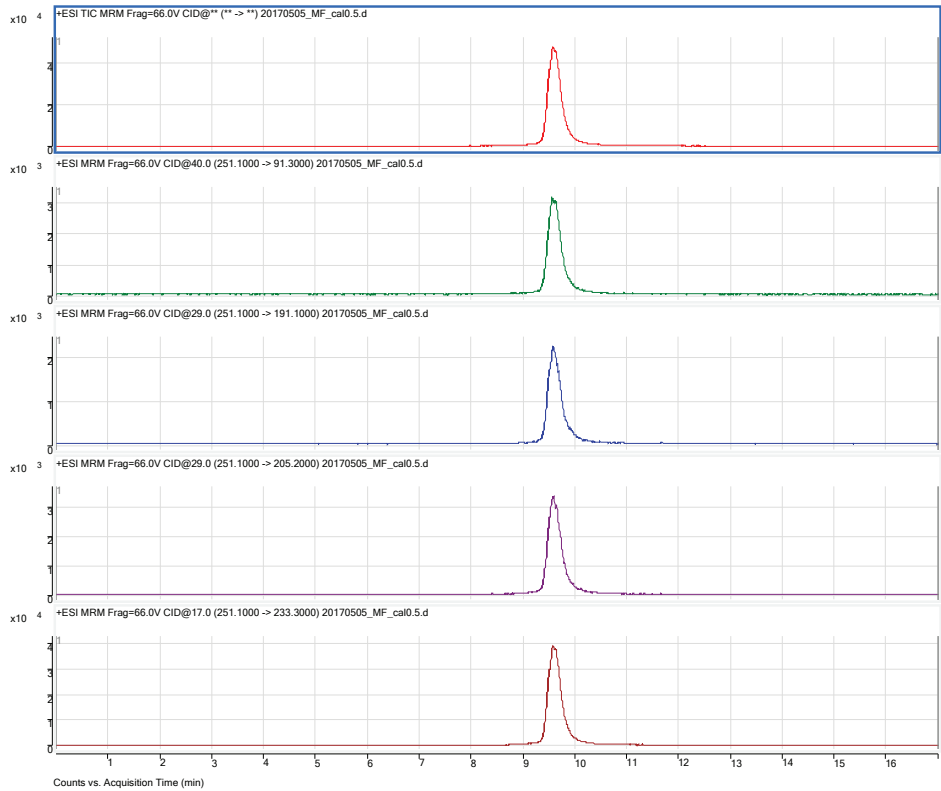
After SRM is established, it is necessary to find out the chromatographic conditions for the best separation of the studied analytes. It is necessary to emphasize that the total ion chromatogram (TIC) does not need to have a good separation resolution, because SRM chromatograms, which contained only one peak and are suitable for further quantitative analyses, are extracted from it.

Table 4. SRM transitions

Compound		Precursor Ion	MS1 Res	Product Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc. Voltage	Polarity
Citrinin	q1	251.1	Wide	205.2	Unit	66	29	7	Positive
Citrinin	q2	251.1	Wide	191.1	Unit	66	29	7	Positive
Citrinin	q3	251.1	Wide	91.3	Unit	66	40	7	Positive
Citrinin	Q	251.1	Wide	233.3	Unit	66	17	7	Positive

The molecular weight of citrinin is 250.25 g/mol, the identification of the target mycotoxin through the selection of specific MRM transitions from 251.1 (used as qualification ion) to 233.3 (used as quantification ion) m/z was carried out at the constant Frag. (66 V) and CE of 40, 25, 17, 10, and 5 V.

As the response, i.e. the peak area, is the largest at 17 V, the other CE values are not taken into consideration (Figure 4).



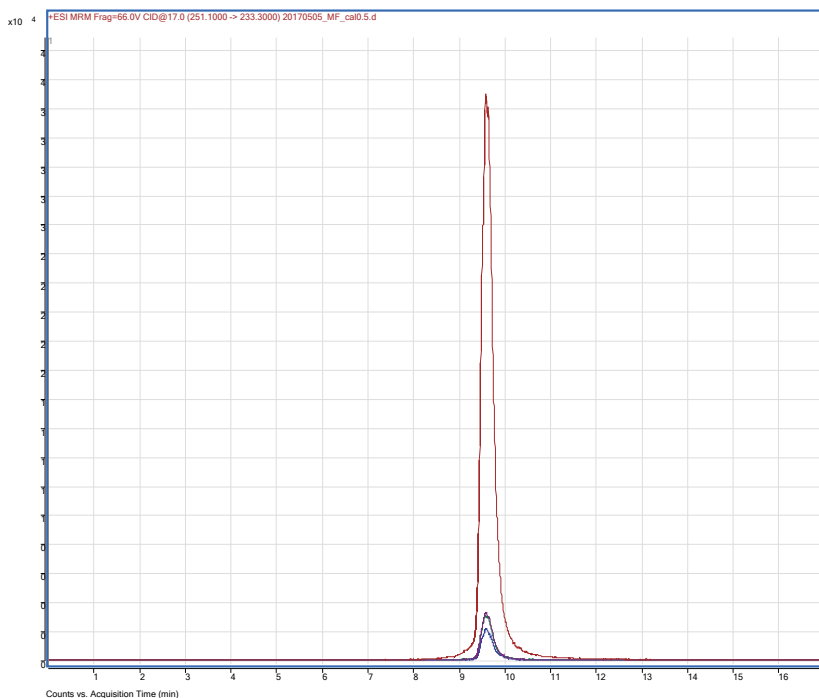


Figure 4. MRM citrinin transitions

The mechanism of citrinin fragmentation was given in Figure 5 (Shu and Lin, 2002).

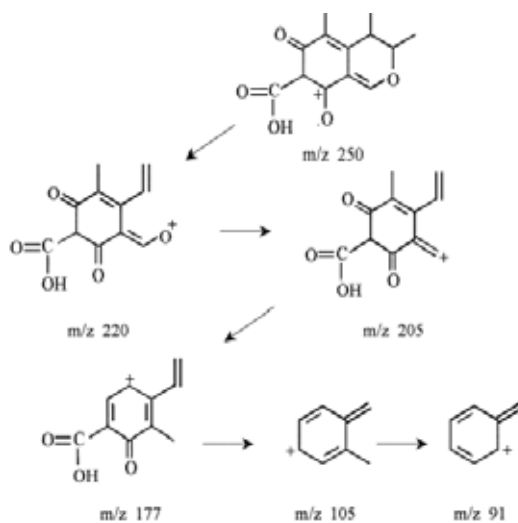


Figure 5. Fragmentation pattern of citrinin

## CONCLUSION

Before doing the calibration or quantification of CIN it is necessary to establish the acquisition parameters of mass spectrometry: to determine the reaction to ion monitoring (SRM), find fragmentation energies (Frag.) and collision cell energies (CE) at which the response of the studied mycotoxin will be the highest for set conditions. The best response is obtained at the collision cell energy of 66 V. Collision cell energies of 17 V give the best CIN signal.

## ACKNOWLEDGEMENT

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## АКВИЗИЦИОНИ ПАРАМЕТРИ ТРОСТРУКОГ КВАДРОПОЛА LC/MS ОДРЕЂИВАЊА ЦИТРИНИНА

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**РЕЗИМЕ:** Анализа ЦИН-а представља велики изазов из разлога што га је потребно детектовати у веома ниским концентрацијама у различитим узорцима. Пре квантификације ЦИН-а потребно је поставити аквизиционе параметре LC-MS/MS, који укључују одређивање реакције праћења јона (СРМ), проналажење енергије фрагментације (Фраг.) и енергије колизионе ћелије (ЦЕ) при којој ће одговор ЦИН-а бити највећи за дате услове. Најбољи одговори ЦИН-а добијају се при Фраг. од 66 V и ЦЕ од 17 и 29 V.

**КЉУЧНЕ РЕЧИ:** ЦИН, аквизициони параметри, LC-MS/MS



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## SPATIAL DISTRIBUTION OF GENETS IN POPULATION OF SAPROTROPHIC FUNGI *Marasmius rotula* ON MT. STARA PLANINA

**ABSTRACT:** This study was conducted to determine the size and spatial distribution of mycelial individuals of *Marasmius rotula* at one locality on Mt. Stara planina in the Republic of Serbia. Total of 12 sporocarps were collected from investigated locality (Vidlič, Stara planina). Sporocarps were distributed in four groups and distances between them were approximately 10–30 meters. Genomic DNA was extracted from each sporocarp and used for inter-simple sequence repeat (ISSR) polymorphism analysis using (GTG)<sub>5</sub> and (GACA)<sub>4</sub> primers. Both primers showed reproducible band patterns on agarose gels and sporocarps with identical band patterns were considered to belong to the same individual (genet) and were grouped accordingly. Grouping with both primers determined that 12 analyzed sporocarps belong to 4 distinct genets (A, B, C, D). Approximate genet diameters were 2 m for two genets (A, B) and 15 m for one genet (C) while diameter of one genet (D) was not possible to determine since it was represented only by one sporocarp. The results presented here are the first data about size and spatial distribution of genets of *M. rotula*. To determine whether the obtained genet sizes are general trait of an analyzed species or a special trait appeared as an effect of environmental conditions, more information on the genet distribution of other *M. rotula* populations is needed.

**KEYWORDS:** genet distribution, ISSR, *Marasmius rotula*, population

## INTRODUCTION

Saprotrophic, litter exploiting basidiomycetes are the primary decomposer organisms of lignocellulose material in nitrogen-limited boreal and temperate forests. Although these species are in a minority of ectomycorrhiza, they are the most abundant fungi in those nitrogen-limited environments (Tlalka *et al.* 2008).

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Despite their crucial role in those environments, very little is known about population structures of this group of fungi.

*Marasmius rotula* (Scop.; Fr.) Fr. is one of the most abundant and wide-spread saprotrophic basidiomycete in the boreal and temperate zones of the Northern Hemisphere. It usually produces plenty of sporocarps in most deciduous forests, which makes it suitable for investigation of saprotrophic fungal populations. Its sporocarps are mostly found in groups on wood (branches, stumps, trunks, bark) of broadleaved trees, on cupulas of *Fagus*, but it was also collected from basal parts of grass, twigs and needles of *Pinus*. It produces relatively small sporocarps with white hemispherical-convex pileus usually 4 to 18 mm in diameter. Lamellae are rather crowded (17–21), broadly adnexed to the collarium. Stipe is 20–60 x 0,25–0,75 mm, filiform, white or pale brown at apex, downwards red-brown to almost black-brown at base (Antonín and Noordeloos, 1993).

The mycelial nature of most fungi affects the definition and interpretations of fungal individuals. One cannot be sure if collected sporocarps from one area have been obtained from one individual or from several individuals. In earlier studies (Rayner and Todd, 1979; Thompson and Rayner, 1982; Huss, 1993) somatic incompatibility (SI) tests were used for discriminating distinct genets – genetically unique fungal individuals (Burchhardt *et al.* 2011). This laborious and time consuming method was later replaced by isozyme and molecular tools which reflect the distribution of mycelial individuals more accurately, especially in in-breeding populations (Jacobson *et al.* 1993; Guillaumin *et al.* 1996; Anderson *et al.* 1998). Molecular analysis of sporocarps (e.g. ISSR, RAPD) can allow identification of distinct genets of fungi and make analyses of fungal biodiversity possible in the same manner as for animals and most plants (Zak and Willig, 2004). Size and distribution of fungal genets are usually estimated by mapping analyzed sporocarps on site, determining which sporocarps have genetically identical genotypes and then measuring the distances between those identical sporocarps (Burchhardt *et al.* 2011). Since the presence of sporocarps indicates the presence of the parent mycelium in soil, the absence of sporocarps does not necessarily mean that mycelia are absent from soil (Dahlberg *et al.* 1997; Sawyer *et al.* 1999) and this must be considered when estimating numbers and sizes of genets on investigated area.

While there was several studies about size and distribution of genotypes of ectomycorrhizal fungi (Anderson *et al.* 1998; Sawyer *et al.* 1999; Zhou *et al.* 2000, Burchhardt *et al.* 2011), data about genets of saprotrophic fungi estimated by molecular methods, can not be found in available literature. The aim of the present study was to investigate the distribution of genets of saprotrophic fungi, *M. rotula* in beech forest on Mt. Stara planina, Serbia.

## MATERIAL AND METHODS

### Sporocarp sampling

Twelve sporocarps of *M. rotula* were collected from one locality (Vidlič) on Mt. Stara planina (43°10'36.65"N, 22°43'02.41"E) in the indigenous beech

stand. All sporocarps were collected on the same day in September, 2015. On site, sporocarps were mapped by measuring physical distance (in meters) between them and schematic map of their positions was drawn. Sporocarps were distributed in four groups and distances between them were approximately 10–30 meters. Investigated area was approximately 2,500 m<sup>2</sup>.

### Molecular analysis

DNA was extracted from around 20 mg of dried sporocarp tissue. Samples were crushed into powder using mortar and pestle with a small amount of liquid nitrogen. Extraction of DNA was performed using CTAB protocol (Doyle and Doyle, 1987).

PCR amplifications were performed using two inter-simple sequence repeat motif primers: (GTG)<sub>5</sub> and (GACA)<sub>4</sub>. Amplification for both primers was carried out in 50 µL reactions volumes which contained *ca* 100 ng of DNA, 5 µL of 10X DreamTaqpufer, 0.2 mM primer (GTG)<sub>5</sub> (0.4 mM for (GACA)<sub>4</sub>), 0.2 mM of dNTPs Mix (Thermo Fisher Scientific, Massachusetts) and 1.25 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, Massachusetts). After initial denaturation at 95 °C for 2 min, cycling conditions consisted of 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min for (GTG)<sub>5</sub> primer and 40 cycles of 94 °C for 1min, 48° C for 1 min and 72 °C for 1 min for (GACA)<sub>4</sub> primer followed by a final extension at 72 °C for 10 min (Zhou *et al.* 1999, slightly modified). A negative control, containing no fungal DNA, was included in each PCR reaction run.

Products of amplification were separated by electrophoresis in 1.5% agarose gels premixed with 2 µL (1 mg/ml) of ethidium bromide using 1 kb molecular weight marker (GeneRuler 1 kb DNA Ladder, Thermo Fisher Scientific, Massachusetts). Electrophoresis was carried out in TBE buffer. Gels were visualized and documented using BioDocAnalyze System (Analytik Jena AG, Germany).

Gel bands patterns were analyzed visually by naked eye. The sporocarps with the same PCR profile patterns for each primer were considered to belong to a same individual (genet) and were grouped accordingly.

## RESULTS AND DISCUSSION

Reproducible fingerprints (patterns) were obtained with both primers for all collected sporocaps (Figure 1 and Figure 2). In PCR reaction with (GTG)<sub>5</sub> primer, 5 to 9 fragments were amplified and their sizes were from 825 to 2,500 bases, while amplification with (GACA)<sub>4</sub> primer gave 2 to 8 fragments from 500 to 2,500 bases. In previous study (Sawyer *et al.* 2003) sporocarps of some *Amanita* species produced 6 to 17 fragments of 200 to 1,900 bases with (GTG)<sub>5</sub> primer and 10 to 22 fragments of 250 to 2,600 bases with (GACA)<sub>4</sub> primer, while in *Cortinarius rotundisporus* 11 to 24 fragments of 300 to 2,700 bases and 16 to 26 fragments of 300 to 2,900 bases were amplified with the same

primers respectively (Sawyer *et al.* 1999). This data implies that number and size of fragments are specific for each species of fungi, but fragments are usually not bigger than 3,000 bases.

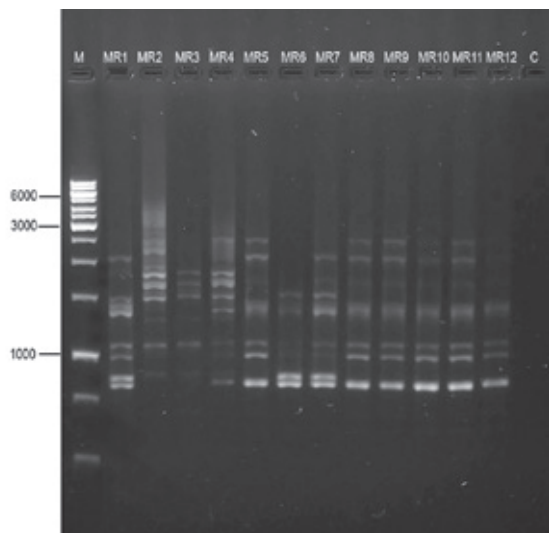


Figure 1. (GTG)<sub>5</sub> band patterns of *M. rotula* sporocarps

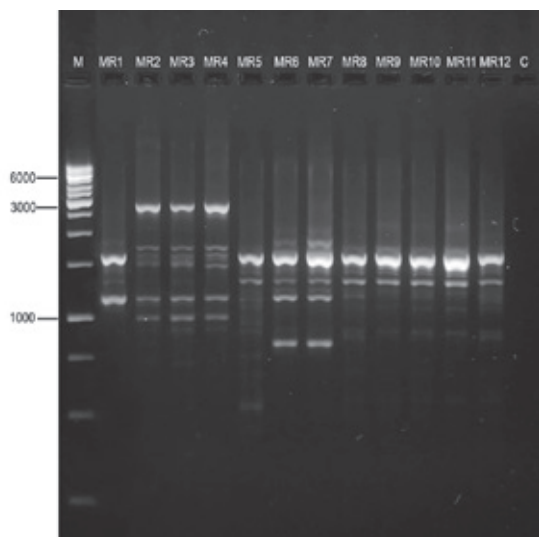


Figure 2. Band patterns of *M. rotula* sporocarp samples after amplification with (GACA)<sub>4</sub> primers

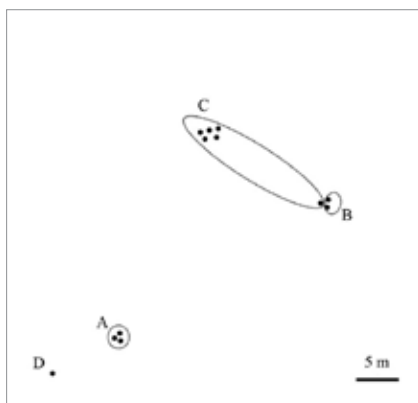
Both primers used for PCR amplification in this study produced ISSR patterns which enabled all analyzed sporocarps to be grouped in the identical

groups (Table 1). Twelve sporocarps were grouped into four genets: A (presented with three sporocarps), B (presented with two sporocarps), C (presented with six sporocarps) and D (presented with one sporocarp).

*Table 1.* Inter-simple sequence repeat (ISSR) groups determined by identical band patterns of the two primers for sporocarps of *M. rotula*.

(GTG) <sub>5</sub>	(GACA) <sub>4</sub>	genet
MA1	MA1	D
MR2	MR2	A
MR3	MR3	
MR4	MR4	
MR5	MR5	
MR8	MR8	C
MR9	MR9	
MR10	MR10	
MR11	MR11	
MR12	MR12	
MR6	MR6	B
MR7	MR7	

Since all sporocarps were mapped on investigated locality, it was possible to determine approximate genet diameters (Figure 3). Genet A was represented by 3 sporocarps found in relatively close proximity with longest distance between them of 2 m, thus diameter of this genet is at least 2 m. Genet B was presented by 2 sporocarps found 2 m from each other thus diameter of this genet was 2 m at least. Genet C had diameter of 15 m and was represented by 6 sporocarps. 1 of these 6 sporocarps was found relatively close to sporocarps of genet B and it would be expected that they also belong to genet B. Instead, it was clustered into same individual with 5 sporocarps found approximately 15 m away. Diameter of genet D was not possible to determine since it was represented only by one sporocarp.



*Figure 3.* Schematic map of the positions of the sampled *M. rotula* sporocarps from locality NP Mt. Stara planina. Encircled dots represent sporocarps which belong to the same genotypes.

Given that there are no other available studies on the spatial distribution of *M. rotula* genet, comparison of results obtained in this study was only possible with the data obtained for other species of fungi, mostly ectomycorrhizal. It was determined that individuals of *Pisolithustinctorius* can be from 7 to 30 m in diameter (Anderson *et al.* 1998), *Suillusvariegatus* can have genet up to 20 m (Dahlberg, 1997), while genet of *Cortinarius rotundi sporus* are usually from 9 m to 30 m (Sawyer *et al.* 1999). From given data it can be concluded that individuals of ectomycorrhizal species occupy a larger areas than saprotrophic fungi, probably because of their association with roots of higher plants which can give better support for somatic growth than substrate of saprotrophic species.

Relatively small diameters of *M. rotula* genet may also be explained by their mode of reproduction. Fungi that form small genet are thought to frequently reproduce sexually through basidiospores, while fungi with larger individuals are able to expand somatically over time (Burchhardt *et al.* 2011). Since this principle was determined for ectomycorrhizal fungi, we cannot be sure that the same can be implied for saprophytic fungal species.

Disturbance of habitat may also have effect on fungal genet size. Dahlberg and Stenlid (1995) determined that populations of *S. bovinus* in undisturbed mature forests are dominated by large persistent genet, while in severely disturbed stands smaller genet prevail. Later studies of ectomycorrhizal fungi (Fiore-Donno and Martin, 2001; Redecker *et al.* 2001; Sawyer *et al.* 2003) showed that some taxa prevail as populations of small genet in mature stands that have not been subject to major disturbances, meaning that there was no strict correlation between stand age and level of disturbance and genet size. This may relate to ectomycorrhizal fungi since they are in association with tree roots and not so dependent of nutrients from forest litter like saprotrophic fungi are. It can be assumed that genet of saprotrophic fungi are more affected by habitat disturbances, but further investigation of distribution and genet size of saprotrophic fungal species is needed to support this claim.

## CONCLUSION

The data presented here indicate that analyzed population of *M. rotula* from one locality on Mt. Stara planina consists of relatively small genet which are usually presented with several sporocarps. Since the given results are the first data about size and spatial distribution of genet of *M. rotula*, more information of other *M. rotula* populations is needed to determine whether the obtained genet sizes are general trait of an analyzed species or a special trait appeared as an effect of environmental conditions.

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ПРОСТОРНА ДИСТРИБУЦИЈА ГЕНЕТА У ПОПУЛАЦИЈИ  
САПРОТРОФСKE ГЉИВЕ *Marasmius rotula*  
НА СТАРОЈ ПЛАНИНИ

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**РЕЗИМЕ:** Истраживање представљено у овом раду вршено је ради утврђивања просторне дистрибуције јединки врсте гљиве *Marasmius rotula* на једном локалитету на Старој планини (Република Србија). Са истраживаног локалитета (Видлич, Стара планина) сакупљено је дванаест плодноносних тела која су на терену била груписана у четири групе, а релативна удаљеност између група била је 10 до 30 м. Из сакупљених плодноносних тела изолована је ДНК која је анализирана употребом ISSR (eng. inter-simple sequence repeat) методе. За ову методу коришћени су (GTG)<sub>5</sub> и (GACA)<sub>4</sub> прајмери, који су се након раздвајања на агарозном гелу показали као репродуцибилни. За плодносна тела која су показала идентичне ISSR фрагменте на гелу сматрано је да припадају истој јединки (генету) и на основу тога су груписана. Резултати анализе ISSR фрагмената добијени са оба прајмера показали су да се 12 анализираних плодноносних тела груписало у 4 генета (А, В, С, D). Утврђено је да је пречник генета А и В био приближно 2 м док је генет С имао пречник око 15 м. Дијаметар генета D није било могуће утврдити с обзиром да је био представљен само са једним плодноносним телом. Резултати добијени у овом раду представљају прве податке о величини и просторној дистрибуцији генета код сапротрофне врсте гљиве *M. rotula*. Да би утврдили да ли добијени подаци о генетима представљају особину испитиване врсте или су превасходно резултат фактора средине, потребно је још података о дистрибуцији генета у различитим популацијама врсте *M. rotula*.

**КЉУЧНЕ РЕЧИ:** дистрибуција генета, ISSR, *Marasmius rotula*, популација

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## *Ganoderma lucidum* – FROM TRADITION TO MODERN MEDICINE

**ABSTRACT:** *Ganoderma lucidum* has a long tradition of use in folk medicine of the Far East, which is documented in the oldest Chinese pharmacopoeia, written in the first century B.C, declaring it a superior medicine. The healing properties of *G. lucidum* reflected on folk names such as: Reishi, Mannentake, Ling Zhi etc., which mean “herb of spiritual power”, “mushroom of immortality” or “10,000-year mushroom”, respectively. It has been known, for thousands of years, that this species extends life span, increases youthful vigour and vitality and it was used in the treatments of hepatitis, kidneys’ disease, hypertension, arthritis, asthma, bronchitis, arteriosclerosis, ulcers and various types of cancer. However, Western civilisation did not discover its healing properties until the 20<sup>th</sup> century. Modern scientific researches and numerous clinical trials, conducted in recent decades, have confirmed the ancient knowledge of Eastern nations and given them a scientific basis. These studies have demonstrated many biological activities of *G. lucidum* extracts and compounds, including: immunomodulating, antioxidative, cytotoxic, hypoglycaemic, anti-inflammatory, antiallergic, antimicrobial, etc. It has been reported that its extracts play important role in detoxification of the body and protection of the liver, as well as in reducing cardiovascular problems, stress and anxiety. However, its most important effect is undoubtedly immunostimulating one as it is the basis of many other positive effects. The Japanese government introduced *G. lucidum* on the official list of auxiliary agents for the treatments of various cancers, Alzheimer’s disease, diabetes and chronic bronchitis. Many chemical components have been isolated from *G. lucidum*, but polysaccharides and terpenoids are the main carriers of its bioactivities.

**KEYWORDS:** *Ganoderma lucidum*, traditional usage, modern studies, bioactivities

## *Ganoderma lucidum* IN TRADITIONAL MEDICINE

*Ganoderma lucidum* (Curt.: Fr.) P. Karst. has been the important part of the traditional medicine in the Far East, especially in China and Japan, for several thousands of years. The people of these countries appreciated *G. lucidum*, not only because of its medicinal but also for its spiritual power. It was considered as a symbol of good luck, prosperity, good health, longevity and immortality.

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*G. lucidum* was also believed to have the power to keep from evil and was treasured in homes as a talisman (Wasser and Weis, 1997). In the ancient China, it was known as “God’s herb”, because it was believed to prolong life, enhance the youthful spirit and maintain vitality. Its fruiting bodies were rarely found in nature, so anyone who brought it to the imperial court was richly awarded (Wasser and Weis, 1997). *G. lucidum* was a part of the daily diet of monks and Taoist magi throughout Asia because of the belief that it calmed the mind, prevented tension, strengthened the nervous system and heart, improved memory and concentration, strengthened willpower, and therefore helped the achievement of wisdom. The importance of *G. lucidum* in the Far East tradition is best demonstrated by the fact that as the “King of Herbs” it was celebrated in many stories and legends and depicted in the art works, the paintings, embroidered fabrics and carvings, along with the Gods and immortals. It was a common theme on dishes, furniture, and even ladies’ hairpins, perfume bottles, always as a symbol of longevity and good fortune (Osuji *et al.* 2016). The significance of *G. lucidum* in traditional oriental medicine is testified by the oldest Chinese medical record, “Shen Nung Pen Tsa’o Ching”, written more than 2,000 years ago. This document presents the first world’s pharmacopoeia which was based on studies of medicinal plants and fungi, conducted by the founder of traditional Chinese medicine, Shen Nung, about 5,000 years ago. Namely, 365 plants and fungal species were described and classified based to their medicinal properties into the superior ones, which included about 120, the above-average ones (the same 120) and the average ones with about 125 species. The group of superior medications, otherwise called “God’s weeds”, included those used for maintaining the vitality, preserving mental health and increasing longevity. The members of the second group, above-average one, were taken as tonics, while those from the average category were used to treat certain diseases. That pharmacopoeia also emphasized that patients should be careful with the amount of average and above-average medications which are taken and that they should not be used continuously. On the contrary, the superior class of drugs can be taken in unlimited quantities, without any negative side effects. The top place on the list of superior remedies is reserved for *G. lucidum* (Wasser and Weis, 1997).

According to the traditional beliefs, *G. lucidum* can possess one of 5 flavors: bitter, salty, sour, sweet and hot, and each of them is aligned with one of the internal organs (heart, kidney, liver, lung and spleen) (Denisova, 2001). Likewise, in Japan, it is thought that depending on the colour and taste of *G. lucidum* fruiting bodies, they heal different organs. Thus, gray and the acidic ones improve vision; red and bitter regulate the functioning of the internal organs and improve memory; yellow and sweet ones affect the spleen and “soothing soul”; red and hot act on the lung and increase the courage and boost will; black and the salty ones protect kidneys; and sweet ones improve hearing, act on joints and muscles and improve the complexion. One Chinese doctor has also written: “The superior treatment consists of treating the disease before it occurs, the average treatment means treatment when the disease is detected and inferior treatment cures the disease that has already manifested itself”. The highest value of *G. lucidum* is in the fact that it can be successfully used

in all three stages of treatment. This species has been used for several thousands of years in the treatment of hepatitis, kidney disease, hypertension, arthritis, asthma, bronchitis, arteriosclerosis, ulcers and various types of cancer (Berović *et al.* 2003).

### *Ganoderma lucidum* IN THE MODERN MEDICINE

Although the Far East traditional medicine has highly valued *G. lucidum* for several millennia, its healing properties were unknown to Western civilisation until the 20<sup>th</sup> century. The current researches and numerous clinical trials conducted in recent decades have confirmed the ancient knowledge of the Eastern nation and given them a scientific basis. These studies have demonstrated many biological activities of *G. lucidum*, including immunomodulating, cytotoxic, antioxidative, antimicrobial, anti-inflammatory, hypoglycemic antiallergic, neuroprotective, etc. (Chang *et al.* 2006; Zhou *et al.* 2010; Bishop *et al.* 2015). However, its most important effect is undoubtedly immunomodulating, which is the basis of many other activities, and therefore Japanese government has put this species on the official list of auxiliary agents for the treatment of various cancers, Alzheimer's disease, diabetes etc. According to Mizuno *et al.* (1995a, b, c), *G. lucidum* also helps in treatments of numerous disorders, such as neurasthenia, dyspnea, insomnia, chronic hepatitis, pyelonephritis, high blood cholesterol level, hypertension, coronary heart disease, leukopenia, rhinitis, chronic bronchitis, bronchial asthma, gastropathy, and duodenum ulcer. A number of reports indicate that this fungus increases the resistance of laboratory mice to the exposure to radiation and therefore its extracts are used as a component of suntan lotion to protect against UV radiation (Wasser and Weis, 1997). It was also demonstrated that it increases the resistance of the animals to the effects of muscarine and nicotine so it is also used as an antidote in poisonings with different poisonous mushrooms (Wasser and Weis, 1997).

The results of the clinical trial including 2,000 patients suffering from chronic bronchitis who had been treated with *G. lucidum* tablets for 2 weeks, showed an improvement of the clinical picture in about 60%–90% of patients (Chang and But, 1986). Likewise, it was confirmed that the extract of this species decreased the blood and plasma viscosities in patients with hypertension and hyperlipidemia who were recovering from cerebral thrombosis (Wasser and Weis, 1997). The usage of *G. lucidum* in the treatment of hepatitis, in particular in the case of severe liver damage, has proved rather effective. Namely, in 355 patients who suffered from hepatitis B and used a preparation Wulingdan Pill with a high content of *G. lucidum* fruiting bodies, the improvement was noted in 92% of patients (Chang and But, 1986).

Since *G. lucidum* fruiting bodies are rare and the demand for them is great, nowadays this species is successfully commercially cultivated and it is available in various forms on the world market. Currently, *G. lucidum* is a popular dietary supplement, with annual global market value amounting to \$1.5 billion (Liu *et al.* 2010).

## THE BIOACTIVITY OF CRUDE *Ganoderma lucidum* EXTRACTS

A wide spectrum of bioactivities (immunomodulatory, antioxidative, cytotoxic, antimicrobial, genoprotective etc.) was demonstrated for *G. lucidum* extracts, their efficiency depending on extractant (water, ethanol, methanol, chloroform, ethyl acetate etc.), the fungal part (basidiocarp, mycelium, spores, cultivation broth) and extraction technique. It has been shown that alcohol extracts (ethanol, methanol) contain compounds which reduce the blood cholesterol and glucose levels, regulate blood pressure and inhibit the release of histamine from the cells, and have cytotoxic, antiviral and hepatoprotective effects (Lu *et al.* 2004).

Kuo *et al.* (2006) noted stimulatory effect of *G. lucidum* mycelial extract on TNF- $\alpha$ , IFN- $\gamma$  and IL-6 production, which led to improvement of innate immune response. Numerous studies have also confirmed a considerable antioxidative potential of various extracts of *G. lucidum* basidiocarps, mycelium and cultivation broth (Mau *et al.* 2002; Sun *et al.* 2004; Tseng *et al.* 2008; Čilerdžić *et al.* 2014; 2016a,b). Namely, overproduction of reactive oxygen species, caused by numerous environmental factors and lifestyle, exceed the defence capacity of an organism and leads to the oxidative stress that could be the trigger of many diseases and disorders. Despite the existence of numerous commercial, synthetic antioxidants, finding new natural antioxidants without negative side effects is very important. Mau *et al.* (2002) and Saltarelli *et al.* (2009) have reported that the extracts may significantly enhance the non-enzymatic and enzymatic antioxidative response and remarkably reduce the level of lipid peroxidation. The extract property to inhibit lipid peroxidation was also base for improvement of heart function (Wong *et al.* 2004).

*G. lucidum* basidiocarp ethanol extract, cultivated under laboratory conditions on different substrates showed a great genoprotective as well as cytotoxic activity against human cervix adenocarcinoma HeLa and human lung adenocarcinoma epithelial A549 cell lines (Čilerdžić *et al.* 2014; 2016c). Likewise, Müller *et al.* (2006) noted significant apoptosis effect of methanol basidiocarp extract on leukemia, lymphoma and multiple myeloma cells, and Harhaji Trajković *et al.* (2009) antiproliferate effect on melanoma, fibrosarcoma, and astrocytoma cell lines.

Uncontrolled usage of commercial antibiotics and antimycotics in the treatment of infectious diseases leads to the appearance of numerous resistant strains of microorganisms. On the other hand, these antimicrobial medicaments also induce various side effects, and finding of new antimicrobial agents of biological origin is the trend of current science. Various *G. lucidum* extracts showed an outstanding antimicrobial potential against Gram – and Gram + bacteria as well as numerous micromycetes, which depends on extractant and extraction method that affect extract composition (Sridhar *et al.* 2011). Several studies demonstrated higher antimicrobial potential of compounds insoluble in water so their content is higher in extracts obtained by non-polar organic solvents, such as chloroform. Thus, Keypour *et al.* (2008) noted that chloroform basidiocarp extract strongly inhibited development of *Bacillus subtilis* and

*Staphylococcus aureus*, while methanol extract had inhibitory effect on *Escherichia coli*, *Salmonella* spp. and *B. subtilis*. The ethanol extract of basidiocarps obtained on alternative substrate showed a considerable antibacterial and antifungal capacity against *S. aureus* and *Micrococcus flavus*, *Acremonium strictum*, *Aspergillus glaucus* and *Trichoderma viride* (Čilerdžić *et al.* 2014). Klaus and Nikšić (2007) reported that bacteriolytic enzymes i.e. lysozymes and acid protease are carriers of antimicrobial activity in aqueous extract.

Peculiarly, it should be emphasized that the latest findings indicate the positive effect of *G. lucidum* extracts on inhibition of acetylcholinesterase and tyrosinase, enzymes which high activities cause appearance of neurodegenerative disorders, especially the most frequent Alzheimer's and Parkinson's diseases (Hasnat *et al.* 2013; Taofiq *et al.* 2016).

## THE BIOLOGICALLY ACTIVE COMPOUNDS OF *Ganoderma lucidum*

A wide range of different chemical compounds with medicinal properties have been isolated from the fruiting bodies, mycelium and spores of *G. lucidum*, but the most important ones are polysaccharides, triterpenoids, phenols and proteins (Berović *et al.* 2003; Paterson, 2006; Leskošek-Čukalović *et al.* 2010).

### Polysaccharides

Polysaccharides are the main biologically active macromolecules and the most responsible for the therapeutic application of *G. lucidum* acting as immunomodulators and carcinostatics (Berović *et al.* 2003). They are present in fruiting bodies, mycelia, spores and cultivation broth of *G. lucidum*. More than 200 different polysaccharides were isolated from *G. lucidum*, 50 of them showing antitumor activity and some hypoglycemic effect (Wasser and Weis, 1997; Fang and Zhong, 2002). Most of the biologically active polysaccharides are 1,3- $\beta$ -D- and 1,6-  $\beta$ -D-glucans consisting of a large number of D-glucose molecules linked by glycosidic bonds and known as homopolysaccharides (Yang *et al.* 2000; Paterson, 2006). They are branched and side chains occur after a certain number of glucose units in the main chain. Numerous studies have shown a positive correlation between the degree of polysaccharide branching and its immunomodulating effect as well as the length of the side chain and bioactivity degree (Zhang *et al.* 2001). Likewise, the majority of anticancer glucans are insoluble in water and have an average molecular weight of 1050 kDa (Paterson, 2006). However, Hsieh and Yang (2004) demonstrated a strong anti-tumor activity of water soluble *G. lucidum* polysaccharides against Sarcoma 180, which inhibited proliferation of even 95% cells.

The most of the researches conducted with *G. lucidum* polysaccharides are dedicated to their immunomodulating activities (Berović *et al.* 2003). Thus, Wasser and Weis (1997) demonstrated that 1,3- $\beta$ -D-glucan is the main carcino-

static agent, which is not toxic for the organism contrary to the conventional chemotherapy and with effect based on the strengthening of the host immune system. It was shown that the inhibition of tumor growth is carried out through the enhancement of the host immune response by stimulating the production of the cytokinin, interleukin (IL), tumor necrosis factor (TNF) and interferon (IFN). Namely, incubation of human macrophages and T-lymphocytes with polysaccharides isolated from fresh *G. lucidum* fruiting bodies caused increasing production of IL-1, IL-6, TNF- $\alpha$  and IFN- $\gamma$  (Wasser and Weis, 1997). However, several researches showed that the antitumor activity of pure polysaccharides extracts is lower than crude extracts which support the fact that other substances also contribute to the bioactivity of *G. lucidum* (Liu *et al.* 2002).

### Terpenoids

Terpenoids, besides polysaccharides, are the most important bioactive metabolites and ones of the main carriers of *G. lucidum* medicinal properties. Until now, 130 *G. lucidum* terpenoids have been isolated and characterized from the basidiocarps, mycelium and spores (Paterson, 2006). According to the number of carbon atoms, structure and functional groups, terpenoids were classified into the non-volatile triterpenoids (C30) and a less volatile diterpenes (C20) (Leskošek-Čukalović, 2010). Anticancer activity of *G. lucidum* triterpenoids is based on direct cytotoxicity against tumor cells, contrary to the polysaccharides with activity based on the strengthening of the organism immune response (Paterson, 2006). Thus, Ganoderic acid T, purified from the methanol extract of *G. lucidum* mycelium, showed cytotoxicity on human lung cancer cell line (95-D), in a dose-dependent manner, *via* apoptosis induction and cell cycle arrest (Tang *et al.* 2006). Some triterpenoids have inhibition effect on the growth of human hepatoma Huh-7 cells causing cell cycle arrest in G2 phase, without any effect on the normal liver cells (Lin *et al.* 2003).

*G. lucidum* triterpenoids also have significant antiviral activity, i.e. they play an important role in the inhibition of HIV-1 and HIV-2 protease, as well as HIV-1 reverse transcriptase. It was demonstrated that Ganosporeic acid, isolated from *G. lucidum* spores, is active hepatoprotective agent (Paterson, 2006).

### Proteins

The bioactive proteins have also been isolated and characterized from *G. lucidum*. The immunomodulatory protein known as Ling Zhi-8 (LZ-8) constructed of 110 amino acid residues, with the molecular mass of 12 kDa has been isolated from mycelium (Tanaka *et al.* 1989). The recent studies showed that peptides present one of the main carriers of antioxidant activity and a polysaccharide-peptide complexes (Gl-PP) have antitumor and antiangiogenesis activities (Sun *et al.* 2004; Cao and Lin, 2006). *G. lucidum* protein, ganodermin,

has antifungal activity against various micromycetes, especially against some phytopathogens, such as *Botrytis cinerea*, *Fusarium oxysporum* and *Physalospora piricola*, while glycoproteins have a direct antiviral effect on a herpes simplex virus types 1 and 2 (Liu *et al.* 2002; Paterson, 2006).

### Phenolic compounds

Phenolic compounds are also important biologically active compounds of *G. lucidum*. The dominant phenolic compounds isolated from *G. lucidum* are gallic and protocatechuic acids (Stajić *et al.* 2015). Numerous studies present that the healing properties of some medicinal mushrooms are in direct correlation with their chemical composition, particularly with the content of the phenolic compounds (Yaltirak *et al.* 2009). Polyphenols are the main antioxidative agents because they act either as free radicals' scavengers or carriers of metal ions (Leopoldini *et al.* 2011). Čilerdžić *et al.* (2014; 2016b) noted high correlation between amount of these compounds and antioxidative capacity of various *G. lucidum* extracts.

### FUTURE PERSPECTIVES OF *Ganoderma lucidum* IN MEDICINE

Despite a long history of usage in traditional medicine and current studies that proved medicinal potential of *G. lucidum*, a number of challenges are still in front of scientists with the aim of its usage in a clinical practice. Firstly, the clarifying of the taxonomy is necessary, since other *Ganoderma* species can be mistaken with *G. lucidum*. Further studies on the identification of active ingredients as well as safe doses ranges should be declared for each disease. The important research goal should also be the optimisation of *G. lucidum* cultivation conditions in order to increase yield and to maximise active constituents' production. Finally, the most important and the most challenging tasks are extensive preclinical and clinical trials which will provide a convincing evidence of the effectiveness of *G. lucidum* based medications. In conclusion, after all findings, we can expect that the slogan "*G. lucidum* a day keeps the doctor away" will be used.

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### *Ganoderma lucidum* – ОД ТРАДИЦИЈЕ ДО МОДЕРНЕ МЕДИЦИНЕ

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**РЕЗИМЕ:** *Ganoderma lucidum* има дугу традицију употребе у народној медицини Далеког истока, што је документовано у најстаријој кинеској фармакопеји, написаној у I веку пре нове ере, у којој је проглашена за супериорни лек. Лековита својства *G. lucidum* су утицала на бројна народна имена као што су: Reishi, Mannentake, Ling Zhi са значењем „биљка духовне моћи“, „гљива бесмртности“ или „10000-годишња гљива“. За *G. lucidum* се хиљадама година веровало да продужује животни век, повећава младалачку снагу и виталност и користила се у лечењу хепатитиса, болести бубрега, хипертензије, артритиса, астме, бронхитиса, артериосклерозе, чирева и разних врста канцера. Међутим, западна цивилизација је открила лековита својства *G. lucidum* тек у 20. веку. Савремена научна истраживања и бројне клиничке студије спроведене у последњих неколико деценија потврдила су древна знања далекоисточних народа и дала им научну потпору. Резултати ових проучавања су показали многе биолошке активности екстракта и једињења *G. lucidum*, као што су имуномодулаторну, антиоксидативну, цитотоксичну, хипогликемијску, антиинфламаторну, антиалергијску, антимикробну и др. Доказано је да екстракти *G. lucidum* имају важну улогу у детоксикацији организма и заштити јетре, као и у смањењу кардиоваскуларних проблема, стреса и

анксиозности. Међутим, њен најважнији ефекат је несумњиво имуностимулаторни који је основа многих других позитивних ефеката. Јапанска влада је увела *G. lucidum* на званичну листу помоћних средстава за лечење различитих врста канцера, Алцхајмерове болести, дијабетеса и хроничног бронхитиса. Многа је-дињења су изолована из плодноносних тела, мицелије, спора и култивационог медијума *G. lucidum* али се полисахариди и терпеноиди сматрају главним носиоцима биолошких активности.

КЉУЧНЕ РЕЧИ: *Ganoderma lucidum*, традиционална примена, савремена истраживања, биоактивности



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## ANTIOXIDATIVE ACTIVITY OF *Lenzites warnieri* BASIDIOCARPS

**ABSTRACT:** Considering that mushrooms synthesize different kinds of compounds with antioxidative activity and that search for natural antioxidants is a topical study area, testing of unstudied species is fully justified. The aim of the study was to evaluate antioxidative capacity of *Lenzites warnieri* basidiocarps using different solvents. Antioxidative potential of 96% ethanolic, 70% ethanolic and methanolic extracts was evaluated by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) bleaching assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. Additionally, total content of phenols and flavonoids in extracts was determined as galic acid equivalent (GAE) and quercetin equivalent (QE), respectively. Presented as EC<sub>50</sub>, 70% ethanolic extracts showed the highest antioxidative capacity by DPPH assay ( $3.08 \pm 0.49$  mg/mL) and 96% ethanolic extract by ABTS assay ( $3.08 \pm 0.24$  mg/mL). Methanolic extract exhibited the lowest antioxidative activity in both assays ( $6.02 \pm 0.99$  mg/mL and  $4.92 \pm 0.38$  mg/mL, respectively). Results showed that antioxidative capacity of extracts depended on solvents and assay used, indicating that ethanolic extracts were with higher capacity in free radicals neutralization. The highest content of total phenols was detected in 70% ethanolic extract ( $37.45 \pm 0.36$  µg GAE/mg of dried extract) while the lowest amount was noted in methanolic extract ( $22.73 \pm 0.05$  µg GAE/mg of dried extract). Total flavonoid contents were negligible and ranged between  $1.91 \pm 0.10$  and  $2.24 \pm 0.13$  µg QE/mg of dried extract. The obtained results indicate that *Lenzites warnieri* possess significant antioxidative capacity which is mainly correlated to phenols present in the extracts.

**KEYWORDS:** *Lenzites warnieri*, antioxidative activity, basidiocarps, extracts

## INTRODUCTION

Mushrooms have been used for thousands of years as food but also in traditional medicine as a reach source of biologically active compounds (Wasser, 2010; Roupas *et al.* 2012). However, this great pharmacological potential is still underutilized since, approximately, only 5% of the species are well studied and comprehensive studies are needed for both unstudied and already examined species (Wasser, 2002).

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Species of the genus *Lenzites* has been used in traditional Chinese medicine for treatment of several diseases and disorders such as haunch and femoral pain, acropathy, apoplexy and cold (Ren *et al.* 2006). Medicinal effects have been also demonstrated in conventional medicine and until nowadays, there have been several studies on the various properties of *Lenzites* spp. basidiocarps and mycelium extracts as scavengers of free radicals, antimicrobials, anti-oxidant, anti-viral and immunosuppressant (Liu *et al.* 2014; Milovanović *et al.* 2015; Hussin *et al.* 2016). The contemporary life style brings human and animal populations to face with abundant presence of free radicals, endogenously or in the environment, which further leads to appearance of oxidative stress which is a basis of aging and the initiation of various diseases and disorders (Limón-Pacheco and Gonsebatt, 2009). The capacity of organisms to defend against free radicals can be improved by various synthetic or natural antioxidants. From this aspect, macromycetes can be of a great interest due to the synthesis of different antioxidative compounds such as phenols, tocopherols, carotenoids, ascorbic acid, etc. (Stajić *et al.* 2013).

*Lenzites warnieri* Mont. & Durieu (Polyporaceae) is a white rot plant pathogen which can be found on living wood of several deciduous trees such as alder, cottonwood, elm and willow, preferring wet habitats such as riparian woodland or fens and warm temperatures (Ryvarden and Gilbertson, 1993). Species primarily inhabit Central and Southern Europe but also parts of Asia and Northern Africa (TortiĆ, 1972; Winterhoff, 1986). Formation of resupinate fruiting bodies occurs in autumn while sporulation is expected in following spring (Ryvarden and Gilbertson, 1993).

Since described activities might have been responsible for medicinal effects of *Lenzites* spp. in traditional practice, the goal of the study was to evaluate possible antioxidative potential of basidiocarp extracts of unstudied *Lenzites warnieri*.

## MATERIALS AND METHODS

### *Organism and extraction*

The basidiocarps were collected in Gornje Podunavlje Special Nature Reserve, Serbia, and identified as *Lenzites warnieri* according to the macroscopic features and the micro morphology of the reproductive structures (Ryvarden and Gilbertson, 1993; Vukojević and Hadžić, 2013).

Fruiting bodies (3.0 g) were air-dried, grinded to powder in laboratory blender (Waring Commercial 8010S, USA). Extraction was carried out with 96% ethanol, 70% ethanol and methanol (90.0 mL) on a magnetic stirrer (150 rpm, 30 °C) for 72 hours. The resultant extracts were then centrifuged (20 °C, 3,000 rpm, 10 min), and supernatants filtrated through Whatmann No. 4 filter paper. The filtrates were concentrated under reduced pressure in a rotary evaporator (Büchi R-114, Germany) at 40 °C to dryness and redissolved in 96% ethanol

to an initial concentration of 16.0 mg/mL. The extraction yield was determined as the ratio of dry fungal biomass before extraction and dry extract weight after the evaporation process.

### *Determination of antioxidative activity*

Antioxidative activities of basidiocarp extracts were determined spectrophotometrically (CECIL CE2501) by DPPH and ABTS tests.

#### **DPPH assay**

The free radical scavenging activity of extracts was based on the reduction of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH<sup>•</sup>) (Blois, 1958). The reaction mixtures composed of 1,800.0  $\mu$ L of 4% methanol solution of DPPH<sup>•</sup> and 200  $\mu$ L of extract at defined concentration (series of double dilutions with ethanol from 16.0 mg/mL to 0.125 mg/mL) were mixed and shaken vigorously. After 30 min of incubation in the dark, absorbance was measured at 517 nm against methanol as a blank. The negative control contained all the reaction reagents except the extract. Scavenging effect was calculated using the equation:

$$\text{DPPH scavenging effect (\%)} = ((A_c - A_s)/A_c) \times 100$$

where  $A_c$  – absorbance of the negative control;  $A_s$  – absorbance of the reaction mixture (with samples at different concentrations).  $EC_{50}$  values (mg extract/mL) represent the concentration of test samples or L-ascorbic acid (standard antioxidant) providing 50% inhibition of DPPH radicals were calculated by interpolation of DPPH<sup>•</sup> absorbance curve at 517 nm from linear regression analysis.

#### **ABTS assay**

This test was based on measuring the level of ABTS<sup>•+</sup> stock solution color change in presence of antioxidants according to the procedure of Miller *et al.* (1993). The initial solution of ABTS cation radicals was prepared by dissolution of 9.0  $\mu$ g ABTS in 2.5 mL dH<sub>2</sub>O and addition of 44.0  $\mu$ L 140 mM potassium persulphate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) solution, 12 to 16 hours before use, while the stock solution was prepared immediately prior to measurement by dilution of the initial solution with dH<sub>2</sub>O and adjustment of solution absorbance on  $0.700 \pm 0.020$  at 734 nm. The reaction mixture (1,500.0  $\mu$ L of the ABTS stock solution and 15.0  $\mu$ L of extract of concentration of 1.0 mg/mL) was incubated at room temperature for 4 min and absorbance change was measured at 734 nm. Distilled water was used as a blank. Extract concentration required for ABTS cation radicals reduction, equivalent to reduction of 1.0 mg/mL ascorbic acid (AAEC) was determined using equation of calibration curve for ascorbic acid.  $EC_{50}$  value (mg extract/mL) presents effective extract concentration that scavenges 50% of ABTS cation radicals and it is obtained by linear regression analysis.

### *Determination of phenol and flavonoid contents*

The total contents of soluble phenolic compounds in the extracts of fruiting bodies were estimated using Folin-Ciocalteu reagent and gallic acid as standards according to the method described by Singleton and Rossi (1965). The reaction mixture (1,000.0  $\mu\text{L}$  of 10% Folin-Ciocalteu reagent and 200.0  $\mu\text{L}$  of sample in concentration of 1.0 mg/mL) was incubated for 6 min in dark and thereafter 800.0  $\mu\text{L}$  of 7.5%  $\text{Na}_2\text{CO}_3$  was added. The mixture was then vortexed and incubated on a rotary shaker (100 rpm) in dark at room temperature for 2 hours. The absorbance was measured spectrophotometrically at 740 nm against a blank (mixture without extract). The total concentration of phenols was presented as  $\mu\text{g}$  of gallic acid equivalent (GAE) per mg of dried extract using equation of calibration curve for GAE.

Total flavonoid contents in extracts were determined using quercetin as standard according to the method of Park *et al.* (1997). The reaction mixture (1,000.0  $\mu\text{L}$  of extract in concentration of 1.0 mg/mL, 4,100.0  $\mu\text{L}$  of 80% ethanol, 100.0  $\mu\text{L}$  of 10%  $\text{Al}(\text{NO}_3)_3 \times 9\text{H}_2\text{O}$  and 100.0  $\mu\text{L}$  of 1.0 M water solution of potassium acetate) was incubated in dark on a rotary shaker (100 rpm) and room temperature for 40 min and thereafter absorbance was measured at 415 nm against blank (mixture which contained ethanol instead extract). Total flavonoid content was expressed as  $\mu\text{g}$  of quercetin equivalent (QE) per mg of dried extract using equation of calibration curve for QE.

### *Statistical analysis*

All measurements were carried out in triplicate and the results are expressed as mean  $\pm$  standard error. One-way analysis of variance (ANOVA) and Tukey's HSD post-hoc test were performed to test any significant differences among means. Statistical significance was declared at  $P < 0.01$ . All statistical analyses were done using software STATISTICA, version 6.0 (StatSoft, Inc., Tulsa, USA).

## RESULTS AND DISCUSSION

Extraction yields of *Lenzites warnieri* basidiocarps differed statistically depending on solvent used for the extraction ( $P < 0.01$ ). The highest extraction yield was noticed when 96% ethanol was used as solvent ( $4.53 \pm 0.05\%$ ) while the lowest yield was obtained in methanol ( $3.57 \pm 0.06\%$ ) (Table 1).

Table 1. Extractions Yields (%) of *Lenzites warnieri* basidiocarps

Solvent	Extraction Yield (%)
96% Ethanol	$4.53 \pm 0.05$
70% Ethanol	$4.23 \pm 0.04$
Methanol	$3.57 \pm 0.06$

Previous studies showed that biomass extractability strongly depends on species, strain and solvent used for the extraction (Knežević *et al.* 2015). Thus, it can be said that the obtained extraction yields using alcohols as solvents are expected for *Lenzites* spp. basidiocarps or fungi belonging to family Polyporaceae, which usually ranges from 2.0% to 10.0% (Liu *et al.* 2014; Knežević *et al.* 2015; Ogidi *et al.* 2015). Compared to results of Oyetayo (2009) who obtained extraction yield of 2.3% for *Lenzites* sp. basidiocarps using ethanol as solvent, material used in our study exhibited double higher extractability. However, Liu *et al.* (2013) showed almost the same extractability of *L. betulina* basidiocarps in 96% ethanol as it was demonstrated for *L. warnieri* in our study which was approximately 4.5%. Additionally, for fruiting bodies of *L. warnieri* ethanol in both applied concentrations was better solvent than methanol. The most likely explanation for this is the polarity of major compounds extractable in alcohols, i.e. less polar compounds were present in higher quantities and dissolved in ethanol with higher yields (Anwar and Przybylski, 2012).

Antioxidative activity of *L. warnieri* extracts was determined using two parallel assays for the evaluation of free-radical scavenging activity, i.e., DPPH and ABTS tests (Table 2). Tested extracts showed significant antioxidative potentials which varied depending on solvent used for the extraction but also applied assay. Scavenging activities of extracts, presented by EC<sub>50</sub> values, ranged from 3.08 ± 0.49 mg/mL to 6.57 ± 0.27 mg/mL. The highest antioxidative capacities were noticed for both 96% ethanolic extract in ABTS assay (3.08 ± 0.24 mg/mL) and 70% ethanolic extract in DPPH assay (3.08 ± 0.49 mg/mL). The lowest activity was detected for 96% ethanolic extract in DPPH assay (6.57 ± 0.27 mg/mL). L-ascorbic acid was far better free-radical scavenger compared to extracts, i.e. EC<sub>50</sub> value was 0.035 ± 0.001 mg/mL (Table 2).

Table 2. Antioxidative activities of *Lenzites warnieri* basidiocarp extracts and commercial antioxidant

Extracts and synthetic antioxidant	EC50 (mg/mL)	
	DPPH assay	ABTS assay
96% Ethanolic	6.57 ± 0.27	3.08 ± 0.24
70% Ethanolic	3.08 ± 0.49	4.47 ± 0.34
Methanolic	6.02 ± 0.99	4.92 ± 0.38
L-ascorbic acid	0.035 ± 0.001	0.247 ± 0.012

The tested extracts were good antioxidative agents, but compared to L-ascorbic acid, they showed lower scavenging activity. Results also indicated that neutralization of free radicals depended on two factors and solvent used for the extraction coupled with assay applied for the evaluation of antioxidative activity. Regarding this, our results showed that scavenging effect of 70% ethanolic extract on DPPH radicals significantly differed ( $P < 0.01$ ) from 96% ethanolic and methanolic extract which did not show any statistically significant difference in radicals neutralization. On the other side, there was no difference in ABTS cation radicals' neutralization between 70% ethanolic and methanolic

extracts. Previous studies have also showed the antioxidative potential of *Lenzites* species (Lee *et al.* 1996; Oyetayo 2009; Liu *et al.* 2013; Milovanović *et al.* 2015). Compared to results of Oyetayo (2009), who demonstrated DPPH radical scavenging of *L. betulina* ethanolic extract for approximately 40% at concentration of 1.0 mg/mL, our results for *L. warnieri* were quite similar ( $32.4 \pm 0.2$  % at 1.0 mg/mL of ethanolic extract – data not shown). Some detailed studies showed that separate fractions obtained from *L. betulina* basidiocarps exhibited much higher antioxidative capacity than crude extract (Liu *et al.* 2013). This study also showed that, compared to mycelial extracts of *L. betulina*, fruiting bodies of *L. warnieri* possess higher capacity in DPPH radical scavenging (Milovanović *et al.* 2015).

Total phenol contents in extracts were significant compared to total flavonoid contents which were very low (Table 3). The highest amount of phenols was obtained after extraction with 70% ethanol ( $37.45 \pm 0.36$  µg GAE/mg of dried extract) while the lowest amount was detected in methanol extract ( $22.73 \pm 0.05$  µg GAE/mg of dried extract). Total amounts of flavonoids were negligible and ranged between  $1.91 \pm 0.10$  and  $2.24 \pm 0.13$  µg QE/mg of dried extract (Table 3).

Table 3. Phenol and flavonoid contents in extracts of *Lenzites warnieri* basidiocarps

Extracts	Total phenol content (µg GAE/mg of dried extract)	Total flavonoid content (µg QE/mg of dried extract)
96% Ethanolic	$35.33 \pm 0.53$	$2.24 \pm 0.13$
70% Ethanolic	$37.45 \pm 0.36$	$2.11 \pm 0.06$
Methanolic	$22.73 \pm 0.05$	$1.91 \pm 0.10$

The degree of correlation between the DPPH and ABTS free radicals scavenging activity of the extracts and phenol contents was very low with  $R^2$  of 0.238 and 0.344, respectively. Accordingly, there was no correlation between DPPH radicals scavenging activity and flavonoids ( $R^2 = 0.0$ ). However, the level of ABTS cation radicals neutralization and flavonoids content was highly correlated ( $R^2 = 0.837$ ).

According to the large number of previous studies, phenols and tocopherols have the main roles in the antioxidative activity of mushrooms (Stajić *et al.* 2013). These compounds are abundant and important constituents of basidiocarps and our results showed significant total phenol content compared to flavonoids. The mechanism of antioxidative action of phenols is based on the presence of hydroxyl groups acting as reducing agents, metal chelators, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al.* 1996). Results for *L. warnieri* phenolic contents were comparable with those of Oyetayo *et al.* (2013) who detected similar content of phenols in *L. betulina* ethanolic extract (35.3 and 30.0 µg GAE/mg of extract, respectively). Even though the detection of total phenol content is the first step in characterization of extracts, search for specific compounds in basidiocarps is a promising approach in evaluation of antioxidative potential of macro fungi. Some compounds isolated from

*L. betulina* basidiocarps, such as betulinan A and B, can significantly inhibit lipid peroxidation (Smolibowska *et al.* 2016). Furthermore, according to Lee *et al.* (1996) betulinan A isolated from *L. betulina* basidiocarps was about four times more active as a radical scavenger than vitamin E in inhibition of lipid peroxidation. Despite low correlation between total phenol contents and radicals scavenging activity, which implies that some other compounds were responsible for neutralization of DPPH and ABTS free radicals, characterization of phenolic compounds which can be isolated from *L. warnieri* basidiocarps will be of a great importance. Additionally, even though total flavonoid content in extracts was characterized as negligible, they were significantly responsible for ABTS cation radicals' neutralization.

## CONCLUSION

Results showed for the first time that *L. warnieri* basidiocarps possess significant antioxidative capacity correlated to phenol content in the extracts. Further studies should focus on isolation, purification and determination or description of specific bioactive compounds with free-radical scavenging activity.

## ACKNOWLEDGEMENTS

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## АНТИОКСИДАТИВНА АКТИВНОСТ ПЛОДОНОСНИХ ТЕЛА *Lenzites warnieri*

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**РЕЗИМЕ:** С обзиром на чињеницу да гљиве синтетишу различите врсте је-дињења с различитим антиоксидативним потенцијалом и да је потрага за новим природним антиоксидансима тренд у данашњим истраживањима, може се рећи да је потпуно оправдано тестирање нових врста. Циљ истраживања био је да се испита антиоксидативни капацитет екстракта плодноносних тела *Lenzites warnieri* користећи различите екстрактанте. Антиоксидативни капацитет 96% етанолног, 70% етанолног и метанолног екстракта је одређиван АВТС (2,2'-азини-бис(3-етилбензотиазол-лин-6-сулфонска киселина)) и DPPH (2,2-дифенил-2-пикрил-хидразил) тестом. Такође је одређена укупна концентрација фенола и флавоноида у екстрактима као еквивалената галне киселине (GAE) односно кверцетина (QE). На основу EC<sub>50</sub> вредности, 70% етанолни екстракт је показао највиши антиоксидативни капацитет DPPH тестом ( $3.08 \pm 0.49$  mg/mL) а 96% етанолни екстракт АВТС тестом ( $3.08 \pm 0.24$  mg/mL). Метанолни екстракт имао је најслабију антиоксидативну активност коришћењем оба теста ( $6.02 \pm 0.99$  mg/mL односно  $4.92 \pm 0.38$  mg/mL). Резултати су показали да антиоксидативни капацитет екстракта зависи од екстрактанта и примењеног теста, па су тако етанолни екстракти имали већи капацитет у неутрализацији слободних радикала. Највећа концентрација укупних фенола је детектована у 70% етанолном екстракту ( $37.45 \pm 0.36$  µg GAE/mg сувог екстракта) док је најнижа забележена у метанолном екстракту ( $22.73 \pm 0.05$  µg GAE/mg сувог екстракта). Укупна количина флавоноида је била занемарљиво мала и кретала се у опсегу од  $1.91 \pm 0.10$  до  $2.24 \pm 0.13$  µg QE/mg сувог екстракта. Добијени резултати показују да *Lenzites warnieri* има значајан антиоксидативни капацитет који је највећим делом у корелацији са садржајем фенола у екстракту.

**КЉУЧНЕ РЕЧИ:** *Lenzites warnieri*, антиоксидативна активност, плодносна тела, екстракти



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## ANTIOXIDANT PROPERTIES OF SOYBEAN SEEDLINGS INOCULATED WITH *Trichoderma asperellum*

**ABSTRACT:** This study was conducted in order to assess the effect of inoculation of soybean (*Glycine max* L.) seeds with *Trichoderma asperellum*, followed by mites (*Tetranychus urticae*) exposure on lipid peroxidation (LP) process and the activity of antioxidant enzymes. *T. urticae* is an occasional pest of soybean that causes biotic stress. Biotic stress leads to overproduction of reactive oxygen species (ROS) which may cause damage to vital biomolecules. Enzymatic antioxidant defense systems protect plants against oxidative stress. *T. asperellum* is commonly used as biocontrol agent against plant pathogens. It has been suggested that previous inoculation of seeds with *T. asperellum* may cause induced resistance against biotic stress. The aim of this study was to determine LP intensity and antioxidant enzymes activity in inoculated and non-inoculated soybean seedlings with and without exposure to mites. Noticeably higher LP intensity was detected in non-inoculated group treated with mites compared to control group. Inoculated soybean seedlings treated with mites had lower LP intensity compared to non-inoculated group. Also, it has been noticed that inoculation with *Trichoderma asperellum* itself, produced mild stress in plants. In addition, positive correlation between enzymes activity and LP was noticed. The level of oxidative stress in plants was followed by the change of LP intensity. According to results obtained, it was concluded that the greatest oxidative stress occurred in non-inoculated group treated with mites and that inoculation successfully reduced oxidative stress. The results indicate that inoculation of soybean seeds with *T. asperellum* improves resistance of soybean seedlings against mites attack.

**KEYWORDS:** antioxidant systems, *Glycine max* L., oxidative stress, *Tetranychus urticae*, three-way-interaction, *Trichoderma asperellum*

## INTRODUCTION

Biotic and abiotic stresses are the main problems that are affecting agricultural losses (Borges *et al.* 2014). Biotic stress is the pressure posed on plants by living organisms (Volkov, 2006). *Tetranychus urticae* Koch, commonly known as two-spotted spider mite, is a rather polyphagous pest. This mite, with

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a worldwide distribution, feeds on various species of plants, attacking more than 200 host plants, vegetables, ornamentals, fruit trees, soybean, cotton, beans etc. The two-spotted spider mite has been considered as a pest on soybean, causing serious damage (Raymjou *et al.* 2009).

The need for high and quality agricultural production has led to an excessive use of chemical fertilizers, causing serious environmental pollution. Biofertilizers and biopesticides are used as an alternative for maintaining high production with low ecological impact. Different soil-borne bacteria and fungi are able to colonize roots and may have beneficial effects on the plant (Hermosa *et al.* 2012). *Trichoderma* species are free-living fungi in soil and root ecosystems (Contreras-Cornejo *et al.* 2009) and one of the most commonly used biological control agents against plant pathogens (Segarra *et al.* 2007). Colonization of roots by certain bacteria and fungi may cause induced systemic resistance (ISR). It was recently proposed that *Trichoderma*, a genus of filamentous fungi, also affects ISR in plants (Shoresh *et al.* 2005). During colonization of roots, the remarkable range of lifestyles and interaction between *T. asperellum* and other fungi, animals and plants is displayed (Druzhinina *et al.* 2011).

In response to pest invasion, plants produce reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Farouk and Osman, 2012). ROS are highly reactive and in absence of any protective mechanism they can seriously damage vital biomolecules such as lipids, protein and nucleic acids (Meloni *et al.* 2009). Consequence of lipid peroxidation (LP) is a loss of membrane integrity. One of the final products of lipid peroxidation is malondialdehyde (MDA), which is considered as indicator of oxidative damage (Kotchoni *et al.* 2006). To mitigate the oxidative damage induced by ROS, plants have developed enzymatic antioxidant defense systems, which include enzymes such as superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX) (Azevedo Neto *et al.* 2006). SOD catalyses the dismutation of  $O_2^-$  to  $H_2O_2$ , catalase (CAT) dismutates  $H_2O_2$  to oxygen and water (Gara *et al.* 2003) and GPX decomposes  $H_2O_2$  using guaiacol as substrate (Azevedo Neto *et al.* 2006).

The purpose of this work was to study the effect of inoculation of soybean (*Glycine max* L.) seeds with *Trichoderma asperellum*, followed by mites (*Tetranychus urticae*) exposure, on lipid peroxidation (LP) and antioxidant enzymes activity in inoculated and non-inoculated soybean seedlings after insect attack. Secondly, the aim was to assess beneficial affect of inoculation itself, before mites attack.

## MATERIALS AND METHODS

### *Material and experimental design*

The experimental design included two groups of soybean plants – a control and another, inoculated with *T. asperellum*. Soybean seeds (*Glycine max* L.) cv. Maximus were obtained from the Institute of Field and Vegetable Crops, Novi Sad (Serbia). Seeds were inoculated before sowing by submersing into

inoculum of *T. asperellum* (120 seeds were submersed into 60 ml of inoculum for 30 minutes). Control seeds were submersed in water. Seeds were cultivated in a controlled climate chamber at 28 °C, 60% relative humidity, a photoperiod of 18 h and a light intensity of 10,000 lx, in plastic pots containing sterile soil. Young plants were grown for three weeks, after that period one half of control and one half of inoculated soybean plants were exposed to mites (10–15 individuals per plant) during another 12 days. After 33 days, plant material (leaves and roots) for biochemical analyses were sampled.

### *Inoculum preparation*

*Trichoderma* sp. strains were isolated using Potato-Dextrose agar (Difco) by employing the serial dilution plate technique. The media were solidified with 1.6% agar and pH 5.4 was adjusted with 1 M HCl before autoclaving at 121 °C for 15 min and added chloramphenicol at 1 g per liter. The plates were incubated for 7–14 days at 28 °C and then checked for sporulation (optical microscope, Olympus, KHC, Japan). Selected *Trichoderma* sp. strains were grown on the same medium. The plates were incubated at 20±2 °C and sporulation results were recorded after 14 days by visual assessment. The plates were flooded with sterile physiological solution and the resulting spore suspension was harvested. The spore suspension was adjusted to  $1 \times 10^6$  spores ml<sup>-1</sup>, determined by spore counting in Neubauer chamber.

### *Insect*

*Tetranychus urticae* Koch, commonly known as two-spotted spider mite, has been considered as a pest on soybean, causing serious damage (Razmjou *et al.* 2008). For experimental use *T. urticae* was taken from the Faculty of Agriculture, University of Novi Sad.

### *Biochemical assays*

For the determination of the oxidative stress parameters, one g of fresh plant material was homogenized with 10 ml of phosphate buffer (0.1 M, pH 7.0). After centrifugation at 15,000 g for 10 min at 4 °C aliquots of the supernatant were used for biochemical assays. Lipid peroxidation was measured at 532 nm using the thiobarbituric acid (TBA) test (Mandal *et al.* 2008). The total amount of TBA-reactive substances was given as nmol malondialdehyde (MDA) equivalents g<sup>-1</sup> fresh weight. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed according to the method by Mandal *et al.* (2008) slightly modified by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. One unit of the SOD activity was defined as the amount of enzymes required to inhibit reduction of NBT by 50%. Catalase (CAT) (EC 1.11.1.6)

activity was determined according to Sathya and Bjorn (2010). The decomposition of  $H_2O_2$  was followed as a decrease in absorbance at 240 nm. Peroxidase (EC 1.11.1.7) activity was measured using guaiacol (guaiacol peroxidase; GPx) as substrate according to Morkunas and Gmerek (2007). The activity of the enzymes was expressed as  $U\ g^{-1}$  of fresh weight.

### *Statistical analyses*

Values of the biochemical parameters were expressed as standard error of the mean of determinations made in triplicates and tested by ANOVA followed by comparison of the mean by Duncan's multiple range test ( $P < 0.05$ ). Data were analyzed using STATISTICA for Windows version 11.0.

## RESULTS AND DISCUSSION

This work was performed to reveal how isolate of *T. asperellum* affects antioxidant properties of soybean plant response to biotic stress such as mites attack. On the other hand, second aim was to reveal how stressful was inoculation itself for plants, without exposure to mites. Leaves and roots extracts were sampled.

Biotic and abiotic stress leads to overproduction of reactive oxygen species (ROS). ROS affect many cellular functions by damaging biomolecules and causing lipid peroxidation (Esfandiari *et al.* 2007). The main end-product of LP is MDA. LP was followed up by detected amount of MDA. Significantly higher LP intensity was detected in non-inoculated group treated with mites compared to control group. Hildebrand *et al.* (2014) recorded increase in LP of two soybean genotypes injured by *T. urticae*. Inoculated soybean seedlings treated with mites had lower LP intensity compared to non-inoculated group (Figure 1).

Bi and Felton (1995) proposed that an oxidative response also occurs following attack by herbivores. The antioxidant enzymes represent a first line of defense against ROS (Sharma *et al.* 2012). Since SOD is an inducible enzyme whose activity is elevated under conditions of biotic stress, the level of oxidative stress in plants was observed by the change in the SOD activity. Furthermore, CAT and GPx activity were followed. Positive correlation between enzymes activity (SOD and GPx) and LP was noticed. The statistically significant increase in activity of SOD and GPx was also detected in non-inoculated group treated with mites compared to control group. Gupta *et al.* (1993) recorded an increase in SOD activity under oxidative stress. Data presented by Farouk and Osman (2012) indicate significant increase in peroxidase activity with mites invasion compared to control. Inoculated soybean seedlings treated with mites had lower enzymes activity compared to non-inoculated group (Figure 2 and Figure 3). In leaves, the statistically significant decrease in CAT activity was recorded in both groups treated with mites compared to control. Farouk and Osman (2012) showed significant decrease in CAT activity due to mites infestation. Haber-Weiss reaction generates hydroxyl radicals from  $H_2O_2$ , which could explain decreased

catalase activity in infested plants (Farouk and Osman, 2012). In addition, the inhibition of enzymes activity may be consequence of enhanced oxidative stress caused by biotic stress, in this case – mite attack (Dewanjee *et al.* 2014). On the other hand, in roots statistically significant decrease in CAT activity was detected in inoculated group, comparing both infested groups (Figure 4).

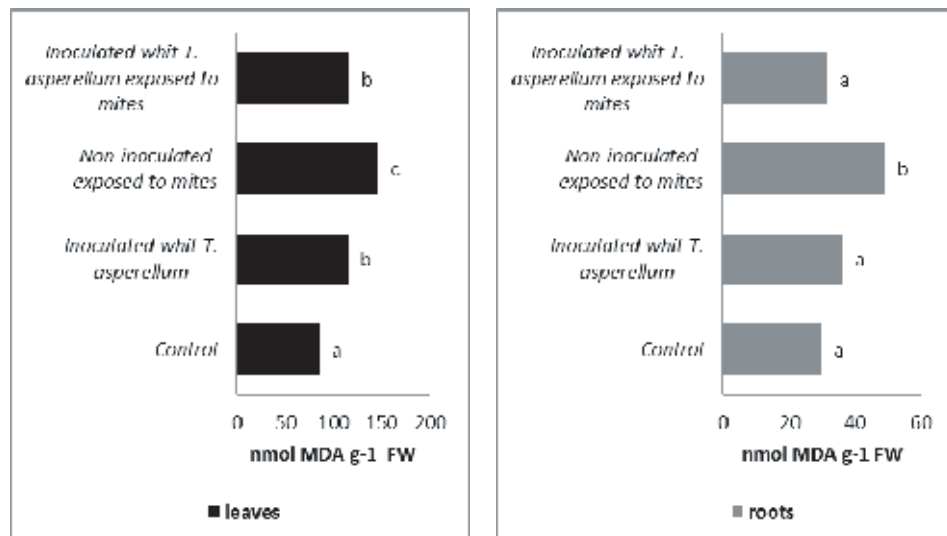


Figure 1. LP intensity (nmol MDA g<sup>-1</sup> fresh weight) in leaves and roots of soybean seedling. Results marked with different letters differ significantly at P<0.05 (Duncan's test).

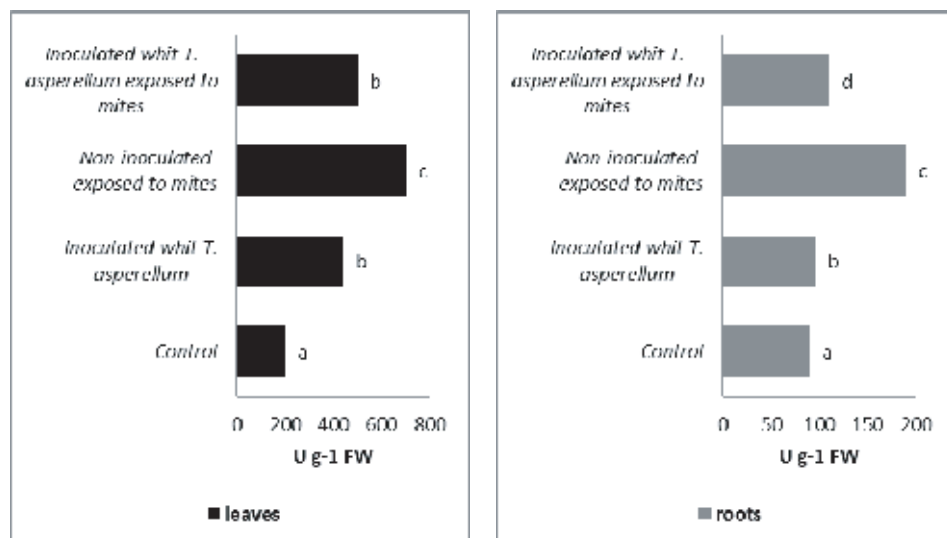


Figure 2. SOD activity (U g<sup>-1</sup> fresh weight) in leaves and roots of soybean seedlings. Results marked with different letters differ significantly at P<0.05 (Duncan's test).

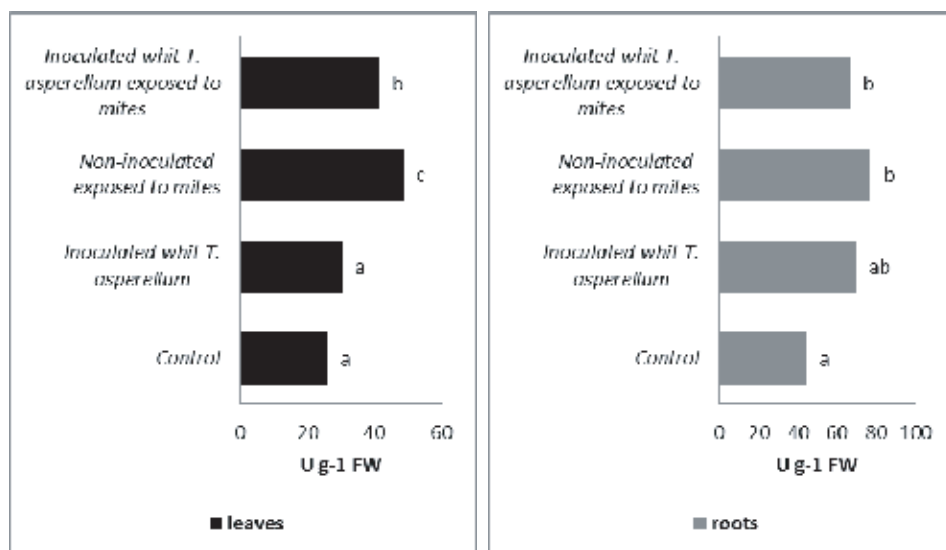


Figure 3. GPx activity (U g<sup>-1</sup> fresh weight) in leaves and roots of soybean seedlings. Results marked with different letters differ significantly at P<0.05 (Duncan's test).

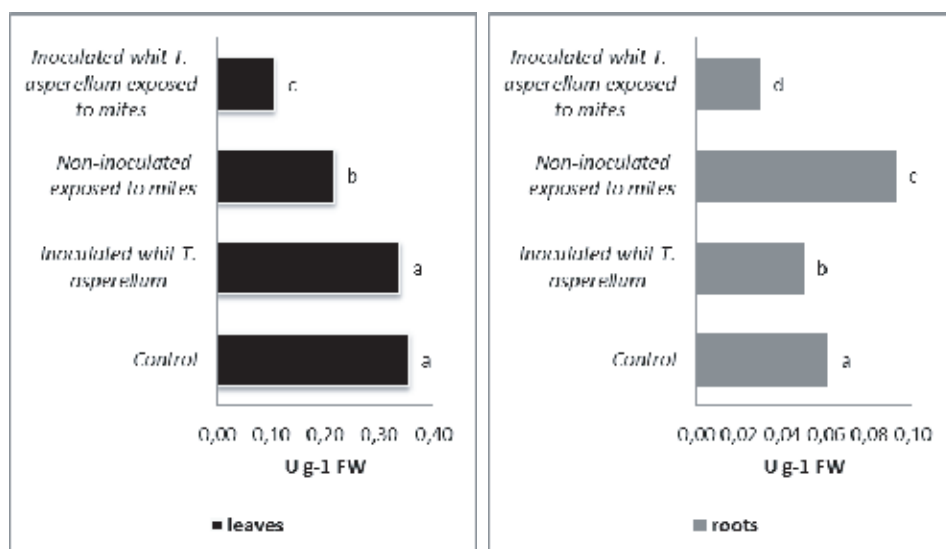


Figure 4. CAT activity (U g<sup>-1</sup> fresh weight) in leaves and roots of soybean seedlings. Results marked with different letters differ significantly at P<0.05 (Duncan's test).

Plant symbiont, opportunistic, avirulent organisms such as *Trichoderma* strains are promising fungal biological agents (Brotman *et al.* 2013). The beneficial effects of *Trichoderma* are based on complex of different mechanisms

such as mycoparasitism, competition, production of antibiotic etc. (El\_Komy *et al.* 2016). *Trichoderma* strains can stimulate plant growth by suppressing plant diseases or insect herbivory, directly through antagonism or indirectly by eliciting a plant-mediated resistance response. Systemic defense responses that are triggered by beneficial microorganisms are controlled by a signaling network which includes plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Van Wees *et al.* 2008). Relatively little is known about defense mechanisms and it is assumed that activation of a signaling cascade activates callose deposition, programmed cell death, production and accumulation of antimicrobial reactive oxygen species and secondary metabolites (Brotman *et al.* 2013). Results from this study showed small increase in LP intensity, SOD and GPx activity in inoculated plants without exposure to mites, compared to control. Thus, mild stress was detected. Lichtenthaler (1996) stated that stress is a factor that improves resistance and adaptive evolution.

## CONCLUSIONS

According to obtained results, it could be concluded that inoculation with *T. asperellum* decreases LP intensity, SOD and GPx activity in group of plants treated with mites. Following the increase of LP, SOD and GPx activity, it was concluded that the greatest oxidative stress occurred in non-inoculated group treated with mites and that inoculation successfully reduced oxidative stress. In addition, CAT activity decreased in both infested groups, which could be explained by saturation of scavenging system in such stress conditions. Secondly, it has been noticed that inoculation with plant growth-promoting fungi *T. asperellum* itself, produced mild stress in plants. Thus, it seems that mild oxidative stress was beneficial to soybean seedlings. Under such conditions (plant-microorganism interaction after inoculation) plants developed molecular mechanisms to better cope with next stress factor, such as insect attack, becoming more resistant to increased production of ROS. The obtained results support the idea that inoculation of soybean seeds with *T. asperellum* improves resistance of soybean seedlings against mites attack.

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## АНТИОКСИДАТИВНА СВОЈСТВА САДНИЦА СОЈЕ ИНОКУЛИСАНИХ СА *Trichoderma asperellum*

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**РЕЗИМЕ:** Студија је спроведена у циљу процене ефекта инокулације семена соје (*Glycine max* L.) са *Trichoderma asperellum* на липидну пероксидацију и активност антиоксидативних ензима праћена нападом гриња (*Tetranychus urticae*). *T. urticae* је уобичајна штеточина која напада соју и узрокује биотички стрес. Биотички стрес доводи до хиперпродукције реактивних кисеоничних врста (ROS) које могу проузроковати оштећења виталних биомолекула. Ензимски антиоксидативни одбрамбени систем штити биљке од оксидативног стреса. *T. asperellum* се често користи као биоконтролни агенс против биљних патогена. Сугестирано

је да претходна инокулација семена са *T. asperellum* може изазвати индуковану резистенцију против биотичког стреса. Циљ ове студије био је да се утврди интензитет липидне пероксидације и активности антиоксидативних ензима у инокулисаним и неинокулисаним садницама соје са и без излагања грињама. Приметно већи интензитет липидне пероксидације је детектован у неинокулисаној групи третираној грињама у односу на контролну групу. Инокулисане саднице третиране грињама имале су мањи интензитет липидне пероксидације у поређењу са неинокулисаном групом. Такође је уочено да сама инокулација са *T. asperellum* производи благи стрес код биљака. Поред тога, позитивна корелација између липидне пероксидације и ензимске активности је примећена. Ниво оксидативног стреса у биљкама је праћен променом интензитета липидне пероксидације. Према добијеним резултатима закључено је да се највећи оксидативни стрес догодио у неинокулисаној групи третираној грињама и да је инокулација успешно редуковала оксидативни стрес. Резултати показују да инокулација семена са *T. asperellum* поспешује отпорност садница соје против напада гриња.

КЉУЧНЕ РЕЧИ: антиоксидативни системи, *Glycine max* L., оксидативни стрес, *Tetranychus urticae*, *Trichoderma asperellum*, трострука интеракција

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## EFFECT OF NITROGEN SOURCES ON THE PRODUCTION OF ANTIFUNGAL METABOLITES BY *Streptomyces hygroscopicus*

**ABSTRACT:** Biotechnologically produced antifungal compounds for control of plant diseases caused by phytopathogenic fungi represent a promising alternative to synthetic pesticides. Fungi from the genera *Alternaria* and *Fusarium* are listed among important storage pathogens of apple fruits. *A. alternata* causes significant annual losses of apple fruit. Also, *F. avenaceum* is one of the most commonly encountered *Fusarium* species identified as the causal agent of a wet apple core rot. Species of the *Streptomyces* genera are soil bacteria that produce significant quantities of bioactive compounds in appropriate media. Defining the culture medium composition is the basis of bioprocesses development. Nitrogen source is critical component of cultivation medium and also the most useful tool for stimulation of the antifungal metabolites production. The aim of this study was to select the best nitrogen sources in medium for the production of antifungal compounds effective against *A. alternata* and *F. avenaceum* by *S. hygroscopicus*. Activity of the cultivation liquids on *A. alternata* and *F. avenaceum* isolates was tested *in vitro* using well diffusion method. The results indicate that maximum inhibition zone was reached in medium with soybean meal as nitrogen source with inhibition diameter more than 35 mm for both tested fungi.

**KEYWORDS:** *Alternaria alternata*, antifungal metabolites, *Fusarium avenaceum*, nitrogen source, *Streptomyces hygroscopicus*

## INTRODUCTION

The increasing concern for environmental protection and demand for organic farming prompt research towards alternative control measures, such as the use of natural antagonists to biologically control plant pathogens (Kani-*ni et al.* 2013). Biological control methods have been recommended to protect

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vegetable and fruit crops from diseases and pests, due to the adverse impact of chemical pesticides. Apple fruits are available on the market all year round and their safety is of great importance for the consumers. Unfortunately, many phytopathogenic fungi can cause apple diseases. *Alternaria* spp. are cosmopolitan fungi and can occur in a variety of food products, where they can produce several types of biologically active metabolites or mycotoxins harmful to human health (Pavon *et al.* 2015). Several different *Alternaria* spp. are associated with moldy core in apples while dry core rot is mostly linked to a single species, *A. alternata* (Serdani *et al.* 2002). They can grow at low temperature, hence they are generally associated with extensive spoilage during refrigerated transport and storage (Lopez *et al.* 2016).

*Fusarium* rot of apples and other fruits occurs while they are stored and shelved (Tadijan *et al.* 2016). *F. avenaceum* apple disease is characterized by a white, rose or reddish mycelium developing initially in the apple core and a light-brown wet rot spreading destructively into the surrounding cortex of infected apples (Sorensen *et al.* 2009). Chemical treatment of ripe fruit has many serious side-effects, such as leaving residues and sometimes causing fruit injury, in addition to the presence of offensive odors under modified storage conditions (Batta, 2004; Grahovac *et al.* 2014).

Microbial antagonists have been widely studied as biological control agents for the last several years, and *Streptomyces* spp. are known to be greatest sources of bioactive metabolites (Singh and Rai, 2012; Grahovac *et al.* 2015). *S. hygroscopicus* is actinomycetes that showed very good potential for production of different antimicrobial components (Lee *et al.* 1997; Sadhasivam *et al.* 2010; Shamim *et al.* 2004). Nature and concentration of some components in fermentation medium have a significant effect on anti-mycotic agents production. Influence of particular nutrients on the anti-mycotic biosynthesis is determined by the chemical structures of anti-mycotic substances. When carbon or nitrogen source is a limiting factor, growth is rapidly reduced and anti-mycotic biosynthesis takes place in the stationary phase. In other cases, anti-mycotic production is associated with the growth phase. Thus, some nitrogen sources can be incorporated in anti-mycotic molecules as precursors or their amino groups transfer to specific intermediate products (Gesheva *et al.* 2005).

The culture medium should provide energy, carbon and nitrogen sources, and minerals for cellular growth and natural product biosynthesis. As a nitrogen source, some *Streptomyces* spp. can use amino acids like aspartate, arginine, and histidine (Lee *et al.* 1997) or phenylalanine, isoleucine, methionine, and tyrosine (Singh *et al.* 2008). *Streptomyces* spp. can also use some organic and inorganic nitrogen sources for growth and production of antimicrobial components. Ripa *et al.* concluded that the highest activity of *Streptomyces* sp. RUPA-08PR was obtained with yeast extract as a nitrogen source (Ripa *et al.* 2009). Adinarayana *et al.* in their research discovered that among the nitrogen sources, corn steep was best followed by soybean meal and sodium glutamate, while sodium nitrate and soya peptone showed similar titers (Adinarayana *et al.* 2003). According to Ueki *et al.* soybean meal is also considered a suitable medium component for antibiotic production by *S. capoamus* (Singh and Rai,

2012). In general, cultivation medium should contain nutrients easily available in the market and, if possible, relatively inexpensive (Ortiz *et al.* 2007).

In accordance with the previous facts, there is diversity in nitrogen sources that can be metabolized by different *Streptomyces* species. Thus, each research should start with defining the culture medium composition, especially carbon and nitrogen sources as the most important macronutrients for each bioprocess. In the present study, the ability of *Streptomyces hygroscopicus* to assimilate different nitrogen sources and produce high-value metabolic compounds with antifungal activity against *Alternaria alternata* and *Fusarium avenaceum* was investigated.

## MATERIALS AND METHODS

### *Fungal pathogen*

Isolates of *A. alternata* and *F. avenaceum* were obtained from apple fruit samples expressing rot symptoms. Apple samples were collected during 2012 from ultra low oxygen storages in the Province of Vojvodina, Serbia. The pathogen was identified according to pathogenic, morphological, and ecological characteristics. The isolates were initially grown on PDA (potato dextrose agar) plates for seven days. After seven days, a small amount of mycelium of each isolate was added to flasks containing 50 ml of potato dextrose broth. The flasks were incubated for 48 hours on a rotary shaker (150 rpm) at 25 °C. Before use, culture liquid was filtered through the double layer of sterile cheesecloth.

### *Antifungal component production*

Microorganism producer, *S. hygroscopicus*, was isolated from the natural environment and stored in the Microbial Culture Collection of the Faculty of Technology in Novi Sad. The medium used for the growth of production microorganism had the following composition (g/L): glucose (15.0), soybean meal (10.0), CaCO<sub>3</sub>, (3.0), NaCl, (3.0), MgSO<sub>4</sub>, (0.5), (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, (0.5), and K<sub>2</sub>HPO<sub>4</sub>, (1.0). The pH of the medium was adjusted to 7.2 ± 0.1 prior to autoclaving.

For the preparation of the fermentation medium, the following ten nitrogen sources were used: yeast extract, peptone, soybean meal, L-aspartic acid, meat extract, NH<sub>4</sub>Cl, NH<sub>4</sub>NO<sub>3</sub>, NaNO<sub>3</sub>, KNO<sub>3</sub>, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Other components were the same as in the medium used for growth. The pH of medium was adjusted to 7.2±0.1 prior to autoclaving. The isolate was grown in a 100 ml shake flask containing 30 ml of the culture medium. The fermentation medium was inoculated with 10% (v/v) of a preculture after 48 hours of growth and incubated at 26 ± 1°C for 7 days under conditions of spontaneous aeration. Rotary shaker (IKA KS 4000i Control Incubating Shaker) at 150 rpm was used for the mixing of fluids during the cultivation. After cultivation, the sample of the cultivation medium was centrifuged at 10,000 g for 10 min (Eppendorf Centrifuge 5804,

Germany) and the supernatant of the cultivation medium was used for *in vitro* antagonistic activity assay.

#### *In vitro* antagonistic activity assay

*In vitro* antagonistic activity assay was performed in Petri plates using well diffusion method (Segy, 1983). In short, two layers of PDA medium were spread on plates. The first layer consisted of 2% PDA medium. After solidification, a new layer composed of 1.2% PDA and filtered fungal culture liquid (35%) was added. Three wells with a diameter of 10 mm represented one treatment. For each treatment, 100  $\mu$ l of test liquid was added in each well. The treatments included: supernatant of *S. hygroscopicus* cultivation medium and sterile distilled water as negative control treatment. After 72 hours of incubation at 27 °C, the diameter (mm) of mycelia growth inhibition zone around wells was measured.

#### *Data analysis*

The obtained data were processed by factorial ANOVA using Software *Statistica* 12 (Statistica, 2012). Duncan's multiple range test was used to test significance of differences ( $p \leq 0.05$ ) between mean values of measured diameter of inhibition zones.

### RESULTS AND DISCUSSIONS

Designing an appropriate fermentation medium is crucial in the production of antifungal agents. Two most critical components of nutritional medium for any fermentation process – carbon and nitrogen sources – are the most useful tools to improve or to stimulate the production of antifungal metabolites. Nitrogen source is required for the synthesis of microorganism cells and as an energy source. Also, biomass yield and cell morphology are strongly influenced by nitrogen source.

In order to examine the growth of *S. hygroscopicus* and production of antifungal metabolites against *A. alternata* and *F. avenaceum* on different nitrogen sources, shake flask cultures were carried out. The selection of mostly used nitrogen sources in biotechnological production was tested (Wu *et al.* 2008; Ripa *et al.* 2009). As expected, the evaluated treatments had significant ( $p < 0.001$ ) influence on the mycelial growth inhibition zone diameter (mm) for both tested fungi. Results presented in Table 1 and Table 2 are mean values of three repetitions of inhibition zone diameter. The mean values with the same lowercase letters in the column inhibition zone diameter (mm) are not significantly different at 5% level of probability.

Table 1. Effect of different nitrogen sources on the inhibition zone diameter after 72 hours incubation at 27 °C and significance of differences at 5% level probability for *A. alternata*

Nitrogen source	Inhibition zone diameter (mm)
L-aspartic acid	23.33 <sup>a</sup>
KNO <sub>3</sub>	24.33 <sup>a</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25.00 <sup>a</sup>
NH <sub>4</sub> Cl	25.00 <sup>a</sup>
Meat extract	25.33 <sup>a</sup>
NaNO <sub>3</sub>	25.67 <sup>a</sup>
NH <sub>4</sub> NO <sub>3</sub>	29.33 <sup>b</sup>
Peptone	29.33 <sup>b</sup>
Yeast extract	29.33 <sup>b</sup>
Soybean meal	38.33 <sup>c</sup>

Duncan's multiple range test showed that the highest efficiency against *A. alternata* isolate was obtained by *S. hygroscopicus* cultivated in medium containing soybean meal as a nitrogen source (inhibition zone diameter was 38.33 mm), compared to all other nitrogen sources (Table 1). Singh *et al.* in their research confirmed that of all examined nitrogen sources, soybean meal was found to be the best nitrogen source for growth, as well as for antimicrobial agent production by *Streptomyces* sp. A2D (Singh *et al.* 2009). Also, Wu *et al.* reported that soybean meal was an excellent nitrogen source for production of fungichromin by *S. padanus* PMS-702 (Wu *et al.* 2008). In general, nitrogen from soybean meal derivatives are extensively utilized in the fermentation processes (Portera and Jones, 2003; Ortiz *et al.* 2007).

Minimum inhibition zone diameter was obtained with L-aspartic acid and KNO<sub>3</sub> as nitrogen sources in the medium. Similarly, Adinarayana *et al.* in their research confirmed that, compared to other tested nitrogen sources, lower neomycin titres were observed with L-aspartic acid using *S. marinensis* as microorganism producer (Adinarayana *et al.* 2003). Medium supplemented with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and NH<sub>4</sub>Cl gave similar results followed by meat extract and NaNO<sub>3</sub>. Peptone, yeast extract and NH<sub>4</sub>NO<sub>3</sub> gave the same inhibition zone diameter of 29.33 mm.

However, some nitrogen sources have better influence on the biomass growth, thus prolonging exponential phase and shortening the stationary phase of production. Considering that antifungal components are usually secondary metabolites, shorter stationary phase can reduce production of antifungal substances. On the other hand, some nitrogen sources can be incorporated in anti-fungal molecules as precursors and stimulate antifungal components production (Gesheva *et al.* 2005).

Therefore, the results shown in Table 1 indicate that the tested isolate of *S. hygroscopicus* shows great potential as a tool for the biological control of *Alternaria* rot in apple, and the medium containing few different nitrogen sources ensures its high activity (inhibition zone diameter > 25 mm).

Table 2. Effect of different nitrogen sources on the inhibition zone diameter after 72 hours incubation at 27 °C and significance of differences at 5% level probability for *F. avenaceum*

Nitrogen source	Inhibition zone diameter (mm)
KNO <sub>3</sub>	19.67 <sup>a</sup>
NaNO <sub>3</sub>	22.00 <sup>ab</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	22.00 <sup>bc</sup>
L-aspartic acid	25.67 <sup>cd</sup>
NH <sub>4</sub> NO <sub>3</sub>	25.67 <sup>cd</sup>
NH <sub>4</sub> Cl	25.67 <sup>cd</sup>
Peptone	26.67 <sup>cd</sup>
Meat extract	27.33 <sup>d</sup>
Yeast extract	27.33 <sup>d</sup>
Soybean meal	35.67 <sup>e</sup>

Similar results were obtained for test microorganism *F. avenaceum* (Table 2). The minimum inhibition zone diameter was observed with medium containing KNO<sub>3</sub> as a nitrogen source followed by NaNO<sub>3</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The inhibition zone diameter over 25 mm was obtained with cultivation medium containing L-aspartic acid, NH<sub>4</sub>NO<sub>3</sub>, NH<sub>4</sub>Cl, peptone, meat extract, and yeast extract. Medium containing soybean meal as a nitrogen source showed statistically bigger inhibition zone diameter (35.67 mm) compared to all other nitrogen sources.

Inhibition zones formed around wells with 100 µl of *S. hygroscopicus* cultivated in medium with soybean meal as a nitrogen source in the medium are shown in Figure 1. Figure 1A represents test fungi *A. alternata* and Figure 1B represents test fungi *F. avenaceum* after 72 hours of incubation at 27 °C. Figures A2 and B2 are control for each test fungi.

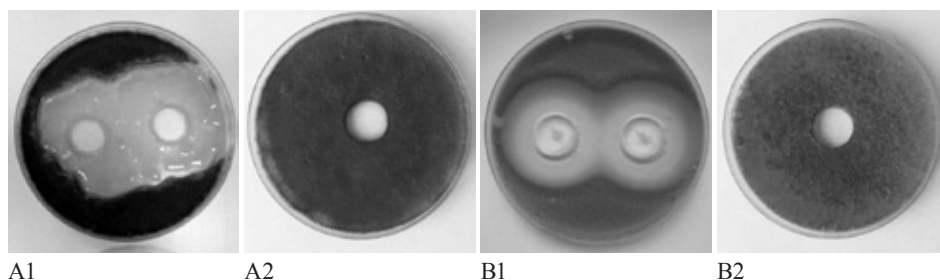


Figure 1. Inhibition zones formed around wells with 100 µl of *S. hygroscopicus* with soybean meal as a nitrogen source for *A. alternata* (A1) and *F. avenaceum* (B1). A2 and B2 are control plates.

The results presented in Table 1 and Table 2 show that organic sources of nitrogen as peptone, yeast extract, and soybean meal gave better inhibition zone diameter for both tested fungi compared to inorganic sources. Also, Abou-Zeid *et al.* concluded that soybean meal and peptone are the best organic nitrogen

sources for production of oxytetracycline by a strain *S. rimosus* 93060 (Abou-Zeid *et al.* 1981).

Thus, the composition of the soybean meal is important factor for the production of antifungal agents, because soybean meal is a complex mixture of proteins (about 50%), carbohydrates (oligosaccharides and fiber 30%), fat (1%), and lecithin (1.8%) (Cheryan *et al.* 1997). Asparagine, glutamine, leucine, arginine, lysine, and serine are the most common amino acids present in soybean meal and it can be assumed that some of these acids are the key nitrogen donors for biosynthesis of antifungal compounds (Portera and Jones, 2003). The largest part of soybean meal consists of proteins, which represent a nitrogen source, followed by carbohydrates and then lipids. The lipid substances in soybean meal can contribute to the stability of the cell wall during fermentation, reducing the rate of cell lysis and allowing better fermentation (Cheryan *et al.* 1997).

However, soybean meal is a cheap raw material, which is certainly an advantage compared to the other organic nitrogen sources, and this fact is particularly important for biotechnological production.

## CONCLUSION

The present study showed that naturally occurring actinomycete have a great potential to assimilate different nitrogen sources and produce high-value metabolic compounds with antifungal activity against *A. alternata* and *F. avenaceum*. In this case, both inorganic and organic nitrogen sources produced reasonable amount of antifungal metabolites but the highest activity was obtained with organic sources, especially soybean meal.

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## УТИЦАЈ ИЗВОРА АЗОТА НА ПРОИЗВОДЊУ АНТИФУНГАЛНИХ МЕТАБОЛИТА ПРИМЕНОМ *Streptomyces hygroscopicus*

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**РЕЗИМЕ:** Забрана примене синтетичких фунгицида у време зрења и бербе, брза појава резистентних јединки и штетан утицај на здравље људи, довели су до повећања интереса и јавности и струке за увођење алтернативних мера заштите биља где заслужено место налазе биолошки препарати. Плодови јабуке су током читаве године присутни у исхрани деце и одраслих те је квалитет и здравствена безбедност ових намирница од изузетног значаја. Током вегетације и током читавог периода складиштења, транспорта и продаје, плодови јабуке су подложни инфекцији различитим патогеним гљивама. Међу значајним, али не и довољно испитаним изазивачима болести јабука помињу се гљиве из родова *Alternaria* и *Fusarium*. *Alternaria alternata* проузрокује значајне годишње губитке плодова јабука. Такође, *Fusarium avenaceum*, врста која је потенцијални произвођач неколико микотоксина, доминантан је узрочник трулежи плода јабуке након складиштења. Од свих биопестицида доступних на светском тржишту, биопестициди на бази микроорганизама имају удео од 30%. Актиномицете рода *Streptomyces* представљају потенцијално значајну групу микроорганизама за производњу биоактивних компоненти на одговарајућој хранљивој подлози. Дефинисање култивационог медијума представља кључан корак у развоју биопроцеса, а одабир адекватног извора азота може значајно да утиче на стимулацију производње антифунгалних метаболита. Циљ овог рада био је одабир најбољег извора азота у подлози за производњу антифунгалних метаболита применом *Streptomyces hygroscopicus*. Као тест гљиве коришћене су *Alternaria alternata* и *Fusarium avenaceum*. Активност култивационе течности на изолате *A. alternata* и *F. avenaceum* одређена је *in vitro*, дифузионом – бунар методом. Резултати су показали да се максимална зона инхибиције постиже у подлози са сојиним брашном као извором азота код обе тест гљиве (пречник зоне инхибиције већи од 35 mm).

**КЉУЧНЕ РЕЧИ:** *Alternaria alternata*, антифунгални метаболити, *Fusarium avenaceum*, извор азота, *Streptomyces hygroscopicus*



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## BACTERICIDAL ACTIVITIES OF SELECTED MACROFUNGI EXTRACTS AGAINST *Staphylococcus aureus*

**ABSTRACT:** The increasing of the antibiotic resistance exhibited by pathogenic microorganisms has resulted in research directed toward evaluation of novel sources of antimicrobial compounds. Previous studies have indicated that macrofungi, as a specific response to the natural hostile environment, produce secondary metabolites with antimicrobial properties. In this study, antimicrobial activities of the extracts from six wild mushrooms: *Amanita echinocephala*, *Russula medulata*, *Cerena unicolor*, *Hericium erinaceus*, *Ishnoderma benzoinum* and *Laetiporus sulphureus* were evaluated against Gram-positive bacterium *Staphylococcus aureus*. The antimicrobial potential of the methanolic mushroom extracts was investigated by the microdilution method. Antimicrobial activity was observed in all species included in the study. All the extracts that demonstrated inhibitory activities were further tested for bactericidal activity and minimum bactericidal concentration (MBC) values were determined. The tested microorganism was most sensitive to the examined extracts from the polypore fungi *C. unicolor* and *H. erinaceus*. The highest bactericidal activity was obtained in the extracts from the species *C. unicolor* (MBC=1.563 mg/mL). The experimental results revealed that the methanolic extract of *C. unicolor* possessed significant bactericidal activity. The findings suggest the potential use of this wild mushroom as antimicrobial agent.

**KEYWORDS:** mushroom, antimicrobial activity, microdilution method, minimum bactericidal concentration (MBC)

## INTRODUCTION

The emergence of multiple antibiotics resistance in bacterial pathogens is a common global problem posing enormous public health concerns in the past four decades. *Staphylococcus aureus* is the pathogen of great interest because of

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its virulence, ability to cause an array of life threatening infections and capacity to adapt to environmental conditions (Lowy, 2003). The non-availability and high cost of new antibiotic generation with limited effectiveness has led to the search of more effective antimicrobial agents among resources of natural origin, with the aim to discover a source for production of new antimicrobial drugs.

Higher fungi or macrofungi are characterized by the production of macroscopic fruiting bodies, which have long been appreciated not only for the unique taste, but also for their nutritional value (Kalač, 2012). In addition, higher fungi synthesize a multitude of metabolites that have different roles in the fungal survival. Studies have displayed that some of these metabolites have antibacterial and antifungal properties (Rai *et al.* 2005; Stamets, 2002), while a higher antimicrobial activity of mushroom extracts was observed against Gram-positive bacteria (Yamac & Bilgili, 2006; Nikolovska Nedelkoska *et al.* 2013). Considering that fungi and humans share common microbial pathogens (e.g. *Escherichia coli*, *S. aureus*, and *Pseudomonas aeruginosa*), antimicrobial compounds that are produced by fungi could be of benefit for humans. Several studies have shown that mushroom extracts and isolates demonstrate promising antimicrobial properties which can be employed to combat several diseases caused by pathogenic bacteria including drug-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (Ameri *et al.* 2011; Alves *et al.* 2012a; Karaman *et al.* 2012).

Therefore, considering the previous reports on the antimicrobial activity of macrofungi and the constant need for the development of new antimicrobial agents, this study aimed to examine the antimicrobial potential of the methanolic extracts from selected macromycetes: *Amanita echinocephala*, *Russula medulata*, *Cerena unicolor*, *Hericium erinaceus*, *Ishnoderma benzoinum* and *Laetiporus sulphureus* against Gram-positive bacterium *Staphylococcus aureus*.

## MATERIAL AND METHODS

### Fruiting body selection

Samples of the wild macromycetes *Amanita echinocephala*, *Russula medulata*, *Cerena unicolor*, *Hericium erinaceus*, *Ishnoderma benzoinum* and *Laetiporus sulphureus* were collected from different locations and habitats in the Republic of Macedonia. Geographical location and natural habitat of the mushroom specimens are shown in Table 1. Taxonomic identification was made in the Mycological Laboratory at the Institute of Biology, Faculty of Natural Sciences and Mathematics in Skopje, by implementing standard methods of microscopic and chemical techniques (coloring of fruit bodies and spores), as well as using appropriate literature. The representative voucher specimens were deposited at the Macedonian Collection of Fungi (MCF) of the Institute of Biology (Table 1).

Table 1. Geographical location and natural habitat of the mushroom species studied for antimicrobial potential

Species	Habitat	Geographical location	Collection number
<i>Amanita echinocephala</i>	mycorrhizal (on ground in park)	Botanical garden, Skopje	MAK 10/13309
<i>Russula medulata</i>	mycorrhizal (on ground in park)	Gazi Baba, Skopje	MAK 10/13305
<i>Cerena unicolor</i>	saprotrophic (on living beech trunks in conifer forest)	Suva Gora Mt.	MAK 11/13368
<i>Hericium erinaceus</i>	saprotrophic (on living oak trunks in deciduous forest)	Sk. Crna Gora Mt.	MAK 11/13360
<i>Ishnoderma benzoinum</i>	saprotrophic (on stump of pine trees)	Suva Gora Mt.	MAK 11/13252
<i>Laetiporus sulphureus</i>	parasitic (on living black locust trunks)	Kozle, Skopje	MAK 11/13361

### Preparation of methanolic extracts of mushrooms

The fruiting bodies were cleaned to remove any residual compost/soil and subsequently air-dried in the oven at 40 °C. Dried specimens were ground to fine powder and extracted by stirring with 80% (v/v) methanol in ultrasonic bath for 30 min at 4 °C, and then centrifuged at 12,000 rpm for 15 min. Supernatants were used for the evaluation of antimicrobial potential of the samples. The organic solvent in the extracts was evaporated to dryness under vacuum. The yields of methanolic extracts of the fruiting bodies are presented in Table 2.

Table 2. Yield of mushroom methanolic extracts

sample	mushroom species	yield of extracts <sup>a</sup> (g/100 g of dry mushroom)
1	<i>Amanita echinocephala</i>	33.082 ± 3.356
2	<i>Russula medulata</i>	4.167 ± 0.577
3	<i>Cerena unicolor</i>	20.800 ± 1.131
4	<i>Hericium erinaceus</i>	17.333 ± 0.764
5	<i>Ishnoderma benzoinum</i>	15.000 ± 1.323
6	<i>Laetiporus sulphureus</i>	33.833 ± 4.254

<sup>a</sup> Each value is the mean of three replicate determinations ± standard deviation.

The tested extracts were dissolved in 10% (v/v) DMSO in sterile water. A solvent control test was performed to study the effect of DMSO on the growth of microorganisms. The test approved that DMSO had no inhibitory effect on the tested organisms.

## *In vitro* antimicrobial assay

### *Test microorganism*

Antimicrobial activities of methanol extracts were tested against Gram-positive bacterium *Staphylococcus aureus* ATCC 6538.

The microorganism was provided from the collection of the Microbiology Laboratory at the Faculty of Natural Sciences and Mathematics in Skopje.

### *Suspension preparation*

Microbial suspension was prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparison with 0.5 McFarland's standard (Andrews, 2005). The initial suspension contained about  $10^8$  colony forming units (CFU)/mL. Additionally, 1:100 dilutions of initial suspension were prepared into sterile 0.9% saline.

### *Microdilution method*

The antibacterial activities of the mushroom extracts were assessed using the microdilution method with resazurin as an indicator of microbial growth (Sarker *et al.* 2007). The antimicrobial assay was performed by using a sterile 96-well plate, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined. The test plates were prepared by dispensing 50  $\mu$ L of Mueller-Hinton broth into each well. A volume of 50  $\mu$ L from the stock solution of tested mushroom extracts was added into the first row of the plate and then two-fold serial dilutions of extracts were performed. Each test plate included growth control and sterility control. MIC was defined as the lowest concentration of tested extracts that prevented a resazurin color change from blue to pink. All tests were performed in triplicate and MIC values were constant.

The extracts that demonstrated inhibitory activities were further tested for bactericidal activity. A sample from each well that tested positive for inhibitory activity was inoculated on fresh sterile Mueller-Hinton agar (MHA) plates and incubated additionally for 24 h at 37 °C. Absence of colonies was regarded as positive for bactericidal activity, while growth of colonies was regarded as negative. MBC was defined as the lowest concentration of the mushroom extract that results in microbial death. All tests were performed in triplicate and MBC values were constant.

## RESULTS AND DISCUSSION

The antimicrobial activity of the tested mushroom extracts was quantitatively assessed and minimum bactericidal concentration (MBC) values were determined. The bactericidal activity of the mushroom extracts is shown in Table 3. Antimicrobial activity was observed in all species included in the study, with MBC values ranging from 1.563 to 31.250 mg/mL.

Table 3. Minimum bactericidal concentration (MBC) of methanolic extracts from mushroom samples

	Samples					
	<i>A. echinocephala</i>	<i>C. unicolor</i>	<i>H. erinaceus</i>	<i>I. benzoinum</i>	<i>L. sulphureus</i>	<i>R. medulata</i>
MBC	25	1.563	2.344	31.250	15.625	25

<sup>a</sup> Minimum bactericidal concentration (MBC) values given as mg/mL

The results showed that *Cerena unicolor* fruiting body extract exhibited the strongest bactericidal activity against the tested bacterium with the lowest MBC value (1.563 mg/mL), followed by the extracts of *Hericium erinaceus* and *Laetiporus sulphureus* (2.344 mg/mL and 15.625 mg/mL, respectively). The methanolic extracts of *Amanita echinocephala* and *Russula medulata* demonstrated antimicrobial potential with the same MBC value of 25 mg/mL. In this study the highest MBC value (31.250 mg/mL), which corresponded to the lowest bactericidal potential against tested *S. aureus*, was observed in methanolic extract of polypore fungus *Ishnoderma benzoinum*.

According to these results, all extracts included in the study showed antimicrobial properties, while the variation in the antimicrobial activity among the species could be attributed to the fungal genomics and environmental factors (Keller *et al.* 2005), that resulted in the presence of different components with antimicrobial properties within species. Based on the evidence reported in the literature, various taxonomic mushroom groups have been investigated for their antimicrobial activities and many low- and high-molecular-weight compounds with antimicrobial properties were identified. Numerous secondary metabolites, such as sesquiterpenes and other terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolines, primary metabolites such as oxalic acid, and high-molecular-weight compounds, mainly peptides and proteins, are among the identified antimicrobial compounds in mushrooms (Alves *et al.* 2012b).

In this study the examined extracts from the polypore fungi *Cerena unicolor*, *Hericium erinaceus*, and *Laetiporus sulphureus* exhibited the most potent bactericidal activity against tested microorganism. These results are in accordance with earlier reported data that confirm the antimicrobial activity of these macrofungi species (Jaszek *et al.* 2013; Demiri and Yamaç, 2008; Zjawiony, 2004; Wong *et al.* 2009). Jaszek *et al.* (2013) evaluated the antibacterial activity of the isolated bioactive fractions from *C. unicolor* and showed that the low molecular subfraction of secondary metabolites possesses potent inhibitory effect against *Staphylococcus aureus*. Demiri and Yamaç (2008) reported relatively high antimicrobial activity for the crude exopolysaccharides of *C. unicolor* and *L. sulphureus*, while the highest antimicrobial activity against the tested cultures in their study they observed for mycelial extracts from *C. unicolor*. Several studies have shown the antimicrobial potential of mushroom species *H. erinaceus* and some bioactive molecules with antimicrobial activity against pathogenic microorganisms have been identified (Okamoto *et al.* 1993; Kim *et al.* 2000; Wong *et al.* 2009). All these observations were in accordance with the results of bactericidal activity reported here. Taking into account all these observations,

further studies could be focused on *in vitro* biological activity of extracts from *Cerena unicolor*, *Hericium erinaceus* and *Laetiporus sulphureus*.

## CONCLUSIONS

Results from the present study showed that *Amanita echinocephala*, *Russula medulata*, *Cerena unicolor*, *Hericium erinaceus*, *Ishnoderma benzoinum* and *Laetiporus sulphureus* extracts could be considered a potential sources of various compounds with bactericidal properties against Gram-positive bacterium *Staphylococcus aureus*. Particularly, extracts from the species *C. unicolor* and *H. erinaceus* exhibited most promising antimicrobial activity against *S. aureus* that may serve as potential candidates for the development of novel antibiotics. Further studies are needed toward the chemical characterization of specific classes of antimicrobial compounds of these selected mushrooms and understanding the possible mechanisms for their utilization in food and pharmaceutical industries.

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## БАКТЕРИЦИДНА АКТИВНОСТ ОДАБРАНИХ ЕКСТРАКТАТА МАКРОГЉИВА ПРЕМА *Staphylococcus aureus*

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**РЕЗИМЕ:** Пораст резистентности патогених врста микроорганизама резултирала је усмеравањем истраживање ка изналагању нових извора антимикробних једињења. Предходне студије указују да макрогљиве као специфичан одговор на услове средине продукују секундарне метаболите с антимикробним особинама. У оквиру ове студије, испитивана је антимикробна активност екстраката пореклом из шест врста дивљих гљива: *Amanita echinocephala*, *Russula medulata*, *Cerena unicolor*, *Hericium erinaceus*, *Ishnoderma benzoinum* и *Laetiporus sulphureus* на Gram позитивни микроорганизам *Staphylococcus aureus*. Антимикробни потенцијал

метанолних екстраката гљива је испитиван применом микродилуционе методе. Антимикробну активност су показале све врсте укључене у студију. Сви испитивани екстракти су показали антимикробну активност, а након испитивања бактерицидне активности одређена је минимална бактерицидна концентрација (МВС). Тестирани микроорганизам је највећу осетљивост показао на екстракте пореклом од полипорних гљива *C. unicolor* и *H. erinaceus*. Највећа антимикробна активност постигнута је с екстрактом врсте *C. unicolor* (МВС=1.563 mg/mL). Резултати истраживања указују да метанолни екстракт *C. unicolor* поседује значајну бактерицидну активност. Резултати ових истраживања сугеришу на потенцијалну употребу ове дивље гљиве као извора антимикробних једињења.

КЉУЧНЕ РЕЧИ: гљиве, антимикробна активност, микродилуциони метод, минимална бактерицидна концентрација (МВС)

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## ANTIFUNGAL ACTIVITY OF *Mentha piperita* AND *Carum carvi* ESSENTIAL OILS

**ABSTRACT:** Aromatic plants are one of the most important sources of biologically active secondary metabolites, which possess various antimicrobial characteristics. The aim of this work was to examine the effect of antifungal activities of mint and caraway essential oils against the selected fungi. Eight species of molds were selected for antifungal testing: *Alternaria alternata*, *Aspegillus flavus*, *A. niger*, *A. versicolor*, *Eurotium herbariorum*, *Penicillium aurantiogriseum*, *P. chrysogenum* and *P. expansum*. Testing of essential oils antifungal activity against the selected species was conducted using the disc diffusion method by adding mint and caraway essential oils (0.5, 1, 5, and 10 µl per disc). Antifungal activity of essential oils was expressed by the diameter of inhibition zone (mm). The most powerful effect of mint essential oil was recorded against *E. herbariorum*, as its growth was completely inhibited by the quantity of 5 µl. The weakest inhibitory effect was observed against *P. chrysogenum* (inhibition zone 13.67 mm) by the quantity of 10 µl. The most powerful antifungal activity of caraway was observed against *E. herbariorum* as growth was completely inhibited by the quantity of 10 µl. The weakest inhibitory effect was observed against *A. niger* (inhibition zone 28 mm) by the quantity of 10 µl.

**KEYWORDS:** antifungal activity, mint, caraway, essential oils

## INTRODUCTION

The biggest risk, from the health aspects, associated with the mold contamination in food products, is a possibility of a mycotoxin synthesis. On the other hand, The World Health Organization (WHO) is expressing concern due to an excessive application of synthetic fungicides. Search of an alternative antifungal resource provides the possibility of essential oil usage, also as extracts,

in food protection from mycotoxigenic molds and their toxic products (Da Cruz Cabral, 2013).

Essential oils are secondary metabolites of higher plants. The plants which contain the major evaporative odor products – essential oils, are called aromatic plants. Aromatic plants are mostly present in different species of the labiate family *Lamiaceae*, *Apiaceae*, *Asteraceae*, and other families such as *Rutaceae*, *Lauraceae* and *Myrtaceae* (Mišan *et al.* 2013).

Essential oils can be composed of more than 60 components. The major components are present in up to 85% of essential oil, whereas other components are present only in traces. Phenolic compounds are especially responsible for antimicrobial activity of essential oils. There are some indications that minor components of essential oils pose a key role in the antimicrobial activity, probably due to synergistic activity with other components. This claim has been proved in the case of sage, some species of thyme and oregano (Burt, 2004; Tajkarimi *et al.* 2010).

Effect of essential oils on molds can be followed at the macromorphological level, as well as at the cellular level. Some of macromorphological changes are: lack of sporulation or pigmentation, change in the number of conidia, increased branching of hyphae or change in their size. It has been considered that changes like these are consequence of oil activities on enzymatic reaction of the cell wall synthesis, which affects mold growth and morphogenesis, also as the pulling back of cytoplasm in hyphae, whereby the mycelium death occurs (Rasooli, 2004; Carmo *et al.* 2008).

Essential oils can inhibit synthesis of DNA, RNA, proteins and polysaccharides in fungi and bacterial cells, where they can cause changes, similar to the mechanism of antibiotic activity.

Mint essential oil shows a powerful antimicrobial activity against large number of pathogenic Gram-positive and Gram-negative bacteria, as well as against different species of molds (Tajkarimi *et al.* 2010). Caraway has a wide application in pharmaceutical and food industry, considering the fact that it possesses antitumor, antiproliferative, antihyperglycemic and antimicrobial activity (Agrahari and Singh, 2014).

## MATERIAL AND METHODS

### *Essential oils*

Essential oils of mint (*Mentha piperita* L.), Serbia, (36681113) and caraway (*Carum carvi* L.), Lithuanian, (05560213) were used for the purpose of antifungal testing. Identification of the mentioned plant species was performed at the Institute of Medicinal Plant Research “Dr. Josif Pančić”, Serbia. Essential oil was distilled from the dried and milled above-ground parts of the plant in the Clevenger apparatus during 2.5 h. Oils were extracted by the procedure of hydrodistillation in accordance with the standard procedure described in the Sixth European pharmacopoeia (European Pharmacopoeia, 2008).

## *Culture of molds*

Eight isolates of molds were selected for antifungal research: *Alternaria alternata*, *Aspegillus flavus*, *A. niger*, *A. versicolor*, *Eurotium herbariorum*, *Penicillium aurantiogriseum*, *P. chrysogenum* and *P. expansum*. Identification of isolated species was performed in accordance with the rules described by Samson *et al.* (2004), Samson and Frisvald (2004) and Pitt and Hocking (2009), on the basis of macromorphological characteristics of reproductive structures (size, shape, color, surface of the cell wall, branching, sexual and asexual structures).

## *Preparation of conidia suspensions*

Seven days old cultures grown on angled Sabouraud dextrose agar (SDA) (HiMedia, Mumbai, India) were used for preparation of the mold conidia suspensions. Conidia suspensions were prepared in physiological solution which contained 0.1% Tween 80 (HiMedia, Mumbai, India). Concentration of  $10^6$  conidia/ml was adjusted by hemocytometer (Bürker-Türk chamber) (Precise, Peillonnex, France).

## *Disc diffusion method*

Testing of essential oils antifungal activity against the selected molds growth was conducted by disc diffusion method (Leboffe and Pierce, 2005). Previously prepared Petri plates (Ø 90 mm) were filled by 20 ml of SDA. Well dried surface was inoculated with the conidia suspension (final inoculum  $10^6$  conidia/ml) along the surface, by the sterile swab. The suspension was equally spread in order to obtain a homogeneous growth. Inoculated Petri plates were left for 15 minutes at room temperature to achieve a good absorption of the suspension. After 15 minutes, sterile discs with diameter of 6 mm (HiMedia, Mumbai, India) were placed over inoculated plates. Furthermore, a selected quantity of essential oils (0.5, 1, 5, and 10 µl) was applied by micropipette onto discs.

Thereafter, inoculated plates were incubated at 25 °C for 48 hours. Inhibition of growth was evaluated by the measuring of inhibition zone diameter (including the diameter of the disc) in mm. As a negative control, sterile distilled water was used. On the basis of measured inhibition zones and comparison of the obtained values, the effects of mint and caraway essential oil on the mold isolates growth was characterized.

## *Statistical analysis*

Antimicrobial testing was performed in triplicate. MC Excel (Microsoft office Professional Plus 2010, Microsoft, USA) was used for calculating average values and standard deviation of disc diffusion method.

## RESULTS AND DISSCUSION

### Inhibitory activities of essential oils against mold growth

The mint essential oil, in the case of disc diffusion method, exhibited antifungal activities against every tested isolate. The inhibitory effect was presented in Table 1.

Table 1. Antifungal effect of mint essential oil against mold growth

Molds	Inhibition zone diameter (mm)				
	Vol. (μl/disc)	0.5	1	5	10
<i>Alternaria alternata</i>		*	*	23.33±0.58	w.g.
<i>Aspergillus flavus</i>		7.0 ±0.0	8.33±0.58	14.67±0.58	25.67±0.58
<i>Aspergillus niger</i>		7.33±0.58	9.67±0.58	17.33±1.15	20.33±1.53
<i>Aspergillus versicolor</i>		*	*	*	w.g.
<i>Eurotium herbariorum</i>		10.33±0.58	11.33±1.15	w.g.	w.g.
<i>Penicillium aurantiogriseum</i>		10.33±0.58	12.67±0.58	19.67±0.58	44.67±0.58
<i>Penicillium chrysogenum</i>		0*	6.67±0.58	9.33±0.58	13.67±0.58
<i>Penicillium expansum</i>		14.33±0.58	15.33±0.58	19.33±0.58	27.67±0.58

w. g. – without growth; \* – reduced growth; 0\* – without inhibition zone

The inhibition zone was not observed when the smallest quantity of essential oil was applied (0.5 μl), only against *P. chrysogenum*. Regarding two species, *A. alternata* and *A. versicolor*, it was observed a slightly reduced growth across the whole surface of Petri dish without inhibition zone around the disc. The largest inhibition zone was measured against *P. expansum* growth (14.33 mm). The quantity of 1 μl showed inhibitory activity against all tested molds. The reduced growth was observed against *A. alternata* and *A. versicolor*. The lowest inhibition zone was recorded against *A. flavus* (8.33 mm), while a higher level of inhibition was noticed against *P. expansum* growth (15.33 mm). Higher inhibitory activity was detected when larger volume of essential oil (5 μl) was applied to all tested isolates. The lowest inhibitory zone was recorded against *P. chrysogenum* (9.33 mm), and the highest against *A. alternata* growth (23.33 mm). Inhibitory effect on *A. versicolor* was detected as the growth reduction, however, still without forming of clear inhibition zone around the disc. In the case of *E. herbariorum*, the complete growth inhibition was observed. When the highest quantity of essential oil was applied (10 μl), the complete inhibition of *A. alternata* and *A. versicolor* growth occurred. Inhibition zone of other species was in the range from 13.67 mm (*P. chrysogenum*) to 44.67 mm (*P. aurantiogriseum*). It can be concluded that the mint essential oil had the strongest impact on *E. herbariorum*, and the weakest on *P. chrysogenum*.

Moghtader (2013) reported that mint essential oil showed antifungal effect on *A. niger*. By the oil treatment from 1,600 ppm to 200 ppm, using the method

of double dilution, significant differences in inhibition zones from 26 mm to 8 mm were recorded, respectively. The mentioned author linked a good antifungal effect with the menthol content (38.33%) in tested oil.

According to Helal *et al.* (2006), by 50  $\mu$ l (agar diffusion method) treatment of mint essential oil against *A. flavus* and *A. niger*, inhibition zones were 29 and 31 mm, whereas against *Penicillium digitatum* and *P. puberulum* were 33 and 34 mm, respectively. Kazemi *et al.* (2012) reported that mint essential oil, in 1  $\mu$ g/ml concentration, shows antifungal effect on *A. niger*, *A. flavus*, and *A. fumigatus*, whereby 20 mm inhibition zones were recorded. However, the effect was slightly lower on *Mucor* sp. with inhibition zones of 15 mm.

Essential oil of caraway, in the case of the disc diffusion method of 0.5, 1, 5, and 10  $\mu$ l, exhibited antifungal activity against every tested isolate. Inhibitory effects are presented in Table 2.

Table 2. Antifungal effect of caraway essential oil on mold growth

Molds	Inhibition zone diameter (mm)				
	Vol. (μl/disc)	0.5	1	5	10
<i>Alternaria alternata</i>		12.33±2.08	19.33±0.58	41.33±1.15	44.33±2.08
<i>Aspergillus flavus</i>		17.33±0.58	24.67±0.58	32.67±0.58	39.33±1.15
<i>Aspergillus niger</i>		10.33±0.58	11.33±0.58	23.0±1.0	28.0±1.0
<i>Aspergillus versicolor</i>		18.67±0.58	26.0±1.73	45.67±1.53	52.0±2.0
<i>Eurotium herbariorum</i>		17.33±1.15	21.33±0.58	40.33±1.53	w.g.
<i>Penicillium aurantiogriseum</i>		20.33±2.08	26.0±1.73	36.33±0.58	43.33±1.53
<i>Penicillium chrysogenum</i>		33.67±0.58	37.67±1.53	44.0±1.0	43.67±0.58
<i>Penicillium expansum</i>		18.67±1.15	20.67±1.15	34.0±1.73	47.0±1.0

w. g. – without growth

Inhibitory activity using the smallest quantity (0.5  $\mu$ l) was recorded against all isolates, and the highest inhibition zone was observed against *P. chrysogenum* (33.67 mm). By using higher concentrations of caraway essential oil (1 and 5  $\mu$ l), a greater antifungal effect was observed on all tested isolates. The total inhibition was registered against *E. herbariorum* when using the highest quantity of oil (10  $\mu$ l), while the highest inhibition zone was observed against *A. versicolor* (52 mm), and the lowest against *A. niger* (Table 2).

Application of the caraway essential oil in the quantity of 50  $\mu$ l, by the agar diffusion method, did not show inhibition zones against *A. flavus*, while in the case of *A. niger*, *P. digitatum*, and *P. puberulum*, inhibition zones were 22, 18 and 27 mm, respectively (Helal *et al.* 2006). Mironescu *et al.* (2009) published similar results of a screening disc diffusion method that showed a better antifungal effect of mint essential oil compared to caraway essential oil against the tested isolate of *A. alternata*. According to Ertürk (2006), the crude extracts from *M. piperita* showed antifungal activity against *A. niger* with inhibition zone diameters of 18 mm/15  $\mu$ l.

## CONCLUSION

Essential oils of mint and caraway demonstrated antifungal activity against every tested isolates. The most powerful inhibitory effect mint essential oil in the quantity of 5 µl demonstrated against *E. herbariorum* as complete growth inhibition. The weakest inhibitory effect was observed against *P. chrysogenum* (inhibition zone 13.67 mm) in the quantity of 10 µl. The most powerful antifungal activity of caraway was observed against *E. herbariorum* as a complete growth inhibition. The weakest inhibitory effect was observed against *A. niger* (inhibition zone 28 mm) in the quantity of 10 µl.

## ACKNOWLEDGEMENTS

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## АНТИФУНГАЛНА АКТИВНОСТ ЕТАРСКОГ УЉА *Mentha piperita* И *Carum carvi*

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**РЕЗИМЕ:** Ароматичне биљне врсте представљају неке од најважнијих извора биолошки активних секундарних метаболита који поседују различите антимикробне карактеристике. Циљ овога рада био да се испита антифунгална активност етарског уља менте и кима према одређеним лабораторијским изолатима плесни. За испитивање антифунгалне активности изабрано је осам врста плесни: *Alternaria alternata*, *Aspegillus flavus*, *A. niger*, *A. versicolor*, *Eurotium herbariorum*, *Penicillium aurantiogriseum*, *P. chrysogenum* и *P. expansum*. Испитивање антифунгалне активности етарских уља на одабране врсте плесни изведено је диск дифузионом методом додавањем етарских уља у количинама од 0,5; 1; 5 и 10  $\mu$ l по диску. Антифунгална активност етарских уља процењивана је мерењем дијаметра зоне инхибиције. Најјачи ефекат етарског уља менте забележен је у случају *E. herbariorum* при чему је уочена потпуна инхибиција при количини од 5  $\mu$ l. У случају *P. chrysogenum* уочен је најслабији ефекат при чему је измерена зона инхибиције износила 13,67 mm при количини од 10  $\mu$ l. Етарско уља кима показало је најјачи ефекат у случају *E. herbariorum* при чему је забележена потпуна инхибиција. У случају *A. niger* забележена је најслабија антифунгална активност етарског уља кима при чему је измерена зона инхибиције била 28 mm при количини од 10  $\mu$ l уља.

**КЉУЧНЕ РЕЧИ:** антифунгална активност, етарска уља, ким, мента



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## COMPARISON OF ANTIOXIDANT CAPACITIES OF TWO *Ganoderma lucidum* STRAINS OF DIFFERENT GEOGRAPHICAL ORIGINS

**ABSTRACT:** *Ganoderma lucidum*, used in a traditional Chinese medicine, represents one of the most important medicinal mushrooms in the world, whose fruiting bodies and spores have been traditionally used because of a wide spectrum of biological activities such as antidiabetic, antioxidative, antiproliferative, cardioprotective, etc. Its ethnomedicinal importance in some parts of the Balkan region (Serbia and Croatia) is almost totally unknown and there should be more scientific investigations carried out. The aim of this work was to make a comparative study of antioxidative activities and total phenolic content of ethanol and hot water extracts of *G. lucidum*, collected from forests in Serbia (Morovićke šume, Fruška Gora) and Croatia (Donji Lapac, Plješevica). The present study was carried out to evaluate antioxidant potential of examined extracts via scavenging potential on ABTS, DPPH, OH<sup>•</sup> and NO<sup>•</sup> radicals, as well as of chelating effects via FRAP assay, together with determination of their total phenolic content. Results showed that both GLS extracts possessed better antiradical activities ( $IC_{50}=0.23\pm0.01$  for H<sub>2</sub>O and  $2.75\pm0.01$  µg/mL for EtOH for OH<sup>•</sup> and DPPH assay, respectively) than in the ABTS assay ( $151.40\pm1.07$  mg TE/g d.w. for EtOH), while the phenolic content was generally equal in extracts of Serbian and Croatian samples ( $60.74\pm0.57$  mg GAE/g d.w. for EtOH and  $77.10\pm0.27$  mg GAE/g d.w. for H<sub>2</sub>O, respectively). Therefore, these extracts could be considered as a good source of natural antioxidants. These results showed that examined *G. lucidum* extracts (especially H<sub>2</sub>O) contain high amount of phenolic content which could significantly enhance the antiradical potential and reduce potential on iron ions. This is the first study reporting the comparison of antioxidant activities and phenolic contents of two different extracts between two *G. lucidum* strains from two different geographical origins from the Balkan region.

**KEYWORDS:** antioxidant capacity, *Ganoderma lucidum*, phenols, reducing power, radical reduction

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## INTRODUCTION

In the last decades there has been a growing interest in the study of antioxidant activities of different foods possessing abundant free radicals scavengers and having an effect on many reactions in food systems (Klaus *et al.* 2011). It is also well documented that the implication of free radicals in oxidative stress represents one of the main causing agents in many diseases (Kozarski *et al.* 2015). Antioxidant compounds have the ability to trap free radicals and thus inhibit the oxidative mechanisms that lead to various degenerative disorders, including cancerogenesis, neurodegenerative and cardiovascular diseases (Ferreira *et al.* 2009; Janjušević *et al.* 2017; Nagaraj *et al.* 2014).

All organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against free radicals, but there are various exogenous antioxidants ( $\alpha$ -tocopherol, ascorbic acid, carotenoids, glutathione, and polyphenols) which can be found in whole grains, fruits, vegetables, teas, spices, and herbs (Nagaraj *et al.* 2014). Mushrooms are also organisms attractive as a source of antioxidants, which demonstrate antiradical activities and also reducing capacities (Ferreira *et al.* 2009; Karaman *et al.* 2014; Rašeta, 2016). Some advantages of using mushrooms in food as a good source of bioactive compounds are the following: the fruiting bodies can be produced in much less time, the mycelium may be rapidly produced in liquid culture, and the extracellular culture medium can be easily manipulated in order to produce optimal quantities of active products (Tešanović *et al.* 2017).

Mushrooms contain a variety of complex compounds that can be derived from both secondary and primary metabolism such as phenolics, polyketides, polysaccharides, triterpenoids, and steroids that characterize each type of fungal species and possess specific medicinal effects including antimicrobial, antioxidant, antiproliferative, etc. (Brakhage and Schroeckh, 2011; Klaus *et al.* 2011; Kozarski *et al.* 2015; Novaković *et al.* 2016; Rašeta, 2016; Rašeta *et al.* 2016; Zhang *et al.* 2016).

The presence and composition of all of these compounds in fungi mainly depend not only on genetic determinants, but also on ecological factors, including particular environmental conditions in specific habitats and their lifestyle (saprotrophic, parasitic and symbiotic/e.g. mycorrhizal) (Chaumonton *et al.* 1993).

A recent review on the chemical composition and biological properties of *Ganoderma* species has described this genus as a therapeutic biofactory (Paterson, 2006). A great deal of work on *Ganoderma* species has been carried out on one peculiar species, namely *G. lucidum* (Lingzhi, Munnertake, Sachitake, Reishi, and Youngzhi), which is the best known medicinal mushroom all over the world (Paterson, 2006). For hundreds of years, this species has been regarded as a traditional folk medicine using mushroom for the prevention and treatment of various human diseases, such as hepatitis, hypertension, chronic bronchitis, bronchial asthma, cancer, and others (Baby *et al.* 2015; Boh *et al.* 2007; Paterson, 2006; Rašeta, 2016).

In recent years, phytochemical studies of *G. lucidum* resulted in an impressively large number of more than 400 different bioactive compounds isolated (Baby *et al.* 2015): polysaccharides (Chiu *et al.* 2017; Kozarski *et al.* 2014), triterpenoids (Baby *et al.* 2015; Boh *et al.* 2007; Wang *et al.* 2006), and phenolic compounds (Rašeta, 2016; Rašeta *et al.* 2016; Stilinović *et al.* 2014; Yildiz *et al.* 2015). Based on literature data, many of these compounds have been already used as antioxidant agents, especially phenolic compounds that could be the major determinants of their antioxidant potentials (Balasundram *et al.* 2006; Ferreira *et al.* 2009). This was demonstrated in recent studies through the high correlation coefficient of analysed antioxidant activities and TP contents (Novaković *et al.* 2016; Rašeta *et al.* 2016).

The aim of the present study was to analyse antioxidative activities of the ethanol and hot water extracts, as well as their phenolic contents. Two different strains of wild medicinal mushrooms *G. lucidum* originating from Serbia and Croatia were compared using four radical scavengers and the reducing power assays *in vitro*.

## MATERIAL AND METHODS

### *Fungal material*

Fruiting bodies of *G. lucidum* were collected during the autumn of 2010 (Morovičke šume, Serbia) and the spring of 2016 (Donji Lapac, Croatia). After the identification of the species, voucher specimens were identified by MSc Eleonora Bošković at the Herbarium BUNS, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad under number (12-00715 and 12-00724). Air-dried samples were ground to a fine powder and stored in dark bottles at room temperature prior to analysis.

### *Preparations of extracts*

For the preparation of ethanolic extracts (EtOH), 20 g of powdered fungal material were extracted by maceration with 96% EtOH (JT Baker, Netherlands) during 72 h at room temperature (Sekljalnik S400 W Chopper, Gorenje).

For the preparation of the hot water extracts (H<sub>2</sub>O), 20 g of fungal material were mixed with 200 ml of boiling water and incubated at 80 °C for 60 min in water bath (Elektromedicina, Slovenia). After filtration (Whatman No. 4 Filter paper), EtOH was evaporated *in vacuo* (Büchi R-210; Büchi Labortechnik AG, Switzerland) at 35 °C and crude residue was dissolved in distilled water (10 mg/mL), whereas H<sub>2</sub>O was stored at -20 °C and then lyophilized (Christ Alpha 1-2 LD Freeze Dryer, Switzerland) for 72–96 h in ice condenser at -55 °C. Dried extracts were dissolved in distilled water to obtain 100 mg/mL stock solutions.

## ANTIOXIDANT ACTIVITY

### *ABTS assay*

The ABTS assay (Arnao *et al.* 2001) was used to measure radical scavenging activity of the extracts and standard compound, propyl gallate (PG). 10 ml of each extract or standard compound (trolox, vitamin E analogue) (except in a control probe when only the solvents were used) was added to 290  $\mu$ l of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) or distilled water (corrections of work sample). Absorbance was measured spectrophotometrically at 515 nm after 30 min of reaction. The same procedure was repeated with PG as a positive control. The absorbance was measured at 734 nm after 5 min of reaction. All the samples were made in triplicate and mean values of ABTS scavenging activity were expressed as milligram trolox equivalents (TE) per gram of dry weight (mg TE/g d.w.), calculated according to the standard curve.

### *DPPH assay*

The DPPH assay was performed as described before (Espin *et al.* 2000), following the transformation of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical to its reduced, neutral form (DPPH-H). 10  $\mu$ l of each extract or standard compound (except in a control probe when only the solvents were used) was added to 60  $\mu$ l of DPPH reagent and mixed with 180  $\mu$ l of methanol. Absorbance was measured spectrophotometrically at 515 nm after 30 min of reaction. The same procedure was repeated with PG as a positive control. The antioxidant activity was expressed as a concentration of the sample that inhibited the DPPH radical formation by 50% (IC<sub>50</sub>), and the experiment was performed in triplicate.

### *OH assay*

The hydroxyl radical scavenging activity was determined according to the method of Halliwell *et al.* (1987). 10  $\mu$ l of each extract or standard compound (except in a control probe when only the solvents were used), was mixed with 100  $\mu$ l of 0.015% H<sub>2</sub>O<sub>2</sub>, 100  $\mu$ l of 10 mmol/L FeSO<sub>4</sub>, 100  $\mu$ l of 0.05 mol/L 2-deoxy-D-ribose, and 2.7 ml of phosphate buffer (pH=7.4). After incubation at room temperature for 60 min, 200  $\mu$ l of 0.1 M EDTA and 2 ml TBA reagent were added to the mixture and heated at 100 °C for 10 min. After cooling, aliquots of 250  $\mu$ l were transferred to the plate, and absorbance was measured at 532 nm. The antioxidant activity was expressed as IC<sub>50</sub> ( $\mu$ g/mL), and the experiment was performed in triplicate.

### *NO assay*

Nitric oxide scavenging capacity was determined according to the method of Green *et al.* (1982). 30  $\mu$ l of each extract and PG (except in a control probe when only the solvents were used) was added to 500  $\mu$ l SNP and 500  $\mu$ l of phosphate buffer (pH=7.4). Test tubes were incubated at room temperature for 90 min under light exposure. After the incubation, 1 ml of Griess reagent was added to all samples. Aliquots of 250  $\mu$ l were transferred to the plate and absorbance was measured at 546 nm. The antioxidant activity was expressed as IC<sub>50</sub> ( $\mu$ g/mL), and the experiment was performed in triplicate.

#### *FRAP assay*

The Ferric reducing antioxidant power (FRAP) assay was done according to Benzie and Strain (1999). A volume of 10  $\mu\text{L}$  of the sample was transferred to a 96 microwell plate with 225  $\mu\text{L}$  of FRAP reagent and 22.5  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ . The absorbance was determined at 593 nm after incubation of 6 min. All the samples were made in triplicate and mean values were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of dry weight (mg AAE/g d.w.), calculated according to the standard calibration curve.

#### *Determination of total phenolic content*

Total phenolic content in the analysed extracts was determined according to the method by Singleton *et al.* (1999). 25  $\mu\text{L}$  of each extract or standard compound (gallic acid) (except in a control probe when only the solvents were used) was added to 125  $\mu\text{L}$  of Folin-Ciocalteu (FC) reagent and mixed after 10 min with 100  $\mu\text{L}$  of sodium carbonate. Absorbance was measured spectrophotometrically (Thermo Scientific Multiskan Spectrum Microplate Photometers – Photometer with Curvette Holder, Model 51118650) at 760 nm after 2h of reaction.

#### *Statistical Analysis*

The results were expressed as mean values  $\pm$  standard deviation (SD).  $\text{IC}_{50}$  values ( $\mu\text{g/mL}$ ) were determined by the linear regression analysis of RSC (Microsoft Excel for Windows, v. 2010 and Origin 8). Statistical analysis was performed using one-way analysis of variance ANOVA with post hoc Turkey's test (STATISTICA; version 10.0; [www.statsoft.com](http://www.statsoft.com)). Correlation coefficient (Microsoft Excel for Windows, v. 2010) was used to estimate the relationship between the antioxidant activity of extracts and TP contents.

## RESULTS AND DISCUSSION

#### *Antioxidant activity and TP content*

Antioxidant activity is manifested in a wide variety of actions, such as inhibition of oxidizing form of enzymes, chelating of metal ions, transfer of H-atom or electrons to radicals, singlet oxygen deactivation, etc. (Halliwell, 1996). Antioxidant compounds found in fruiting bodies, mycelium and extracellular broth so far have been confirmed to be mainly phenolic compounds (phenolic acids and flavonoids), followed by other compounds such as ergothioneines, glycosides, polysaccharides, and vitamins (tocopherols, carotenoids, and ascorbic acid) (Ferreira *et al.* 2009; Kozarski *et al.* 2015). Mushroom antioxidants can exhibit their protective properties at different stages of the oxidation process and by different mechanisms: primary (scavenging of free radicals and chain breaking)

and secondary (regeneration of primary antioxidants, inhibition of lipid peroxidation and quenching of singlet oxygen) (Brewer, 2011; Kozarski *et al.* 2015).

A whole range of scientific data reported high antioxidant potentials of different types of *G. lucidum* extracts (Deepalakshmi *et al.* 2013; Ferreira *et al.* 2009; Rašeta *et al.* 2016; Saltarelli *et al.* 2009; Stilinović *et al.* 2014; Tel *et al.* 2015; XiaoPing *et al.* 2009; Yildiz *et al.* 2015; Zhang *et al.* 2016), but still there is no data for the strain from Croatia. The obtained results for the TP content and antioxidant activity of analysed extracts are shown in Table 1. Although both types of extracts possessed antioxidant properties, H<sub>2</sub>O extracts demonstrated higher antioxidant activity than EtOH extracts, mainly for GLS in OH, DPPH, and FRAP assays ( $IC_{50}=0.23\pm0.01$  µg/mL,  $IC_{50}=2.75\pm0.01$  µg/mL and  $696.38\pm1.33$  mg AAE/g d.w., respectively) and NO assay for GLC ( $IC_{50}=62.16\pm1.14$  µg/mL). We compared activities of analysed extracts using standard compound PG and all obtained results are statistically significant with the exception of OH assay ( $IC_{50}=0.66\pm0.01$  µg/mL for PG and  $IC_{50}=0.23\pm0.01$  µg/mL for H<sub>2</sub>O of Croatian samples, respectively) (Table 1).

The ABTS radical reactions involve electron transfer and the process take place at a faster rate when compared to DPPH radicals (Arnao *et al.* 2001). In the present study, EtOH extracts of *G. lucidum* strain from Serbia (GLS) and Croatia (GLC) showed weaker activity in ABTS' reduction ( $151.40\pm1.07$  mg TE/g d.w. and  $107.55\pm1.11$  mg TE/g d.w., respectively) in comparison with PG ( $711.53\pm1.94$  mg TE/g d.w.). In summary, the ABTS radical scavenging activity was more active in EtOH extracts, which is in high correlation with TP content ( $R^2=0.93-0.98$ ) (Table 1).

In the present study, analyzed extracts showed concentration-dependent activity in DPPH and FRAP assays, which may be attributed to its hydrogen-donating ability via high reducing power ability confirmed for both H<sub>2</sub>O extracts GLS and GLC ( $696.38\pm1.33$  mg AAE/g d.w. and  $127.00\pm0.29$  mg AAE/g d.w., respectively). The reducing properties are generally high for H<sub>2</sub>O extract ( $696.38\pm1.33$  mg AAE/g d.w. for GLS and  $127.00\pm0.27$  mg AAE/g d.w. for GLC, respectively) and associated with the TP content with the exception of EtOH extract of GLS (Table 1).

Hydroxyl radical is the most reactive among free radical species and obtained results of antiradical activities were the highest for the GLS ( $IC_{50}=0.23\pm0.01$  µg/mL for H<sub>2</sub>O and  $IC_{50}=05.35\pm0.01$  µg/mL for EtOH extract) which was in the range of activity for PG (Table 1).

In the present study, scavenging activity on NO radicals was relatively low ( $IC_{50}=765.28\pm0.71 - 1108.30\pm2.82$  µg/mL) with the exception of H<sub>2</sub>O extract for GLC ( $IC_{50}=62.16\pm1.14$  µg/mL) (Table 1).

EtOH extracts of GLS had high amount of TP ( $60.74\pm0.57$  mg GAE/g d.w.) and our previous study showed high correlation between TP content and obtained phenolic profile via LC-MS/MS, where the content of protocatechuic, p-hydroxybenzoic, p-coumaric, and quinic acids dominated (Rašeta *et al.* 2016), while H<sub>2</sub>O extracts of GLC had the highest TP content ( $77.10\pm0.27$  mg GAE/g d.w.) and its phenolic profile has been unknown so far.

Obtained results showed lower activities for analyzed EtOH extracts for ABTS assay than MeOH extracts of *G. lucidum* from Turkey (Tel *et al.* 2015), EtOH extracts of the same species from India and Italy (Deepalakshmi *et al.* 2013; Saltarelli *et al.* 2009) and ~ 5 times higher activity than MeOH extracts (Rani *et al.* 2015). Products which scavenge DPPH *in vitro* may scavenge polyaromatic hydrocarbon cations *in vivo* (Deepalakshmi *et al.* 2013).

Obtained results for DPPH and FRAP assays showed mostly higher values than the values obtained from the literature data (Deepalakshmi *et al.* 2013; Rani *et al.* 2015; Rašeta *et al.* 2016; Saltarelli *et al.* 2009; Tel *et al.* 2015).

Our results are in accordance with data presented in previous studies for OH<sup>-</sup> assay (Deepalakshmi *et al.* 2013; Mohsin *et al.* 2011).

Generally, obtained results showed that all analyzed extracts, except H<sub>2</sub>O/GLS, contained high TP content (Table 1). These results showed higher values than in previous studies for the same fungal species from Korea and Serbia (Hasnat *et al.* 2013; Kim *et al.* 2014; Veljović *et al.* 2017). Phenolic compounds may be the key components accounting for the demonstrated results and manifested antioxidant activities, statistically determined through correlations (Table 1) (Novaković *et al.* 2016).

Table 1. Antioxidant activity, TP content and correlations between TP and antioxidant activities of two *G. lucidum* fungal strains

Assay	GLS		GLC		PG
	EtOH	H <sub>2</sub> O	EtOH	H <sub>2</sub> O	
<b>ABTS</b> (mg TE/g d.w.)	151.40±1.07 <sup>a</sup>	23.30±2.15 <sup>d</sup>	107.55±1.11 <sup>b</sup>	102.42±0.60 <sup>c</sup>	711.53±1.94 <sup>a</sup>
<b>DPPH</b> (µg/mL)	3.64±0.15 <sup>b</sup>	2.75±0.01 <sup>b</sup>	56.51±2.80 <sup>d</sup>	37.16±0.29 <sup>c</sup>	0.53±0.02 <sup>a</sup>
<b>OH</b> (µg/mL)	5.35±0.01 <sup>b</sup>	0.23±0.01 <sup>a</sup>	95.68±2.15 <sup>c</sup>	173.95±0.33 <sup>d</sup>	0.66±0.01 <sup>a</sup>
<b>NO</b> (µg/mL)	765.28±0.71 <sup>c</sup>	1108.30±2.82 <sup>d</sup>	1014.65±0.01 <sup>c</sup>	62.16±1.14 <sup>b</sup>	0.48±0.01 <sup>a</sup>
<b>FRAP</b> (mg AAE/g d.w.)	111.52±0.94 <sup>c</sup>	696.38±1.33 <sup>a</sup>	76.46±0.73 <sup>d</sup>	127.00±0.29 <sup>b</sup>	–
<b>TP</b> (mg GAE/g d.w.)	60.74±0.57 <sup>b</sup>	11.55±0.30 <sup>d</sup>	42.78±0.32 <sup>c</sup>	77.10±0.27 <sup>a</sup>	
<b>R<sup>2</sup>*</b>					
<b>ABTS</b> TP	0.98*	0.80*	0.93*	0.96*	
<b>DPPH</b> TP	0.94*	0.93*	1.00*	1.00*	
<b>OH</b> TP	0.13	1.00*	0.96*	0.98*	
<b>NO</b> TP	0.94*	0.49	0.98*	1.00*	
<b>FRAP</b> TP	0.29	0.76*	0.71*	0.87*	

Legend: GLS – *G. lucidum* (Serbia); GLC – *G. lucidum* (Croatia); PG – propyl gallate (synthetic antioxidant); TP – total phenol content

Data are reported as mean ± standard deviation of triplicates.

<sup>a,b,c,d,e</sup> – different letters in the same row indicate significant difference between extracts (p<0.01)

R<sup>2</sup> – correlation coefficient between antioxidant activity and TP content, \* – all values are statistically significant (p<0.05)

The correlation between the TP and antioxidant capacity was significant (specifically for GLC extracts), and these results suggested that a significant amount of phenolic compounds was strongly linked to antioxidant and reducing power of analyzed fungal extracts. Furthermore, the chemical characterizations and determinations of biopotentials of autochthonous species from different geographical regions could have a great importance for future use of mushrooms as potential medicinal supplements.

## CONCLUSION

In the present study, two types of *Ganoderma lucidum* extracts (EtOH, H<sub>2</sub>O) from different localities (Serbia-GLS and Croatia-GLC) were investigated for their antioxidant, reducing power potential and total phenolic content.

Our results suggest that *G. lucidum* and their extracts could be very good sources of naturally-derived antioxidants. The obtained assays showed that H<sub>2</sub>O extracts in analyzed GLS and GLC extracts had mainly better antioxidant activities than EtOH, with the exception of ABTS assay for both strains (151.40±1.07 mg TE/g d.w. for GLS and 107.55±1.11 mg TE/g d.w. for GLC, respectively), OH assay for GLS (IC<sub>50</sub>=0.23±0.01 µg/mL), and NO assay for GLS (IC<sub>50</sub>=765.28±0.71 µg/mL). PG, as a derivative of gallic acid, is essential phenol which is used in foodstuffs to protect fats against oxidative rancidity and showed the most powerful antioxidant activity with the exception of scavenging of OH radical (IC<sub>50</sub>=0.66 µg/mL and IC<sub>50</sub>=0.23 µg/mL for GLS H<sub>2</sub>O extract, respectively).

The antioxidant activity of analyzed extracts had high correlation with the content of their TP, therefore the fungal phenols may act as antioxidants.

This study suggests that H<sub>2</sub>O extract of *G. lucidum* generally possesses better antioxidant activity, which firmly supports further studies with an aim to promote consumption of H<sub>2</sub>O extracts in the form of tea or soups of *G. lucidum* in the Balkan region, which represent food supplements rich in phenolics with valuable health benefits for humans.

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## ПОРЕЂЕЊЕ АНТИОКСИДАНТНИХ КАПАЦИТЕТА ДВА СОЈА *Ganoderma lucidum* РАЗЛИЧИТОГ ГЕОГРАФСКОГ ПОРЕКЛА

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**РЕЗИМЕ:** *Ganoderma lucidum* представља једну од најзначајних медицинских гљива на свету, чије се плодно тело и споре традиционално употребљавају у ки-неској медицини због широког спектра биолошких активности: антидијабетске, антиоксидативне, антипролиферативне, кардиопротективне. Међутим, још је непознат етномедицински значај у појединим деловима Балкана, као што су Србија и Хрватска. Циљ овог рада је упоредна студија антиоксидантне активности и садржаја укупних фенола етанолних и водених екстраката *G. lucidum* из Србије (Моровићке шуме, Фрушка гора) и Хрватске (Доњи Лапац, Пљешевца). Одређена је антиоксидантна активност екстраката праћењем потенцијала редукције ABTS, DPPH и OH радикала, хватања NO као и редукције Fe<sup>3+</sup> у Fe<sup>2+</sup> FRAP тестом, као и носиоци те активности, тј. садржај укупних фенола. Резултати указују да су екстракти узорака из Србије добро редуковали OH<sup>•</sup> и DPPH<sup>•</sup> (IC<sub>50</sub>=0.23±0.01 за H<sub>2</sub>O односно 2.75±0.01 µg/mL за EtOH, потом ABTS<sup>•+</sup> (151.40±1.07 mg TE/g с.м. за EtOH), док је садржај фенола мало скоро исти и у српским и у хрватским узорцима (77.10 ±0.27 mg GAE/g d.w. за H<sub>2</sub>O односно 60.74±0.57 mg GAE/g d.w. за EtOH) те се ови екстракти могу сматрати значајним изворима природних антиоксиданата. Ови резултати показују да испитивани екстракти (нарочито H<sub>2</sub>O) садрже висок садржај фенола, који су главни носиоци антиоксидативне активности. Ово је прва студија у којој је саопштено поређење антиоксидативне активности и садржаја фенола екстраката два соја *G. lucidum* различитог географског порекла са територије Балкана.

**КЉУЧНЕ РЕЧИ:** антиоксидантни капацитет, *Ganoderma lucidum*, феноли, редукциона моћ, редукција радикала



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## ASSESSMENT OF DIESEL FUEL UPTAKE BY FUNGI ISOLATED FROM PETROLEUM CONTAMINATED SOIL

**ABSTRACT:** The aim of this research was to isolate and identify the fungi from petroleum-contaminated soil, to test their ability to grow in the presence of diesel fuel and uptake it in solid and broth media. Among 16 isolated and identified filamentous fungi, six were tested to determine their growth on media enriched with diesel fuel as a sole carbon source. All tested isolates showed different degree of diesel fuel utilization. Colony diameter was measured, where the highest colony growth dynamics ratio was demonstrated for *Penicillium* sp. (74.16%) and *Cladosporium* sp. (71.43%), and the lowest for *Absidia spinosa* (23.15%). Results of dry mass measurements demonstrated the highest potential of *A. strictum* (71.43%) to utilize diesel fuel in broth medium. All other tested isolates showed insignificant dry mass production ratio values. The highest utilization degree was documented for *A. strictum* (60.28 %) and the lowest for *Penicillium* sp. (25.18%).

**KEYWORDS:** diesel fuel utilization, fungi, soil

## INTRODUCTION

Diesel represents a heavy petroleum fraction used as a fuel in diesel engines. Petroleum or crude oil is, like all fossil fuels, a complex mixture of polyaromatic hydrocarbons (PAHs) and hundreds of different hydrocarbon components like paraffins, naphthenes, phenols, etc. (Al-Jawhari, 2014). In high concentrations, hydrocarbons from this petroleum product mixture are highly toxic for most organisms. However, petroleum products are dominant in the world economy, which consequently leads to distribution of these pollutants in ecosystems, especially in industrial areas (Adekunle and Adebambo, 2007). Leakage of these

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products into soil occurs often in regions with oil production and processing, where they can change physical and chemical characteristics of the soil and have a strong impact on the environment. Nowadays, petroleum-contaminated soil is becoming a global problem, as polluted grounds lack agricultural, industrial and recreational properties, and often become a source of surface and underground water pollution (AI-Jawhari, 2014).

There are several soil purification methods including incineration, washing, chemical treatment and bioremediation. Bioremediation is defined as utilization of plants and microorganisms to remove or detoxify environmental contaminants (Mohsenzadeh *et al.* 2012). The ability to actively degrade specific petroleum fractions has been proven for many microorganisms. This process has been intensively studied over the past two decades, urged by cost-effective, sustainable with natural environment, and *in situ* alternative to more expensive engineering-based remediation technologies (AI-Jawhari, 2014; Mohsenzadeh *et al.* 2012). However, bioremediation is quite a time-consuming process, and some bacteria need many years in order to obtain adequate results (Adekunle and Adebambo, 2007; Thomas *et al.* 1998). Hence, the usage of combined cultures of bacteria and fungi was proposed, especially because different components of petroleum are differently susceptible to degradation (AI-Jawhari, 2014). It has been shown that fungi have stronger petroleum degradation ability than bacteria and fungi combined, and that mycoremediation is a more effective method than traditional microbiological techniques (Thomas *et al.* 1998).

Previous researches have shown that some fungal species are resistant to petroleum product pollution and have the ability to degrade it in order to purify the soil. These researches were usually conducted in polluted areas of countries rich in oil, like Saudi Arabia or Iran, where petroleum product pollution caused significant treat to environment. Fungal species usually isolated from oil polluted soils were: *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosus*, *Penicillium notatum* and *Ulocladium atrum*. It was also shown that *Pleurotus ostreatus*, a widely known edible and medical mushroom, had the ability to degrade PAH in soil (Mohsenzadeh *et al.* 2012).

Soils in the vicinity of gas stations are assumed to contain a lot of petroleum products and also to be habitats of microorganisms that can degrade these products (Popov *et al.* 2008). The aim of this study was to isolate and identify the soil microfungi from immediate surroundings of gas stations in selected urban area, as well as to test their ability to degrade diesel fuel.

## MATERIAL AND METHODS

### *Isolation and identification of microfungi*

Soil was sampled using the sterile scoop in immediate surroundings of gas stations in the center of town Valjevo. Samples were transferred to the laboratory in sterile plastic bags, than diluted in sterile distilled water (1g/ml)

and inoculated on standard mycological media Malt extract agar (MEA) and Czapeck agar (CzA), in triplicates. Primary isolates were incubated in thermostat (Memmert UE500) at  $25\pm 1$  °C, and the growth of fungal colonies was successively measured for seven days. Morphologically different colonies were reinoculated on sterile MEA and incubated under the same conditions, in order to get axenic cultures. After the incubation period, isolates were identified using dichotomous keys, based on colony morphology and microscopic features of reproductive structures (Samson *et al.* 2010; Watanabe, 2002). During the preparation of microscopic slides, hyaline fungal structures were dyed with Lactophenol Cotton Blue and then observed under light microscope (Zeiss Axio Imager M.1 with AxioVision Release 4.6 software). All isolates were reinoculated on MEA slants and deposited in Mycotheca of the Department of Algology, Mycology and Lichenology at the Faculty of Biology, University of Belgrade.

### *Diesel degradation in solid media*

Fungal-induced diesel degradation was tested using method described by Adekunle and Adebambo (2007). Minimal salt agar (MSA) medium (0.1 g  $\text{MgSO}_4$ ; 4 g  $\text{NH}_4\text{NO}_3$ ; 0.53 g  $\text{KH}_2\text{PO}_4$ ; 2 g  $\text{NaH}_2\text{PO}_4$ ; 0.17 g  $\text{CaSO}_4$ ; 17 g agar-agar) was enriched with 30 ml of diesel fuel as a sole carbon source. Originally isolated microfungi were inoculated on prepared media and incubated in thermostat at  $25\pm 1$  °C. During a period of seven days, growth dynamic, morphology, especially color of colonies and sporulation intensity, were successively observed and compared with control cultures grown on MSA enriched with equal amount of glucose (30 g) and expressed as colony growth dynamics ratio (DR) according to formula:

$$DR = \frac{d_s}{d_c} \times 100\%$$

where  $d_s$  represents the colony diameter of tested sample, and  $d_c$  colony diameter of control sample.

### *Diesel degradation in broth media*

Minimal salt broth (MSB) medium (0.1 g  $\text{MgSO}_4$ ; 4 g  $\text{NH}_4\text{NO}_3$ ; 0.53 g  $\text{KH}_2\text{PO}_4$ ; 2 g  $\text{NaH}_2\text{PO}_4$ ; 0.17 g  $\text{CaSO}_4$ ) was used for testing the ability of diesel degradation in liquid cultures. Glucose enriched media (glucose conc. 30g/l) were added in 12 glass flasks which were used as a control group (100 ml media each), and diesel enriched media (diesel conc. 30 ml/l) were added in another 12 flasks (also 100 ml media each). Inoculation was carried out in duplicate and cultures were incubated for 3 weeks on rotary shaker (Heidolph Titromax 1000), at the room temperature. After that, media were filtrated using cellulose

filter paper (Whatman, Ø 5 µm). The precipitate was then transferred to the drying chamber (Binder) at 80 °C, and after 24 h its dry mass was measured and expressed as dry mass production ratio (MR) according to formula:

$$MR = \frac{m_s}{m_c} \times 100\%$$

where  $m_s$  represents dry mass of tested sample, and  $m_c$  dry mass of control sample.

Remaining filtrate was used for absorbance measuring on spectrophotometer (CECIL CE 2501 2000), in order to quantify utilization of diesel fuel. Filtrate aliquots (1 ml) were pipetted into the cuvettes and absorbance was measured spectrophotometrically ( $\lambda = 330$  nm). Utilization degree (UD) was calculated using the following formula:

$$UD = [1 - \frac{\lambda_s}{\lambda_c}] \times 100\%$$

where  $\lambda_s$  represents the absorbance of tested sample and  $\lambda_c$  absorbance of control sample.

## RESULTS

A total of 16 filamentous fungi were isolated from the collected soil samples. Most of the isolates were hyphomycetes, with genus *Penicillium* as dominant, represented with six different species. For further testing, six isolates were chosen: *Absidia spinosa*, *Acremonium strictum*, *Cladosporium* sp., *Fusarium* sp. 1, *Fusarium* sp. 2, and *Penicillium* sp. (Figure 1).

Growth dynamics ratio on solid media is presented in Figure 2. The highest value was demonstrated for *Penicillium* sp. (74.16%) and *Cladosporium* sp. (71.43%), and the lowest for *A. spinosa* (23.15%).

Morpho-physiological differences were documented for tested fungal isolates in treatment and control group, based on their macroscopic and microscopic characteristics. Lack of sporulation and change in colony color were documented for *Fusarium* sp. 1, while *A. spinosa* showed lack of zygospores production in MSA media enriched with diesel. Furthermore, morphological differences were documented for *Penicillium* sp. colonies, which were intensively pigmented on diesel enriched media. On the other hand, *Cladosporium* sp. colonies were paler with conspicuous concentric zonation.

Dry mass production ratio of isolated microfungi is shown in Figure 2. The lowest value was documented for *Penicillium* sp. (0.46%) and *Cladosporium* sp. (0.47%) and the highest for *A. strictum* (71.43%).

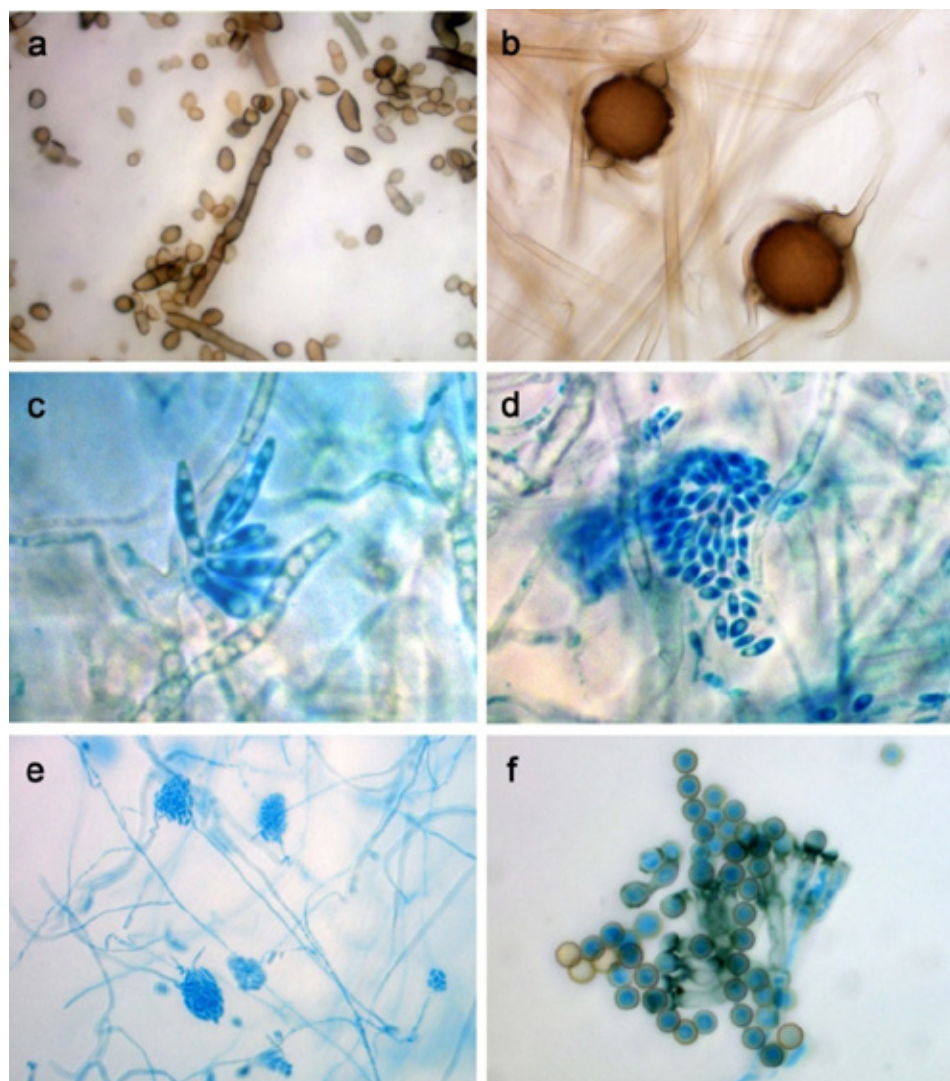


Figure 1. Tested isolates of soil microfungi: a) *Cladosporium* sp., conidiophore, ramoconidia and conidia; b) *Absidia spinosa*, zygospores; c) *Fusarium* sp. 1, macroconidia; d) *Fusarium* sp. 2, microconidia; e) *Acremonium strictum*, slime conidial heads; f) *Penicillium* sp., conidial chains.

Results of spectrophotometric analysis are shown in Figure 2. The highest utilization degree in broth media (60.28%) was demonstrated for *A. strictum*, and the lowest for *Penicillium* sp. (25.18%).

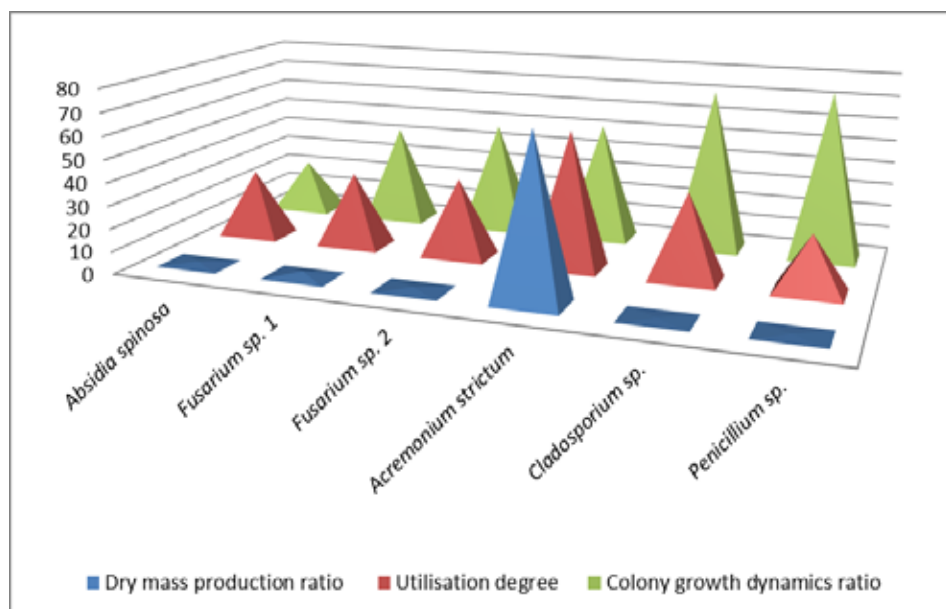


Figure 2. Ratios (%) of dry mass production, utilization degree (in MSB) and colony growth dynamics (on MSA) of tested fungi after 7 days of growth

## DISCUSSION

Fungi isolated from petroleum-contaminated soil, members of genera *Absidia*, *Acremonium*, *Cladosporium*, *Fusarium* and *Penicillium* are known as typical colonizers of different soil layers (Dix and Webster, 1994; Watanabe, 2010). In this research, the most prevailing was genus *Penicillium* with 6 documented species. Al-Jawhari (2014) documented *Penicillium* species as the most frequent isolates from soils near an oil refinery in Iraq.

All isolates selected for *in vitro* testing showed different potentials for diesel fuel uptake. *A. strictum* displayed the highest UD in broth media and moderate DR. Additionally, this isolate exhibited the highest MR which corresponds to its high UD value. This demonstrates high utilization potential of diesel fuel for this isolate. April *et al.* (1999) documented a high mycelial growth rate of *Acremonium* species in presence of liquid hydrocarbons, but the same isolates did not display degradation potential. In contrast to *A. strictum*, other tested isolates in our study showed insignificant MR values. Moderate UD and DR values were documented for both tested *Fusarium* species which demonstrated medium potential for diesel fuel uptake. Cekic *et al.* (2004) and Chaudhry *et al.* (2012) reported *Fusarium* species as successful bioremediators of carbohydrate pollutants in the oil refinery wastewater and soil environments. *Penicillium* and *Cladosporium* isolates showed the highest DR values on solid media, contrary to the lowest documented MRs. *Cladosporium* isolate displayed

moderate, while *Penicillium* isolate displayed the lowest UD. April *et al.* (1999) reported *Penicillium* and *Aspergillus* species as the most documented fungi with the ability to utilize various carbohydrates. These authors documented the ability of carbohydrate degradation on solid media for 22 *Penicillium* soil isolates. *Cladosporium resinae*, among others, was reported as one of the most important microorganism in the natural environment with the ability to degrade oil products (Walker *et al.* 1973). Documented discrepancy in growth rates of *Penicillium* and *Cladosporium* isolates, depending on media type, can be explained by different cultivation conditions. In the research presented here, *A. spinosa* showed very low values of all monitored parameters in solid and broth media. As opposed to our results, zygomycetes are often documented as successful carbohydrate utilizers (Adekunle and Adebambo, 2007; Chaudhry *et al.* 2012). Adekunle and Adebambo (2007) demonstrated the highest diesel and kerosene degradation degree for *Rhizopus* species.

Chaudhry *et al.* (2012) reported that *Aspergillus*, *Fusarium* and *Penicillium* species were the most efficient carbohydrate degraders because of the enzymes they produce during their exponential growth phase. By enzyme catalysis, C-C bonds in carbohydrate chains are broken, and that results in forming of lower molecular mass compounds, which fungi can uptake by osmosis.

## CONCLUSION

Results presented in this research suggest that all of the tested isolates have the potential, in varying degrees, to successfully utilize diesel fuel. They also represent a good starting point for further research on fungal diversity, as well as for testing diesel utilization by different isolates. According to George-Okafor *et al.* (2009), *Aspergillus* species are the most successful in degrading of different carbohydrates, comparing to the other tested species. Therefore, further research should expand on testing ability of diesel fuel utilization by *Aspergillus* species.

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## СПОСОБНОСТ ГЉИВА ИЗОЛОВАНИХ ИЗ ЗЕМЉИШТА КОНТАМИНИРАНОГ НАФТНИМ ДЕРИВАТИМА ДА КОРИСТЕ ДИЗЕЛ ГОРИВО

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РЕЗИМЕ: Циљ рада била је изолација и идентификација микромицета из земљишта које је контаминирано нафтним дериватима као и утврђивање потенцијала изолата да расту у присуству дизел горива и да га користе као извор угљеника

у агаризованом и течном медијуму. Од 16 изолованих и идентификованих филаментозних микромицета, шест је одабрано за *in vitro* тестирање. Тестирана је способност изолата да расту на чврстом и у течном медијуму обогаћеним дизел горивом као јединим извором угљеника. Сви тестирани изолати показали су различит степен метаболисања дизел горива као извора угљеника. Мерењем дијаметра колонија на агаризованој подлози констатовано је да *Penicillium* sp. (74.16%) и *Cladosporium* sp. (71.43%) показују највећу динамику раста колонија, а *Absidia spinosa* (23.15%) најмању. Резултати мерења суве масе изолата гајених у течном медијуму показали су да *A. strictum* има највећи потенцијал метаболисања дизел горива. За остале тестиране изолате показана је незнатна продукција суве масе. Највиши степен коришћења дизел горива, изражен као UD параметар, забележен је код *A. strictum* (60,28%) а најнижи код *Penicillium* sp. (25,18%).

КЉУЧНЕ РЕЧИ: гљиве, земљиште, коришћење дизел горива



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## ANTIFUNGAL ACTIVITY OF MACROFUNGI EXTRACTS ON PHYTOPATHOGENIC FUNGAL STRAINS OF GENERA *Fusarium* sp. AND *Alternaria* sp.

**ABSTRACT:** During the last decades, intensive application of synthetic fungicides in the agricultural crop protection practice caused growing concern for the existence of toxic chemical residues in food as well as in the whole environment. Instead of using synthetic fungicides, it is suggested that crop protection be carried out by using preparations based on compounds of natural origin (secondary metabolites of plants or microorganisms, including macrofungi from Basidiomycota) as biological control agents. The potential of macrofungal species as biocontrol agents was analyzed in this investigation of eight autochthonous species from different locations in Serbia. Both the terricolous species: *Coprinus comatus*, *Coprinellus truncorum*, *Amanita strobiliformis*, *Hydnum repandum* and the lignicolous species: *Flammulina velutipes*, *Stereum subtomentosum*, *Trametes versicolor* and *Bjerkandera adusta* were examined, with an aim to detect some novel sources of antifungal agents. This study surveyed antifungal activity of selected macrofungal extracts (MeOH, EtOH and CHCl<sub>3</sub>) against phytopathogenic *Fusarium* and *Alternaria* strains isolated from garlic, soybean and rice: *F. proliferatum*, *F. verticillioides*, *F. proliferatum*, *F. graminearum* and *A. padwickii*. Microdilution method in 96 well micro-plates was applied for the estimation of antifungal effects of macrofungi extracts in the range from 24.75 to 198.00 mg/ml and determination of minimal inhibitory (MIC) and minimal fungicidal concentration (MFC). EtOH extract of mycorrhizal species *H. repandum* showed antifungal activity against all analyzed phytopathogenic strains, with the strongest effect on *Fusarium* strains (MIC 24.75 mg/ml; MFC 24.75 mg/ml). Among others, MeOH extracts of *S. subtomentosum* and *C. micaceus* showed similar effects while only *B. adusta* showed slight effect on *Fusarium* strains (MIC 24.75–99.00 mg/ml; MFC 24.75–99.00 mg/ml) and none effect on *A. padwickii*. The obtained results indicate the possibility of using examined extracts as efficient antifungal agents and provide the basis for the new formulations of biocontrol agents against phytopathogenic fungi in the future.

**KEYWORDS:** Biocontrol, antifungal activity, *Fusarium*, *Alternaria*, *Hydnum repandum*

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## INTRODUCTION

Intensive application of synthetic fungicides in combat against phytopathogenic fungi has recently caused considerable concern, primarily due to toxic and carcinogenic chemical compounds found in food after fungicide application, as well as pollution caused by poor biodegradability of these compounds, and the development of pathogenic strains resistant to common commercial fungicides (Montesinos, 2003; Živković, 2016).

Interest in introducing alternative plant protection measures is therefore immense, especially concerning the research on novel organic fungicides, which are a form of biological control and an important natural phenomenon. Biological control is a way of protection of agro-ecosystems from harmful organisms, mainly pathogens, carried out by applying various agents and bio-pesticides such as microorganisms (bacteria, yeasts, macro-fungi) and their metabolic products or plants and plant extracts. Possibilities of applying macro-fungal extracts for biological control of invasive organisms, especially phytopathogenic fungi, are therefore one of the top priorities in scientific research.

The term “macrofungi” relates to the fungi visible to the naked eye i.e. containing large fruiting bodies, which mainly belong to phyla Basidiomycota, Ascomycota (Chang and Miles, 2004). Macrofungi produce biologically active compounds, products of primary or secondary metabolism, with different activity: antifungal, antimicrobial, antioxidant, anti diabetogenic, anticarcinogenic, immunomodulatory (Kiho *et al.* 1996; Kim *et al.* 1999; Wasser, 2002; Lindequist *et al.* 2005; Karaman *et al.* 2009; Karaman *et al.* 2014). Macrofungi produce antimicrobial and antifungal metabolites so as to survive in their natural environment, thus the different macrofungal species (*Ganoderma carnosum*, *Hydnum repandum*, *Hygrophorus agathosmus*, *Lenzites betulina*, *Lepista nuda*, *Leucoagaricus pudicus*, *Paxillus involutus*, *Polyporus arcularius*, *Rhizopogon roseolus*, *Sarcodon imbricatus*, *Trametes versicolor*) can be used as a new source of natural compounds with antifungal activity (Yamaç and Bilgili, 2006).

Antifungal activity has been confirmed in 50 different species so far, including mostly the wild, edible macrofungi originating from Turkey, Portugal, China, Japan, Brasil, Hungary, Ireland and Malesia, namely *Flammulina velutipes*, *Hydnum repandum*, *Lentinus edodes*, *Ganoderma lucidum*, as well as the antifungal activity of various macrofungal extracts (Hirasawa *et al.* 1999; Hatvani, 2001; Smania *et al.* 2007; Hearst *et al.* 2009; Öztürk *et al.* 2011; Teoh *et al.* 2012; Alves *et al.* 2013; Heleno *et al.* 2013). Majority of the studies focus on antifungal activity towards one species of the human pathogen *Candida albicans* (Rosa *et al.* 2003; Kalyoncu i Oskay, 2008; Kalyoncu *et al.* 2010; Ozen *et al.* 2011), while others deal with phytopathogenic fungi *Fusarium verticillioides*, *Botrytis cinerea*, *Fusarium oxysporum*, *Physalospora piricola*, *Mycosphaerella arachidicola* (Wang, 2004; Lindequist *et al.* 2005; Wang, 2006; Gilardoni *et al.* 2007; Wang *et al.* 2012).

Since studies of autochthonous fungi of different geographical origin are important for discovering new isolates with prospective antifungal activity, the aim of the study was to draw attention to the use of “raw” extracts of the

selected macrofungi, such as *Coprinus comatus*, *Coprinellus truncorum*, *Amanita strobiliformis*, *Hydnum repandum*, *Flammulina velutipes*, *Stereum subtomentosum*, *Trametes versicolor*, and *Bjerkandera adusta*, in biocontrol of phytopathogenic fungi, namely *Fusarium* and *Alternaria* isolated from garlic, soybean and rice, as a prospective mode of plant protection.

## MATERIAL AND METHODS

### Preparing the suspension of filamentous phytopathogenic fungi

Isolation of five phytopathogenic fungi was carried out on a nutrient Potato Dextrose Agar agar (PDA) using the infested plant parts which showed symptoms of rot (Table 1). After growth and sporulation, fungi were isolated from the monospore cultures in order to obtain uniform isolates (Figure 1). Tested and confirmed, such obtained isolates are phytopathogenic (Ignjatov *et al.* 2016).

Table 1. Isolated phytopathogenic fungi and host plants\*

Code	Pathogen	Hosts
BL1	<i>Fusarium proliferatum</i>	Garlic
BL4	<i>Fusarium verticillioides</i>	Garlic
BL5	<i>Fusarium proliferatum</i>	Garlic
S1	<i>Fusarium graminearum</i>	Soybean
ALT	<i>Alternaria padwickii</i>	Rice

\* fungal collection – dr Maja Ignjatov

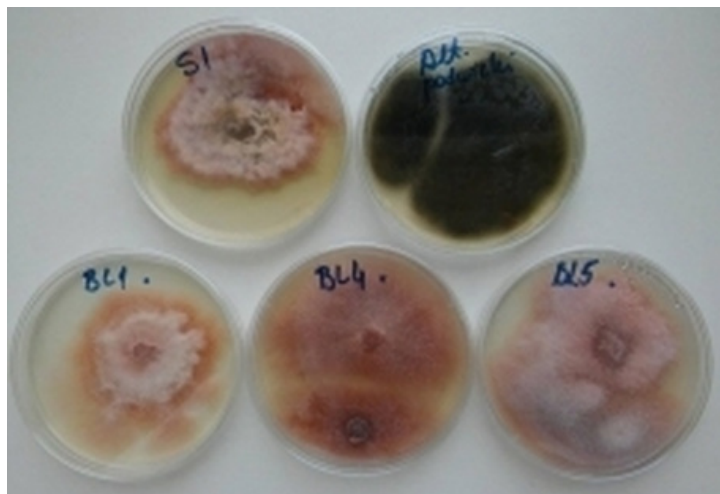


Figure 1. Analyzed phytopathogenic fungi: *F. graminearum* S1, *A. padwickii*, *F. proliferatum* BL1, *F. verticillioides* BL4, *F. proliferatum* BL5 (collection – dr Maja Ignjatov)

Cultures were grown on PDA, which proved to promote fast growth and sporulation, within 7 days at 27 °C. After incubation under sterile conditions, a sample of the cultivated fungi was taken and suspended in sterile distilled water. Suspension of spores of *F. proliferatum* (BL1), *F. verticillioides* (BL4), *F. proliferatum* (BL5) was at the density of  $1.5 \times 10^7$  cells per ml (c/ml), while the observed density in *F. graminearum* (S1) and *A. padwickii* was  $1.5 \times 10^6$  c/ml. Bürker Türk chamber (hemocytometer) and a microscope were used in order to obtain the adequate inoculum turbidity and the desired density. The number of spores in the chamber was determined by direct counting using a microscope (Olympus, BX51, Japan) in specific chamber cubes. The number of spores in a chamber is used to calculate the concentration or spore density in a suspension, using the formula:

$$\text{Mixture cell concentration} = \frac{(\text{no. of counted cells})}{(\text{no. of chambers}) \times (\text{chamber volume})} \times 1000$$

Suspensions with adequate density were obtained ( $1.5 \times 10^7$  i  $1.5 \times 10^6$  c/ml) using this formula.

#### Preparation of MeOH, EtOH, CHCl<sub>3</sub> macrofungal extracts

Eight autochthonous macrofungi were collected from different locations in Serbia (Fruska Gora, Tara and Sremski Karlovci), four of which were terricolous: *Coprinus comatus* (O.F. Müll.) Pers. 1797, *Coprinellus truncorum* (Scop.) Redhead, Vilgalys & Moncalvo 2001, *Amanita strobiliformis* (Paulet ex Vittad.) Bertill. 1866, *Hydnum repandum* L. 1753, and four lignicolous species: *Flammulina velutipes* (Curtis) Singer 1951, *Stereum subtomentosum* Pouzar 1964, *Trametes versicolor* (L.) Lloyd 1921, *Bjerkandera adusta* (Willd.) P. Karst. 1879.

Fungal fruiting bodies were first cleaned and lyophilized at -80 °C, under vacuum. The lyophilized mass obtained from the samples was measured (OHAUS explorer, ex 224M), and then extracted in the ratio of 1:10, using different solvents (70% MeOH, 80% EtOH, and 100% CHCl<sub>3</sub>). Extraction was carried out in a mechanical mixture for 72 h (New Brunswick Scientific, Edison, USA) (100 rpm). Thereafter, the obtained extracts were filtered on a vacuum pump using filters paper Watman No. 1 (Fironi, Italy).

MeOH and EtOH extracts were then evaporated on a Rotavapor unit (Büchi, R-210, Switzerland) at 50 °C, while the CHCl<sub>3</sub> extracts were evaporated at 40 °C. Thereafter, the leftover dried mass thus obtained was dissolved in a specific solvent so as to obtain the final concentrations of the extracts, i.e. 10%, 20% and 40% (w/w).

The following concentrations of extracts were obtained: 10% MeOH (*T. versicolor*), 20% MeOH (*F. velutipes*, *S. subtomentosum*, *H. repandum*, *B. adusta*), 40% MeOH (*A. strobiliformis*, *C. comatus*, *C. truncorum*), 20% EtOH (*H. repandum*, *A. strobiliformis*, *C. comatus*, *C. truncorum*), and 20% CHCl<sub>3</sub>

(*C. comatus*, *C. truncorum*). The prepared extracts were kept in a refrigerator at +4 °C, until the next use.

#### Testing antifungal activity of MeOH, EtOH, CHCl<sub>3</sub> macrofungal extracts using the microdilution method

Antifungal activity of analyzed macrofungi was determined *in vitro* by microdilution method in 96 well microtiter plates (Spektar, Čačak, Serbia), so as to determine the minimal inhibitory and fungicide concentrations (MIC and MFC). Sterile polypropylene microtiter plates were used for this purpose. The total volume of a well was 101 µl. The amount of 50 µl nutrient broth (Malt broth, Torlak, Serbia) was applied into each well and 1 µl of phytopathogenic fungal spore suspension in 3 different double solutions (100%, 50% and 25%).

Microtiter plates were incubated for 72 h at 27 °C, and the results were read visually. The first concentration of extract without visible growth was taken as minimal inhibitory concentration (MIC), while minimal fungicide concentration (MFC) was determined after reading the MIC values, by transferring the whole volume of a well onto petri Malt Agar plates (Torlak, Serbia). The total volume was transferred to the Malt agar plates without any turbidity noticed. After incubation for 72 h at 27 °C, the results previously monitored by counting colonies were read.

## RESULTS AND DISCUSSION

The results obtained by the microdilution method were presented (Table 2) through a parallel review of MICs and MFCs of macrofungal extracts towards phytopathogenic fungal strains: four *Fusarium* (*F. proliferatum* – BL1, *F. verticillioides* – BL4, *F. proliferatum* – BL5, *F. graminearum* – S1) and one *Alternaria* (*A. padwickii* – ALT).

Among the tested macrofungal extracts, antifungal activity to all the tested phytopathogenic fungi was displayed by the EtOH extracts of *H. repandum*, the strongest to strains *Fusarium* sp. (MIC 24.75 mg/ml; MFC 24.75 mg/ml), while somewhat lower to *A. padwickii* (MIC 24.75 mg/ml; MFC 49.50 mg/ml). On the other hand, the MeOH extract of the same species exhibited activity to *A. padwickii* (MIC 24.75 mg/ml), as well.

Besides, the EtOH extract of *H. repandum*, antifungal activity to all phytopathogenic isolates was exhibited by MeOH extracts of *S. subtomentosum* (MIC 49.50–99.00 mg/ml; MFC 49.50–99.00 mg/ml) and *C. truncorum* (MIC 99.00–198.00; MFC 99.00–198.00 mg/ml). MeOH extract of *B. adusta* had effect only on *Fusarium* (MIC 24.75–99.00; MFC 24.75–99.00 mg/ml), while the MeOH extracts of *C. comatus*, *T. versicolor*, *F. velutipes*, as well as EtOH extracts of *C. comatus*, *A. strobiliformis*, and the CHCl<sub>3</sub> extract of *C. comatus* had antifungal effects on the phytopathogenic isolate *A. padwickii*.

Table 2. MIC and MFC values (mg/ml) analyzed for MeOH, EtOH, and CHCl<sub>3</sub> macro-fungal extracts towards phytopathogenic fungi (moulds)

Moulds→  Fungal extracts ↓	<i>Fusarium proliferatum</i> (BL1)		<i>Fusarium verticillioides</i> (BL4)		<i>Fusarium proliferatum</i> (BL5)		<i>Fusarium graminearum</i> (S1)		<i>Alternaria padwickii</i> (ALT)	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
	concentration (mg/ml)									
	MeOH									
CcMeOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
CtMeOH	198.00	198.00	198.00	198.00	198.00	198.00	198.00	198.00	99.00	99.00
AsMeOH	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BaMeOH	99.00	99.00	49.95	49.95	49.95	49.95	24.75	24.75	nd	nd
HrMeOH	nd	nd	nd	nd	nd	nd	nd	nd	24.75	nd
TvMeOH	nd	nd	nd	nd	nd	nd	nd	nd	49.50	nd
FvMeOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
SsMeOH	49.50	49.50	99.00	99.00	99.00	99.00	49.50	49.50	99.00	99.00
	EtOH									
CcEtOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
CtEtOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
AsEtOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
HrEtOH	24.75	24.75	24.75	24.75	24.75	24.75	24.75	24.75	24.75	49.50
	CHCl <sub>3</sub>									
CcCHCl <sub>3</sub>	nd	nd	nd	nd	nd	nd	nd	nd	24.75	nd
CtCHCl <sub>3</sub>	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

\*nd – not detected, CcMeOH – MeOH extract *C. comatus*, CtMeOH – MeOH extract *C. truncorum*, AsMeOH- MeOH extract *A. strobiliformis*, BaMeOH – MeOH extract *B. adusta*, HrMeOH – MeOH extract *H. repandum*, TvMeOH – MeOH extract *T. versicolor*, FvMeOH – MeOH extract *F. velutipes*, SsMeOH – MeOH extract *S. subtommentosum*, CcEtOH – EtOH extract *C. comatus*, CtEtOH – EtOH extract *C. truncorum*, AsEtOH – EtOH extract *A. strobiliformis*, HrEtOH- EtOH extract *H. repandum*, CcCHCl<sub>3</sub> – CHCl<sub>3</sub> extract *C. comatus*, CtCHCl<sub>3</sub> – CHCl<sub>3</sub> extract *C. truncorum*

Only in case of the MeOH extract of *A. strobiliformis* and CHCl<sub>3</sub> extract of *C. micaceus*, no antifungal effects on any of the tested phytopathogenic isolates were observed.

According to Aqueveque *et al.* (2016), MeOH extract of two species of the *Stereum* genus showed weak antifungal activity, exhibiting very weak antifungal activity to *B. cinerea* in only 7 out of 36 strains tested. In our study, MeOH extract of *S. subtommentosum* had a strong antifungal effect on all the tested phytopathogenic strains reaching MIC at 49.50–99.00 mg/ml and MFC at 49.50–99.00 mg/ml.

MeOH extracts of the both cultivated and wild *C. comatus* in the study of Stojković *et al.* (2013) exhibited strong antifungal effect on *Trichoderma viride* (MIC 0.25–1.50 mg/ml and MFC 1.50–3 mg/ml) and *Aspergillus versicolor* (MIC 0.20–0.75 mg/ml and MFC 1.50–3 mg/ml). Slightly weaker antifungal

activity to *Aspergillus fumigatus* and *Penicillium verrucosum* was observed, while the lowest was detected for the MeOH extract of wild *Aspergillus niger*. In the present study, the MeOH extract of *C. comatus* exhibited no antifungal activity on *Fusarium*, whereas it was detected on *Alternaria* strain tested (MIC 99.00 mg/ml). According to Ehssan and Saadabi (2012), EtOH extract of *C. comatus* from Soudan did not exhibit antifungal activity on phytopathogenic strain *A. niger*. The result is partially in accordance with our results obtained in the study which showed generally low antifungal activity of the EtOH extract of *C. comatus* (Table 2). EtOH extract of *Hydnum repandum* in the study of Yamaç *et al.* (2006) exhibited low antibacterial activity, (eight bacterial strains without activity and only one with < 10 mm) using the disc diffusion method, whereas high antifungal activity was observed in our study on *Fusarium* strains (MIC 24.75 mg/ml; MFC 24.75 mg/ml).

According to Alves *et al.* (2012) proteins and polysaccharides ( $\beta$ -Glucans) isolated from mushrooms showed antifungal activity. Earlier studies indicate that the diterpenoids and sesquiterpenoids from Basidiomycetes macrofungi showed antifungal activity against some phytopathogenic fungal strains: *Fusarium culmorum*, *Alternaria solani*, *Botrytis cinerea*, *Trichoderma lignorum* (Florianowicz, 1999; Liu, 2007). According to Wang *et al.* (2012) sesquiterpenes, enokipodin F, G and I isolated from *F. velutipes* mycelium presented low activity against *Aspergillus fumigatus* with IC<sub>50</sub> values  $229.1 \pm 3.6$ ,  $233.4 \pm 3.8$ ,  $235.1 \pm 4.2$   $\mu$ M respectively and the result is in accordance with our study which showed generally low antifungal activity of the MeOH extract of *F. velutipes* on all phytopathogenic strains.

## CONCLUSION

Antifungal activity in all the tested phytopathogenic fungi was exhibited by the EtOH extract of *H. repandum*, with the strongest activity to *Fusarium* strains (MIC 24.75 mg/ml; MFC 24.75 mg/ml) and weaker activity to *A. padwickii* (MIC 24.75 mg/ml; MFC 49.50 mg/ml). Antifungal activity to all tested phytopathogenic fungi was found for MeOH extracts of *S. subtomentosum* and *C. truncorum*, but in higher concentrations (MIC 49.50–99.00 mg/ml; MFC 49.50–99.00 mg/ml and MIC 99.00–198.00; MFC 99.00–198.00 mg/ml, respectively). MeOH extract of the macrofungi *B. adusta* exhibited high antifungal activity to *Fusarium* (MIC 24.75–99.00 and MFC 24.75–99.00 mg/ml), while no activity to *A. padwickii* was observed. In extracts not exhibiting activity to *Fusarium* phytopathogenic fungi, minimal inhibitory concentrations to *A. padwickii* were detected. MIC of 24.75 mg/ml was detected in the MeOH extract of *H. repandum* and the CHCl<sub>3</sub> extract of *C. truncorum*, at the concentration of 49.50 mg/ml in metanol extract of *T. versicolor*, 99.00 mg/ml in MeOH and EtOH extract of *C. comatus*, EtOH extract of *C. truncorum*, MeOH extract of *F. velutipes*, and EtOH extract of *A. strobiliformis*.

Based on the results obtained in this study, we can conclude that some macrofungal extracts such as EtOH of *H. repandum*, MeOH of *S. subtomentosum*,

MeOH of *C. truncorum* and MeOH of *B. adusta* are potentially efficient antifungal agents, and can therefore be the basis of the formulations for preparations used in biocontrol against phytopathogenic fungi.

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## АНТИФУНГАЛНА АКТИВНОСТ ЕКСТРАКТА МАКРОГЉИВА НА ФИТОПАТОГЕНЕ СОЈЕВЕ ГЉИВА РОДОВА *Fusarium* sp. И *Alternaria* sp.

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**РЕЗИМЕ:** Током последњих деценија интезивна примена синтетичких фунгицида у заштити пољопривредних усева изазива све већу забринутост људи због присуства токсичних хемијских остатака у прехранбеним производима као и целој околини. Уместо коришћења синтетичких фунгицида, препоручује се да се заштита усева врши коришћењем препарата на бази једињења природног порекла (секундарни метаболити биљака или микроорганизама укључујући и макрогљиве из раздела Basidiomycota) као агенсе биолошке контроле. У овом раду истраживан је потенцијал агенаса биолошке контроле за осам аутохтоних врста макрогљива с различитих локалитета у Србији. Обе, териколне врсте: *Coprinus comatus*, *Coprinellus truncorum*, *Amanita strobiliformis*, *Hydnum repandum* и лигниколне врсте: *Flammulina velutipes*, *Stereum subtomentosum*, *Trametes versicolor* и *Bjerkandera adusta* су истражене с циљем да се открију неки нови извори антифунгалних агенаса. У оквиру овог рада истражена је антифунгална активност одабраних екстраката макрогљива (метанолни, етанолни и хлороформски) против фитопатогених сојева *Fusarium* и *Alternaria* изолованих с белог лука, соје и пиринча: *F. proliferatum*, *F. verticillioides*, *F. proliferatum*, *F. graminearum* и *A. padwickii*. За процену антифунгалног ефекта екстраката макрогљива употребљена је микродилуциона метода микротитар плочама с 96 велова у опсегу концентрација од 24,75 до 198,00 mg/ml и детерминацију минималне инхибиторне (МИС) и минималне фунгицидне концентрације (МФС). Етанолни екстракт микоризне врсте *H. repandum* показао је антифунгалну активност према свим анализираним фитопатогеним сојевима, са најјачим ефектом према сојевима *Fusarium* (МИС 24,75 mg/ml; МФС 24,75 mg/ml). Између осталог, сличан ефекат показали су и метанолни екстракти *S. subtomentosum* и *C. micaceus*, док је само *B. adusta* имала благи ефекат на сојеве *Fusarium* (МИС 24,75–99,00 mg/ml; МФС 24,75–99,00 mg/ml), али не и на *A. padwickii*. Добијени резултати указују на могућност коришћења испитаних екстраката као веома ефикасних антифунгалних агенаса и самим тим они представљају основу за нове формулације биоконтролних агенаса против фитопатогених гљива у будућности.

**КЉУЧНЕ РЕЧИ:** биоконтрол, антифунгална активност, *Fusarium*, *Alternaria*, *Hydnum repandum*

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## MORPHO-ANATOMICAL CHARACTERIZATION OF *Tuber macrosporum*/*Corylus avellana* MYCORRHIZAS FROM CULTIVATED SEEDLINGS: CASE REPORT

**ABSTRACT:** This study investigated the presence of mycorrhizae on seedlings from part of ten-year-old truffles plantation (about 3,000 m<sup>2</sup>) located in Eastern Serbia. This study is observation of the presence of ectomycorrhizal fungus from genus *Tuber* during its symbiotic stage on the roots of *Corylus avellana* L. Ten root samples were collected (randomly) and observed macroscopically and microscopically analyzed. There were changes in morphology and anatomy of the infected roots of *C. avellana*. Mantle was clearly observed to cover the roots and the mycelia formed the Harting net. Among arbitrary selected seedlings, there were found mature fruiting bodies on the surface of the soil. The truffles, identified as *Tuber macrosporum* Vittad, were found in the immediate vicinity of the hazelnut trees. There has been no previous information of mycorrhizae *Tuber macrosporum*/*Corylus avellana* on artificially created truffles plantation in Serbia.

**KEYWORDS:** *Tuber* spp., *Corylus avellana* L., ectomycorrhizae, morpho-anatomical characters, ascocarp, ascospore

## INTRODUCTION

The mycorrhiza is a widespread symbiotic association between land plants (roots) and fungi. About 8,000 plant species form ectomycorrhiza, one type of mycorrhiza, were characterized by the presence of hyphal sheath or mantle on the surface of a root, Harting net (hyphae nets between cortex root cells) and

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extraradical mycelium (Agerer, 1995). Ectomycorrhizal associations are usually mutual relationships in which a fungus provides water and nutrients for its plant partner and receives assimilates from its host (Finlay, 2008). In this type of mycorrhiza symbiosis, both higher plants from families such as Pinaceae, Betulaceae, Fabaceae, Dipterocarpaceae, Fagaceae and Myrtaceae are involved (Brundrett, 2009) and the fungi that belong mostly to the phyla Basidiomycota and Ascomycota. Ectomycorrhizal associations are formed by estimated 20,000–25,000 fungal species (Rinaldi *et al.* 2008).

The genus *Tuber* belongs to Ascomycotina, Pezizales, Tuberaceae family. These fungi establish an ectomycorrhizal symbiosis with trees and shrubs and as a result of this symbiosis hypogeous ascocarps – fruit body (truffles) is produced in order to accomplish their life cycle (Mello *et al.* 2006). The genus contains 180–230 species, subspecies and varieties distributed worldwide (Bonito *et al.* 2010). In Europe, around 32 species are considered to be valid (Ceruti *et al.* 2003). Truffles have been collected and consumed by humans for centuries. Because of their specific taste and smell, as well as the special conditions in which they can grow, these edible fungi are among the most expensive ones in the world. The most hunted and prized truffles species are the white species *Tuber magnatum* Pico and the black ones are *T. melanosporum* Vittad. and *T. aestivum* Vittad. (Wang and Marcone, 2011).

This first study on hypogeous fungi (truffles and truffle-like fungi) in Serbia started in 1992. Collected material, fruit bodies of *Tuber* species had been founded and identified and extensively studied during the last decade of the twentieth century (Milenković *et al.* 1992; Glamočlija, 1996; Glamočlija *et al.* 1997; Glamočlija, 1999a). According to the earlier reports and new investigations, Marjanovic *et al.* (2010) provided expanded list of species of the genus *Tuber* founded in Serbia and showed the first molecular verification of the *Tuber* spp. samples originating from Balkan Peninsula. Recently, Milenković *et al.* (2015) founded new truffle species from Serbia.

Despite the current interest in truffles in Serbia, there is not much information about artificially established truffle plantations. In publications (Glamočlija, 1999b; 2000), given results represent a successful mycorrhization roots system of four species of oaks and hazel trees with ascospore suspension of different species of truffles. Plants had been grown in a greenhouse and after monitoring the development of mycorrhiza at the structural and ultra structural level during 4, 8, 10 and 36 months, they were set in the experimental well. These presented data have no prior results relating to the period of observation of ectomycorrhizae after 36 months.

Information given in the daily press about the existence of 150 truffle plantations with a total area of 33 hectares have not been officially verified by government institutions. The owners of land that is suitable for the growth of artificially mycorrhized host plants with *Tuber* spp plant them, but there is no official company that deals with the control of production, transport and sales

of inoculated plants in Serbia. The register orchards of truffles in Serbia have not been created yet.

Thus, the objective of this study was the morphological and anatomical characterization of an unknown ectomycorrhizas of host species *Corylus avellana* from cultivated seedlings from part of a ten-year-old truffles plantation and identification of valuable fruit body after that period.

## MATERIALS AND METHODS

### *Identification of the truffle-ground, geographical data*

In autumn 2016, we were invited to visit and determine what kind of mycorrhizae is situated in the plantation near the town of Požarevac (Eastern Serbia). The truffle-ground (about 3,000 m<sup>2</sup>) is placed in Kličevac 44°44'01.0"N 21°17'18.3"E where a plantation was established during the autumn 2007. According to the owner, he bought commercially produced seedlings of *Corylus avellana* inoculated with the black truffle.

### *Sample source*

Twenty seedlings *C. avellana* from a small part of the plot were observed. Ten root samples were collected (randomly) from 10 cm depth increments of the root system, rinsed in water and observed under a stereo dissecting microscope (Leica WILD M3Z, Germany) in order to find ectomycorrhizal roots.

The three fresh mature ascocarps of truffles, each characterized by a gray-brown surface occur at about 1-5 cm depth. The fruit bodies were washed in tap water with a brush and air-dried afterwards. Each truffle was examined macroscopically and microscopically and identified by morphological methods, according to Montecchi and Lazzari (1993). The dried voucher specimens are deposited at the Fungal Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research "Siniša Stanković".

### *Light microscopy*

Root tips 5–10 mm long and pieces of fruit body (10 mm x 5 mm) were fixed in FAA (formalin-acetic acid-ethanol 10:5:85), dehydrated in a graded ethanol series and embedded in paraffin wax at 58 °C. Sections (8  $\mu$ m thick) were stained with haematoxylin (cross sections of fruit bodies), alcian blue and Schiff's reagent (cross sections of root tips) and examined under a Zeiss Axiovert microscope (Carl Zeiss GmbH, Göttingen, Germany).

## RESULTS AND DISCUSSION

### *Identification based on the morphological features of ascocarps collected from plantation of Corylus avellana*

The fruit body has ascocarp: globose, regular shape, lobed, with a diameter of 2–5 cm. Peridium: brownish to black rigid with very short and flat warts. Gleba: grey brown to brown-lilac and purple-brown, with thick, branching and winding white veins (Figure 1). The globose to subglobose asci of 132–135 (130) x 75–82 (80)  $\mu\text{m}$  size contain 1-3 yellowish-brown spores (generally two) (Figure 2.). The ellipsoid spores were 58–62 (60) x 70–74 (70)  $\mu\text{m}$ , covered with reticulate-alveolate, polygonal, dense, closed and small meshes (Figure 2a). Odor of mature ascocarp was intensive and specific and had aromatic resemblance of garlic while its aroma was similar to the white truffle *Tuber magnatum* Pico (Benucci *et al.* 2016). The shape and size of all small fruit bodies found on parts of experimental orchards are in accordance with those reported for *Tuber macrosporum* Vitt.

“Garlic truffle” (*T. macrosporum*) is a common species in Serbia, collected from October to December in hilly terrains of low mountains. The hosts of *T. macrosporum* in Serbia are oak (*Quercus robur* L.), native poplars (*Populus alba* L., *P. nigra* L.), ash trees (*Fraxinus angustifolia* L., *F. exselsior* L.) and maple (*Acer campestre* L.) (Glamočlija *et al.* 1997; Marjanovic *et al.* 2010; Đurđević *et al.* 2015).

*T. macrosporum* has a wide distribution in Balkan Peninsula being common in Serbia but also occurs in Croatia and Slovenia. In Europe, harvested ascocarps of *T. macrosporum* are common in Hungary and Romania, less frequent in Italy and rare in France and Great Britain but occur in Switzerland, Germany and the Ukraine. Recently, it has been reported in Slovakia, Poland and Turkey (Benucci *et al.* 2016).

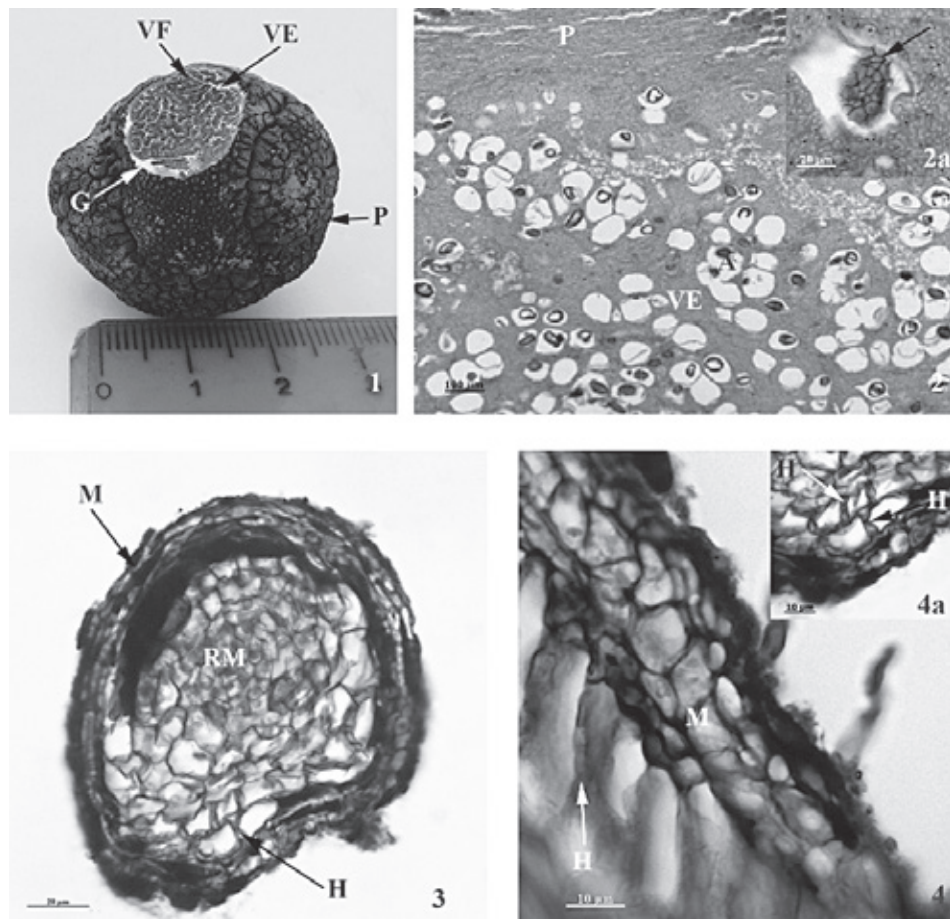
In contrast, the world’s most hunted and expensive truffles species (*T. magnatum*, *T. melanosporum* and *T. aestivum*) *T. macrosporum* has limited reputation and market (Zambonelli *et al.* 2015). This attractive species with small fruit bodies and specific organoleptic features can merit more attention.

This is the first report of the valuable hypogeous fruit bodies *T. macrosporum* originated from truffle plantations in Serbia.

### *Identification based on the morphological features of mycorrhizal roots collected from plantation of Corylus avellana*

Mycorrhizal roots were thick, cylindrical with rounded tips and developed a white mantle. *Tuber macrosporum* ectomycorrhizae were simple or ramified in monopodial-pinnate or monopodial-pyramidal pattern. Mycelia proliferate on the root surface and form the multi-layered mantle (Figure 3). According to Agerer (2001), mantles can be divided into two main groups depending on the hyphal distribution and organization: plectenchymatous and pseudoparenchymatous.

The mantle, formed on the surface of the *Corylus avellana* roots was pseudo-parenchymatous, composed of angular/epidermoid cells that formed puzzle-like pattern and probably contained glycogen. Piche *et al.* (1981) using the PAS reaction (Schiff's reaction) showed that the inner mantle and Harting net formed on short roots of *Pinus strobus* contained PAS-positive material, presumably glycogen.



Figures 1-4.

Legend of the figure:

Figure 1. Fruit body of *Tuber macrosporum*; Figure 2. Cross section of *T. macrosporum* fruit body; Figure 2a. Oval ascus with ascospores. Note reticular ornamented; Figure 3. Cross section of the ectomycorrhizal roots tip of *Corylus avellana*. Note mantle and Hartig net; Figure 4. Pseudoparenchymatous mantle;

Figure 4a. Detail: pariepidermal Harting net

P-peridium; G-gleba; VE-venae externae; VF-venae fertillae; A-ascus; M-mantle; H- Harting net; RM-root apical meristem

Pseudoparenchymatous mantles have short-celled, inflated, compactly packed hyphae, that look like a true parenchyma. From a phylogenetic point of view, hyphal organization in pseudoparenchymatous mantles is considered more advanced (Agerer, 1995). Cells of ectomycorrhizal mantle of *T. macrosporum* varied in shape and size (Figure 4). Benucci *et al.* (2012) showed significant differences in size and shape within the same ectomycorrhizas. Hyphae penetrate between epidermal cells to form a Hartig net (Figure 3, 4, 4a). The Hartig net, the zone of contact between the plant and the fungus, plays the key role in the transfer of nutrients between both partners (Corrêa *et al.* 2012).

The high economic value of truffles has stimulated researchers to find the most efficient methods for cultivating them. *T. macrosporum* ectomycorrhizae with hornbeam seedlings were published first by Giovannetti and Fontana (1980–1981). The other authors (Zambonelli *et al.* 1993; Granetti, 1995; Agerer and Rambold, 2004–2008) photographed and described ectomycorrhizae oaks and hazel seedlings inoculated by *T. macrosporum* spores. Later Benucci *et al.* (2012) described morphologically *T. macrosporum* on *Quercus robur* L., *Quercus cerris* L. and *Corylus avellana* L. and identified its DNA through the use of species-specific primers (Benucci *et al.* 2016).

Our results of morphological and anatomical characteristic of ectomycorrhizas structures from *C. avellana* roots are consistent with those presented in Benucci *et al.* (2012).

The successful cultivation of *T. macrosporum* on experimental orchards have been established in Italy (Vezzola, 2005). Among common host plants for *T. macrosporum* cultivation, hazelnut *C. avellana* with vigorous growth, with its tendency to form a well developed root system and its well-known capacity to form ectomycorrhizae is especially suitable. Previous studies carried out in Serbia demonstrated the feasibility of producing mycorrhizal plants of *C. avellana* inoculated with black truffle (Glamočlija, 1996).

In the European countries as well as in the Southern Hemisphere and New Zealand, programs of using the seedlings inoculated with truffle fungi are well developed. Over 40 years, an enormous progress has been made (Murat, 2015). In Serbia, there have been no scientific data on the state of private truffieries so far.

## CONCLUSION

Root samples of hazel trees (*Corylus avellana* L.) and samples of fruiting bodies of truffles were collected from part of truffle-ground. The presence of *Tuber macrosporum* Vittad from natural habitats is well documented in Serbia while the existence of this fungus on an artificially established truffle plantation has not been documented.

We found that ectomycorrhizae of *Tuber macrosporum* were present on roots of seedlings *Corylus avellana* from part of ten-year-old plantation. Further research will be carried out on other parts of this private truffieries.

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МОРФО-АНАТОМСКА КАРАКТЕРИЗАЦИЈА МИКОРИЗЕ  
*Tuber macrosporum/Corylus avellana* ИЗОЛОВАНЕ СА  
ГАЈЕНИХ САДНИЦА: ПРИКАЗ СЛУЧАЈА

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**РЕЗИМЕ:** Испитивано је присуство микоризе на садницама из дела десет година старог тартуфишта (3.000 m<sup>2</sup>) у источној Србији. Овај рад представља први извештај о утврђивању присуства ектомикоризне гљиве из рода *Tuber* током симбиотске фазе на кореновима *Corylus avellana* L. Десет узорака коренова сакупљани су методом случајног узорка и макроскопск посматрани и анализирани микроскопским методама. Уочене су промене у морфологији и анатомији измењених коренова *C. avellana*. На површини корена јасно се уочава омотач од хифа – мантила, и мицелија која формира Хартингову мрежу. У непосредној близини насумично одабраних садница леске, на површини земљишта пронађена су зрела плодносна тела црног тартуфа – идентификована као *Tuber macrosporum* Vittad. Према нашим сазнањима до сада нису постојале информације о микоризи *Tuber macrosporum/Corylus avellana* на вештачки успостављеном тартуфишту у Србији.

**КЉУЧНЕ РЕЧИ:** *Tuber* spp., *Corylus avellana* L, ектомикориза, морфо-анатомска анализа, аскокарп, аскоспоре



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## ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AND FRUITS SUPPLEMENT IN REDUCED NITRITE SALTS CONDITION

**ABSTRACT:** Because of the growing negative perception of consumers related to the use of meat products produced by conventional curing methods, organic and natural products are increasingly accepted by consumers. Such products contain a large number of natural products derived from plants, spices, as well as their derivatives in form of essential oils, extracts, concentrates, and so on. These derivatives contain large number of active substances which are known to inhibit the metabolic processes of bacteria, yeasts and molds. Therefore, the goal of this paper was to investigate the synergistic antimicrobial activity of the models with a reduced presence of nitrite salt in aqueous solution, emulsions of essential oils in varying concentrations *in vivo* via antibiogram tests on pathogenic microorganisms.

The effect of the six model groups was analyzed. Two groups were fruit powder solutions in concentrations of 0.2% to 1.2% (Acerola powder and fruit powder mix), while the other four groups were models of aqueous emulsion of essential oil in concentrations ranging from 0.05% to 1.2% (tea tree, clove, oregano, and cinnamon essential oils). In all models reduced amount of the sodium salt of 1.80%, 0.0075% nitrite salt and the liquid derivative as a natural source of the nitrate salt of 3% were used. Antibiogram tests were performed on five pathogenic bacteria (*C. perfringens*, *E. coli*, *S. enterica*, *L. monocytogenes*, and *S. aureus*). All antibiogram tests were performed according to Kirby-Bauer disk diffusion protocol.

Results of antibiograms showed that without the presence of additional antimicrobial agents, in model systems with reduced content of salts, inhibition zones were not detected. Additionally, models with essential oils of tea tree oil and oregano had the widest inhibition zone diameters, ranging from 17.76±0.48mm for *E. coli* up to 42.50±0.13mm for *S. aureus*.

**KEYWORDS:** antibiograms, essential oils, food pathogens, natural antimicrobials, organic meat products

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## INTRODUCTION

In the production of organic meat products, various natural derivatives, such as plant extracts, dried fruit and vegetable powders (Sebranek *et al.* 2012), various fibers from fruits, vegetables and cereals, vegetable proteins, unsaturated fat and others (Weiss *et al.* 2010; Grasso *et al.* 2014) are widely used. Plants, spices, as well as their derivatives, essential oils, extracts, concentrates, etc. contain many active substances that are known to inhibit the metabolic processes of bacteria, yeasts and molds (Falow *et al.* 2014). Antimicrobial compounds in plant materials are predominantly found in essential oils of various leaf fractions, flowers and flower buds, heads, rhizomes, fruits or other parts of the plant (Sultanbawa, 2011; Pradeep, 2012). Active substances from plants may be fatal for microorganism cells or inhibit the production of metabolites (e.g. mycotoxins) (Navajas *et al.* 2012). A small number of active compounds in essential oils can have synergistic effects with other components, which as a final result produce natural supplements with certain antimicrobial activities (De Oliveira *et al.* 2012; Reihani *et al.* 2014; Guerra-Rosas *et al.* 2017).

Basically, all bioactive compounds can be divided into two large groups – the first, larger group, consists of terpenes and terpenoids, while the other consists of aromatic compounds (phenylpropanoid) (Dinesh and Cheorun, 2013). The most common terpenes in herbal products are: p-cymene, terpinene, limonene, pinene, and sabinene (Sultanbawa, 2011; Sang-Jo *et al.* 2017). Terpenoids can be divided into alcohols, esters, aldehydes, ketones, ethers and phenols (Manzoor *et al.* 2014). Geraniol, menthol, linalool, citronellol, thymol, carvacrol, geranyl acetate, eugenol acetate and 1,8-cineole are the best known representatives of terpenoids, which are most commonly found in various essential oils (Dinesh and Cheorun, 2013; Senanayake, 2013). Cinnamaldehyde, cinnamyl alcohol, kavicol, eugenol, estragole, eugenol methyl cinnamate and methyl group are phenylpropanoids (Tingting *et al.* 2012; Hyun-Joo *et al.* 2014; Siroli *et al.* 2015).

Therefore, the aim of this study was to investigate the synergistic antimicrobial activity models in aqueous solutions/emulsions of natural antimicrobials (fruit concentrates and essential oils), which are used or potentially could be used in the development of organic meat products, by antibiogram tests on pathogen microorganisms.

## MATERIALS AND METHODS

Models of antibiograms (A1 to A30) were tested *in vivo* with reference strains of pathogenic microorganisms (*Clostridium perfringens*, *Escherichia coli*, *Salmonella enterica*, *Listeria* and *Staphylococcus aureus*). Essential oils or derivatives of fruits as potential antimicrobial agents were used in an appropriate range of concentrations as shown in Table 1. In this way, the concentration range of the fruit products (Acerola powder manufacturers “Raps<sup>®</sup>”, Belgium and commercial product of mixed fruit powder “Superfruits” from the company

“Biotona<sup>®</sup>”) and essential oils, which can be used in organic meat products, was covered, as well as a wider range of the tested product concentrations. This was performed with the main goal to test the antimicrobial activity of natural antioxidants/preservatives in synergy with the commonly used salts (NaCl, KCl) and in the presence of nitrite salt. In addition to these, in all models was used a “liquid supplement” (1/3 Kombucha ferment and 2/3 leaf beet juice (*Eruca sativa*)) in the amount of 3% as a natural source of nitrate salt purchased from Raps<sup>®</sup>, Belgium.

Table 1. Models of antibiograms for different concentrations of fruit mixtures and essential oils

Models %	Water	NaNO <sub>2</sub>	NaCl	L.s.	Ac.	Sf.	T.t.	Cl.	Or.	Cin.	Total %
A 1	94.9925	0.0075	1.8	3	0.2						100
A 2	94.7925	0.0075	1.8	3	0.4						100
A 3	94.5925	0.0075	1.8	3	0.6						100
A 4	94.3925	0.0075	1.8	3	0.8						100
A 5	93.9925	0.0075	1.8	3	1.2						100
A 6	94.9925	0.0075	1.8	3		0.2					100
A 7	94.7925	0.0075	1.8	3		0.4					100
A 8	94.5925	0.0075	1.8	3		0.6					100
A 9	94.3925	0.0075	1.8	3		0.8					100
A 10	93.9925	0.0075	1.8	3		1.2					100
A 11	95.1425	0.0075	1.8	3			0.05				100
A 12	95.0425	0.0075	1.8	3			0.15				100
A 13	94.8925	0.0075	1.8	3			0.3				100
A 14	94.5925	0.0075	1.8	3			0.6				100
A 15	93.9925	0.0075	1.8	3			1.2				100
A 16	95.1425	0.0075	1.8	3				0.05			100
A 17	95.0425	0.0075	1.8	3				0.15			100
A 18	94.8925	0.0075	1.8	3				0.3			100
A 19	94.5925	0.0075	1.8	3				0.6			100
A 20	93.9925	0.0075	1.8	3				1.2			100
A 21	95.1425	0.0075	1.8	3					0.05		100
A 22	95.0425	0.0075	1.8	3					0.15		100
A 23	94.8925	0.0075	1.8	3					0.3		100
A 24	94.5925	0.0075	1.8	3					0.6		100
A 25	93.9925	0.0075	1.8	3					1.2		100
A 26	95.1425	0.0075	1.8	3						0.05	100
A 27	95.0425	0.0075	1.8	3						0.15	100
A 28	94.8925	0.0075	1.8	3						0.3	100
A 29	94.5925	0.0075	1.8	3						0.6	100
A 30	93.9925	0.0075	1.8	3						1.2	100

L.s. – Liquid supplement; Ac. – Acerola powder; Sf. – Superfruit powder; T.t. – Tea tree oil; Cl. – Clove oil; Or. – Oregano oil; Cin. – Cinnamon oil.

## ANTIBIOGRAMS

Antibiograms of pathogens were carried out according to the Kirby-Bauer disk diffusion protocol (Hudzicki, 2009). The tests were conducted with reference pathogens *Escherichia coli* WDCM 0001, *Clostridium perfringens* WDCM 00007, *Staphylococcus aureus* WDCM 00032, *Salmonella enterica* WDCM 00030, and *Listeria monocytogenes* WDCM 00020. All tests were performed on Mueller-Hinton agar media with a paper disc of 9mm in diameter. The 0.1 cm<sup>3</sup> of testing, reference pathogen was applied to previously dried sterile Mueller-Hinton agar surface plate. The starting concentration of the reference pathogen was matched by a 0.5 McFarland standard. Afterwards, on agar plates, paper disks were placed and infused with 50 µl of a tested model (A). Prepared plates were incubated at +37 °C for 24h. *Clostridium perfringens* WDCM 00007 was incubated under anaerobic conditions, while the rest were incubated aerobically. After incubation, diameter of inhibition zones around the disc paper was measured and recorded in millimeter. The higher the inhibiting effect of the tested model was (A), it corresponded to a larger diameter of the inhibition zone.

## STATISTICAL ANALYSIS

All experiments were performed with a model of minimum four replications. The results were processed by the PCA analysis of XLSTAT trial with the aim of reducing the large number of variables on a limited number of related factors (F) with the significance of  $p \leq 0.05$ . Associated factors, usually F1 and F2, described over 95% of the total variance of the observed variables.

## RESULTS AND DISCUSSION

The results of the detected inhibition zone with observed models (A) are shown in Table 2.

Table 2. Inhibition zone diameter (mm) of testing model (A) with the reference pathogen

Models	<i>C. perfringens</i>		<i>E. coli</i>		<i>S. enterica</i>		<i>L. monocytogenes</i>		<i>S. aureus</i>	
	$\bar{X}$ (mm)	±Sd	$\bar{X}$ (mm)	±Sd	$\bar{X}$ (mm)	±Sd	$\bar{X}$ (mm)	±Sd	$\bar{X}$ (mm)	±Sd
A1-A10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A11	0.00	0.00	0.00	0.00	14.12	0.82	13.67	0.31	12.07	0.56
A12	10.32	0.37	10.08	0.12	14.89	0.58	14.73	0.41	12.98	0.31
A13	12.05	1.17	14.83	0.46	15.28	0.62	15.92	0.67	13.09	0.59
A14	14.89	0.38	21.36	0.92	15.96	0.71	20.07	0.12	14.12	0.62
A15	17.73	0.29	23.87	1.26	20.73	0.83	24.31	1.37	15.97	0.72
A16	10.03	0.85	11.89	0.41	10.21	0.18	13.25	0.58	0.00	0.00

A17	14.21	1.15	14.25	0.63	11.87	0.62	14.46	0.84	12.25	0.48
A18	14.96	0.63	14.31	0.36	13.26	0.67	15.73	0.91	13.75	0.33
A19	16.27	0.89	14.56	0.67	13.58	0.48	16.18	0.39	14.50	0.19
A20	24.58	1.18	15.02	0.72	15.25	0.72	17.27	0.87	15.18	0.21
A21	16.37	0.82	14.92	0.69	14.85	0.67	15.76	0.81	15.83	0.32
A22	18.21	0.26	15.36	0.71	16.89	0.86	16.84	0.74	19.68	0.17
A23	20.52	0.59	16.87	0.83	17.26	0.56	17.36	0.31	24.25	1.24
A24	24.81	1.23	17.76	0.48	19.37	0.89	18.92	0.12	30.75	1.12
A25	35.07	1.89	20.81	0.94	28.84	1.84	21.89	0.28	42.50	0.13
A26	11.78	0.46	0.00	0.00	0.00	0.00	11.96	0.39	0.00	0.00
A27	13.87	0.91	0.00	0.00	11.78	0.62	13.48	0.17	11.39	0.41
A28	16.67	0.85	10.09	0.24	12.59	0.56	15.68	0.89	12.85	0.52
A29	17.88	0.94	12.28	0.57	12.97	0.81	15.95	0.19	14.73	0.38
A30	18.84	0.31	14.35	0.69	14.50	0.39	16.12	0.74	15.17	0.58

The results of PCA analysis of detected inhibition zone diameters (mm) are shown in Table 3 and Figure 1.

Table 3. Correlation coefficients (“loadings”) of the main components (F1 and F2) of the PCA analysis of inhibition zone diameter (mm)

Variable	F1	F2
$\phi$ (mm) – <i>C. perfringens</i>	0.958	0.036
$\phi$ (mm) – <i>E. coli</i>	0.918	-0.307
$\phi$ (mm) – <i>S. enterica</i>	0.975	-0.009
$\phi$ (mm) – <i>L. monocytogenes</i>	0.943	-0.238
$\phi$ (mm) – <i>S. aureus</i>	0.948	0.304
Eigenvalue	356.741	19.966
Total variance (%)	90.086	5.042

$\phi$  (mm) – Diameter of inhibition zones

Table 3 and Figure 1 show that the inhibition zones depend on the tested model (A) and are strongly correlated with component F1, which describes the antimicrobial potential of tested models in antibiograms. Component F1 describes the 90.09% of the total variance. All models located on the right side of Biplots, Figure 1, are strongly antimicrobial, with antimicrobial activity significantly different ( $p \leq 0.05$ ) in relation to the models (A) located on the left side of Biplot. On the far right side is model A25 with the oregano essential oil in a concentration of 1.2%. Models with oregano essential oil even at lower concentrations proved to be potent inhibitors of the tested bacteria. The diameters of inhibition zone depended on the concentration of the essential oil and the type of testing, reference pathogenic microorganisms, ranged up to  $42.50 \pm 0.13$  mm (A25), Table 3, for the reference strain *Staphylococcus aureus*.

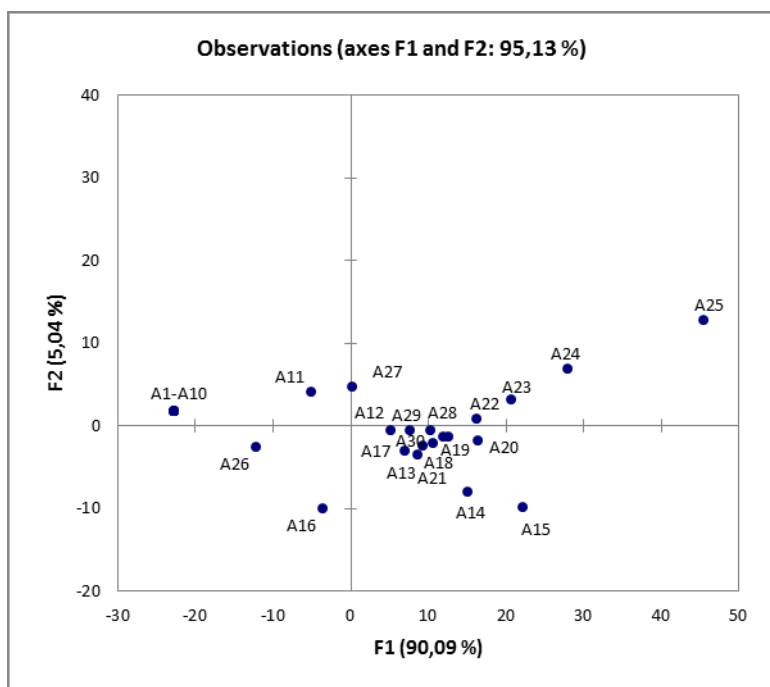


Figure 1. “Biplots” main components (F1-anti-microbial potential) of the PCA analysis of the tested model (A) inhibition zone diameter (mm)

From the right to the left side of Biplot in Figure 1, the model A15 is a model in which the tea tree essential oil was added in concentration of 1.2%. This model, in addition to models with oregano essential oil, has a significantly potent ( $p \leq 0.05$ ) antimicrobial activity, compared to models located on the left side of Biplot. On the far left side on Biplot are positioned models A1 to A10 with Acerola powder and fruit mix powder “Superfruit”. These models did not show antimicrobial activity in the tested concentration range, since zones of inhibition were not detected.

Tea tree essential oil shows a wide range of antimicrobial activity to Gram-positive and Gram-negative bacteria, *Bacillus spp.*, and fungi (Bakkali *et al.* 2008). Oussalah *et al.* (2007) and Goñi *et al.* (2009) studied the effect of cinnamon and clove essential oil on a large number of pathogenic microorganisms such as *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Listeria monocytogenes*. The scanning electron microscope images showed significant damage on the surface of cell membranes of pathogens (Souza *et al.* 2013; Rai *et al.* 2017). The essential oil of oregano showed a strong antimicrobial activity against *E. coli* O157:H7, *L. Monocytogenes*, and *S. Typhimurium*. Oregano essential oil exhibited potent microbicidal activity against pathogenic microorganisms in concentrations of up to 2% (Sang-Jo *et al.* 2017). Inhibition zones of *S. enteritidis*, *E. coli* O157:H7, *L. Monocytogenes*, and *S. aureus* growth,

proportional to concentration applied at higher concentrations of essential oils, yielded wider zones of inhibition (Khanjar *et al.* 2013; De Jesus *et al.* 2016).

The results of this experiment are in great correlation with previously mentioned papers related to antimicrobial activity of tested essential oils.

## CONCLUSION

All tested essential oils showed high antimicrobial activity against tested reference strains of bacteria. Statistically significant potent ( $p \leq 0.05$ ) inhibition of tested pathogens was achieved in the models with oregano essential oil (A21 to A25). Besides oregano, the model A15 with tea tree essential oil showed high antimicrobial activity with inhibition zone from  $15.97 \pm 0.72$  mm (*S. aureus*) up to  $24.31 \pm 1.37$  mm (*L. monocytogenes*). The synergistic antimicrobial activity of essential oils in the presence of reduced concentration of inorganic salts was not observed. Furthermore, in models in which fruit supplements were used, zones of inhibition were not detected. System of reduced content of salt (sodium chloride, nitrite, etc.), without the additional presence of essential oils has no inhibitory effect on tested bacteria.

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## АНТИМИКРОБНА АКТИВНОСТ ЕТЕРИЧНИХ УЉА И ВОЋНИХ ПРЕПАРАТА У УСЛОВИМА РЕДУКОВАНОГ САДРЖАЈА НИТРИТНИХ СОЛИ

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**РЕЗИМЕ:** Због све веће негативне перцепције потрошача која је везана за употребу производа од меса који су произведени по конвенционалним методама саламуриња, производи од меса који носе ознаку на декларацији као органски и природни производи, уживају све веће повјерење и прихватање код потрошача. Такви производи садрже велики број природних препарата добијених из биљака, зачина, као и њихових деривата у облику етеричних уља, екстраката, концентрата итд. Ови препарати садрже велики број активних супстанци за које се поуздано зна да инхибирају метаболичке процесе бактерија, квасаца и плјесни. Стога је циљ овог рада био да се испита синергијска антимикробна активност модел система с редукованим присуством нитритних соли водених раствора и емулзија етеричних уља у различитим концентрацијама *in vivo* путем антибиограмских тестова на патогеним микроорганизмима. Испитиван је утицај шест група модела – од тога двије групе су биле прави раствори воћних препарата у концентрацијама од 0,2% до 1,2% (ацерола у праху и воћни микс), а остале четири групе модела представљали су водене емулзије етеричних уља у распону концентрација од 0,05% до 1,2% (етерична уља чајеваца, каранфилића, оригана и цимета). У свим моделима коришћене су редуковане количине кухињске соли (1,80%), нитритне

соли (0,0075%) и течни препарат као природни извор нитратних соли (3%). Урађени су антибиограмски тестови *in vivo* на патогеним микроорганизмима (*C. perfringens*, *E. coli*, *S. enterica*, *L. monocytogenes* и *S. aureus*). Сви антибиограмски тестови изведени су према Kirby-Bauer диск дифузионом протоколу. Резултати антибиограмских тестова су показали да без присуства додатних антимикробних препарата у модел системима с редукованим садржајем соли, нема никаквог инхибирања пораста испитиваних патогена. Такође, модели с етеричним уљима чајевца и оригана имали су најјаче изражене зоне инхибиције, које су се кретале од  $17,76 \pm 0,48$  mm за *E. coli* до  $42,50 \pm 0,13$  mm за *S. aureus*.

**КЉУЧНЕ РЕЧИ:** антибиограмски тестови, етерична уља, патогени у храни, природни антимикроби, органска храна

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## ANTIFUNGAL ACTIVITY OF INDIGENOUS *Bacillus* spp. ISOLATED FROM SOIL

**ABSTRACT:** Biocontrol using plant growth-promoting rhizobacteria (PGPR) represents an alternative approach to disease management, since PGPR are known to promote growth and reduce diseases in various crops. Among the different PGPR, members of the genus *Bacillus* are preferred for most biotechnological uses due to their capability to form extremely resistant spores and produce a wide variety of metabolites with antimicrobial activity. The objective of this research was to identify antagonistic bacteria for management of the plant diseases. Eleven isolates of *Bacillus* spp. were obtained from the soil samples collected from different localities in the Province of Vojvodina. The antifungal activity of bacterial isolates against five fungal species was examined using a dual plate assay. *Bacillus* isolates exhibited the highest antifungal activity against *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *cepae* and *Alternaria padwickii*, while they had the least antagonistic effect on *Fusarium verticillioides* and *Fusarium graminearum*. Molecular identification showed that effective bacterial isolates were identified as *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11), *Bacillus subtilis* (B5, B7) and *Bacillus megaterium* (B8, B9). The highest antagonistic activity was exhibited by isolates B5 (from 39% to 62% reduction in fungal growth) and B7 (from 40% to 71% reduction in fungal growth). These isolates of *B. subtilis* could be used as potential biocontrol agents of plant diseases.

**KEYWORDS:** *Bacillus*, biocontrol, *Fusarium*, *Alternaria*, antifungal activity, isolation, soil

## INTRODUCTION

*Fusarium* and *Alternaria* species are among major pathogens that infect plants throughout the year at all growth stages and cause destructive and eco-

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nomically damaging diseases responsible for high yield reductions (James, 1981). Control of plant diseases is largely based on genetic resistance in host plants, cultural practices and synthetic pesticides (Lazarovits *et al.* 2014). Beside environmental impact and potential health risk related to the chemical pesticides application in agriculture, chemical control also creates imbalances in the microbial community, which may be unfavorable to the activity of beneficial organisms and lead to the development of resistant strains of pathogens (Aktar *et al.* 2009).

The need for alternative control strategies, particularly those involving biological control, has greatly increased over the past two decades. Biocontrol using plant growth-promoting rhizobacteria (PGPR) represents an alternative approach to disease management, since PGPR are known to promote growth and reduce disease in crops (Lugtenberg and Kamilova, 2009). The most common approach to biological control consists of selecting antagonistic microorganisms and developing a biological control product (Alabouvette *et al.* 2006).

Several antagonistic microorganisms have been tested for their ability to inhibit phytopathogenic fungi, including *Fusarium* and *Alternaria* species (Jain and Pandey, 2016; Li *et al.* 2017). Although some fungal antagonists showed effective inhibition, bacterial antagonists mainly from the genus *Bacillus* have shown by far the most promising results (Pane and Zaccardelli, 2015; Zalila-Kolsi *et al.* 2016). Due to their capability to form extremely resistant spores and produce a wide variety of metabolites with antimicrobial activity, members of the *Bacillus* genus are generally found in soil. *Bacillus* spp. strains inhibit pathogen growth primarily through the production of antibiotics, cell wall degrading enzymes, competition for nutrients and/or inducing systemic resistance (Lugtenberg *et al.* 2013).

The objective of this study was to isolate *Bacillus* spp. from soil and to examine their *in vitro* antifungal activity toward *Fusarium* and *Alternaria* species.

## MATERIAL AND METHODS

### *Soil sample collection*

Different soil samples were randomly collected from various parts of the Province of Vojvodina (northern Serbia). Several diverse locations were selected for the collection of soil samples, which included the rhizosphere of plants, agricultural and non-agricultural soils. Soil samples differed in their cropping and tillage history, physical and chemical properties. Samples were taken up to a depth of 20 cm. After removing approximately 3 cm of the soil surface, as well as large roots and stones, the remainder was passed through an autoclave-sterilized brass sieve with a 2 mm aperture size and then stored at 4 °C until further examination.

### *Isolation of Bacillus spp.*

Soil dilutions were prepared with 1 gram of each soil sample suspended in 9 mL of 0.85% NaCl in sterile test tubes. A 0.1 ml aliquot of each dilution

( $10^{-3}$ – $10^{-6}$ ) was spread aseptically on Nutrient Agar (NA) and incubated at 30 °C for 24 hour. After the incubation, colonies showing resemblance with *Bacillus* spp., roughly identified based on their morphology, were transferred and recultivated five times to obtain pure cultures. The bacterial isolates were characterized by their morphological and biochemical characteristics using standard methods (Jarak and Đurić, 2006).

### *Antifungal activity assay*

Antifungal activity of *Bacillus* spp. isolates against five fungal isolates was tested *in vitro* using a dual plate assay (Zhao *et al.* 2010). In addition to three fungal isolates identified from garlic cloves (*Fusarium proliferatum*, *Fusarium verticillioides*, *Fusarium oxysporum* f. sp. *cepae*) (Ignjatov *et al.* 2016a), another two were isolated from seeds of soybean (*Fusarium graminearum*) (Ignjatov *et al.* 2016b) and rice (*Alternaria padwickii*). Bacterial isolates were grown for 24h in nutrient broth (NB) at 30 °C, while potato dextrose agar (PDA) was used for the cultivation of fungi. The mycelial plugs (6 mm in diameter) of each fungus were sampled from the 7-day-old cultures and aseptically transferred on the PDA, about 25 mm from the edge of each Petri dish. A broth culture of the tested bacteria was then streaked 30 mm away from the mycelial plugs in the same dish. The controls consisted of cultures of the tested fungi without the presence of *Bacillus* spp. isolates. All dual cultures and controls were incubated for 7 days at 25 °C. Antifungal activity assay was done in three repetitions for each treatment. The percent of growth inhibition (PGI) was calculated using the following formula:  $PGI (\%) = [(KR-R1)/KR] \times 100$ , where KR represents the fungal growth (measured in mm) in the control dishes, and R1 is the fungal growth in the treated dishes (Dimkić *et al.* 2015).

### *Molecular species identification*

*Bacillus* isolates for DNA extraction were grown on NA plates for 24 h. DNA was extracted using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's recommendations. For the amplification of 16S rDNA gene fragments, primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) were used (Weisburg *et al.* 1991). The polymerase chain reaction (PCR) was done in 25- $\mu$ l aliquots using S-thermal cycler (Eppendorf, Germany). The PCR reactions were performed with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C primer annealing for 1 min, and 72 °C extension for 30 s, followed by a final extension step at 72 °C for 7 min. Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide. Purification and sequencing of the PCR-amplified DNA fragments were done in the company MACROGEN, Seoul, South Korea (<http://dna.macrogen.com>). FinchTV Version 1.4.0. was used for sequence analysis and nucleotide sequences were

filed in the GenBank Database at the National Center for Biotechnology Information (NCBI).

### *Statistical analysis*

Data were subjected to analysis of variance (ANOVA) using software STATISTICA 12.6 (Statsoft, Tulsa, Oklahoma, USA). Means were separated using Tukey's HSD (honest significant difference) test at the  $P < 0.05$  level.

## RESULTS AND DISCUSSION

This study confirmed the presence of *Bacillus* spp. in soil samples collected from diverse locations in Vojvodina. Because of their fast growth and ability to sporulate under unfavorable conditions, *Bacillus* spp. isolates are attractive candidates for application as biocontrol agents. Analysis of antagonistic activity of newly-isolated strains against phytopathogenic fungi showed that *Bacillus* spp. isolates exhibited the highest antifungal activity against *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *cepae* and *Alternaria padwickii*, while they had the least antagonistic effect on *Fusarium verticillioides* and *Fusarium graminearum* (Table 1). The highest antagonistic activity was exhibited by isolates B5 (39–62%) and B7 (40–71%) which inhibited the growth of all tested fungal isolates except *F. verticillioides* (Figure 1). Antagonistic effect toward *F. proliferatum* and *A. padwickii* was also observed through confrontation with the isolates B2 (35–42%), B3 (31–38%), and B11 (3–37%). Isolate B8 exhibited antifungal activity against *F. proliferatum* (45%), while isolate B9 inhibited the growth of *F. proliferatum* (42%) and *Fusarium oxysporum* f. sp. *cepae* (33%). Antifungal activity of isolates B1, B4 and B10 was not detected. Significant variability within the same fungal species was found in different isolates, except for *F. graminearum*. The results obtained in this study showed different sensitivity of fungal species tested. Different degrees of fungal inhibition by individual *Bacillus* spp. isolates were also observed.

Similar findings about fungal growth inhibition and possible application of *Bacillus* spp. isolates as biocontrol agents have been found in numerous studies. Isolates of *Bacillus* spp. showed strong *in vitro* inhibition, as well as plant disease suppression of *Fusarium*, *Alternaria*, *Rhizoctonia*, *Aspergillus*, *Cryphonectria*, *Phytophthora*, etc. (Mnif and Ghrib, 2015). Dimkić *et al.* (2015) reported that *Fusarium* species were more resistant to *Bacillus* spp. isolates, while *Alternaria* were among the most sensitive fungi tested. *Bacillus* spp. isolates efficient in biocontrol of various plant pathogens have been mostly found positive for production of lytic enzymes and lipopeptide antibiotics (Abdallah *et al.* 2017). Beside their role in biocontrol, *Bacillus* species can enhance plant nutrition and promote plant growth and development via associative nitrogen fixation, phosphate solubilization, production of phytohormones and siderophores, or enzymatic activities (Borriss, 2011). Therefore, the use of *Bacillus* spp.

as biopesticides and biofertilizers is a promising approach which may result in reduced application of chemical pesticides and fertilizers and improved quality of agricultural products.

Table 1. Antifungal activity of *Bacillus* spp. isolates

Isolate	<i>Fusarium proliferatum</i>	<i>Fusarium verticillioides</i>	<i>Fusarium oxysporum</i> f. sp. <i>cepae</i>	<i>Fusarium graminearum</i>	<i>Alternaria padwickii</i>
PGI (%) $\pm$ SD					
B1	nd	nd	nd	nd	nd
B2	41.57 $\pm$ 2.96 b	nd	nd	nd	35.29 $\pm$ 1.18 c
B3	30.98 $\pm$ 2.72 c	nd	nd	nd	38.04 $\pm$ 0.68 c
B4	nd	nd	nd	nd	nd
B5	61.57 $\pm$ 2.96 a	nd	46.27 $\pm$ 4.45a	39.20 $\pm$ 1.01 a	58.04 $\pm$ 2.72 b
B6	nd	nd	nd	nd	nd
B7	64.71 $\pm$ 2.04 a	nd	42.94 $\pm$ 2.61 a	39.80 $\pm$ 0.68 a	71.37 $\pm$ 2.96 a
B8	44.71 $\pm$ 3.11 b	nd	nd	nd	nd
B9	41.96 $\pm$ 2.45 b	nd	33.33 $\pm$ 1.80 b	nd	nd
B10	nd	nd	nd	nd	nd
B11	3.14 $\pm$ 0.96 d	nd	nd	nd	36.83 $\pm$ 0.68 c

Mean values of fungal growth inhibition (n = 3) with standard deviation (SD) are shown. Values followed by the same letter within columns are not significantly different ( $P < 0.05$ ), according to Tukey's HSD test. PGI: percent of growth inhibition; nd: not detected.

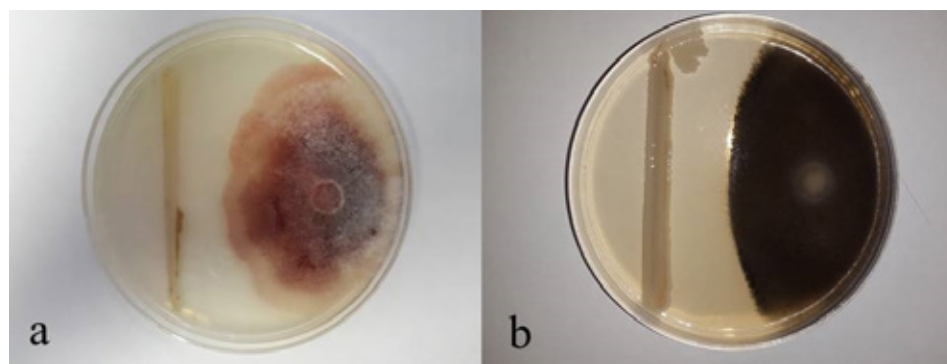


Figure 1. Antifungal activity of *Bacillus subtilis* B7 against *Fusarium proliferatum* (a) and *Alternaria padwickii* (b)

By comparing the sequences with the *Bacillus* ID-database, bacterial isolates effective in fungal growth inhibition were identified as *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11), *Bacillus subtilis* (B5, B7) and *Bacillus megaterium* (B8, B9). Non-effective isolates were identified as *Lysinibacillus fusiformis* (B1, B4, B10) and *Bacillus cereus* (B6) (Table 2).

Table 2. Isolates of *Bacillus* species from soil in Vojvodina (Bjelić *et al.* 2016)

Isolate	Isolation source	Locality	<i>Bacillus</i> species	NCBI
B1	Agricultural soil	Rumenka	<i>Lysinibacillus fusiformis</i>	KU953922
B2	Non-agricultural soil	Banatski Dvor	<i>Bacillus safensis</i>	KU953932
B3	Rhizosphere (wheat)	Bukovac	<i>Bacillus pumilus</i>	KU953923
B4	Non-agricultural soil	Petrovaradin	<i>Lysinibacillus fusiformis</i>	KU953924
B5	Rhizosphere (sunflower)	Bački Petrovac	<i>Bacillus subtilis</i>	KU953925
B6	Non-agricultural soil	Šangaj	<i>Bacillus cereus</i>	KU953926
B7	Rhizosphere (maize)	Rimski Šančevi	<i>Bacillus subtilis</i>	KU953927
B8	Rhizosphere (pepper)	Rimski Šančevi	<i>Bacillus megaterium</i>	KU953928
B9	Rhizosphere (alfalfa)	Perlez	<i>Bacillus megaterium</i>	KU953929
B10	Non-agricultural soil	Pančevo	<i>Lysinibacillus fusiformis</i>	KU953930
B11	Forest soil	Vršачka kula	<i>Bacillus pumilus</i>	KU953931

*Bacillus*-based plant disease biocontrol products usually contain one or two strains which belong to species of *B. subtilis*, *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens* (Berg, 2009). In this study, the strongest and broadest antagonistic activity against all tested fungi was exhibited by isolates of *B. subtilis*. High genetic heterogeneity of different *Bacillus* species, particularly *B. subtilis* allows to suggest that search and identification of new strains from different sources may expand the number of practically important strains and improve our understanding of mechanisms involved in antagonistic interactions (Mardanov *et al.* 2017).

## CONCLUSION

This study confirmed that most of the isolates of *Bacillus* spp. from the soil were found positive for antifungal activity by *in vitro* test. Significant variability within the tested fungal species was found in different isolates. The most effective isolates, identified as *Bacillus subtilis* (B5 and B7), could be used as potential biocontrol agents of plant diseases. Further selection of these isolates through greenhouse and field trials will be necessary in order to establish their efficiency as biopesticides in different crops.

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## АНТИФУНГАЛНА АКТИВНОСТ ПРИРОДНИХ *Bacillus* spp. ИЗОЛАТА ИЗ ЗЕМЉИШТА

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**РЕЗИМЕ:** Биоконтрола фитопатогена представља алтернативу примени пестицида, с обзиром да бактерије означене термином PGPR (Plant Growth Promoting Rhizobacteria) стимулишу биљни раст и штите биљке од болести. Захваљујући способности да формирају веома резистентне ендоспоре и производе широк спектар антимикробних супстанци, врсте рода *Bacillus* су веома заступљене у земљишту и погодне за примену у биотехнологији. Циљ истраживања био је да се утврди антифунгална активност једанаест *Bacillus* spp. изолата из земљишта с различитих локалитета у Војводини. Способност бактеријских изолата да инхибирају раст пет изолата гљива испитана је методом двојне култивације. Изолати *Bacillus* spp. испојили су највећу антифунгалну активност према *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *cepae*, и *Alternaria padwickii*, док је најмањи антагонистички ефекат утврђен према *Fusarium verticillioides* и *Fusarium graminearum*.

Ефективни изолати идентификовани су као *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11), *Bacillus subtilis* (B5, B7) и *Bacillus megaterium* (B8, B9). Највећу антифунгалну активност испољили су изолати *B. subtilis* B5 (39–62%) и B7 (40–71%). Ови изолати могу се користити као потенцијални агенси за биолошку контролу биљних болести.

КЉУЧНЕ РЕЧИ: *Bacillus*, биоконтрола, *Fusarium*, *Alternaria*, антифунгална активност, изолација, земљиште



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## MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *Fusarium tricinctum* AND *Fusarium acuminatum* AS CAUSAL AGENTS OF GARLIC BULBS ROT IN SERBIA

**ABSTRACT:** Garlic (*Allium sativum* L.) is considered to be one of the oldest crops in the world. During 2016, infected garlic bulbs occurred in storages on several localities of the Province of Vojvodina. Symptomatic cloves showed typical rot symptoms such as softened and spongy areas covered with white fungal growth with deep lesions formed on the cloves which became dry over time. A total of 36 isolates of *Fusarium* species were obtained from diseased cloves of garlic. Colony morphology and microscopic properties of isolated *Fusarium* species were recorded from the cultures grown on PDA and CLA, respectively. Identification of two chosen isolates was performed by sequencing the EF-1 $\alpha$  gene. The TEF sequence of isolate JBL12 showed 100% similarity with several *F. tricinctum* sequences and sequence of JBL539 showed 99% identity with several *F. acuminatum* sequences and they were deposited in the NCBI GenBank. Based on the results of the morphological and molecular identification, isolates JBL12 and JBL539 were identified as *F. tricinctum* and *F. acuminatum*, respectively, as new causal agents of garlic bulbs rot in Serbia. Specific primers were designed for the PCR identification of the *F. tricinctum*.

**KEYWORDS:** garlic (*Allium sativum*), bulb and clove rot, *Fusarium* spp., EF-1 $\alpha$  gene

## INTRODUCTION

Garlic (*Allium sativum* L.), a bulbous vegetable, is considered to be one of the oldest horticultural crops in the world (Moyers, 1996). This garlic species is grown worldwide, particularly in mild climate regions, with the total world annual production being 24 million tons on average. The top producers are China, Egypt, India, Korea and USA. Garlic is cultivated in Serbia, covering more

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than 7,000 ha, mostly concentrated in the northern part of Serbia, Vojvodina Province.

Fungal pathogens such as *Fusarium* spp. can cause significant economic losses at the postharvest stage of garlic (Kim *et al.* 2003; Palmero *et al.* 2013). The significance of *Fusarium* rot of garlic has been increasing in Serbia and according to Lević *et al.* (2009) *F. proliferatum*, *F. oxysporum*, *F. solani* and *F. verticillioides* species were previously identified and isolated from infected cloves of garlic in Serbia. Stanković *et al.* (2007) reported that *F. acuminatum* and *F. equiseti* were isolated from onion, whereas *F. proliferatum*, *F. oxysporum* and *F. solani* were species detected on both onion and garlic in Serbia.

During 2016, infected garlic bulbs occurred in storage and warehouses in several localities of the Province of Vojvodina. Diseased cloves showed typical rot symptoms such as softened and spongy areas covered with white or reddish fungal growth with deep lesions formed on the cloves which became dry and small over time. Symptomatic bulbs were subjected to phytopathological analysis in order to identify the causative agent of the disease.

## MATERIAL AND METHODS

### *Morphological characterization*

To isolate the causative organism, cloves were separated from the bulbs, the margins of the lesions were cut into small pieces, surface-sterilized with 1% NaOCl for 2–3 min, and washed three times with sterile distilled water and plated onto a Potato Dextrose Agar (PDA) medium amended with 300 mg/l streptomycin sulphate (Gerlach and Nirenberg, 1982). Plates were incubated at 26 °C in the dark. Seven days later, *Fusarium* colonies were recognized morphologically and chosen isolates were subcultured in PDA using a single spore technique. A total of 36 isolates of *Fusarium* species were obtained. Colony morphology and microscopic properties of isolated *Fusarium* species were recorded from the cultures grown on PDA and CLA, respectively.

### *DNA extraction and molecular species identification*

To obtain a DNA sequence, a total DNA of the 36 isolates and one positive control FE-3 was extracted directly from the 7 days old mycelium (~ 100 mg wet weight), with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following DNA extraction, the translation elongation factor 1-*alpha* gene region was amplified by PCR with the primer pair EF1 and EF2 (Geiser *et al.* 2004). The amplification was performed on the Eppendorf Mastercycler PCR device, using the modified program by Abdel-Satar *et al.* (2003): 35 repeated cycles: 94 °C 1 min, 53 °C 1 min, 72 °C 2 min. The PCR mixture with a total volume of 25 µl consisted of 2x Eppendorf Master Mix (Taq DNA polymerase 1.25 U, 30mM Tris-HCl, 50mM KCl, 1.5mM

MgCl<sub>2</sub>; 0.1% Igepal-CA630; 0.2 mM dNTP); 0.6 µM of each primer and 1 µl of fungal DNA. Amplification fragments were determined using electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 µg/mL). The expected size of the amplified fragments was estimated by comparison with ready-to-use O'RangeRuler™ 100 bp DNA Ladder (SM0623, Fermentas). The agarose gel was visualised in UV transilluminator and the images were captured with DOC PRINT system (Vilbert Lourmat, USA).

Identification of isolates was performed by sequencing the translation elongation factor EF-1 $\alpha$  gene. Purification and sequencing of the amplified fragments was done in Company MACROGEN, Seoul, South Korea (<http://dna.macrogen.com>). Sequences were analyzed in the program FinchTV Version 1.4.0. Sequence of isolates JBL12 and JBL539 was compared with the previously reported isolates available in the NCBI GenBank and the *Fusarium* ID-database (Geiser *et al.* 2004), using the ClustalW program (Thompson *et al.* 1994) and MEGA5 software (Tamura *et al.* 2011).

### PCR assay specificity

On the basis of the EF-1 $\alpha$  gene fragment sequence, two primer sets were designed for specific identification of *F. tricinctum* using NCBI tool for finding specific primers based on specific sequence of isolate JBL12 (accession no. KX611146). PCR were performed in 25-µl reaction (Table 1), with all investigated isolates of *Fusarium* spp. (JBL1-JBL36). The PCR temperature profile comprised an initial denaturation step at 94 °C for 2 min, 35 cycles at 94 °C 1 min, 60 °C 1 min, 72 °C 2 min and a final extension at 72 °C for 10 min. Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide.

Table 1. Designed new primer sets for specific identification of *Fusarium tricinctum* and PCR conditions

Primer name	5'....'3	PCR components	Final concentration	25 µl reaction
BL12-3FUSTRF	TTCGCTCCCTCACTCGAAAC	2x MMix	1x	12.5 µl
BL12-3FUSTRR	TGAAGGAACCTTTCCGAGC	10 µM Primer F	1 µM	2.5 µl
BL12-5FUSTRF	AGTGCGGTGGTATCGACAAG	10 µM Primer R	1 µM	2.5 µl
BL12-5FUSTRR	GTTTCGAGTGAGGGAGCGAA	DNA	~1,000 ng	1.0 µl
		Nuc.-free water		6.5 µl

## RESULTS

### Morphological characterization

The fungal isolate (JBL539) formed a fast-growing (7 cm in 6 days), abundant, pale ochraceous, whitish-pink and partly carmine aerial mycelium.

This isolate also produced dark to blood – red pigmentation in agar which later turned amber with a dark tan colour at the edge of PDA, which is typical of *F. acuminatum* (Gerlach and Nirenberg, 1982). On CLA macroconidia were abundant, slender, equilaterally curved with elongated apical cell and pedicellate basal cell, mostly three to five septate (rarely 0–1 septate) and measuring 32–44 µm x 3.5–4.7 µm. The fungus formed globose to subglobose chlamydospores, mostly in pairs, chains or clusters. Microconidia were not observed. Based on the colony morphology and the description of fungal structures, the isolated fungus was identified as *F. acuminatum* (Ell. & Kellerm) (Gerlach and Nirenberg, 1982).

The isolate (JBL12), when grown on PDA, rapidly produced abundant, dense, white, aerial mycelium that became pink with age and formed red pigments in the medium. On CLA, macroconidia were abundant, relatively slender, curved to lunate and three to five septate. Microconidia were napiform, oval or pyriform, zero to one septate and commonly clustered in false heads, without chlamydospores. On the basis of fungal morphology, the fungus was identified as *F. tricinctum* (Corda) Saccardo (Gerlach and Nirenberg, 1982).

#### *DNA extraction and molecular species identification*

To confirm the morphological identification, total genomic DNA was extracted from the mycelium of the isolates JBL12 and JBL539 with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the EF-1 $\alpha$  region was successfully detected in all tested samples amplified by PCR with the primer pair EF1 and EF2 and obtaining fragments of predicted size (700bp). The PCR product derived from the isolates JBL12 and JBL539 was directly sequenced in both directions using the EF1/EF2 primer pair as in PCR and deposited in GenBank (Accession No. KX611146, KX752419). The sequences were compared to those in GenBank. TEF sequence of isolate JBL12 showed 100% similarity with several *F. tricinctum* sequences (e.g., HM068307, EU744838, and EU744837) while the EF sequence of JBL539 showed 99% identity with several *F. acuminatum* sequences (e.g., EF531698, KP868658, KJ194170).

#### *PCR assay specificity*

These new designed primers (BL12-3FUSTRF/BL12-3FUSTRR and BL12-5FUSTRF/BL12-5FUSTRR) successfully identified *F. tricinctum* and its separates from other different *Fusarium* species. One clear band of 223bp and 112bp, respectively, were visible in tested sample designated as JBL12 (*F. tricinctum*).

### DISCUSSION

Garlic bulbs are quite perishable because their high moisture content makes them vulnerable to microbial decay as well as physiological deterioration (Eckert

and Ogawa, 1988). Also *Fusarium* sp. are one of the most important pathogens in the growing season and during the storage. The control of *Fusarium* rot of garlic is difficult due to the epiphytic survival and spreading of the pathogen during storage. During 2016, infected garlic bulbs occurred in storages and warehouses on several localities of the Province of Vojvodina. Symptoms appeared during the storage, as spongy, softened, cloves covered with white, light pink or reddish mycelium. Over time the cloves became dry and small.

Based on the results of the molecular identification, macromorphological and micro-morphological characteristics of isolates JBL12 and JBL539 were identified as *F. tricinctum* and *F. acuminatum*, respectively, as new diseases causal agents of garlic bulbs rot in Serbia. Stanković *et al.* (2007) reported that *F. acuminatum* and *F. equiseti* were isolated from onion, whereas *F. proliferatum*, *F. oxysporum* and *F. solani* were species detected on both onion and garlic in Serbia. Recently, *F. tricinctum* has been described as a new pathogen of garlic in Serbia (Ignjatov *et al.* 2017) which, unlike *F. acuminatum* (JBL539), has citriform microconidia and falcate, strongly curved macroconidia, with a well-marked foot cell.

*Fusarium* species caused similar symptoms on stored garlic and it was difficult to distinguish them based on the symptoms and morphological characteristics. Polymerase chain reaction (PCR) with primers designated as EF1 and EF2 were created as choice of a single locus identification tool in *Fusarium* genus (Geiser *et al.* 2004). The presence of a 700 bp amplicon in all investigated isolates was confirmed by comparing the amplified DNA fragments with the marker and positive control. The translation elongation factor 1- $\alpha$  (TEF) gene which encodes an essential part of the protein machinery is highly informative at the species level in *Fusarium* (Geiser *et al.* 2004). Primers EF1 and EF2 were first developed in the fungi to investigate lineages within the *F. oxysporum* complex and these primers amplify an ~700 bp region of TEF in all known fusaria (O'Donnell *et al.* 1998).

Nucleotide sequence differences were found when sequence of the EF-1 $\alpha$  region of the *F. acuminatum* was compared to those of *F. tricinctum*, separating those two species and diverse clusters. Our report on these pathogens provides a basis for epidemiological studies and supports other efforts towards the development of effective disease management strategies for this pathosystem.

The disease tends to occur more frequently in garlic and it is more often a problem in storage than in the field. *Fusarium* species infecting garlic affect the health safety of agricultural workers, especially those associated with processing and store houses, as well as the consumers. Presence of *Fusarium* cloves infection decreases physiological properties of garlic, especially seed health and germination potential.

Using tool for finding specific primers based on specific sequence of *F. tricinctum* (JBL12/NCBI Acc. KX611146), a PCR-based assay was developed for the specific detection of *F. tricinctum*, which has been validated using 36 strains of the *Fusarium* sp. from different garlic varieties and geographical origins. Two primers sets were designed for specific PCR identification: BL12-3FUSTRF/BL12-3FUSTRR and BL12-5FUSTRF/BL12-5FUSTRR. One clear

band of 223 bp and 112 bp was visible in tested isolate JBL12 and no amplicon was recorded in negative control and other strains.

This finding will provide the basis to develop the effective disease management strategies and specific identification of *F. tricinctum* as causal agent of garlic bulb rot in Serbia.

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МОРФОЛОШКА И МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА  
*Fusarium tricinctum* И *Fusarium acuminatum*  
ПРОУЗРОКОВАЧА ТРУЛЕЖИ БЕЛОГ ЛУКА У СРБИЈИ

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**РЕЗИМЕ:** Бели лук (*Allium sativum* L.) сматра се једном од најстаријих биљних врста на свету. Током 2016. године, запажена је појава трулежи белог лука у складиштима и магацинима на неколико локалитета у Војводини. Симптоми су се испољавали у виду лезија, трулежи белог лука као и појавом мицелије на инфицираним ченовима. Циљ рада био је изолација и идентификација *Fusarium* spp. на основу морфолошких и молекуларних карактеристика патогена. Изолацијом је добијено 36 изолата *Fusarium* spp. Детекција и идентификација одабраних изолата потврђена је методом PCR коришћењем прајмера EF1 и EF2 који амплификују производе величине 700bp. У свим проучаваним изолатима формирани су ампликони величине 700bp. Изолација ДНК два одабрана изолата извршена је директно из мицелије гљиве (~100 mg), коришћењем DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Идентификација изолата JBL539 и JBL12 извршена је секвенцирањем EF-1α гена, који су депоновани у NCBI базу података под бројем KX611146 (*F. tricinctum*) и KX752419 (*F. acuminatum*). Креирани су специфични прајмери за PCR идентификацију врсте *F. tricinctum*.

**КЉУЧНЕ РЕЧИ:** бели лук (*Allium sativum*), трулеж луковица и ченова, EF-1 alpha ген



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## DARK-FIELD MICROSCOPE STOOL ANALYSIS – ITS ROLE IN DIAGNOSIS OF YEAST OVERGROWTH IN GUT

**ABSTRACT:** Not long after birth, yeast, predominantly *Candida albicans*, colonizes the epithelium of oral cavity and the whole gastrointestinal tract. *C. albicans* lives in yeast, a non-harming form, as a commensal member of the microbial flora, but may turn into pathogen infective form under certain conditions that encourage its overgrowth. In this phase, it may damage the intestinal wall and enter the bloodstream, causing invasive candidiasis with high mortality rate. It is essential to recognize candidaemia and start the lifesaving therapy on time. Recognizing the risk factors which allow candida to overgrow is the most important step in preventing candida's overgrowth and chronic candidiasis, the previous status of invasive candidiasis. If this recognition is missed, and the overgrowth advances, a question remains how to discover and treat it and in which phase it should be done. A stool culture requires time and proves the presence of live yeast cells only. If the live yeast cells are not present in the stool, the result of the culture will be negative. In this paper, the author presents her experience of stool analysis under dark-field microscope, as a rapid, easy to carry out method for detecting the presence of live or dead yeast cells and yeast overgrowth.

**KEYWORDS:** yeast infection, candida infection, candida overgrowth, chronic candidiasis, stool analysis, dark-field microscope

## INTRODUCTION

The first publication of *Candida* overgrowth in the intestine after antibiotics administration was written by Irene Neuhauser in 1954. In 1972, Iwata Kazuo published his observation of drunken people who never drank alcohol, but had high levels of blood alcohol produced by overgrown candida in their gut. He called this condition “drunkenness without drinking alcohol.” Later, Orian C. Truss published his book “The Missing Diagnosis” in 1983, William G. Crook published his first book “The Yeast Connection” in 1986 and John P Trowbridge's book “The Yeast Syndrome” was also published in 1986. At that time, their observations and findings were not accepted as scientific, but later on,

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more and more researchers have proved that these first works were right. Still, there is no consensus among doctors. Some believe, and the others do not, that yeast can overgrow in gut and cause disturbances in the functions of the whole body, and that candida syndrome or chronic candidiasis really exists.

The yeast, predominantly candida species, in small amount is normally the member of the intestinal microbial flora of a healthy person. *C. albicans* is the most common yeast species isolated from human stool. It colonizes the surfaces of the oral cavity and the whole gastrointestinal tract early after birth (Forbes *et al.* 2001) and remains there in yeast form without any sign of its presence until the intestinal flora maintains its balance and the immune system functions properly. It is commensal, unicellular yeast, but has dimorphic feature – ability to turn from round or oval unicellular budding yeast form to pseudo- or hyphae (filament) and mycelium forming multicellular fungal form. It is also an opportunistic pathogen. It means that under certain circumstances, when the friendly bacteria are reduced, or the cell mediated immune defence is depressed (following antibiotics, steroids, or immunosuppressive therapy, in immunocompromised persons, in diabetic person, in seriously ill patients treated in intensive care unit, and in the case of long term unhealthy nutrition, rich in sugar and refined carbo-hydrates) the benign yeast form begins to overgrow, spreads on the epithelium surface of the gut, turns into fungal form, damages the epithelium, and at the end, may cross through the intestinal mucosa and enter into the bloodstream, causing candidaemia and systemic candidiasis – fungal infection of any organ (Jawetz, Melnick and Adelber, 2001; Miranda, 2009). During the past decade, the incidence of mycotic infections has been increasing such as the mortality of systemic, invasive mycosis.

Before entering the bloodstream, candida overgrows on the mucosal surface of the gastrointestinal tract. The colonies are attached to the mucosa of the intestine wall with adhesin. This attachment is relatively stable and essentially irreversible (Kennedy, 1988). I surmise that the existence of this attachment may be the explanation of why in some patients at high risk with candida overgrowth, the colonies of the yeast/fungus are not present or just few of them is found in the stool, and consequently, the microscopic analysis and the culture are negative. However, after the administration of purgative, live yeast/fungus detaches from the surface and appears in the stool and becomes visible under dark-field microscope, as the dead yeast/fungus is visible after the administration of fungicide.

When overgrown, yeast/fungus may cause local symptoms of the intestine and distal symptoms of malfunction of any organ, as the whole body is affected. In literature this is known as candida syndrome or chronic candidiasis. The longer period they have been overgrowing, the larger surface they occupy, the symptoms become more complex and by time more severe. Therapy will take longer time, months or even years until the stool is cleared from yeast/fungus. If the complex therapy is not long enough, and/or was stopped prematurely, the remained yeast/fungus continues to overgrow and the symptoms reoccur.

As of recently, discovered pathogenesis and symptoms can be learned in the paper (Klein, 2009) and the book (László, 2014) written by the author.

Overgrowing candida irritates the mucosa of the intestine causing inflammation, mucous production, pain, rise in temperature, diarrhoea or constipation. Fermenting the sugar, yeast releases carbon dioxide, which leads to bloating and flatulence without smell, and alcohol damaging the liver or even causing drunkenness. If the covered surface of the mucosa is larger, the food digestion and nutrients absorbance are poor, and some nutrients deficiency occur with their typical symptoms. The damage of the intestine's wall may cause "leaky gut". This allows extraneous substances to enter the bloodstream developing food intolerance and allergy. The histamine blood level is higher causing itching anywhere, rash, overproduction of stomach acid, even asthma. Yeast needs sugar to overgrow, thus sugar, sweets and bread craving is very typical and leads to overweight. Starvation leads to the drop of the blood sugar (hypoglycaemia) with its typical symptoms. Many metabolic products of the yeast are absorbed into the bloodstream causing disturbances all over the body with a lot of different symptoms like chronic fatigue, irritability, attention deficit, headache, loss of memory and concentration, palpitation, cold arms and legs, recurrent infections, hormonal imbalance, fluid retention, and many others.

In most of the patients suffering mostly from chronic health problems, the cause/causes remain unrecognized, as in many cases of gastrointestinal disturbances the cause remains unknown, because the possibility of yeast overgrowth is not considered. Most doctors still refuse to admit that yeast can cause such numerous symptoms or illnesses due to their intestinal overgrowth.

To prove the connection between the candida overgrowth in gut and symptoms is not easy, because presently available diagnostic methods are imperfect. Candida antibodies (IgM, IgG) are present in blood of all people with normal humoral immunity as candida lives in everyone. Early skin prick test to candida is also positive in case of normal cellular immune response. In case of weakened immune system, the blood level of the antibodies is very low or immeasurable, and the early skin prick test to Candida is negative. People with weakened immune system presumably have certain overgrowth of candida. The same problem is with the stool analysis – microscopic and culture. Positive results can be seldom obtained from healthy people. The question is the quantity of yeast in their stool, normally there should be only a few single yeast cells. Rarely, in people with yeast/fungus overgrowth the stool may be yeast free, or just a few single cells can be seen, as the yeast/fungus colonies are strongly attached to the intestine mucosa. However, after the administration of purgative or fungicide, live or dead colonies of yeast/fungus, become visible in the stool by naked eye, as well as by dark-field microscope.

Dark-field illumination is a technique used to observe unstained samples, causing them to appear brightly lit against a dark, almost black background. In bright-field illumination, the object is lit from below the stage, resulting in a larger, contrasted image that can be studied. A dark-field microscope blocks this central light with a condenser so that only oblique rays hit the object. These rays from the side of the field make the samples bright, usually white against the dark background as the aperture of the condenser is larger than the aperture of the lens.

By using the dark-field microscope, the author aims to identify the presence and estimate the quantity of yeast in the stool of the patients suffering from chronic health problems, with increasing number of complaints over a long period of time, but with unidentified causes. The study is prospective, started ten years ago, and is still on-going. Until now the author has examined over 10,000 stool samples and made several thousand photographs. In this paper, the author presents her results of dark-field microscopic stool analysis of the mentioned people. Almost all patients have negative records (findings) of different examination carried out in the past (laboratory, endoscopy, US, etc.). None of them had microscopic stool analysis and almost none of them had candida stool culture. Just few of them had records of candida positive culture of the stool, but no doctors paid attention to the result.

### MATERIAL AND METHODS

The study is carried out in the author's medical office. The samples are taken from the fresh stool delivered on the day of the analysis.

First, the stool specimens are observed by naked eye and then examined with dark-field microscope.

Observation with naked eye:

1. The shape of the stool can be normal, sausage-like, or small globules stick together with or without mucus, covered with mucous (Figure 4), loose or even diarrhoea.
2. On the surface of the stool can be seen white, yellow or brown mucus or patches of different dimension (Figure 2, Figure 3), or the surface is normal, but deep scratching discovers the previously mentioned formations (Figure 1).

Dark-field microscope analysis is carried out with Zeiss Axioscop 40 microscope, with 10x and 40x Zeiss Achromat lenses.

The native specimens are taken from two different parts of the stool, or more if the previous two are yeast free; from the mucus or the patches if present, and placed on the slide (Figure 5).

Frequency of analysis:

In the case of positive findings at the first analysis, the next one will be carried out after two weeks of antifungal treatment. In the cases of negative findings, the patients also receive antifungal treatment and recall for first control examination after two weeks as well. To the author's experience, two weeks of therapy is sufficient for killing some quantity of colonies which then detach from the epithelium and become visible in the stool by naked eye and by microscope.

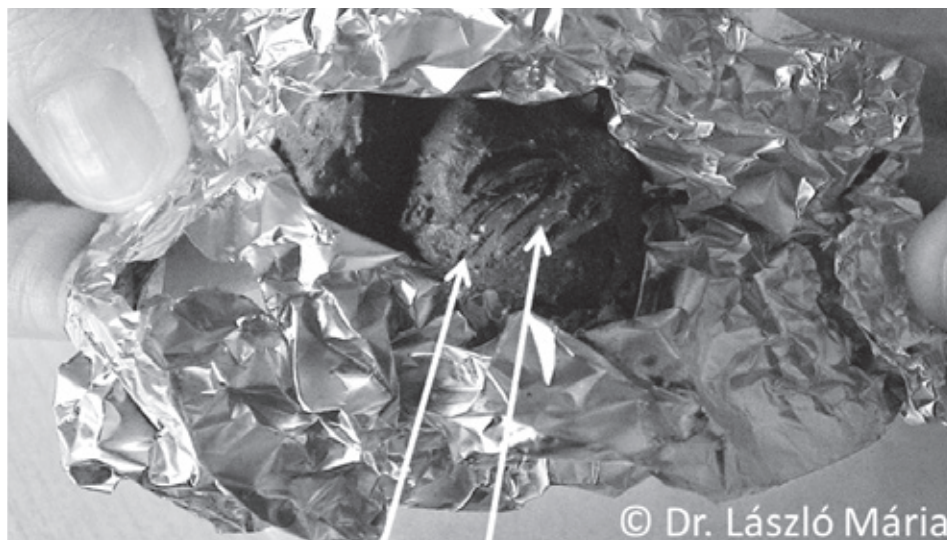
## RESULTS

The microscopic images of the stool may sometimes be yeast/fungal free (Figure 25), due to their stable adhesions to the wall of the intestine, or show different shape, dimensions and quantity of yeast/fungus as follows:

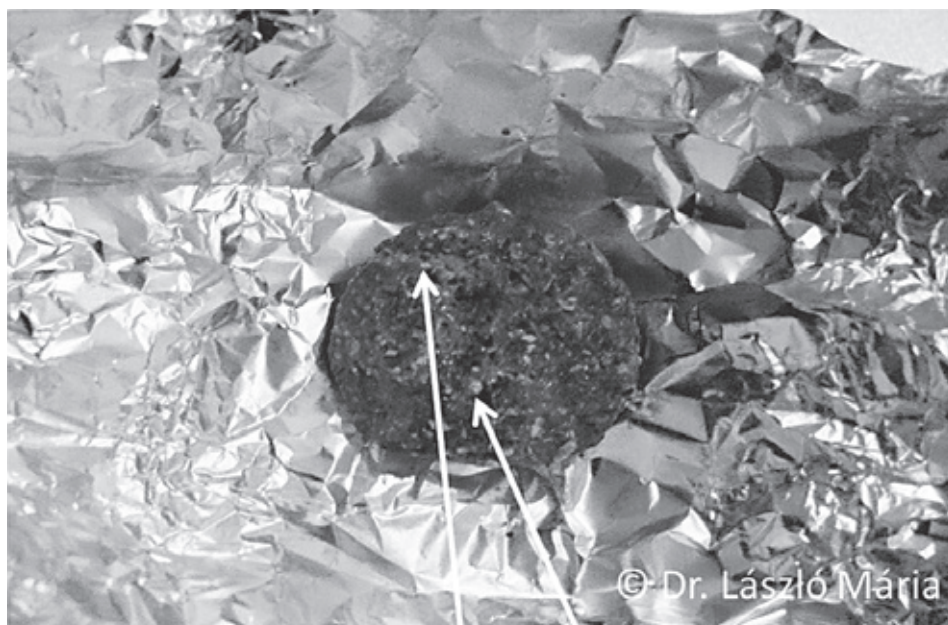
1. A few big single round live yeast cells (Figure 6)
2. A few big round live yeast cells, small colony of small round yeast cells and single hypha (Figure 7)
3. More small round, budding yeast cells (Figure 8)
4. More big round and oval live yeast cells without forming colonies (Figure 9)
5. A lot of small round live yeast cells without forming colonies (Figure 10)
6. A lot of small round live yeast cells with forming colonies (Figure 11)
7. A few big round live yeast cells and small round cells colony (Figure 12)
8. Big round live yeast cells colony with gas (bubbles) production (Figure 13), (in these cases patients complain of bloating and flatulence gas without smell)
9. Round live yeast cells, few or a lot of live conidia (Figure 14, 15, 16)
10. Live mycelia (Fig. 17) and live yeast colony inside the mycelia (Figure 18)

After two weeks of antifungal treatment the findings may be as follows:

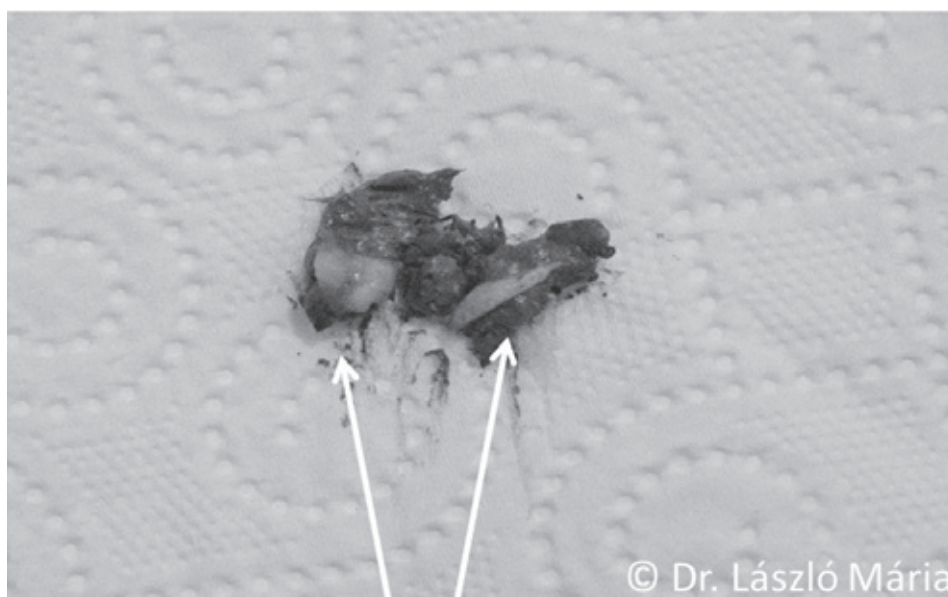
1. Big single round dead yeast cells (Fig. 19) and cracking big oval cells (Figure 20)
2. Big round yeast cells colony with live and dead cells (Figure 21)
3. Big round dead yeast colony with gas production (Figure 22) (these patients complains of very unpleasant bloating and flatulence of stink gas)
4. Dead conidia (Figure 23)
5. Dead mycelia (Figure 24)
6. Yeast free stool (Figure 25)



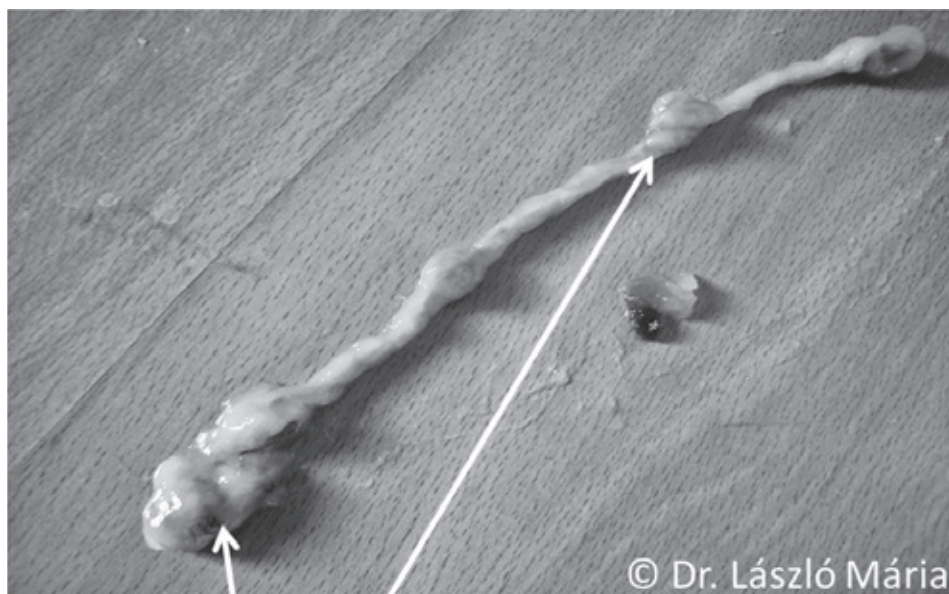
*Figure 1. White spots visible only after scratching*



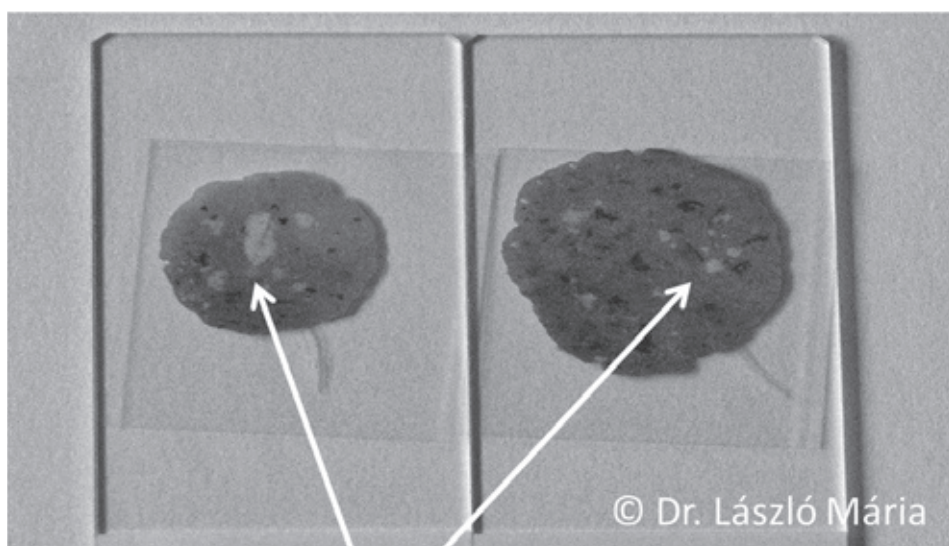
*Figure 2. A lot of white spots on and inside the stool*



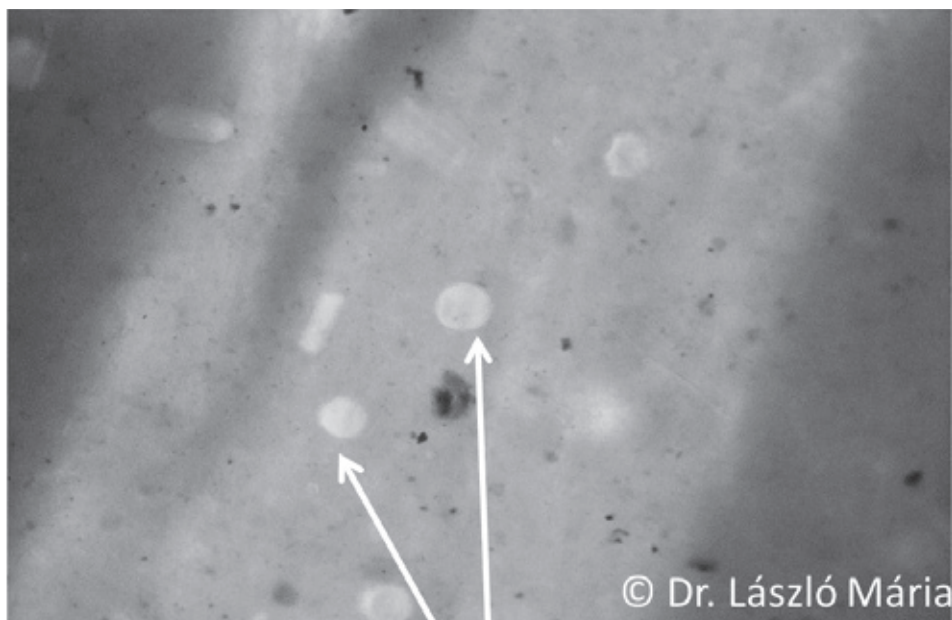
*Figure 3. White mucus in the stool*



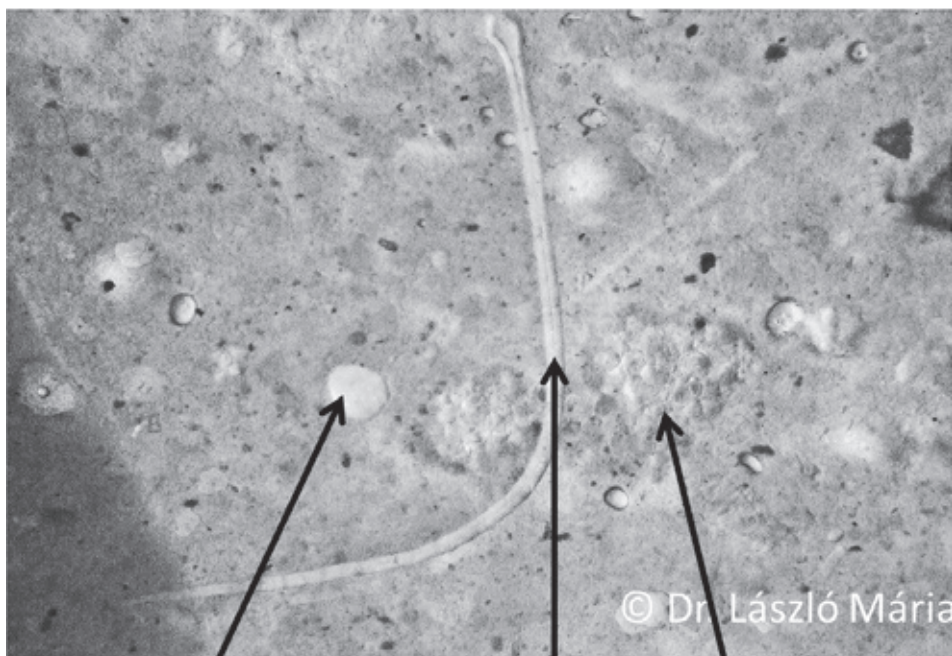
*Figure 4. Small pieces of stool covered with white mucus*



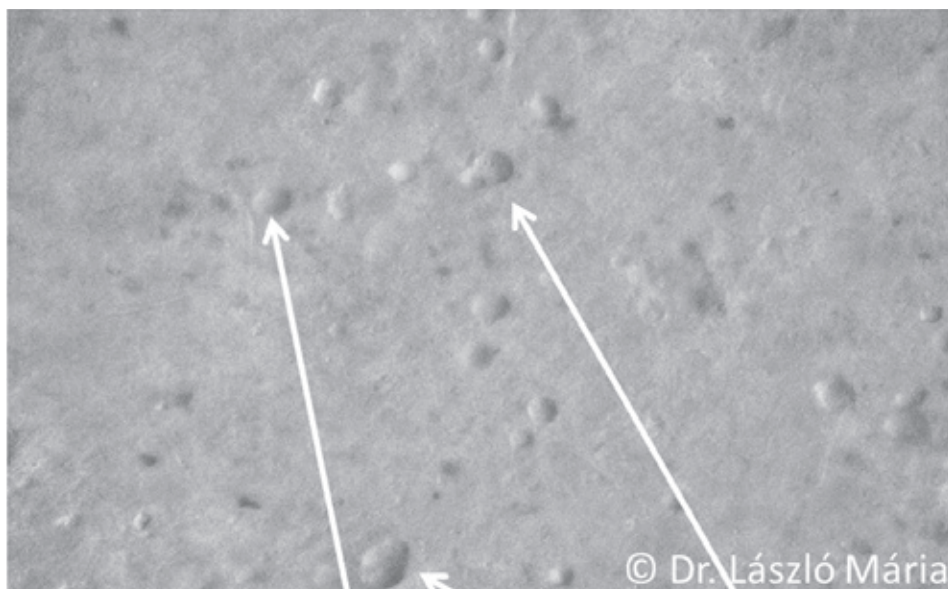
*Figure 5. White spots of different dimension*



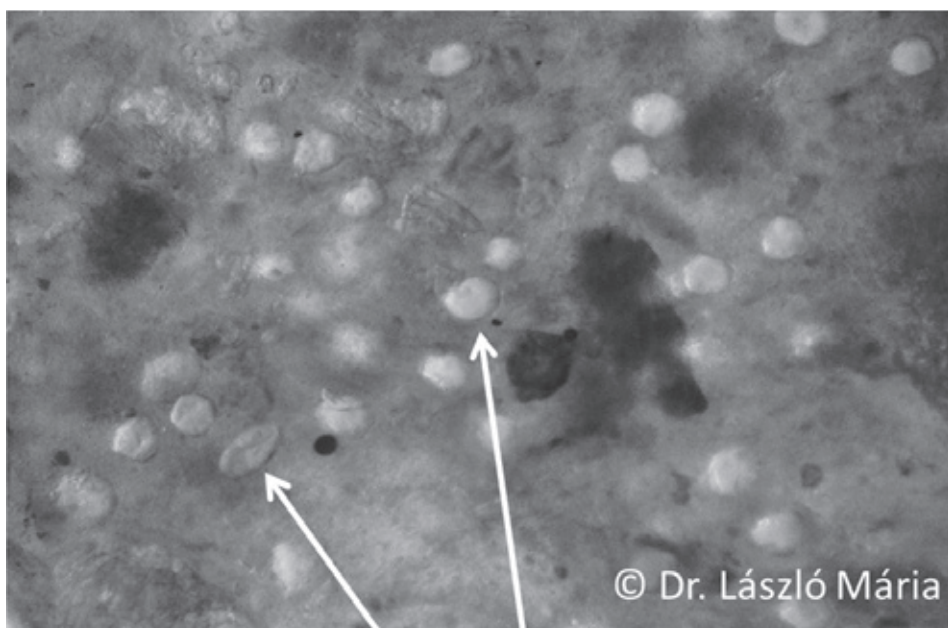
*Figure 6. A few big live single round yeast cells*



*Figure 7. Single big round live yeast cell, single hyphae, small yeast colony*



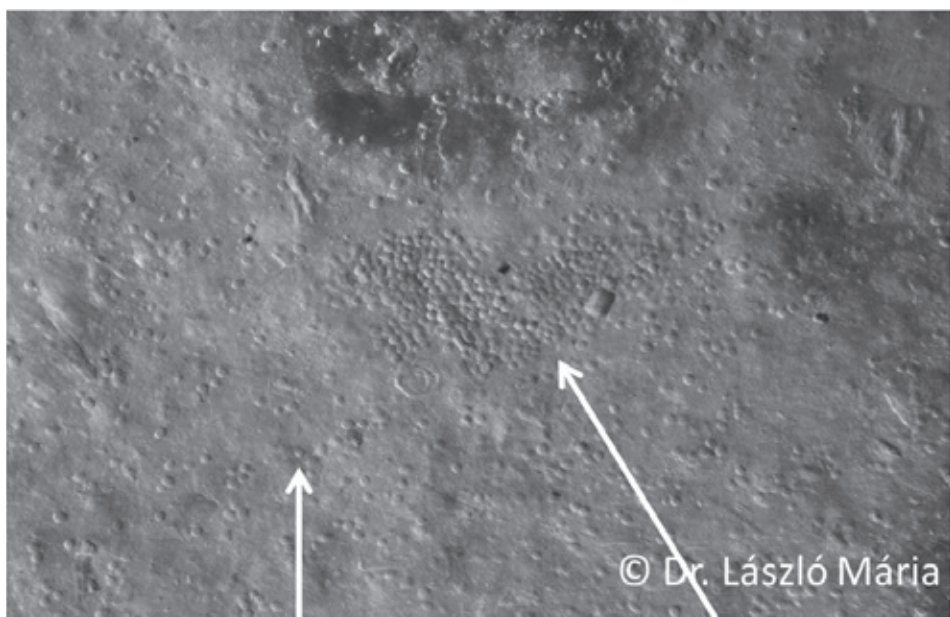
*Figure 8.* More single small round, one big oval live cell and budding



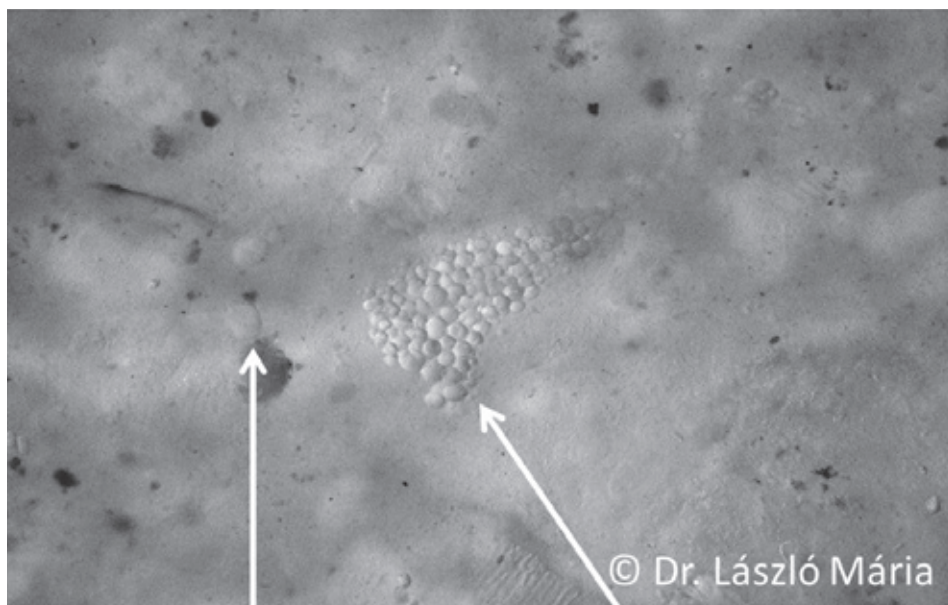
*Figure 9.* More big single live oval and round yeast cells



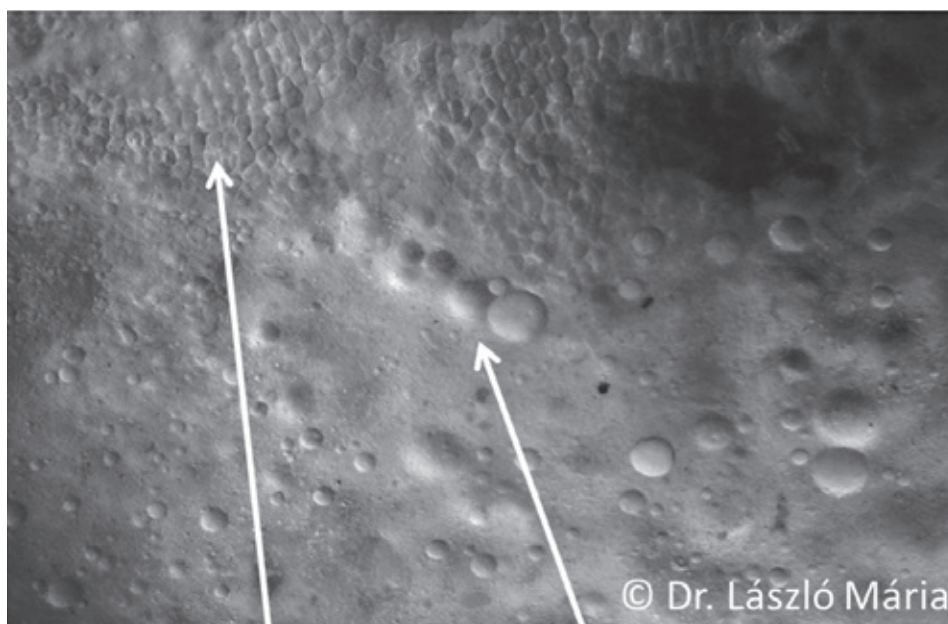
*Figure 10.* A lot of small single live yeast cells without forming colonies



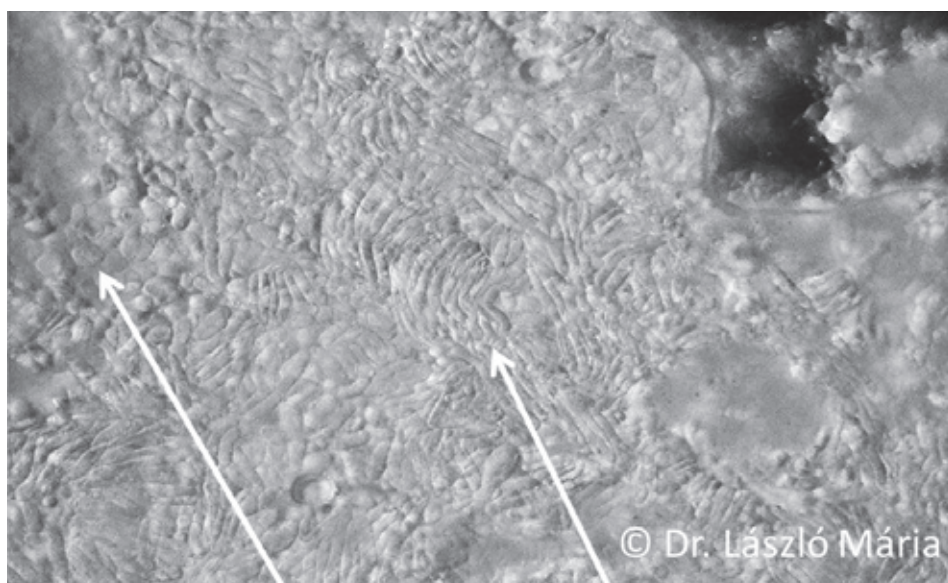
*Figure 11.* A lot of single round live yeast cells, a colony



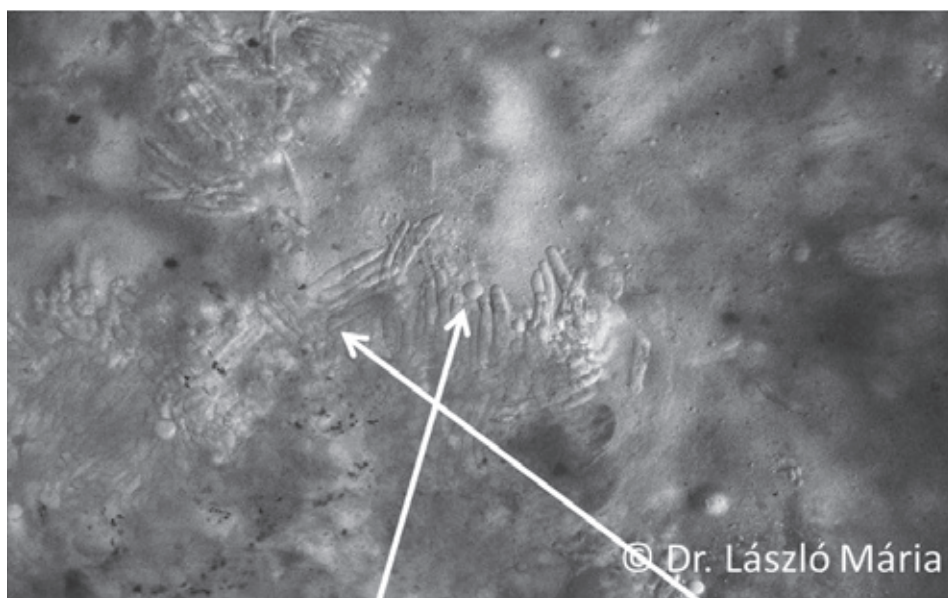
*Figure 12.* A few big single live yeast cells, a small yeast colony



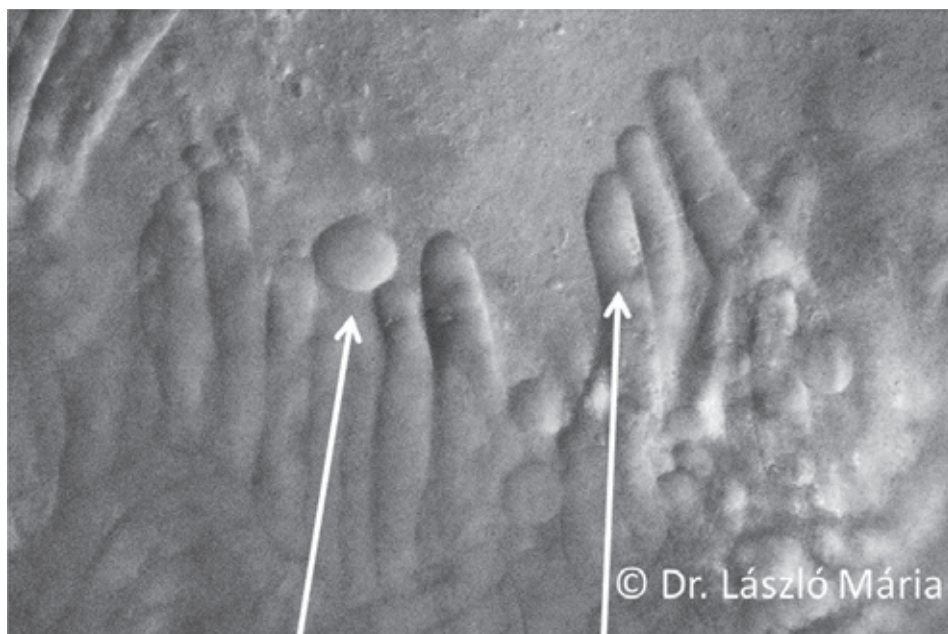
*Figure 13.* Big live yeast colony producing bubbles



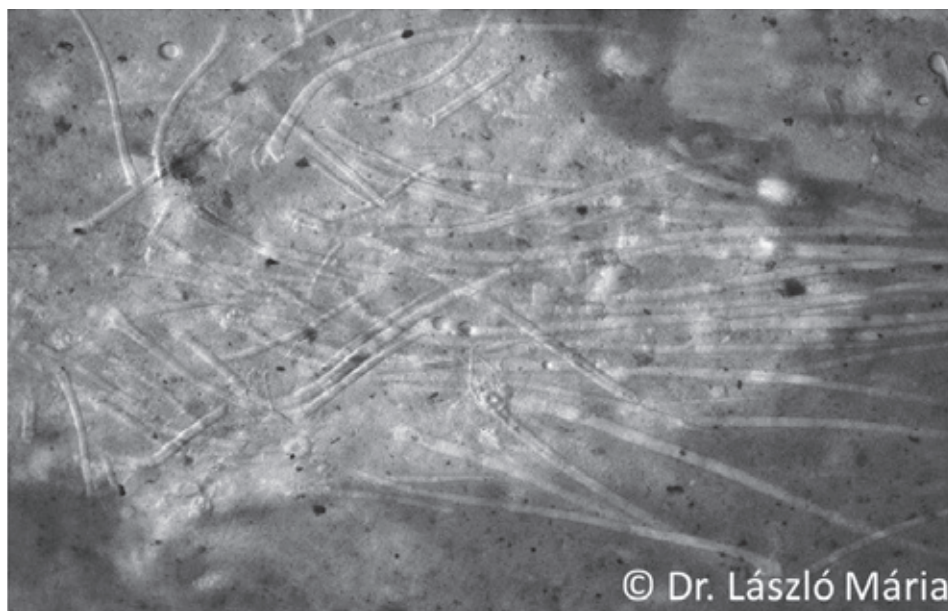
*Figure 14. Live yeast cells and a lot of live conidia*



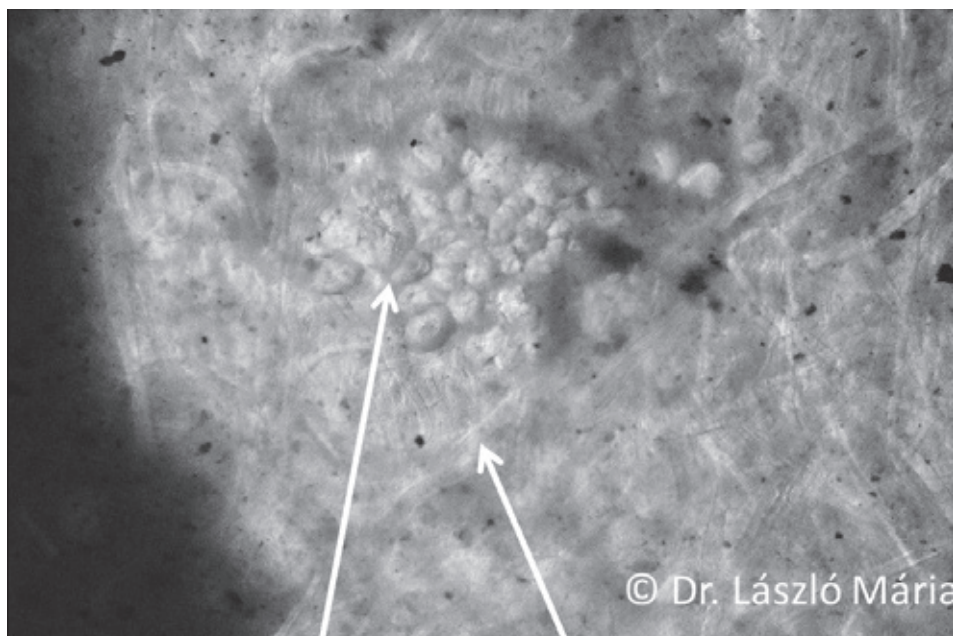
*Figure 15. A few single live yeast cells and a lot of live conidia*



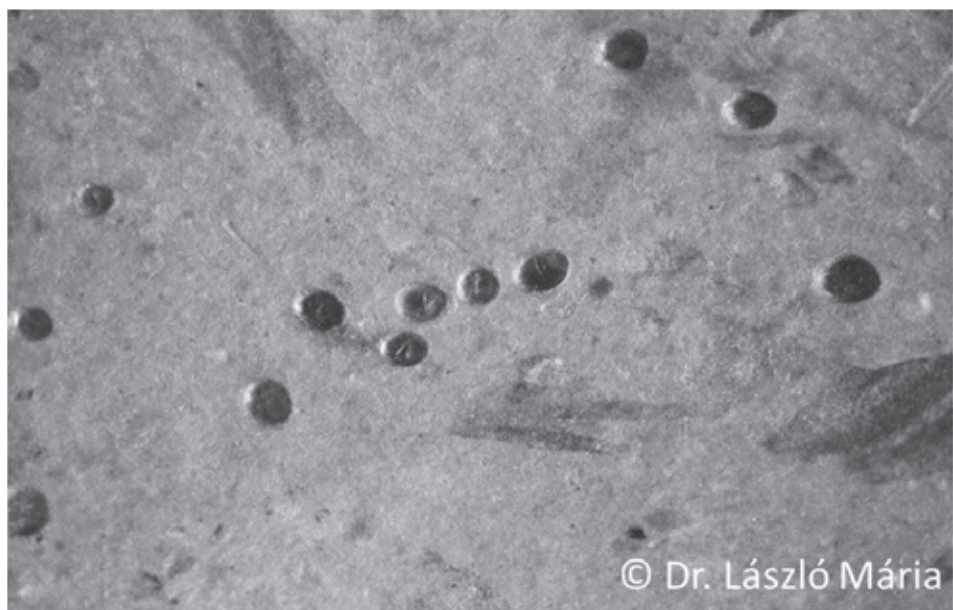
*Figure 16. A single live yeast cell and conidia (enlarged image)*



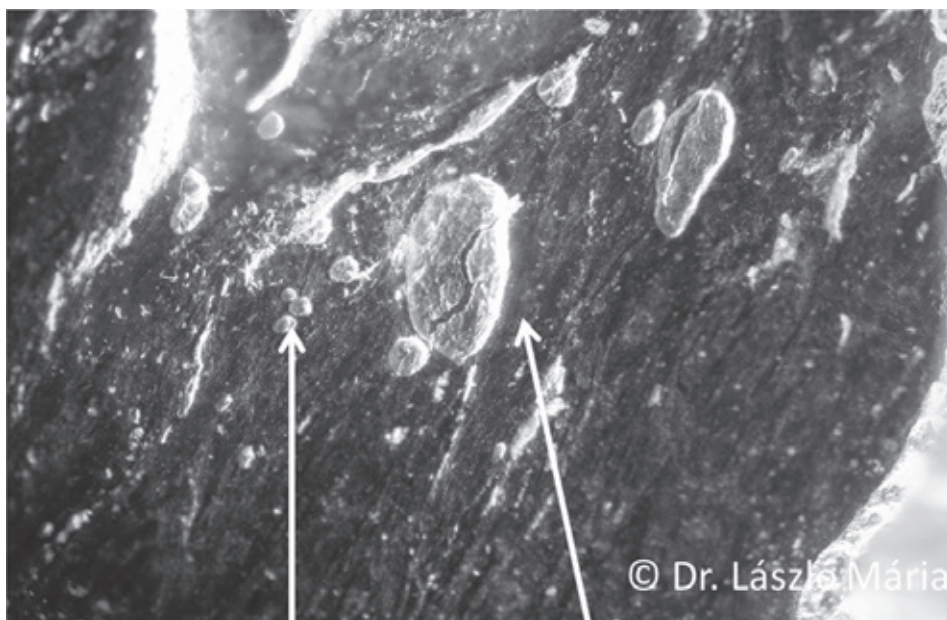
*Figure 17. Live mycelia*



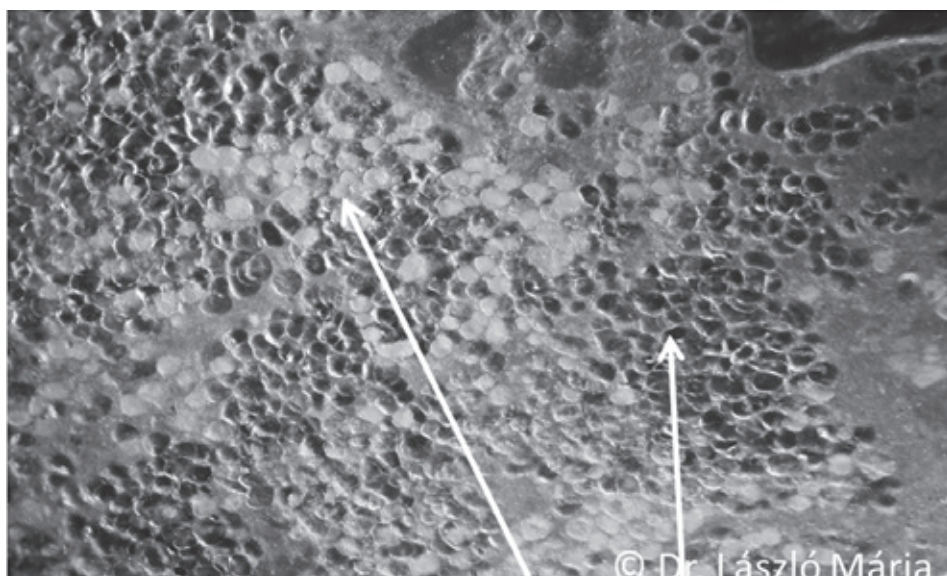
*Figure 18.* Live yeast colony inside the live mycelia



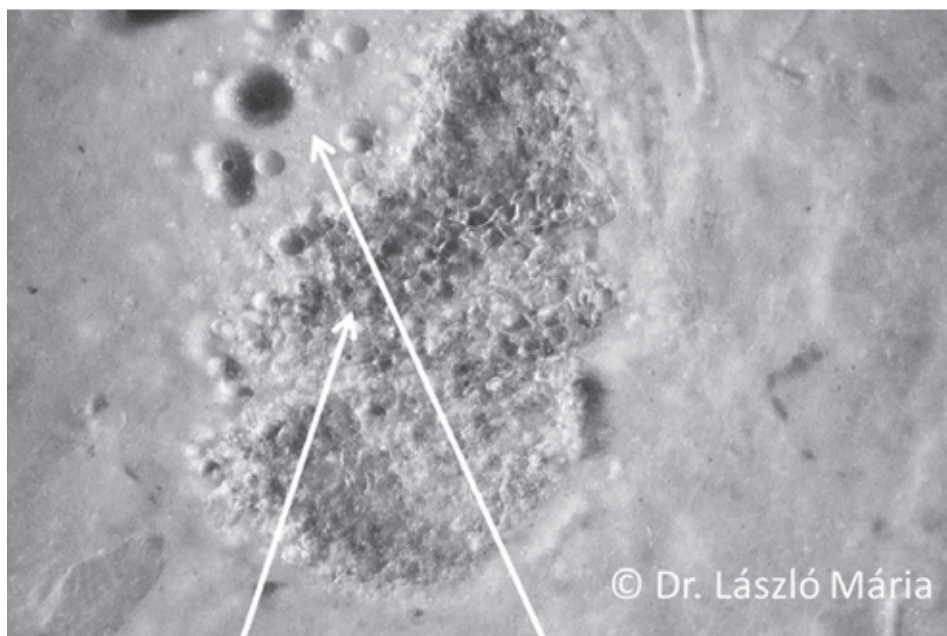
*Figure 19.* Single dead yeast cells



*Figure 20.* Small round live yeast cells and big cracking oval cells



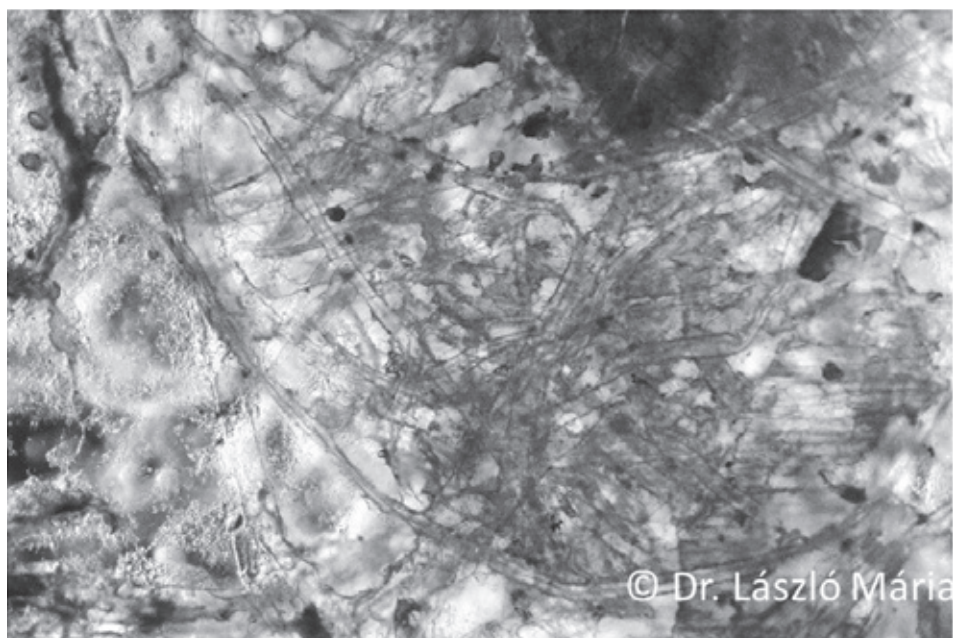
*Figure 21.* Big yeast colony: live (white) and dead (black) cells



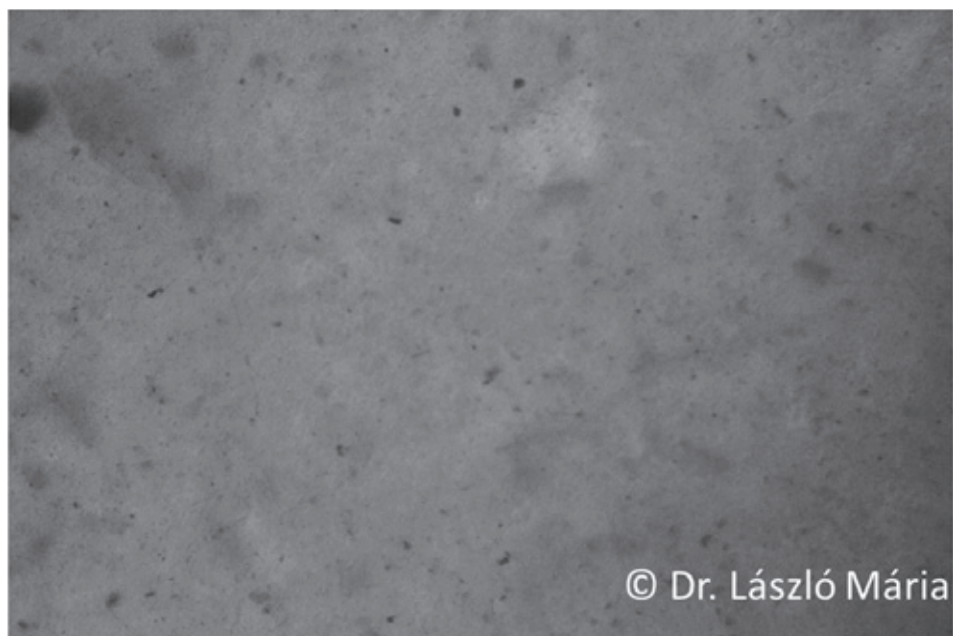
*Figure 22. Dead yeast colony, gas production*



*Figure 23. Dead conidia*



*Figure 24. Dead mycelia*



*Figure 25. Yeast free stool*

## DISCUSSION

In some cases of those who had yeast free stool at the first microscopic examination, after two weeks of antifungal treatment different shape and quantity of live and dead yeast/fungus appears, as it is in the first positive findings. In other cases with the first yeast negative stool, after two weeks of treatment the stool remains yeast negative. The follow-up of these patients should be discontinued, advising them a further search for the cause of their health problems and teaching them how to prevent candida overgrowth. There is no correlation in shape and quantity of live and dead yeast/fungus between the first, second, and all following examinations, but by time, if the treatment is efficient, the live yeast/fungus decreases in number and disappears at the end, while the quantity of dead ones increases at the beginning of the effective therapy and disappears at the end too. During the treatment, sometimes it is possible to see a few very big live and dead colonies, then smaller and yet smaller ones and again a few very big ones, until they all disappear. The next analyses are carried out from time to time during the treatment. Duration between the two analyses depends on the patient's status, complaints, way of therapy and microscopic findings. The dark-field microscope stool analysis is very useful in monitoring the effectiveness of the therapy, and if necessary it can be modified in time. The aim of the treatment is to reduce, minimize, or even clear the patient's symptoms and clear the stool from fungus and reduce the yeast to minimum. The length and the way of therapy is person-dependent, complex and may last from several months to more years. The longer is the yeast overgrowing, the bigger are the colonies, the larger is the territory they occupied and longer lasts the treatment. When the stool is yeast/fungus free (Figure 25), it doesn't mean that the therapy is over. After several months, yeast/fungus can be visible again by microscope, because often the remained yeast begins to overgrow if the treatment was stopped prematurely and the circumstances which encourage the yeast to overgrowth are still present and, of course, the symptoms return. Despite the fact that some doctors do not admit candida overgrowth as a possible source of candidaemia and invasive candidiasis in people at high risk, others emphasize the benefit of prophylactic antifungal treatment (Ozturk, 2006; Damjanovic, 1993) even in people at low risk (Normand, 2005).

## SUMMARY

Native stool analysis under dark-field microscope is an easy-to-carry-out examination, rapid, with no preparation, and may be carried out in general practice.

The freshly sampled stool from a person without any preparation is first observed with the naked eye and then, a small amount of it, taken from two or three different parts, should be placed on the slides, covered and observed under the dark-field microscope.

Rather soon, the doctor gets information whether the candida started to overgrow in patients at risk. Also, he/she is able to follow up the changes in the stool over time, to start with therapy on time and to monitor the effectiveness of therapy, aiming the prevention of candidaemia and invasive candida infection.

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АНАЛИЗА СТОЛИЦЕ МИКРОСКОПОМ С ТАМНИМ ПОЉЕМ –  
ЊЕГОВЕ МОГУЋНОСТИ У ОТКРИВАЊУ ПРЕКОМЕРНО  
РАЗМНОЖЕНИХ ГЉИВИЦА

Марија С. КЛАЈН-ЛАСЛО

„Клајн и Клајн Бт.“

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**РЕЗИМЕ:** Недуго након настанка, гљивице, углавном *Candida albicans*, насељавају слузокожу усне дупље и целог гастроинтестиналног тракта. *Candida albicans* живи у облику гљивица које нису штетне саме по себи, односно као саставни део микробне флоре, али може да постане патогена и инфективна под одређеним околностима које подстичу њено бујање. У тој фази, она може да оштети зид црева и уђе у крвоток узрокујући инвазивну кандидијазу са високом стопом смртности. Од суштинског је значаја препознати обољевање од кандиде и започети на време терапију која може да сачува живот. Препознавање фактора ризика који омогућавају кандиди да буја је најважније у превенцији њеног раста и хроничне кандидијазе, што је претходни степен инвазивне кандидијазе. Ако ово препознавање изостане и бујање унапредује, поставља се питање како стање открити и лечити и у којој фази то урадити. Узгајање културе из узорка столице захтева време, а осим тога доказ је само присуства живих гљивичних ћелија. Ако живе гљивичне ћелије нису присутне у столици, резултат културе ће бити негативан. У овом раду, ауторка представља своје искуство с анализом столице под микроскопом с тамним пољем, као брзом методом лаганом за извођење преко које се утврђује присуство живих или мртвих гљивичних ћелија и бујање гљивица.

**КЉУЧНЕ РЕЧИ:** гљивична инфекција, инфекција кандидом, бујање кандиде, хронична кандидијаза, анализа столице, микроскоп с тамним пољем

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## FARM WATER AS A POSSIBLE SOURCE OF FUNGAL INFECTIONS

**ABSTRACT:** The quality of drinking water depends on the water sources, but also on the quality of the water distribution system which supplies the water on to the final user. In addition, the possibility of contamination of water used for watering animals in the farm buildings depends on the hygienic conditions on farms. Microbiological quality of water on farms in Serbia has not been one of the main focuses of animal breeders, although according to the Food Safety Law water is considered as food. As feed safety for the animals, which includes microbiological analyses, is an important concern of breeder farmers, it is also important to control the water safety in order not to become a cause of the animal health problems. Change of the water quality is not important only from the sanitary epidemiological point of view, but the presence of different microorganisms, especially fungi, can cause changes in taste and smell, as organoleptic properties of water. According to legal regulations, there is no difference between the quality requirements for drinking water relative to the water supply intended for animals. For the aforementioned reasons, the subject of this study is microbiological control of water samples from the drinkers for animals at farms. The aim of the work is to examine which fungi are possibly present in the water and what their number is. In total, 35 samples of water from pig and poultry farms were tested. The method of direct seeding and filtration was used. The presence of different types of mold (*Aspergillus* sp., *Penicillium* sp., *Alternaria* sp., *Mucor* sp. and *Rhizopus* sp., and *Candida* sp.) was determined. The results indicate the necessity of microbiological control of water for watering of farm animals, which implies the analysis for the presence of molds.

**KEYWORDS:** water for watering animals, molds

## INTRODUCTION

The normal functioning of the physiological activities of the living world depends on the presence of nutrients and water. In order to provide the production features of domestic animals, it is important to ensure quality and healthy food and water for feeding animals. According to the Food Safety Law (*Official*

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*Gazette*, 2009) water is considered as a foodstuff and must be healthy and safe as much as all the other nutritive substances that are used for human consumption. In accordance with these legal requirements there is no difference in the quality of water used for the watering of animals in relation to drinking water intended for humans. The Regulation on Hygienic quality of drinking water (*Official Gazette*, 1999) defines limit values of microbiological quality of drinking water. These limits vary depending on the sources used for water supply. In the given lists of microbiological characteristics of drinking water, regardless of whether it is purified, disinfected or bottled water, or the natural waters from closed and opened water sources, they must not contain, in addition to listed bacteria and algae, any other organisms that can alter the appearance of smell and the taste of water. The regulations do not explicitly mention the necessity to control the presence of fungi (molds and yeasts) in water but, without doubt, their presence may potentially affect water quality and thus the health of the animals and the people consuming it.

Supply of farm animals with water has its own characteristics with different aspects. Besides the local rural or suburban water supply system, for supplying water to the farms dug wells within the same farm can be used. Except the aforementioned facts, a part of an aqueduct, located within a farm, can be used for antibiotic treatment of animals when needed. Medicators (Stojanov *et al.* 2003) are part of the water supply system ensuring that the application of antibiotic therapy, carried out for a sufficient period of time with the required concentration of the drug, will provide a therapeutic dose and efficacy of the administered antibiotics. The potential risk of the creation and expansion of biofilms (Wingender and Fleming, 2011), as well as the specifics of the water system on farms, provide a good opportunity for the survival and spread of the fungi. A research on the presence of fungi in drinking water (Hageskal *et al.* 2009) in the past decade, indicates that they are water contaminants. It also indicates that our knowledge of their presence in water and their importance for health have not been sufficiently explored. The finding of fungi in water, including filamentous molds and yeasts, may not have a direct effect on the health. Indirectly, molds could be potentially pathogenic, toxic or allergenic biological agents that harm the health, particularly in immunocompromised individuals (Hageskal *et al.* 2009). Finding fungi in hospital, in aerosols from the shower or tap water, and saunas, allows for the possibility that the infection spread through inhalation. Similar situation is possible on farms.

The hygienic conditions in facilities where animals are bred are far disadvantageous, compared to those in hospitals. For these reasons, the subject of our study is microbiological control of water samples from the drinkers for farm animals. The aim of this paper is to examine which fungi are possibly present in water and what their number is.

## MATERIAL AND METHODS

Water samples processed in the laboratory came as part of regular analysis of the water for watering of farm animals. A total of 23 water samples from

poultry farms and 12 samples from a pig farm were examined. The samples were taken from the drinker in the facilities of a farm. Before the samples were collected in sterile bottles, water from the drinkers is poured out 3 times. In that way, the negative impact of possible contamination of water with residual food that animals can leave during watering was reduced. The taken samples were transferred to the laboratory at 4 °C for 2–4 hours. The samples were divided in two parts. The first part was directly inoculated on a nutrient substrate, while the other part was filtered off, after which the filter was seeded into a nutrient medium. The laboratory methods described in the national laws for samples examination (similar with international methods) are obligatory. Direct seed included microbiological testing according to the Regulation on the method of sampling and laboratory methods for the analysis of drinking water (*Official Gazette*, 1987), and it was added to Sabouraud agar (Biocare) for isolation of fungi. Water was filtered by the Millipore filters and disposable cups (MF – Millipore™, Microfilla Funnel & Filter, 250ml, 0,45µm 47mm) and single-channel filtration system (Millipore), after which the filter was seeded directly into Saburo agar. The seeded plates were incubated at 25 °C for 4 days.

## RESULTS AND DISCUSSION

The microbiological control of drinking water includes the control of the presence of bacteria, viruses or parasites, which can contaminate water and affect human health (Hageskal *et al.* 2009). The problem of control of water used for watering animals in this country is relatively neglected because breeders do not realize the importance of microbiological safety of water used by farm animals. It seems that they are not aware that water is also foodstuff and that it has to be controlled as any other food (Stojanov *et al.* 2015). Table 1 presents the results of microbiological analysis of water. The samples were directly planted on nutrient medium.

Table 1. Results of examination of water from farms – direct seeding

	No. of processed samples	No. of samples that did not correspond to the regulations on the hygienic quality of drinking water	No. of samples positive for the presence of fungi
Samples from poultry facility	23	21	5
Samples from pig facility	12	10	4
Isolated fungal species from water samples: <i>Aspergillus</i> spp., <i>Penicillium</i> spp., <i>Alternaria</i> spp., <i>Rhizopus</i> spp., <i>Mucor</i> . sp. and <i>Candida</i> sp.			

Fungi are divided into several groups according to their systematization. They belong to kingdom *Eumycota* and are classified into the five phyla: *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Chytridiomycota*, and *Glomeromycota*

(Kirk *et al.* 2001; Schübler *et al.* 2001). According to the structure, fungi can be divided into filaments which are called molds, yeasts and mushrooms. Some fungi are naturally adapted to water and therefore can be found in it as normal inhabitants. They are called zoosporic fungi and mainly belong to the phylum *Chytridiomycota*. The fungi which belong to other phyla of *Eumycota* are adapted to the terrestrial conditions and can be found in soil, organic matter and air (Kirk *et al.* 2001). These types of fungi can enter water from the above mentioned substrates, so it can be said that water is not their natural environment. The water analyzed in this paper, certainly, could be contaminated with different types of fungi, because it was taken from the building where animals lived and where there was a large amount of organic matter. With regard to this, it is important to emphasize that the level of humidity in farm facilities is usually very high, which makes favorable conditions for fungi. In this study, it was found that a large number of water samples used for watering animals was microbiologically incorrect. Most commonly present were coliforms and fecal coliforms, as well as fecal streptococci. Unfortunately, there could not be determined the correlation with the finding of fungi. Similar results were reported by Pereira *et al.* (2009) when testing three different samples of drinking water.

In Table 2 are given data on tested samples with the filtration. In this test was used 100 mL water sample. Samples were filtered through a 0,45µm filter. The filter was seeded into the surface of a nutrient agar.

Table 2. Results of farm water examination – filtration method

	No. of processed samples	No. of samples that did not correspond to the regulations on the hygienic quality of drinking water	No. of samples positive for the presence of fungi
Samples from poultry facility	23	23	8
Samples from pig facility	12	12	6
Isolated fungal species from water samples: <i>Aspergillus</i> spp., <i>Penicillium</i> spp., <i>Alternaria</i> spp., <i>Rhizopus</i> spp., <i>Mucor</i> spp. and <i>Candida</i> spp.			

Comparing the results in Table 1 and Table 2, it can be seen that the method of filtration was better than method of direct plating. We found that every water sample from a facility where animals are bred was not in accordance to the criteria of the regulations on hygienic quality of drinking water. This result was expected. However, the method of filtration could not be used to determine the total number of mesophilic bacteria. Due to the large presence of bacteria in the water, determining the total number of bacteria is not possible by the method of filtration. A large number of bacteria which were retained by the filter after the inoculation did not allow determination of the total number of microorganisms.

All kinds of isolated fungi in the tested water samples do not represent the pathogens that would directly jeopardize the health of animals. Similar results

were reported by Goncalves *et al.* (2006) who examined the drinking water in buildings where people live. Some of the isolated fungi can be potentially pathogenic, can represent allergens or possess toxigenic potential. Certain species of *Aspergillus* were present in 49% of samples tested at the University Hospital in Norway (Warris *et al.* 2009). However, *Aspergillus fumigatus* was one of the most common pathogens that caused infections in immunocompromised individuals (Marr *et al.* 2002; White, 2005). Hospitals are facing significant increase in opportunistic infections caused by fungi in patients with reduced functional status of the immune system (Denning, 2006). The use of corticosteroids, chronic diseases, diabetes, HIV infection, and surgical interventions can be some of the predisposing factors that create a favorable conditions for fungal infections. Particular issues are the aging of human population and the increasing number of transplantations and human cancers (Perlroth *et al.* 2007). A special aspect of the issue of the presence of different microorganisms in drinking water is the possibility of the formation of biofilms. Finding biofilms in water supply system can represent temporary or permanent problem. It mainly depends on the species of microorganism found there, as well as on the hygiene standards (Wingender and Flemming, 2011). All the uncertainties and potential risks related to the water system and drinking water in the human population, when compared to water quality and water supply systems within farm, indicate the need for water control and continuous monitoring of the farm water quality.

## CONCLUSION

Our testing included microbiological control of water for watering on pig and poultry farms. In the tested samples, the presence of fungi and bacteria was determined. The presence of fungi in the samples was between 20% and 40% and it was dependent on the method used. The filtration method was better for testing water for the presence of fungi, while the direct seeding method proved to be better for determining the presence of bacteria.

The finding of fungi in the tested samples indicates a possible microbiological disadvantage of the water. In addition, these microorganisms change organoleptic properties because they change the smell and taste of water. Studies indicate the need for microbiological control of water, which includes bacteriological and mycological examination.

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## ВОДА СА ФАРМИ КАО МОГУЋИ ИЗВОР ИНФЕКЦИЈА ГЉИВИЦАМА

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**РЕЗИМЕ:** Квалитет воде за пиће зависи непосредно од самог изворишта која се користи за добијање воде, а посредно од квалитета дистрибутивног система којим се вода транспортује до корисника. Вода која служи за напајање животиња у објектима додатно је оптерећена могућностима контаминације различитим микроорганизмима, укључујући и гљивице, што је условљено хигијенским околностима фарме. Микробиолошка исправност воде на фармама, у нашим условима, најчешће није предмет пажње одгајивача животиња иако се према Закону о безбедности хране вода сматра намирицом. Онако како се одгајивачи – фармери брину о здравственој исправности хране, што обухвата микробиолошке и нутритивне анализе квалитета хране, тако је важно да се и вода контролише како не би постала разлог здравственим проблемима животиња. Промена квалитета воде није важна само са епидемиолошко санитарног аспекта, већ присуство различитих микроорганизама, а посебно, гљивица може довести до промене укуса и мириса што мења органолептичка својства воде. Према наведеним законским одредбама не постоји разлика у квалитету вода које се користе за употребу напајања животиња у односу на воду за пиће намењену за људе. Специфични хигијенски услови у објектима, бунари на фармама који се користе као изворишта напајања животиња и могућност коришћења водоводног система фарме за терапирање оболелих животиња су неке од критичних тачака у сточарској производњи који представљају разлог микробиолошке контроле воде. Из наведених разлога предмет нашег рада је микробиолошка контрола узорка воде из појилица с којих се животиње напајају водом на фарми. Циљ рада је да утврдимо да ли се у испитаним узорцима налазе гљивице и колика је њихова заступљеност. Укупно је прегледано 35 узорка воде са фарми свиња и живине. Коришћен је метод директног засејавања и филтрације. Утврђено је присуство различитих врста плесни (*Aspergillus*, *Penicillium*, *Alternaria*, *Mucor*, *Rizopus* и *Candida*). Резултати указује на неопходност микробиолошке контроле воде за напајање животиња која обухвата и анализе присуства плесни.

**КЉУЧНЕ РЕЧИ:** вода за напајање животиња, плесни



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## FIRST CASE REPORT ON PATHOGENIC FUNGUS *Fonsecaea* sp. Negroni FROM SKIN OF *Pelophylax* kl. *esculentus* L. IN SERBIA

**ABSTRACT:** Non-harmful adhesive tape method was applied directly on the skin surface of edible frog (*Pelophylax* kl. *esculentus*), captured in vernal ponds on the locality “Stevanove ravnice” within the Special Nature Reserve „Deliblatska peščara“, in order to detect fungal dwellers of frogs’ skin. Light microscopy analyses of LactophenolCottonBlue-mounted adhesive tape samples taken from frog’s ventrum revealed the presence of melanized septate hyphae, branched conidiophores with chains of single-celled ovoid conidia, arising directly from the skin, which corresponds to morphological features of dematiaceous hyphomycete – *Fonsecaea* sp. Since members of genus *Fonsecaea* are frequently cited as causative agents of chromomycosis in amphibians, as well as human phaeohyphomycosis, worldwide, it is of great significance to study the presence of this fungal pathogen on amphibians in Serbia in order to make the basic reference data of the incidence of these pathogens in this region.

**KEYWORDS:** adhesive tape method, chromomycosis, dematiaceous fungi, *Fonsecaea* sp., frogs, pathogen

## INTRODUCTION

Since the discovery of amphibian chytrid fungus, *Batrachochytrium dendrobatidis* Longcore, Pessier & D.K. Nichols, pathogen responsible for extinction and rapid decline in frogs’ population worldwide (Berger *et al.* 1999; Woodhams *et al.* 2012), more attention is given to fungal infections of amphibians, in general. The degree of susceptibility to *B. dendrobatidis* varies greatly between species and is attributable to multiple factors including host physiology, environmental conditions and the skin microbial communities (Bletz *et al.* 2013).

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Concerning these factors, skin microbial communities have come to the forefront of research on *B. dendrobatidis* susceptibility due to the role symbiotic bacteria play in host resistance. During metamorphosis, the skin of the body becomes increasingly keratinized and the fungal infection is then able to spread over the skin of susceptible species (Marantelli *et al.* 2004; Rachowicz and Vredenburg, 2004). Apart from *B. dendrobatidis*, causative agent of chytridiomycoses, amphibians (anurans and caudates) are also susceptible to other fungal infections such as mucormycoses, basidiobolomycoses, saprolegniases and chromomycoses (Pessier, 2002; Pare, 2003; Densmore and Green, 2007). Darkly pigmented filamentous fungi, i.e. dematiaceous hyphomycetes, members of genera *Cladosporium*, *Exophiala*, *Fonsecaea*, *Phialophora*, *Scolecobasidium* and *Veronaea*, are causative agents of cutaneous and systemic chromoblastomycoses of post-metamorphic anurans. This presents chronic, cutaneous and subcutaneous infection, characterized by slowly expanding nodules that eventually lead to emerging, cauliflower-like, mutilating and disfiguring eruptions. Infection proceeds with muriform cells in tissue provoking a granulomatous immune response (Pare, 2003; Hosoya *et al.* 2015). Propagules of these fungi (conidia, ascospores, chlamydospores, hyphal fragments) are often present in soil and leaf litter. Seldomly, these saprotrophic fungi act as opportunistic pathogens (Pare, 2003), infecting anurans through traumatic inoculation of fungal propagules, followed by dissemination to internal organs (Pessier, 2002).

Ascomycete *Fonsecaea* is an anamorph member of the family Herpotrichiellaceae, order Chaetothyriales class Eurotiomycetes. This genus contained only three described species, namely *F. pedrosoi* (including its morphological variant *F. compacta*), *F. monophora* and *F. nubica* (de Hoog *et al.* 2004). These species are morphologically indistinguishable and could be separated only on the basis of ITS sequencing. Also, Vicente *et al.* (2012) described and reported novel species in this genus, *F. brasiliensis*, associated with letargic crab disease of mangrove crab, *Ucides cordatus* L.

Relationships between microbial communities and their hosts can be highly complex. Host-microbiome interactions could be influenced by numerous factors which include host traits such as genetics, life history and behavior, as well as broader effects of environmental factors (Ding and Schloss, 2014). Furthermore, the skin of different amphibian species, and even individuals, may vary by both the presence and the type of anti-microbial peptides, mucosal secretions and levels of skin sloughing, which can affect the formation and maintenance of resident microbiota (Meyer *et al.* 2012). Current studies are focused on elucidating the basic biology governing the host-microbiome relationship to confidently and effectively implement microbiome science in conservation efforts (Longo *et al.* 2015).

Special Nature Reserve “Deliblatska peščara” is situated in North Serbia and represents the largest European continental sand. Due to flora and fauna species richness, “Deliblatska peščara” is one of the most important centers of biodiversity in Serbia and Europe and is protected by a Decree of the Government of the Republic of Serbia (*Official Gazette of RS*, no. 3/2002). As the internationally significant bird habitat, it is included in the Ramsar List of Wetlands of International Importance (Josimović and Pucar, 2010). Many rare species

nest in the area of the flood riverbanks of the Danube river where there is one of the largest migratory stations of water birds in Serbia as well as a nesting place of many rare and threatened bird species such as Little egret (*Egretta garzetta* L.), common pochard (*Aythya ferina* L.), pygmy cormorant (*Microcarbo pygmeus* Pallas) etc. This is also one of the areas in Serbia that are inhabited with all three taxa of the *Pelophylax synklepton esculentus* complex (*P. lessonae*, *P. ridibundus* and hybrid species *P. kl. esculentus*). Also, this region represents southern limit of distribution for *P. lessonae*. During their whole life cycle, green frogs represent important food source for water birds and other higher level consumers.

The main aim of this research was to get insight into frog's epidermis-associated mycobiota and detection of potential fungal pathogens via application of the adhesive tape method, already successfully applied in medical and veterinary mycology (Harris, 2000), for the first time on anuran skin, which could lead to recognizing and registration of novel fungal pathogens in this region. Also, investigation of specific biological communities within protected areas, could lead to implementation of more effective management strategies.

## MATERIALS AND METHODS

Specimen of water frog, *Pelophylax synklepton esculentus* complex was captured in vernal ponds on the locality "Stevanove ravnice" within the Special Nature Reserve "Deliblatska peščara" in September 2016. Based on qualitative traits and morphometric parameters (Krizmanić, 2008), the captured individual was identified as male specimen of edible frog *Pelophylax kl. esculentus* L. (Figure 1).



Figure 1. *Pelophylax kl. esculentus* in its natural habitat (Photo K. Breka)

Captured specimen was put in wet denim sack and proceeded to field laboratory. In laboratory conditions, adhesive tape was gently adhered to five different skin areas (dorsal and ventral side, head, fore- and hindlimbs) and removed imperceptibly (Urzi and De Leo, 2001). Adhesive tapes samples were then mounted in standard mycological dye LactophenolCottonBlue (LCB), attached to microscope slides and observed under light microscope (Nikon Eclipse E200, equipped with camera Bresser MikroCam PRO HDMI, Japan). Observed and documented fungal structures were compared with available identification keys in order to identify fungi present in the samples (Larone, 1989).

After the examination, captured frog specimen was safely returned to its original habitat.

## RESULTS AND DISCUSSION

Adhesive type samples taken directly from skin of the ventrum and investigated under light microscopy revealed the presence of well developed mycelium consisted of septate, melanized and loosely branching hyphae, with conidiogenous apparatus producing an asterisk-like appearance (Figure 2a). Abundant septate conidiophores, erected from somatic hyphae, bearing short chains of single celled ovoid conidia ( $\varnothing$  3.5–5 x 1.5–2 $\mu$ m) were frequently observed (Figure 2b). Apparently, the mycelial growth and conidiation were abundant on frog's ventral side and typical conidia with denticles were observed in mass (Figure 2c). These fungal structures were not detected on other parts of frog's skin. According to Larone (1989) documented micromorphological features, including conidia shape and size, type of conidiation and branching of conidiophores, correspond to dematiaceous fungus, *Fonsecaea* sp. Additionally, morphological identification of documented fungal structures was confirmed via online mycological database presented on the website "Mycology online" (<http://www.mycology.adelaide.edu.au/descriptions/hyphomycetes/fonsecaea>). Morphologically, four types of conidial formation were described for *Fonsecaea* species: *Fonsecaea*, *Rhinochrysiella*, *Cladosporium* and *Phialophora* type and morphological features observed on *P. kl. esculentus* correspond to *Fonsecaea* type of conidiation (septate and erect conidiophores, primary conidia produced on swollen denticles, long conidial chains not formed).

In axenic cultures, *Fonsecaea* spp. are characterized by slow growing colonies, displaying flat to heaped and folded shape, with suede-like to downy or olivaceous to black coloration with black reverse. The identification of *Fonsecaea* spp. to species level based solely on morphological criteria is very difficult, due to polymorphism, and hence for proper and more detailed identification of these fungi, isolation of pathogen is required, followed by additional molecular analyses or metagenomics approach. Although, none of known chromomycoses symptoms (ie. cutaneous lesions, nodules, skin ulcers...) were documented during examination of captured frog specimen, the presence of *Fonsecaea* sp. structures, in form of well developed mycelial phase and abundant sporulation directly on frogs skin, is very significant, since *Fonsecaea* spp. is cited as causative

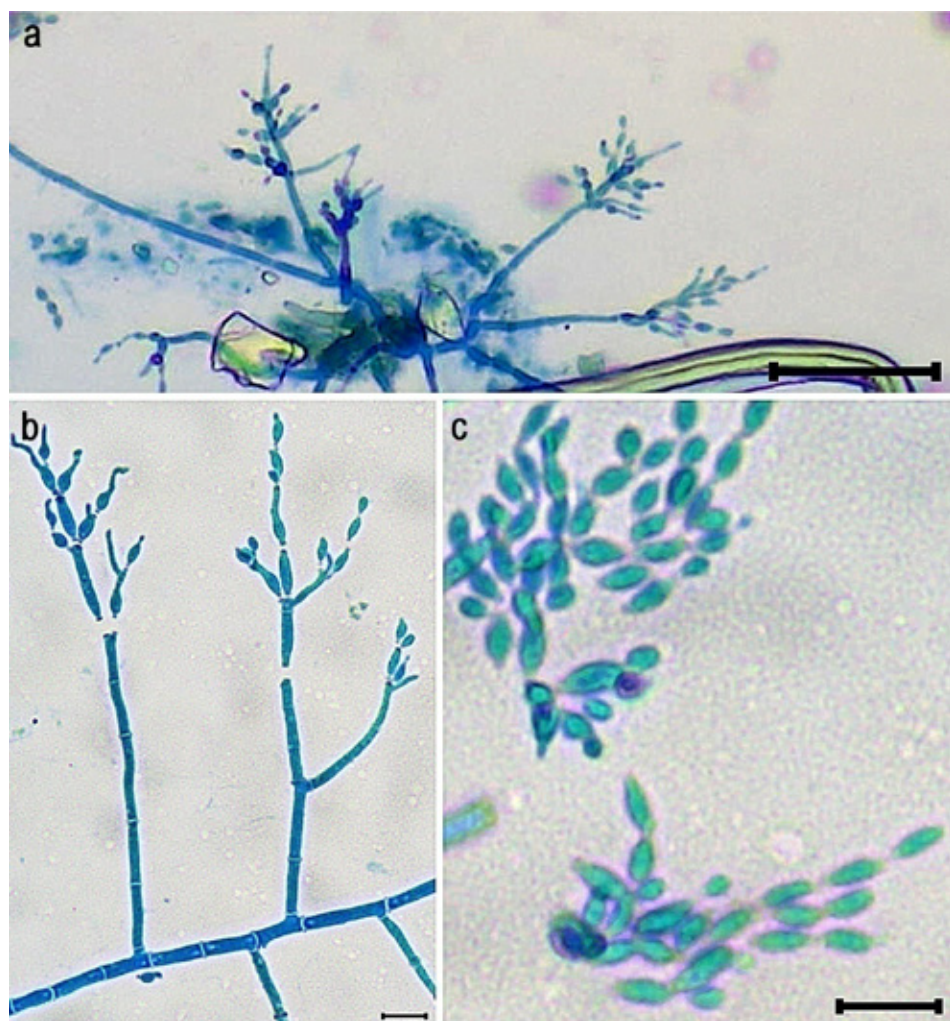


Figure 2. *Fonsecea* sp. growing and sporulating on *Pelophylax kl. esculentus* skin, dyed with LCB; a) mycelium with asterisk-like conidiogenous apparatus (bar represents 50 µm); b) detail of conidiogenous apparatus (bar represents 10 µm); c) single-celled ovoid conidia with denticles in mass (bar represents 10 µm).

agent of chromomycoses of amphibians. Infections caused by *F. pedrosoi* were reported for cane toad, *Rhinella marina* L. (Cicmanec *et al.* 1973) and northern leopard frog *Lithobates pipiens* Schreber (Rush *et al.* 1974). Albeit, infections caused by *F. pedrosoi* and other dematiaceous fungi affect people, via chromoblastomycosis and phaeohyphomycosis, pathogen transmission from amphibians to humans has not yet been reported (Pare, 2003). Likewise, since the dematiaceous hyphomycetes are usually considered as opportunistic or secondary

pathogens of anurans, it could be assumed that examined frog specimen also suffer from some other primary fungal or bacterial infection.

## CONCLUSION

In this research the application of adhesive tape method directly on amphibians' *stratum corneum* was demonstrated for the first time as useful tool for preliminary observation of fungal skin dwellers. Not only transients but also the potential pathogens could be detected via adhesive tape method. This method is completely safe and provides minimal stress to studied animal, so it can be introduced as significant diagnostic tool for detection of epizootic communities of frogs and other amphibians, possibly cyst and zoosporangium of *B. dendrobatidis*, as well. In further researches other amphibian species from different localities in Serbia should be included. This research could be helpful in further studies on amphibian declines and their causes as well as on amphibian conservation, with an emphasis on those that describe methods for monitoring and conserving amphibian populations in Serbia.

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ПРВИ ПРИКАЗ СЛУЧАЈА ПАТОГЕНЕ ГЉИВЕ *Fonsecaea* sp.  
*Negroni* НА КОЖИ *Pelophylax* kl. *esculentus* L. У СРБИЈИ

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**РЕЗИМЕ:** У циљу детекције и идентификације фунгалних колонизатора коже зелене жабе (*Pelophylax* kl. *esculentus*), из ефемерних бара с локалитета „Стеванове Равнице” у оквиру Специјалног резервата природе „Делиблатска пешчара”, извршено је узорковање безбедном методом адхезивне траке. Микроскопска анализа је показала присуство меланизованих, септираних хифа и гранатих конидиофора које се уздижу директно с епидермиса трбушне стране жабе и носе терминално постављене ланце једноћелијских, овалних конидија. Документоване микроморфолошке карактеристике одговарају опису патогене гљиве рода *Fonsecaea*. Овај налаз је значајан обзиром да се врсте овог рода наводе као узрочници хромомикоза водоземаца, као и хуманих феохифомикоза. Како присуство *Fonsecaea* sp. на кожи зелене жабе у Србији није саопштено, од великог је значаја даље проучавање ове патогене гљиве у циљу сакупљања референтних података о њеној дистрибуцији у региону.

**КЉУЧНЕ РЕЧИ:** метода адхезивне траке, хромомикоза, патогена гљива, *Fonsecaea* sp., жабе, патоген

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## EFFECT OF NOZZLE TYPE ON THE FUNGICIDE EFFICACY FOR FUSARIUM HEAD BLIGHT SUPPRESSION ON WHEAT

**ABSTRACT:** Effect of fungicide treatments on Fusarium head blight (FHB) and grain yield of wheat depending on application technique i.e. use of different nozzle types, was evaluated in the study. Nozzles types TJ 11004, Albuz ATR 8004 and Arag TFA 11004 were used for application of systemic fungicide Duett Ultra (0.5 l/ha). FHB intensity (%) was determined on the basis of a visual assessment of the number of infected heads and the percentage of the disease symptoms on the individual head. Differences in grain yield between the treated variants, as well as between the treated and untreated variants, were determined after hand threshing. The lowest percentage of FHB development in wheat and the highest yield were recorded in variants where fungicides were applied by nozzle type ATR 8004. Application technique directly affects the reduction of fusarium head blight in wheat and indirectly it also reduces yield loss.

**KEYWORDS:** application technique, fungicide, fusarium head blight, wheat, yield

## INTRODUCTION

In Republic of Serbia in 2014 wheat was sown on 610,218 ha, and the total production amounted 2,313,947 t, which corresponds with the average yield of 3.79 t/ha (Mladenov, 2015).

The most significant diseases of wheat stem and leaf are powdery mildew, leaf spots and rusts, while the most significant disease of wheat head is fusarium head blight. Under agroecological conditions of Serbia, FHB occurs at variable intensity, depending on weather conditions during flowering and grain forming

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as well as on crop rotation (Balaž, 1989; Balaž, 1990). In certain years, fusariosis occurs at epidemic proportions. On some wheat varieties, infection intensity of up to 50% was recorded (Balaž *et al.* 2010). Yield reduction caused by head fusariosis varies from year to year from 5 to 70% (Korić and Tomasović, 1989). Fungicides for FHB suppression on wheat are applied at the beginning of flowering.

Very good results in the control of FHB can be achieved by timely, foliar fungicide sprays, using sprayers with adequate nozzles. Nozzles are sprayer elements that define droplet size, shape and angle of stream, liquid volume and coverage quality of the treated area. Structure and spectrum of drops in the stream are very important parameters of the nozzles and deposit precision i.e. volume of protective agent applied on the protection object depends on these parameters. Structure and drop spectrum are defined by droplet size in the stream and mostly depend on the shape of the nozzle outlet and operating pressure (Sedlar *et al.* 2014).

## MATERIAL AND METHODS

Field trial at locality Ada, route Brežane, on an area of 3,584 m<sup>2</sup>, was set in production in years 2014 and 2015 to test effects of fungicide Duett Ultra (a.i. 187 g/l epoxiconazole and 310 g/l tiophanate-methyl) on FHB intensity on wheat depending on application technique. The trial consisted of four variants and was set in four replicates (Table 1). Trial area was divided in 16 equal parts and replicates of different variants (different fungicide application techniques) were set in randomized design. Wheat variety in the trial was Euclide, a high-yielding variety originating from France. Sowing was conducted on 15th of November 2014 with the norm of 220 kg/ha.

Duett Ultra at application rate 0.5 l/ha was applied to wheat plants with different nozzle types at the beginning of flowering with an aim to protect wheat heads from fusariosis. Variants and application techniques are presented in Table 1.

Table 1. Nozzle types used in different variants in the trial (Anonimus, 2009, 2015, 2015a)

Variants	Nozzles	Characteristics of the tested nozzles
V <sub>1</sub>	Untreated control	–
V <sub>2</sub>	TJ 11004 (Figure 1)	Standard flat fan nozzle, red color, stream angle 110°, flow rate 1.6 l/min, brassy.
V <sub>3</sub>	ATR 8004 (Figure 2)	Hollowcone nozzle, red color, stream angle 80°, flow rate 1.6 l/min, plastic with ceramic pad.
V <sub>4</sub>	TFA 11004 (Figure 3)	Twin fan air nozzle, red color stream angle 90° with 30° – 30° forward and reverse output, flow rate 2 x 0.8 l/min, plastic with polymer pad.



Figure 1. Stream of the nozzle type TJ 11004 (original)



Figure 2. Stream of the nozzle type ATR 8004 (original)



Figure 3. Stream of the nozzle type TFA 11004 (original)

Two weeks after fungicide application, FHB was assessed to determine fungicide efficacy depending on the nozzle type.

Head infection was evaluated in each variant on 600 heads per replicate (in total 2,400 heads per variant) and the percentages of diseased heads and disease intensity were calculated. Percentage of affected head area i.e. infection category was determined according to the scale – Figure 4 (Steffenson *et al.* 2004).

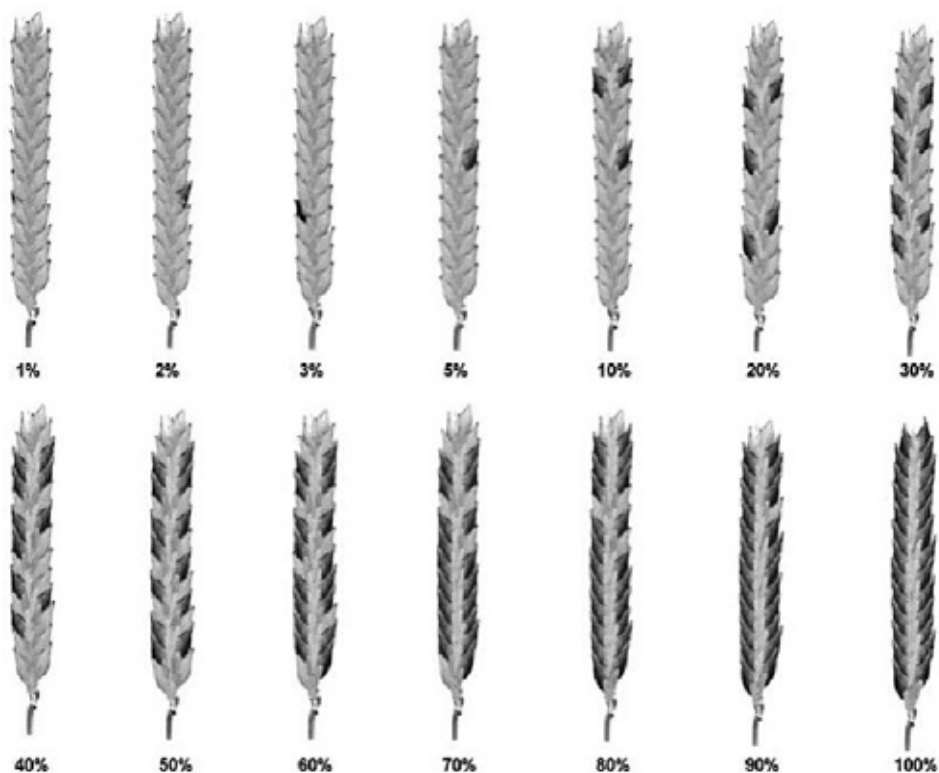


Figure 4. FHB, percentage of head area affected (Steffenson *et al.* 2004).

Disease intensity i.e. disease index was calculated using Mc Kinney formula (Collective of authors, 1983). Fungicide efficacy depending on application technique was determined by Abbot formula (Wentzel, 1963).

Wheat grain yield was determined after hand threshing conducted on 2<sup>nd</sup> of July, 2015. Four points of 1 m<sup>2</sup> were randomly marked on each plot and after hand threshing, the grains were measured and the yield per hectare was calculated. Differences in yield between treated variants and untrated control were determined.

## RESULTS AND DISCUSSION

Wheat flowering in 2015 was carried out in the second decade of May. In this period, four days of precipitation of 11.8 mm were recorded. Average air temperature in May was 17.2 °C. The above mentioned conditions partially favored FHB development.

In control plot, percentage of affected head area was between 0% and 90%. In control plot, 6.91% of wheat heads were infected. Disease index in control plot was 2.05% (Table 2). In fungicide treated variants no symptoms or mild infection of heads were observed. On average, 0.3% of heads were infected. In treated variants, infected heads were in category 1%–10% of infected head area and average disease index was 0.044%. Disease index recorded for each treated variant is shown in Table 2.

*Table2.* Percentage of disease heads, disease intensity and fungicide efficacy depending on application technique

Variant	Average of diseased heads (%)	Disease index (%)	Fungicide efficacy (%)
V1	6.9100	2.05	–
V2	0.7916	0.0935	95.44
V3	0.0416	0.00375	99.82
V4	0.1250	0.036	98.24

The results show that fungicide application in flowering phase of wheat resulted in reduced incidence of FHB (Table 2).

Regarding application technique, the best result in head protection was obtained in variant 3 in which fungicide was applied by ATR 8004 nozzles, with average of 0.0416% of infected heads. Fungicide efficacy in this variant was 99.82%. In variant 4 in which TFA 11004 nozzles were used, percentage of diseased heads was 0.125% on average, while the lowest fungicide efficacy was recorded in variants in which fungicide was applied with nozzle TJ11004 in variant 2 (on average 0.7916% of infected heads).

In Table 3, the obtained grain yield after hand threshing calculated per hectare is presented for each plot. According to total yield obtained on the plots, and depending on fungicide nozzle types, calculated average yields were the

following – V1 (control) – 5,762.5 kg/ha, V2 – 5,825 kg/ha, V3 – 5,875 kg/ha, and in V4 – 5,846.88 kg/ha.

The highest yield was recorded in V3 (ATR 8004) and compared to V4 (TFA 11004) and V2 (TJ 11004) it was 28.12 kg/ha (0.48%) and 50 kg/ha (0.85%) higher, respectively. In variant 3, the yield was 112.5 kg/ha (191%) higher compared to untreated control.

The lowest percentage of head infection and the highest yield were recorded in variant 3 in which the fungicide was applied with the nozzle type ATR 8004.

*Table 3.* Average values of the yield depending on fungicide application technique

Variant	Obtained yield per plot on 4m <sup>2</sup> (g)	Calculated yield per hectare (kg/ha)
V1	2,305	5,762.5
V2	2,330	5,825
V3	2,350	5,875
V4	2,338.75	5,846.88

## CONCLUSION

Adequate pesticide selection and a timely and proper application are significant factors that need to be fulfilled to obtain efficient chemical plant protection. According to the results, it can be concluded that application technique directly affected FHB incidence on wheat heads while it had an indirect effect on yield by controlling disease incidence.

## ACKNOWLEDGEMENT

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## УТИЦАЈ ТИПА РАСПРСКИВАЧА НА ЕФИКАСНОСТ ФУНГИЦИДА ЗА СУЗБИЈАЊЕ ФУЗАРИОЗЕ КЛАСА ПШЕНИЦЕ

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**РЕЗИМЕ:** Утицаји фунгицида у сузбијању фузариозе класа пшенице (FHB) и индиректно на принос зрна проучавани су зависно од примене технике, односно коришћења различитих врста распрскивача. За примену системичног фунгицида Duett Ultra (0.5 l/ha) коришћене су распрскивачи типа TJ 11004, Albus ATR 8004 и Arag TFA 11004. Интензитети фузариозе класа (%) у испитиваним варијантама, у природним условима заразе класа утврђени су на основу визуелне оцене броја заражених класова и површине са симптома болести на појединачним класовима. Такође је утврђен и утицај технике апликације на принос. Разлике у приносима између третираних варијанти међусобно, као и између третираних и нетретираних варијанти утврђене су након ручне вршидбе. Најмањи проценат развоја фузариозе класа пшенице и највећи принос зрна је остварен у варијанти где је фунгицид примењен коришћењем распрскивача ATR 8004. Техника за примену фунгицида имала је директан утицај на смањење фузариозе класа пшенице и индиректно на смањење губитка приноса зрна.

**КЉУЧНЕ РЕЧИ:** пшеница, фузариоза класа, фунгицид, техника апликације, принос

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## RESISTANCE OF EARLY MATURITY MAIZE GENOTYPES TO SOUTHERN CORN LEAF BLIGHT

**ABSTRACT:** In order to study the resistance of early maize genotypes to Southern Corn Leaf Blight (SCLB) or Maydis Leaf Blight (MLB), RCBD experiments with 20 inbred lines and hybrids in 2014 and 16 genotypes in 2015 were conducted at Karaj and Sari Stations. Inoculation was carried out with spore suspension using syringe (3ml/each whorl) firstly at 3–4 leaf stage of maize, and then, sorghum grain inoculated with fungus was applied at 6–8 leaf stage in whorl of each plant using the bazooka technique. Evaluation was done on the basis of disease progress at pollination stage and two weeks after pollination stage with scale 0–5. The results of variance analysis and mean comparison showed that there are different reactions among genotypes to disease. In this study, in 2014, 15% genotypes categorized into resistant group, and these were genotypes: KE 77003/10 × KE 75039, KE 72012/12 × K1263/1 (KSC 400), and K 2331 × KE 75039. Among 16 genotypes in 2015, three hybrids – No. 16 with pedigree (KE 76009/311 × K 1264/5-1), No. 10 with pedigree (K 2331 × KE 75039) and No. 9 with pedigree KSC 400 (KE 72012/12 × K1263/1) – were identified as resistant hybrids that can be a good source of resistance to SCLB.

**KEYWORDS:** Maize, resistance, leaf disease, *Bipolaris maydis*

## INTRODUCTION

One of the most important maize diseases, *Helminthosporium* leaf spot, exists around the world, as well as in Mazandaran, Gilan and Golestan wetlands in Iran (Mehrian *et al.* 2000). Leaf spot diseases affect the reduction of grain yield and nutritional value of forage. Yield losses due to the disease, leaf spot of maize, depending on operating and environmental conditions can be substantial, in this case the yield reduction of 40% or more in tests with strain O inoculation was reported (Fisher *et al.* 1976; Byrnes *et al.* 1989). Two species of leaf spot, *Bipolaris maydis* and *Exserohilum turcicum*, were noted to make a major damage to the crop (Shurtleff, 1980). Symptoms caused by *B. maydis* are like small, diamond-shaped spots, which then grow to over 2 cm in diameter.

Their growth is limited to surrounding leaf veins. In the cases where the infection is severe, the patches can be integrated and cause a wide area of leaf blight. The first epidemics and large damages caused by this pathogen in maize production were recorded in the 1970s in North America. Seed maize production mainly on the basis of cytoplasmic sterility T-type, the occurrence of a new race T, which is specific to the genotypes with the cms-T cytoplasm, and favorable climatic conditions were the causes of these epidemics. However, after the removal of this hybrid, the epidemic of race T in North America reached very low levels (Leonard, 1977). It is considered one of the most important seed-borne diseases of maize that causes much damage to the plant products and is known by the name of southern corn leaf blight – SCLB (White, 1999). SCLB disease is caused by Ascomycetes fungal species named *Cochliobolus heterostrophus* Drechs., anamorphic stage of *B. maydis*. The fungus has three races (O, C and T), but O race is the most common race in most areas where SCLB occurs (White, 1999). Temperature and humidity are the most important factors for the spread of the disease (Shurtleff *et al.* 1985). To control leaf spot disease, agronomic practices such as rotation and elimination of infected plant debris could be used to prevent the spread of disease, but using resistant cultivars is the most important way for SCLB control. Regarding the resistance of cultivars to leaf spot disease, there are several reports of polygenic resistance to *E. turcicum* and there is a high genetic heritability for this type of resistance. Polygenic resistance has also been reported to *B. maydis*. This resistance is quantitative and depends on the size of the spot and correlates with the contaminated leaf tissue of the host. To develop resistant varieties, disease outbreaks are important. In order to be more uniform, the application of artificial infection can be effective and efficient method in breeding programs for resistance to diseases. The most effective way to control leaf spot disease is using genetically resistant hybrids (Carson *et al.* 2004). Some researchers believe that incorporation of additive genes can control maize leaf spot diseases (Burnette and White, 1985; Lim, 1975). In order to map the genetic (QTL) for resistance to SCLB, Carson *et al.* 2004 showed that there is no interaction Increase  $\times$  increase in disease control and the results proved that the heritability of resistance to SCLB is polygenic. In Iran, a few years ago, some researches were carried out under natural conditions to evaluate the resistance of inbred lines and hybrids of maize to SCLB. Zamani and Mehrian (2005), when examining seventeen inbred lines and hybrids for the resistance to SCLB, determined inbred line K3547/212 as the most resistant and inbred line K3653/111 as one of the most susceptible to the disease. They were also used for identification and diagnosis of species by Sivanesan (1987) who pointed out that most of the isolates were identified as *B. maydis*. Zamani and Choukan (2000) when evaluating resistance of 60 hybrids to SCLB, genotype K1259  $\times$  Mo17 identified as a hybrid resistant to this disease. SCLB has increased in recent years in many areas of Iran including the northern regions Gorgan, Sari and Gilan. Therefore, planting resistant varieties is the most economical way to control this leaf disease. The main objectives are to identify resistant genotypes of early maturing maize, as well as to use and apply the maize breeding program in the future.

## MATERIALS AND METHODS

In order to inspect experimental farms and seed production in the various parts of the northern regions Gorgan, Sari and Gilan, some infected leaf samples of SCLB were collected in 2013.

### *Laboratory trials*

Infected leaf samples were placed separately in plastic bags and after the registration were transferred as soon as possible to laboratory conditions. Several samples were selected on the basis of the spots number, as well as spot size and the sporulation. Small pieces of each sample were cut from the margins of the infected leaf tissue and in a 0.5% Clorox solution (undiluted Clorox solution has a 5% of active chlorine) were surface sterilized, then washed with distilled water, and placed on the PDA culture medium. After five days, grown colonies of fungus were studied and a single spore or purification of the isolates was carried out by valid key (Sivanesen, 1987). Pathogenicity of the fungal isolates was tested on seedlings of a susceptible inbred line B73 to SCLB. Out of the total number of tested isolates, five were highly virulent and were selected for evaluating of genotypes resistance.

### *Field trials*

To evaluate the response of inbred lines and hybrids of maize, 20 genotypes of early maturing maize were cultivated in 2014 and 16 in 2015 in Karaj and Sari in a randomized complete block design with three replications. Row spacing of 75 cm and a length of each inbred line two meters at a distance of 25 cm was considered. All operations were done during the growing season and the required records, such as germination, emergence of tassels and silks, were taken. A mixture of five virulent isolates was used as inoculum for artificial inoculation of maize leaf.

Different type of inoculums were prepared for plant inoculation in two phenological stages of maize, one was spore suspension and second was sorghum grain artificially inoculated. Spore suspension was prepared from culture of isolates incubated on autoclaved maize leaves as substrate (Bajet and Renfro, 1994). Green leaves of maize, after washing with cold water, were crushed into pieces of 5 cm to 10 cm and poured in Erlenmeyer flasks and autoclaved twice for 30 minutes in 24 hours. Sterilized maize leaves were inoculated with small PDA plug of isolates and were incubated at 25 °C in dark for 3 weeks. After this period, spores were collected by washing the leaves and spore suspension was adjusted to a concentration of  $3 \times 10^4$  spores per ml. The second type of inoculum was infected sorghum grain (Jeffers, 1994). After washing, sorghum grain was poured into the flask and autoclaved two times. Then, fragments of grown colony of each isolate were poured into the flask and incubated in the light

germinator NUV at 25 °C. After 25–20 days, infected sorghum grain was dried and used for leaf inoculation.

Plants were inoculated with the 1–2 ml of fungal spore suspension by injection in the whorl of the 3–4 leaf stage of maize for the first time, and then repeated in the 6–8 leaf stage of maize with inoculums of artificially infected sorghum grain which was distributed in the plant whorl using bazooka technique.

### *Assessment*

One month after the second inoculation or two weeks after pollination stage of maize, disease assessment was carried out based on disease severity in ten plants per replication. Based on the percentage of leaf surface infection, disease severity was rated on a scale 0–5 (Elliott and Jenkins, 1946). Therefore, 0 – healthy plants with no infection (100% healthy plants and 0% infection), 1 – plants with one or two scattered spots on the lower leaves (infection less or equal to 5%), 2 – plants with a few spots on the lower leaves (infection less or equal to 20%), 3 – plants with a lot of spots on the lower leaves and a few spots on upper leaves (infection less or equal to 30%), 4 – plants with a lot of spots on the lower and central leaves and a few spots on upper leaves (infection less or equal to 50%), and 5 for the plant with a large number of necrotic spots over entire leaves (infection more than 50%), were considered. Finally, after the evaluation and scoring, obtained data were analyzed for disease severity (SCLB), and the response of all genotypes were compared in terms of susceptibility to disease. The resistance to SCLB based on disease severity was determined as follows: R (Resistant) – infection equal to or less than 5%, MR (Moderately Resistant) – infection equal to or less 20%, MS (Moderately Susceptible) – infection equal to or less 30%, S (Susceptible) – infection equal to or less 50%, HS (Highly Susceptible) – infection more than 50%. Accordingly, all inbred lines and hybrids were classified in different groups in order to be identified resistant, moderately resistant and susceptible genotypes.

## RESULTS

Based on analysis of variance, the disease severity affected by virulent isolates of *B. maydis* showed that there is a significant difference among examined genotypes of maize to SCLB at 99% probability level (Table 1).

There is a significant difference at 99% probability for disease severity (DS) among tested genotypes (Table 1). Meanwhile, the effect of the location and interaction of genotype  $\times$  location was significant at 5% and 1% for the disease severity, respectively. This indicates that disease severity in both locations is not the same and there is a significant difference between regions. Therefore, based on the response of early maturity maize genotypes to disease, they were divided into four of five different groups, the results of which are shown in Table 2.

Table 1. Analysis of variance for disease severity (SCLB) of early maturity maize genotypes in Karaj and Sari in 2014

(MS)	d. f.	(S.O.V.)
Leaf disease		
DS		
0.507 *	1	Location (L)
0.132	4	Error (E1)
2.873 **	19	Genotype (A)
1.092**	19	(L × A)
0.164	76	Error (E2)
	23.09%	(%CV)

\* and \*\*: Significant at 5% and 1% probability level, respectively

Table 2. Mean comparison of disease severity (SCLB) and response of early maturity maize genotypes in Karaj and Sari in 2014

Response	Mean of Disease Severity (DS)	Early Genotypes	No. of genotype
S	3.283 <sup>A</sup>	S 61	1.
R	0.767 <sup>B</sup>	K 2331 × KE 75039	2.
R	0.867 <sup>B</sup>	KE 77003/10 × KE 75039	3.
R	0.7833 <sup>B</sup>	KE 72012/12 × K1263/1 (KSC 400)	4.
MS	2.183 <sup>AB</sup>	K 1264/5-1	5.
MS	2.283 <sup>AB</sup>	K 615/1	6.
MS	2.467 <sup>AB</sup>	KE 78052/111	7.
MS	2.133 <sup>AB</sup>	KE 78052/111 × KE 75039	8.
MS	2.467 <sup>AB</sup>	KSC 260	9.
MS	2.600 <sup>AB</sup>	KSC 301	10.
MR	1.117 <sup>B</sup>	K 615/1 × KE 75039	11.
MR	1.033 <sup>AB</sup>	KE 72012/12	12.
MR	1.483 <sup>AB</sup>	KE 75016/321 × K 1264/5-1(KSC350)	13.
MR	1.317 <sup>AB</sup>	KE 75039	14.
MR	1.917 <sup>AB</sup>	KE 76005/111 × K 1264/5-1(KSC290)	15.
MR	1.167 <sup>B</sup>	KE 76009/114 × K 1264/5-1	16.
MR	1.900 <sup>AB</sup>	KE 76009/211 × K 1264/5-1	17.
MR	1.983 <sup>AB</sup>	KE 76009/312 × K 1263/1(KSC380)	18.
MR	1.833 <sup>AB</sup>	KE 76009/312 × K 1264/5-1(KSC405)	19.
MR	1.483 <sup>AB</sup>	KE 78012/22 × KE 75039	20.

In this study, only one genotype (S 61) belonged to susceptible group (S) (Table 2). Six genotypes belonged to moderately susceptible (MS) and ten to moderately resistant group (MR). Among the 20 tested genotypes, hybrids No. 3, 4 and 2 with the pedigree KE 77003/10 × KE 75039, KE 72012/12 × K1263/1, and K 2331 × KE 75039, belonged to resistant group (R), and could be a good source of resistance to SCLB (Table 2).

The obtained results in 2015 for resistance of 16 early maize genotypes are shown in Tables 3 and 4.

*Table 3.* Analysis of variance for disease severity of early maturity maize genotypes in Karaj and Sari in 2015

(MS)	d. f.	(S.O.V.)
Leaf disease		
DS		
1.404 *	1	Location (L)
0.076	4	Error (E1)
4.817 **	15	Genotype (A)
0.484**	15	(L × A)
0.162	60	Error (E2)
	21.11%	(%CV)

\* and \*\*: Significant at 5% and 1% probability level, respectively

There is a significant difference for disease severity (DS) among tested genotypes at 99% probability level (Table 3). Meanwhile, the effect of the regions at 5% level and interaction of genotype × location at 1% level was significant and highly significant for the disease severity, respectively. This indicated that disease severity in both locations was not the same and there was a significant difference between regions. Based on the response of early maturity maize genotypes to disease, they were divided into five different groups, the results of which are shown in Table 4.

*Table 4.* Mean comparison of disease severity and response of early maturity maize genotypes in Karaj and Sari in 2015

Response	Mean of Disease Severity (DS)	Early Genotypes	No.
S	3.100 <sup>B</sup>	KSC 301	1.
R	0.800 <sup>G</sup>	KE 72012/12 × K1263/1 (KSC 400)	2.
R	0.833 <sup>G</sup>	K 2331 × KE 75039	3.
R	0.966 <sup>G</sup>	KE 76009/311 × K 1264/5-1	4.
MS	2.400 <sup>CD</sup>	KSC 201	5.
MS	2.417 <sup>C</sup>	K615/1	6.
MS	2.367 <sup>CD</sup>	KSC260	7.
MS	2.317 <sup>CDE</sup>	KE 78052/111 × KE 75039	8.
MR	1.800 <sup>CDEF</sup>	KSC 250	9.
MR	1.667 <sup>EF</sup>	OH 43/1-42	10.
MR	1.718 <sup>DEF</sup>	K1264/5-1	11.
MR	1.467 <sup>FG</sup>	KE 72012/12	12.
MR	1.833 <sup>CDEF</sup>	KE 75039	13.
MR	1.267 <sup>FG</sup>	KE 78012/22 × KE 75039	14.
MR	1.317 <sup>FG</sup>	K59 × K1263/1	15.
HS	4.233 <sup>A</sup>	S61	16.

In this study, only two genotypes were classified as a highly susceptible (HS) or as a susceptible (S) to *B. maydis* (Table 4). These genotypes were inbred lines S 61 and KSC 301, respectively. Four genotypes belonged to moderately susceptible group (MS), where two inbred lines and two hybrids were classified. Seven genotypes belonged to moderately resistant group (MR) where four inbred lines were classified (OH 43/1-42, K1264/5-1, KE 72012/12 and KE 75039) and two hybrids (KE 78012/22  $\times$  KE 75039 and K59  $\times$  K1263/1). Among the 16 tested genotypes, hybrids No. 4, 3 and 2 with define pedigree, such as KE 76009/311  $\times$  K 1264/5-1, K 2331  $\times$  KE 75039, and KSC 400 (KE 72012/12  $\times$  K1263/1), respectively, were specified as resistant group (R) and could be a good source of resistance to disease SCLB.

## DISCUSSION

Study of the response of 20 genotypes of early maturing maize cultivated in 2014 and 16 in 2015 (inbred lines and hybrids) to SCLB revealed that there was a significant difference among tested genotypes at 1% probability level (Tables 1 and 3). In general, the development of disease due to unfavorable weather conditions (low temperature and % relative humidity) was not satisfactory in 2014, so that the disease severity ranges from 0.76 to 3.28. However, in 2015, the development of disease was satisfactory so that the disease severity varied from 0.8 to 4.23.

Most genotypes were medium resistant (45% in 2014 and 50% in 2015), followed by medium-susceptible (23% in 2014 and 30% in 2015) and the smallest number of genotypes were resistant (20% in both years). Based on necrotic spots formed and score of 3–4 genotypes, S 61 and KSC 301 were classified as the most susceptible inbred line and hybrid, respectively. Resistant hybrid combinations KE 77003/10  $\times$  KE 75039, KE 72012/12  $\times$  K1263/1 (KSC 400), K 2331  $\times$  KE 75039, and KE 76009/311  $\times$  K 1264/5-1 were classified into resistant group (R) and could be a good source of resistance to disease SCLB. It should be noted that genotypes KE 2331  $\times$  K 76009 and KE 75039 / 311  $\times$  K 1264 / 5-1 expressed a high degree of resistance in both years.

Nowadays, extensive researches have been conducted in international centers such as CIMMYT related to the screening of maize germplasm for the resistance to the disease and several methods have been applied to infected plants. In this study, two methods (injection of spore suspension – dropping sorghum grains in whorl of plant), at two different stages of plant phenology, were used and infection carried out uniformly. The advantage of the method is that the structural properties of plant tissue are protected. Jeffers (1994) pointed out the advantage of the use bazooka method compared to the application of the spore suspension as inoculum. Using these methods, CIMMYT has managed to achieve resistant populations to the disease like 21–22 population. Bajet and Renfro (1994) noted that, based on the percentage of disease progression in the leaves in bazooka method, the infection is more severe and the screening

of materials for the disease is more uniform. Hooker (1978) reported that SCLB caused by *B. maydis* has a polygenetic resistance and the resistance depends on the percentage of infected leaf tissue. In this experiment, it was found that if there is a larger number and size of spots the plant is more susceptible to the disease. In our experiment, in inbred line K615/1 all the leaves at the bottom, middle and top of the plant were highly infected and the spots were interconnected in a wide area and created leaf blight. The results of Halseth *et al.* (1991) for leaf spot disease using artificial inoculation during two seasons showed that the additive effect was significant and the most important factor. In a trial, they also concluded that there was mainly an additive effect in the response to the disease (Thompson and Bergquist, 1984). They also reported that the resistance to disease was controlled by recessive genes having additive effect. They also reported that inbred line B73, after screening in seedling and adult stages for the disease, was evaluated and specified as a susceptible inbred line. Carson *et al.* (2004) stated that the inbred line B73 was susceptible to a number of leaf spot pathogens, while the inbred line MO17 had a high level of partial resistance to leaf spot disease. They also reported that the control of leaf spot in many hybrids was the result of some form of a polygenetic resistance and this kind of resistance had mainly a moderate to high inheritance that could be easily performed in breeding programs (Carson *et al.* 2004). In the evaluation of 60 maize hybrid combinations for resistance to SCLB *B. maydis*, Zamani and Choukan (2000) applied bazooka method and identified K 1250 × MO17 as resistant genotypes. By carrying this experiment, it was confirmed that bazooka method was a suitable inoculation method for developing infection in maize and could be used in determination of susceptible and resistant hybrids and inbred lines of maize. According to Bajet and Renfero (1994) by using natural infection, percent of infection remained at a low level and extent of infection, between 8% and 25%, while using artificial infection, especially by bazooka method, it could be increased to 75% of infection and all plants were uniformly infected. Therefore, it is recommended that this method could be applied in the breeding programs to develop resistant genotypes to *B. maydis*.

## CONCLUSION

It is clear that disease severity can be considered as a suitable and stable index for evaluating the resistance of genotypes to *B. maydis* causing Southern Corn Leaf Blight (SCLB), which our results demonstrated. In general, disease development using this technique (bazooka technique) and its facility in applying in maize fields could be an accurate assessment for breeders to release the best and most resistant hybrids and inbred lines for introducing to farmers. Therefore, further investigations should be conducted to determine resistant cultivars to Southern Corn Leaf Blight (SCLB) in Iran.

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## ОТПОРНОСТ ГЕНОТИПОВА РАНОГ КУКУРУЗА НА ЈУЖНУ ПЕГАВОСТ ЛИСТА

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**САЖЕТАК:** Да би се испитала отпорност генотипова раног кукуруза на јужну пегавост листа (Southern Corn Leaf Blight – SCLB или Maydis Leaf Blight – MLB), експерименти по RCBD методи са 20 инбред линија и хибрида у 2014. и 16 генотипова у 2015. години спроведени у пољопривредним станицама у Карацу и Сарију. Инокулација је изведена са суспензијом спора помоћу шприца (3 мл/сваки пршљен), први пут када је кукуруз био у фази 3–4 листа, а затим су зрна сирка инокулирана гљивицама нанета на кукуруз у фази 6–8 листова у пршљен сваке биљке користећи базука технику. Евалуација је обављена на бази напредовања болести у стадијуму опрашивања, као и две недеље након фазе опрашивања, користећи скалу 0–5. Резултати анализе варијансе и поређења средњих вредности показали су да генотипови различито реагују на болест. У овој студији, 15% генотипова у 2014. години означени су као резистентни, а то су били генотипови: KE 77003/10 × KE 75039, KE 72012/12 × K1263 / 1 (KSC 400) и K 2331 × KE 75039. Од 16 генотипова у 2015. години, три хибрида – бр. 16 са педигреом (KE 76009/311 × K 1264 / 5-1), бр. 10 са педигреом (K 2331 × KE 75039) и бр. 9 са педигреом KSC 400 (KE 72012 / 12 × K1263 / 1) – идентификована су као отпорни хибриди који могу бити добар извор отпорности на јужну пегавост листа кукуруза.

**КЉУЧНЕ РЕЧИ:** кукуруз, отпорност, болест листа, *Bipolaris maydis*

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## *Colletotrichum orbiculare* ON WATERMELON: IDENTIFICATION AND *IN VITRO* INHIBITION BY ANTAGONISTIC FUNGI

**ABSTRACT:** Anthracnose caused by the fungus *Colletotrichum orbiculare* is one of the most significant diseases of *Cucurbitaceae*. In Serbia watermelon fruits with typical anthracnose lesions were collected during the year of 2015. Affected fruits showed sunken, dark brown to black lesions with orange conidial masses produced in black acervuli. In an attempt to identify the causal organism, small pieces of necrotic tissue were surface sterilized and placed on potato dextrose agar (PDA). Macroscopic and microscopic morphologically characteristics of three isolates were observed after growth on PDA for 7 days at 25 °C under a 12 h light/dark cycle. Fungal colonies developed white, grey to black dense aerial mycelium. Conidia were hyaline, aseptate, straight and cylindrical to clavate, 9–12.5 µm × 4–5.5 µm. Fungal isolates were also characterized by sequencing of the internal transcribed spacer (ITS) rDNA region using ITS1F/ITS4 primers and β-tubuline 2 gene using T1/Bt2b primers. The nucleotide sequences were deposited in GenBank (ITS Acc. No. KT454386, KT454387 and KT454388; β-tubuline 2 gene Acc. No. KT581236, KT581237 and KT581238). BLAST analysis of ITS and β-tubuline 2 gene sequences showed that our isolates were 100% identical to other *C. orbiculare* in NCBI GenBank. Pathogenicity test was conducted on symptomless, detached watermelon fruits. All tested isolates caused anthracnose lesions on watermelon fruits after 10 days of incubation. *Trichoderma harzianum* (DSM 63059) and *Gliocladium roseum* (DSM 62726) were evaluated *in vitro* for their antagonistic potential against *C. orbiculare*. The results of this study identify *T. harzianum* and *G. roseum* as promising biological control agents (BCAs) for further testing against anthracnose disease on watermelon fruits.

**KEYWORDS:** *Colletotrichum orbiculare*, watermelon, identification, antagonistic fungi

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## INTRODUCTION

Anthrachnose caused by the fungi *Colletotrichum orbiculare* species complex is one of the most significant diseases of cucumber (*Cucumis sativus* L.), melons (*Cucumis melo* L.), pumpkin (*Cucurbita pepo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai). The disease is widespread under both greenhouse and field cultivation and can occur on seedlings, leaves, petioles, stems and fruits of *Cucurbitaceae* and other herbaceous host, belonging to the *Asteraceae*, *Fabaceae* and *Malvaceae* (Farr and Rossman, 2013). In Serbia, *C. lagenarium* (synonym of *C. orbiculare*, von Arx, 1957) has been reported as pathogen on several *Cucurbitaceae* (Spasić, 1963; Stojanović *et al.* 2002).

Differentiation between *Colletotrichum* species based on host range or host of origin may not be a reliable criterion for fungi of this genus (Freeman *et al.* 1998). The host plants of species of the *C. orbiculare* complex can be attacked by other *Colletotrichum* species: *C. melonis* (*C. acutatum* complex), *C. karstii* (*C. boninense* complex) and *C. coccodes* (Damm *et al.* 2012a; Liu *et al.* 2013). However, due to their morphological variability, the ample range of hosting crops and the wide variety of isolates are partially difficult to identify as *Colletotrichum* spp. by traditional taxonomic methods, which must be complemented with molecular techniques and multilocus phylogenetic studies (Whitelaw-Weckert *et al.* 2007; Cannon *et al.* 2012; Damm *et al.* 2012a; Weir *et al.* 2012). In a major taxonomic reorganization of 42 strains of *C. orbiculare* and related species, Damm *et al.* (2013) identified 9 distinct clades within the *C. orbiculare* species complex based on multilocus phylogenetic analysis (ITS, GAPDH, CHS-1, HIS3, ACT, TUB-2 and GS). The results of analysis confirmed the four species previously known as belonging to this species complex: *C. lindemuthianum*, *C. malvarum*, *C. orbiculare* and *C. trifolii*, and recognized four new species from weeds: *C. bidentis*, *C. sidae*, *C. spinosum* and *C. tebeestii*.

Watermelon is susceptible to numerous plant pathogenic fungi. The main concern is related to leaf blight (*Alternaria cucumerina*), gummy stem blight (*Didymella bryoniae*), anthracnose (*Colletotrichum lagenarium*) and fusarium wilt (*Fusarium oxysporum* f.sp. *niveum*) (Bulajić *et al.* 2008). The occurrence of anthracnose on watermelon fruits has been found in Serbia during several last years. Economic losses caused by the disease are mainly attributed to lower fruit quality and marketability.

Controls of anthracnose on watermelon are currently limited to the use of cultural and chemical control methods. In search of alternatives, biological control has emerged as a way of managing this disease. *T. harzianum* and *G. roseum* are the most common fungal biological control agents (BCAs) that have been comprehensively researched and deployed throughout the world (Janisiewicz and Korsten, 2002).

The objectives of the present study were: (a) identifying the species of *Colletotrichum* causing the anthracnose on watermelon fruit using both classical and molecular techniques, and (b) evaluate the antagonistic effect of *T. harzianum* and *G. roseum* against *Colletotrichum* spp. originated from watermelon fruits.

## MATERIAL AND METHODS

### *Isolates*

Watermelon fruits with typical anthracnose lesions were collected during 2015 in the area of Ašanja, Srem district. Symptoms on infected fruits appeared as sunken, dark brown to black lesions with orange conidial masses produced in black acervuli (Figure 1). Pieces of the diseased tissues were sterilized in 3% NaOCl for 3 min, followed by several rinses with sterile distilled water, and placed on PDA in Petri plates at 25 °C for 5 days. Monoconidial cultures were produced for each isolate and maintained on PDA slants at 4 °C.

### *Pathogenicity test*

Pathogenicity tests with three representative isolates (LC1, LC2 and LC3) were conducted on mature and symptomless watermelon fruits. The fruits were cleaned and surface sterilized with ethanol (70%). Mycelial PDA discs of 5 mm were taken from a 14-day-old culture of each isolate and deposited on watermelon fruits superficially wounded with a sterile scalpel. In control fruits, only PDA disks without fungal mycelia were deposited onto wounds. The fruits were then incubated in a plastic container at 25 °C and >95% relative humidity, and examined for lesion development 10 days after inoculation. After 14 days, spores from diseased fruits were aseptically transferred onto PDA plates, which were incubated at 25 °C in darkness. The resultant cultures were checked for colony and spore morphology to confirm Koch's postulates.

### *Morphological identification*

Macroscopic and microscopic morphology characteristics of three isolates were observed after growth on PDA for 7 days at 25 °C under a 12 h light/dark cycle. Appressoria were produced using a slide culture technique (Johnston and Jones, 1997). Microscopic preparations were made in clear lactic acid. Length and width were measured for 100 conidia and shape of characteristic structures (conidiophores, conidia, setae, appressoria) was recorded using Olympus BX51 microscope.

### *Molecular identification*

The selected isolates were transferred on PDA medium and allowed to grow for 7 day at 25 °C. The isolation of DNA was performed with DNeasy Plant Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. All isolates were identified at the species level using a molecular strategy based

on sequences of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and partial sequences of the beta-tubulin 2 gene (TUB2).

The ITS and partial sequences of the TUB2 gene were amplified and sequenced using the primer pairs ITS1-F (Gardes and Bruns, 1993) and ITS4 (White *et al.* 1990); T1 (O'Donnell and Cigelnik, 1997) and Bt-2b (Glass and Donaldson, 1995). The PCRs were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, California) in a total volume of 25 µl. The ITS and TUB2 PCR mixture contained 12.5 µl 2 X PCR Master mix (K071, Fermentas, Lithuania), 9 µl RNase-free water, 1.25 µl each of both forward and reverse primers (100 pmol/µl, Metabion International, Deutschland) and 1 µl template DNA. Amplification conditions of ITS gene constituted an initial denaturation of 3 min at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, 1 min elongation at 72 °C and a final extension of 10 min at 72 °C, while the TUB2 PCR was performed at an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, and a final extension step of 7 min at 72 °C.

Amplified products were analyzed by 1% agarose gel electrophoresis, stained with Midori Green DNA Stain (Nippon Genetics) and visualized under a UV transilluminator. Sequencing in both directions was performed on an automated sequencer (ABI 3730XL Automatic Sequencer Macrogen, Korea). Sequence generated in this study was subjected to a Megablast search analysis at NCBI's GenBank nucleotide database for sequence similarity. Sequence of Serbian representative isolates (LC1, LC2, LC3) was aligned by using ClustalW algorithm implemented in MEGA6 (Tamura *et al.* 2013).

Alignment consisted of ITS and TUB2 sequences from all available isolates of species of the *Colletotrichum* group with outgroup species. Gene regions were aligned separately and concatenated into a single alignment. Sequences were initially aligned using Clustal W algorithm (Thompson *et al.* 1994) and manually adjusted in MEGA6 (Tamura *et al.* 2013). Phylogenetic analyses were constructed by the Neighbor-Joining (NJ) algorithm implemented in MEGA6 using 46 isolates of *C. orbiculare* and related *Colletotrichum* species and the outgroup *C. gloeosporioides* (Table 1). Sequences from isolates LC1, LC2 and LC3 were included in the analysis. The reliability of the obtained tree was evaluated using the bootstrap method based on 1,000 replicates and bootstrap values <50% were omitted.

### Antagonistic activity *in vitro*

*T. harzianum* (DSM 63059) and *G. roseum* (DSM 62726), employed for *in vitro* antagonistic activity were obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). The assay was performed on PDA by dual culture method. Plates inoculated only with three tested isolates served as controls. After 10 days at 25 °C the percent growth inhibition (PGI) was calculated using the formula:  $PGI (\%) = \frac{KR - R1}{KR} \times 100$ , where KR is the colony

diameter in control plate without antagonist, and R1 is the colony diameter in treated plate (Skidmore and Dickinson, 1976). Hyphal interaction and morphology were observed with Olympus BX51 microscope.

Table 1. Stains of *Colletotrichum* spp. with collection details and GenBank accessions. Strains studied in this paper are in bold.

Taxon	Isolate name <sup>a</sup>	Host	Country	GenBank accessions	
				ITS	TUB2
<i>C. bidentis</i>	COAD 1020, CPC 21930	<i>Bidens subalternans</i>	Brazil	KF178481	KF178602
<i>C. gloeosporioides</i>	IMI 356878, CBS 112999, ICMP 17821, STE-U 4295	<i>Citrus sinensis</i>	Italy	JQ005152	JQ005587
<i>C. lindemuthianum</i>	CBS 523.97, LARS 798	<i>Phaseolus coccineus</i>	Costa Rica	JX546815	JX546861
<i>C. lindemuthianum</i>	CBS 524.97, LARS 800	<i>Phaseolus coccineus</i>	Costa Rica	JX546816	JX546862
<i>C. lindemuthianum</i>	CBS 571.97, LARS 83	<i>Phaseolus vulgaris</i>	Brazil	JX546818	JX546864
<i>C. lindemuthianum</i>	CBS 569.97, ATCC 56897, LARS 9	<i>Phaseolus vulgaris</i>	Europe	JX546817	JX546863
<i>C. lindemuthianum</i>	CBS 151.56, IMI 063364, ATCC 12611, UCLAF 230	<i>Phaseolus vulgaris</i>	France	JX546812	JX546858
<i>C. lindemuthianum</i>	CBS 143.31	<i>Phaseolus vulgaris</i>	Germany	JX546808	JX546854
<i>C. lindemuthianum</i>	CBS 144.31	<i>Phaseolus vulgaris</i>	Germany	JQ005779	JQ005863
<i>C. lindemuthianum</i>	CBS 146.31	<i>Phaseolus vulgaris</i>	Germany	JX546809	JX546855
<i>C. lindemuthianum</i>	CBS 147.31	<i>Phaseolus vulgaris</i>	Germany	JX546810	JX546856
<i>C. lindemuthianum</i>	CBS 150.28	<i>Phaseolus vulgaris</i>	Germany	JX546811	JX546857
<i>C. lindemuthianum</i>	CBS 151.28	<i>Phaseolus vulgaris</i>	Germany	GU227800	GU228094
<i>C. lindemuthianum</i>	CBS 152.28	<i>Phaseolus vulgaris</i>	Netherlands	JX546813	JX546859
<i>C. lindemuthianum</i>	CBS 153.28	<i>Phaseolus vulgaris</i>	Netherlands	JX546814	JX546860
<i>C. lindemuthianum</i>	CBS 130841, CIKY1	<i>Phaseolus vulgaris</i>	USA	JX546819	JX546865
<i>C. lindemuthianum</i>	CBS 131.57	<i>Phaseolus vulgaris</i>	USA	JX546805	JX546851
<i>C. lindemuthianum</i>	CBS 132.57	<i>Phaseolus vulgaris</i>	USA	JX546806	JX546852
<i>C. lindemuthianum</i>	CBS 133.57	<i>Phaseolus vulgaris</i>	USA	JX546807	JX546853
<i>C. malvarum</i>	CBS 521.97, LARS 720, Lav-4	<i>Lavatera trimestris</i>	UK	KF178480	KF178601
<i>C. malvarum</i>	CBS 123.24	Malvaceae	unknown	KF178479	KF178600
<i>C. orbiculare</i>	CBS 129432, USYD-2008-01	<i>Benincasa hispida</i>	Australia	KF178469	KF178590
<i>C. orbiculare</i>	CBS 133194, KTU-H1	<i>Cucumis melo</i>	Japan	KF178459	KF178580
<i>C. orbiculare</i>	CBS 133195, KTU-H2	<i>Cucumis melo</i>	Japan	KF178460	KF178581
<i>C. orbiculare</i>	CBS 133196, KTU-H5	<i>Cucumis melo</i>	Japan	KF178461	KF178582
<i>C. orbiculare</i>	CBS 133197, KTU-K5	<i>Cucumis melo</i>	Japan	KF178468	KF178589
<i>C. orbiculare</i>	CBS 133198, KTU-K6	<i>Cucumis melo</i>	Japan	KF178458	KF178579
<i>C. orbiculare</i>	CBS 570.97, LARS 73	<i>Cucumis sativus</i>	UK	KF178466	KF178587
<i>C. orbiculare</i>	CBS 514.97, 104-T, LARS 414	<i>Cucumis sativus</i>	Japan	JQ005778	JQ005862
<i>C. orbiculare</i>	CBS 274.54	<i>Cucumis sativus</i>	Netherlands	KF178462	KF178583
<i>C. orbiculare</i>	CBS 172.59	<i>Cucumis sativus</i>	Netherlands	KF178464	KF178585
<i>C. orbiculare</i>	CBS 173.59, IMI 213974	<i>Cucumis sativus</i>	Netherlands	KF178463	KF178584
<i>C. orbiculare</i>	CBS 122.24	<i>Cucumis sativus</i>	UK	KF178467	KF178588
<i>C. orbiculare</i>	CBS 107.17	unknown	unknown	KF178465	KF178586
<i>C. orbiculare</i>	LC1	<i>Citrullus lanatus</i>	Serbia	KT454386	KT581236
<i>C. orbiculare</i>	LC2	<i>Citrullus lanatus</i>	Serbia	KT454387	KT581237
<i>C. orbiculare</i>	LC3	<i>Citrullus lanatus</i>	Serbia	KT454388	KT581238
<i>C. sidae</i>	CBS 504.97, LARS 76, ATCC 58399, NRRL 8096	<i>Sida spinosa</i>	USA	KF178472	KF178593
<i>C. sidae</i>	CBS 518.97, LARS 629, Cm-4	<i>Sida spinosa</i>	USA	KF178471	KF178592
<i>C. sidae</i>	CBS 574.97, LARS 625, ATCC 96725, 3-1-1, Cm-9	<i>Sida spinosa</i>	USA	KF178471	KF178591
<i>C. spinosum</i>	CBS 113171, IMI 368075, STE-U 5296	<i>Xanthium spinosum</i>	Argentina	KF178475	KF178596
<i>C. spinosum</i>	CBS 515.97, LARS 465, DAR 48942	<i>Xanthium spinosum</i>	Australia	KF178474	KF178595
<i>C. tebeostii</i>	CBS 522.97, LARS 733, S3-43	<i>Malva puzilla</i>	Canada	KF178473	KF178594
<i>C. trifolii</i>	CBS 128554, ICMP 12934, LARS 164, N85 ANW	<i>Medicago sativa</i>	USA	KF178476	KF178597
<i>C. trifolii</i>	CBS 158.83, BBA 70709	<i>Trifolium</i>	USA	KF178478	KF178599
<i>C. trifolii</i>	CBS 425.83	unknown	unknown	KF178477	KF178598

## RESULTS AND DISCUSSION

The symptoms of watermelon fruits begin as small, sunken lesion that have a water-soaked appearance, increase in diameter and coalesce, leaving a large sunken soft area. The necrotic spots can expand and merge to cover the whole affected area. The color of the infected part darkens. Orange conidial masses may occur scattered or in concentric rings on the lesion (Figure 1).

All tested isolates caused anthracnose lesions on watermelon fruit after 10 to 14 days of incubation. No lesions developed on fruit inoculated with non colonized PDA disk. Koch's postulates were fulfilled by reisolation from inoculated watermelon fruits. Conidia shape, size, and colony morphology were identical for the original and recovered isolates.

Macroscopic and microscopic morphology characteristics of isolates LC1, LC2 and LC3 were uniform. Fungal colonies were dense aerial, initially white, becoming gray and then turning black, as the cultures aged on PDA. Colony reverse was gray to dark gray. The cultures developed black acervuli around the center of the colony. Mycelia were branched, septate, and hyaline. Conidiophores and setae formed directly from hyphae. Setae were brown, smooth-walled, 1-5 septate, 30–120  $\mu\text{m}$  long. Conidia were hyaline, aseptate, straight, cylindrical to clavate, with one end round and the other truncate, 9-(10.5)-12.5  $\mu\text{m}$  x 4-(4.5)-5.5  $\mu\text{m}$  (Figure 2). Appressoria were single, dark brown, smooth-walled, ovate or clavate, 5.5-(6)-7.5  $\mu\text{m}$  x 4.5-(5.5)-6  $\mu\text{m}$ .

The morphological characteristics of our isolates are similar to those reported by Damm *et al.* (2013). However, definitive identification of *Colletotrichum* species based on morphology is difficult because isolates have overlapping ranges of conidial and colony characteristics, and because variation in morphology is accepted for isolates within a species (Sutton, 1992).



*Figure 1.* Anthracnose symptoms on watermelon fruit: sunken necrotic lesion with orange conidial masses and black acervuli.

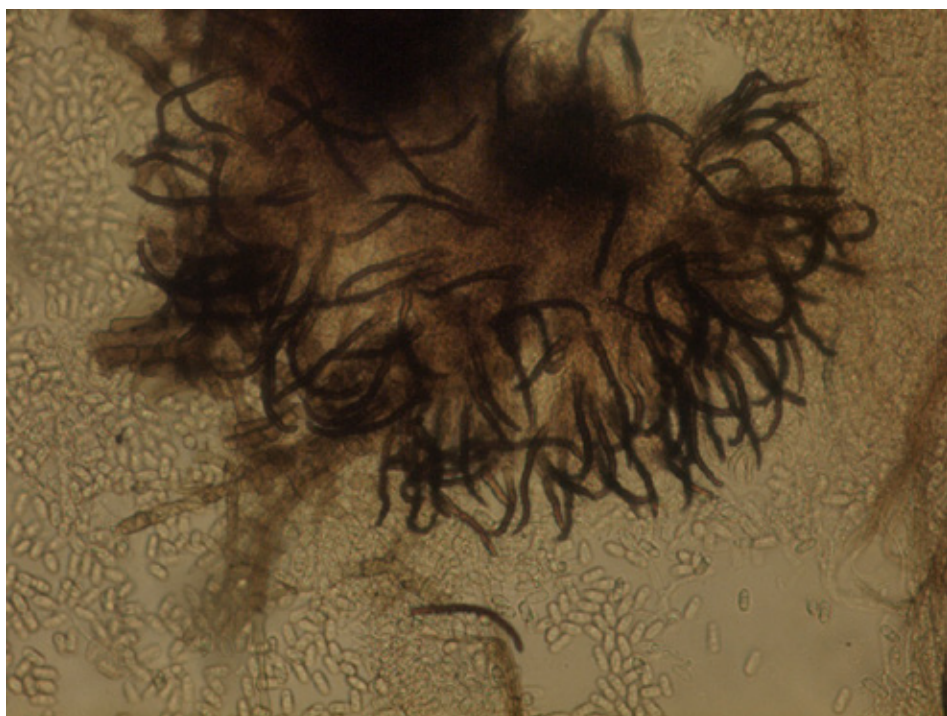


Figure 2. Morphological characteristics of isolate LC1: acervuli, setae and conidia (x400).

PCR amplifications of the ITS and TUB2 gene gave a fragment of the expected size (approximately 600 and 800 bp) and their sequences were used for classification based on a BLAST analysis.

The sequence analysis of ITS region revealed that the Serbian isolates LC1, LC2 and LC3 (GenBank Accession No. KT454386, KT454387 and KT454388) shared 100% identity with *C. orbiculare* isolate deposited in the GenBank from South Korea (JX997422). BLAST analysis of the TUB2 sequences of the three Serbian *C. orbiculare* isolates LC1, LC2 and LC3 (GenBank Accession No. KT581236, KT581237 and KT581238) shared the highest identities with 23 Indian *C. orbiculare* isolates (KP899039-61) from *Citrullus lanatus* and two Japanese *C. orbiculare* isolates (JQ005862 and KF178579).

A neighbor-joining tree (Figure 4) of 46 *Colletotrichum* species and the outgroup (*C. gloeosporioides*) was constructed based on combined alignment of ITS and TUB2 genes. Phylogenetic analysis resulted in detection of three main clades and 9 subclades within the *C. orbiculare* species complex. The first main clade is formed by *C. lindemuthianum* strains and is well supported with a bootstrap support of 99%. The second main lineage is represented by a single strain of *C. bidentis*. The third main clade consists of six subclades: the clades

representing *C. trifolii* and *C. malvarum* are well supported and grouped with each other. A sister clade is formed by *C. orbiculare* containing the largest number of strains with two smaller subclades representing *C. sidae* and *C. tebeestii* as well as a single-clade representing *C. spinosum*.

The results of our study showed that, all of the three isolates obtained from diseased tissues of watermelon in Serbia belonged to the *C. orbiculare*. Our isolates, together with isolates from Japan, the UK and the Netherlands were clustered in the branch of clade *C. orbiculare*, with high bootstrap support of 99%. The overall shape of the *Colletotrichum* reconstructed phylogenetic tree was similar to those previously reported and phylogenetic analysis resulted in the delineation of three main clades as determined by the most recent comprehensive study (Damm *et al.* 2013). Presently however, not all *Colletotrichum* species and species complexes are sufficiently known from DNA sequence data and some of them might have an intermediate position between *C. orbiculare* and other species complexes (Damm *et al.* 2013). Different gene sequences of *Colletotrichum* can be used for the detection of these taxa from *Colletotrichum* at generic level and have been successfully applied in the characterization of several *Colletotrichum* species.

Results from dual culture assay showed that *T. harzianum* had significantly greater inhibitory activity against *C. orbiculare* than the *G. roseum* *in vitro*. *T. harzianum* exhibited the strong antagonism against isolates LC1, LC2 and LC3 with a high PGI value (69%, 67%, and 70% respectively). No distinct inhibition zones were observed between antagonistic fungus and pathogens. Major mechanisms involved in the antagonistic activity of *Trichoderma* spp. were competition for space and nutrients, production of diffusible and/or volatile antibiotics, and hydrolytic enzymes like chitinase and  $\beta$ -1,3-glucanase (Howell, 2003). These hydrolytic enzymes partially degrade the pathogen cell wall and lead to its parasitization (Kubicek *et al.* 2001). Microscopic examination revealed that antagonist caused a wide spectrum of mycelial malformation of all tested *C. orbiculare*: abnormal stunted, highly branched hyphal tips, swollen hyphae and the vacuolar appearance of the mycelium of pathogenic fungi. Similar results were reported by Gupta *et al.* (1995), Howell (2003) and Begum *et al.* (2008).

*G. roseum* presented a moderate antifungal effect *in vitro* on isolates of *C. orbiculare*, LC1 (40%), LC2 (35%) and LC3 (38%). After 10 days of incubation a very weak inhibition zones were observed between *G. roseum* and all tested pathogens (2–3 mm). In these study hyphae of *G. roseum* were never observed to overlap the colony of *C. orbiculare*. In all cases isolates of *Colletotrichum* stopped growing before direct contact was made, presumable in response to diffusible inhibitors released by the antagonist. These results were similar to the results revealed by Lee and Wu (1984).

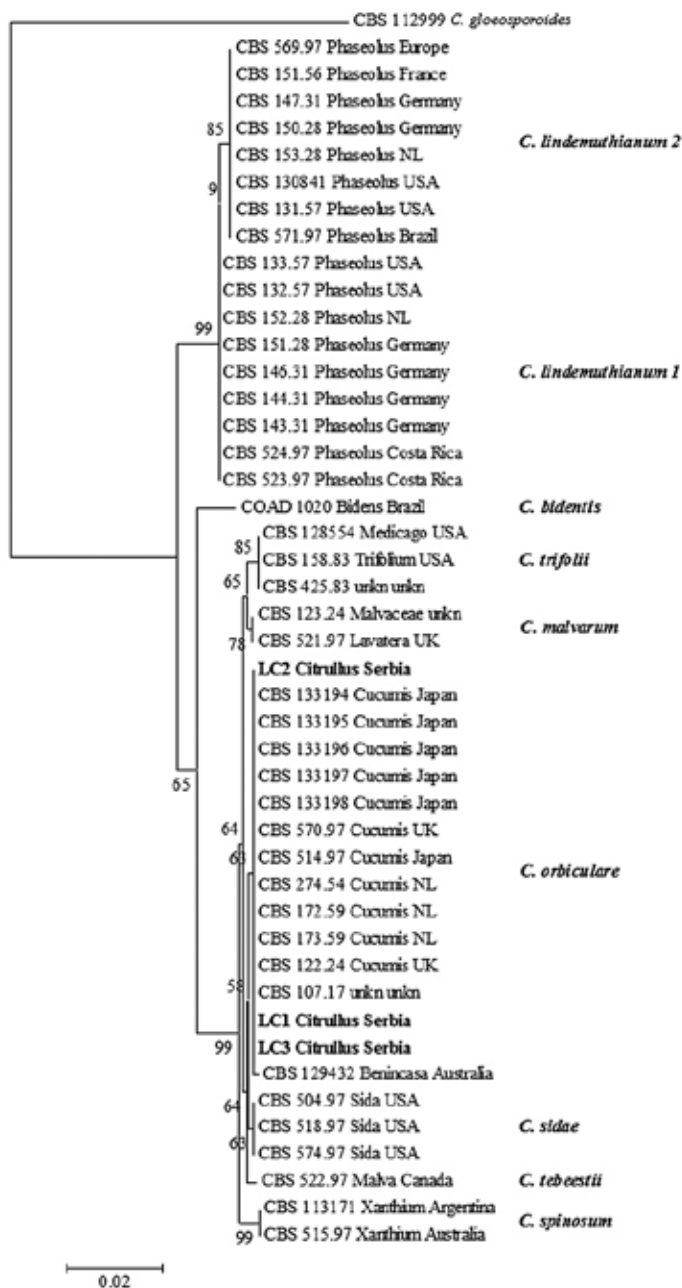


Figure 3. Neighbour-Joining tree based on analysis of combined alignment of ITS and TUB2 genes containing for 46 isolates of *Colletotrichum* species. *Colletotrichum gloeosporioides* CBS 112999 is used as outgroup. Bootstrap analysis was performed with 1,000 replicates and bootstrap values (>50%) are shown next to relevant branches. The Serbian *Colletotrichum* isolates are bolded.

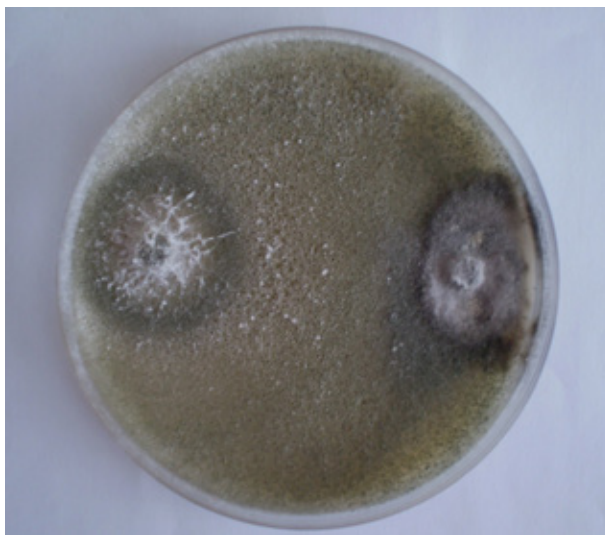


Figure 4. Antagonistic activity of *T. harzianum* against *C. orbiculare* – isolate LC1.

## CONCLUSION

Identification of *Colletotrichum* spp. is a fundamental criterion in the development of more efficient control measures. In the present study all of the three isolates of *Colletotrichum* spp. from watermelon fruits were morphologically identified as *C. orbiculare* and species identification was confirmed by PCR and sequencing. To our knowledge, this is the first molecular and phylogenetic analysis of *C. orbiculare* in Serbia. The results of antagonistic activity *in vitro* identify *T. harzianum* and *G. roseum* as promising BCAs for further testing against anthracnose disease on watermelon fruits.

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## *Colletotrichum orbiculare* СА ЛУБЕНИЦЕ: ИДЕНТИФИКАЦИЈА И IN VITRO ИНХИБИЦИЈА ГЉИВАМА АНТАГОНИСТИМА

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**РЕЗИМЕ:** Антракноза проузрокова на гљивом *Colletotrichum orbiculare* једна је од најзначајнијих болести на биљкама рода *Cucurbitaceae*. У Србији су током 2015. године прикупљени плодови лубенице с типичним антракнозним лезијама. Инфицирани плодови су са улегнутим, тамно браон до црним лезијама и масом наранџастих конидија из ацервула. У циљу идентификације проузроковача болести, с некротичног ткива узети су фрагменти, површински стерилисани и засејани на кромпир декстрозни агар (КДА). Макроскопске и микроскопске морфолошке карактеристике три изолата проучавана су након седам дана инкубације на температури од 25 °C у условима 12<sup>h</sup> светло/мрак. Гљиве формирају колоније беле, сиве до црне боје са густом, ваздушастом мицелијом. Конидије су хијалинске, несептиране, праве, цилиндричне до облика палице, величине 9–12,5 µm × 4–5,5 µm. Карактеризација изолата обављена је секвенцирањем ITS rDNA региона коришћењем прајмера ITS1F/ITS4 и β-tubulin 2 гена помоћу T1/Bt2b прајмера. Нуклеотидне секвенце су депоноване у NCBI банку гена (ITS Acc. No. KT454386, KT454387).

и KT454388;  $\beta$ - тубулин 2 ген Acc. No. KT581236, KT581237 и KT581238). BLAST анализа секвенци ITS и  $\beta$ -tubulin 2 гена је показала да су наши изолати 100% идентични с другим *C. orbiculare* врстама из NCBI базе. Тест патогености је обављен на одабраним, здравим плодовима лубенице. Сви испитивани изолати проузрокују антракнозне лезије на плодовима лубенице, десет дана након инокулације. Антагонистички потенцијал гљива *Trichoderma harzianum* (DSM 63059) и *Gliocladium roseum* (DSM 62726) испитиван је *in vitro* према изолатима *C. orbiculare*. *T. harzianum* и *G. roseum* су резултатима ових истраживања идентификовани као биолошки агенси који се могу успешно укључити у будућа тестирања у циљу сузбијања антракнозе плодова лубенице.

КЉУЧНЕ РЕЧИ: *Colletotrichum orbiculare*, лубеница, идентификација, гљиве антагонисти



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